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Amperometric sensors based on immobilised enzymes

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ABSTRACT. The state, problems, and prospects of the development of one of the new fields of analytical chemistry—"reagent-free" analytical methods—are described and surveyed using as an example enzyme-based amperometric sensors. Questions associated with the design of biosensors and the characteristics of their functioning under various conditions are examined. The applied aspects of the use of enzyme electrodes of this type in various fields of science and the national economy are noted.

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I. Introduction

One of the most important problems in analytical chemistry is the creation of sensitive and selective methods which would make it possible to analyse biological objects. In connection with ecological problems, the task has become of still more urgent interest. The use for these purposes of enzymes—preparations or biological materials containing them—which can be called new generation analytical reagents, proved to be attractive and promising.

Enzymes as biological catalysts exhibit a very high catalytic activity combined with a high specificity. These factors combined with the mild conditions under which enzyme reactions take place, the possibility of recording the useful signal, and also the advances achieved in the field concerned with the creation of stabilised enzyme preparations via immobilisation[1–6] have ensured the development of a new aspect of analytical chemistry—"reagent-free" analytical methods based on the use of biochemical sensors (biosensors).[7]

A biochemical sensor usually consists of some kind of physicochemical detector-sensor (electrochemical, solid-state, opticoelectronic, enthalpimetric, etc.) and a layer of immobilised enzyme fixed on its surface or near it. Many biosensors contain a semipermeable membrane.[7–9] The principle governing their activity is based on the diffusion of the substrate into a thin layer of biocatalyst, in which the enzyme reaction takes place, and on the determination of any component of the enzyme reaction (product or substrate) by some kind of sensor. If electrochemical sensors are used (ion-, gas-, and molecule-selective electrodes, etc.), then, depending on the measured quantity (current or potential), the biosensors (their initial name was "enzyme electrodes") are divided into potentiometric and amperometric.[10] The potentiometric biosensors exhibit a linear dependence of the potential on the logarithm of the concentration of the substance determined, while in the amperometric sensors the analytical signal is the current of the electrochemical reaction, which depends on the concentration of the compound to be determined.[10,11] Differences are observed in the range of the component concentrations determined and in the response time.

Since the first mention of enzyme electrodes in 1967 by Updike and Hicks,[12] the interest in this field of bioelectroanalytical chemistry has continuously grown. The number of publications has increased every year. Symposia and conferences devoted to electrochemical biosensors and related questions are held annually, i.e. the world interest in this problem is extremely high. This is in fact not surprising because enzyme electrodes make it possible to solve successfully a whole series of complex problems in the analysis of enzyme substrates, activators, and inhibitors; they are extremely promising in biochemical analysis, medicine, and pharmacology.[13,14] Furthermore, effective electroanalytical analysers have already been created on the basis of enzyme electrodes which combine the specificity of biocatalysts with the high sensitivity of electrochemical apparatus.[15]

This review is devoted to one of the types of biosensors with amperometric signal indication and covers the literature from 1977 up to the present time. A systematic account of earlier data has been given elsewhere.[7,8,10,11,16] It may be of interest to mention them in connection with the establishment of the field of electroanalytical chemistry and bioelectrocatalysis.[17] A whole series of subsequent reviews were devoted to the description of a large number of enzyme systems and a wide variety of amperometric sensors based on them.[18,19] Most attention has been devoted to the theoretical description and properties of various types of enzyme electrodes[18–24] and their components,[21] questions of the kinetics of enzyme reactions,[25–27] and the conditions under which electrodes operate (*in vivo* and *ex vivo*).[28,29] From year to year, the number of new enzyme electrodes has increased and the range of their applications has expanded.[30] However, despite the wide variety of enzyme sensors, there exist unique methodological approaches to their design and unique requirements as regards the function of the electrodes and also the methods for monitoring the stability and reproducibility of the readings.[31]

Modern enzyme electrodes make it possible to determine with a high sensitivity and selectivity many organic and inorganic substances which are enzyme substrates, inhibitors, and activators, i.e. direct and indirect participants in enzyme reactions. The sensitivity of many biosensors reaches in some cases several picomoles per litre.[30]

Reviews[32,33] as well as proceedings of symposia and conferences[13,34–39] have been devoted to problems of the practical application of enzyme electrodes in clinical research, microbiology, immunology, other branches of medicine, as well as ecology and agriculture. The undoubted economic gain in the application of

enzyme systems in analysis[37,40] and good prospects for the use of biosensors *in vivo* and *in vitro*[38,41] have been noted.

Despite the sudden rise in the interest shown by various investigators in this field of knowledge, which has been reflected in the publications mentioned above, one should note that the bibliography in the reviews, which appeared relatively quickly, reflects the "specific" interest of the authors and is as a rule not repeated, so that each review constitutes a supplement to the others.

II. Amperometric sensors based on various enzyme systems

Analysis of the literature on problems associated with the preparation of biosensors makes it possible to distinguish four main stages in the preparation of any enzyme electrode:[16] the choice of enzyme as a reactant; the preparation of the enzyme; the immobilisation of the enzyme; the construction of the enzyme electrode itself.

It is also necessary to note that the choice of the method of recording the useful signal, i.e. the choice of a particular (in the given instance electrochemical) sensor, is important in the preparation of the electrode. It is possible to make electrodes of three types: type A—dialysis membrane electrode; type B—with physical deposition of the enzyme on the surface; type C—with direct immobilisation of the enzyme on the membrane.[16]

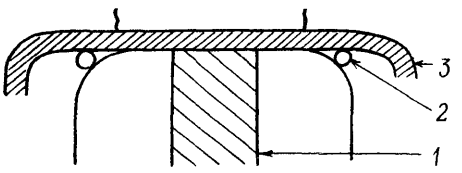


Figure 1. Schematic diagram of an enzyme glucose electrode prepared on the basis of a membrane oxygen sensor:[8] *1*, cathode; *2*, reference electrode; *3*, semipermeable Teflon membrane; 4, enzyme gel layer.

The first biosensor was a type A enzyme electrode for the determination of glucose, which was constructed on the basis of the Clark cell (electrode) where glucose oxidase was retained in the form of a thin layer between two dialysis membranes.[7,8] Subsequently the glucose oxidase solution was replaced by glucose oxidase immobilised in a polyacrylamide gel. The design of one of the first biosensors is illustrated in Fig. 1.[8] At the present time the enzyme electrode is represented by a two-membrane model (Fig. 2),[42] which makes it possible to obtain by theoretical analysis complete and approximate equations characterising the stationary behaviour of monoenzyme electrodes over the entire substrate concentration range. It is possible to estimate the influence of various factors, such as the diffusion layer and the ratio of the thickness of the enzyme-containing layer and of the outer membrane, on the working characteristics of the sensors.

It has also been suggested that the enzyme preparation be fixed on the surface of the stirrer or on the surface of an easily replaced stationary component placed within the enzyme reactor, which improves, according to the authors,[43] a number of analytical characteristics of such designs.

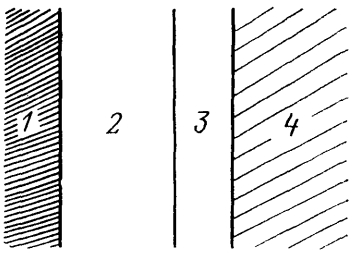


Figure 2. General schematic arrangement of the enzyme electrode:[42] *1*, indicator electrode (planar); *2*, layer of immobilised enzyme; *3*, outer membrane; *4*, substrate solution being analysed.

The operation of enzyme electrodes is based on the direct electron transfer between the active centre of the immobilised enzyme and the surface of the electrochemical sensor, which is frequently modified in some way. However, electron transfer can be effected also with the aid of special mediators.

The principal requirements which must be met by the mediators can be formulated as follows:[44,45] the stage in which the mediator interacts with the active centre of the enzyme must be rapid, i.e. the mediator must in essence behave as an enzyme substrate; the oxidation-reduction potential of the mediator must be low and must be independent of the pH; the mediator must be oxidised (or reduced) electrochemically at the electrode made from the given material under conditions close to reversibility; the electron transferring agent must be stable (its reduced form must not be oxidised, for example, by atmospheric oxygen).

Low-molecular-weight compounds characterised by a high mobility of the electrochemical equilibrium are most often employed as mediators. These are dyes, quinone type compounds, hexacyanoferrates-(II) and -(III), and conducting organic salts, i.e. molecules and ions which exist in solution in two forms corresponding to different oxidation states of one of their constituent elements.[21,25,26] Thus the choice of mediator constitutes a special problem.

1. Biosensors for the determination of glucose

It is noteworthy that the greatest number of various biosensors and the corresponding analytical devices have been created at the present time for the determination of glucose. This is associated with the need to monitor the sugar content in biological liquids not only for the diagnosis but also for the therapy of a whole series of diseases. Soviet designs for the industrial production of such analysers are already available.[9]

All the enzyme electrodes for glucose with amperometric indication, which employ the ability of glucose to be oxidised [Eqn. (1)], are constructed on the basis of Clark oxygen electrodes, peroxide electrodes, or certain other detectors.[46]

Electrodes made of platinum, gold, silver, or rhodium are used as indicator electrodes in Clark oxygen cells and its modifications. The electrode component is as a rule separated from the object analysed and the solution by a thin polymer film which is impermeable to both high- and low-molecular-weight compounds but is permeable to oxygen (Fig. 1).

The principle of the operation of such an electrode is simple: the enzyme catalyses the removal of oxygen from the solution at a rate which depends on the glucose content and this is indicated by the oxygen electrode:

$$\text{glucose} + O_2 + H_2O \xrightarrow{GO} \text{gluconic acid} + H_2O_2; \quad (1)$$

GO = glucose oxidase.

The principal advantage of oxygen sensors is the simplicity of their preparation, high stability, selectivity, and reproducibility of the readings, and a fairly high sensitivity.

The oxidation of hydrogen peroxide [Eqn. (2)] usually follows reaction (1), which can be detected by appropriate electrodes corresponding to hydrogen peroxide:

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e\ . \quad (2)$$

Such sensors are made from various materials (platinum, gold, and nickel, graphite). A distinct diffusion current makes it possible to use reaction (2) for the quantitative determination of glucose (at a platinum electrode, this reaction is observed at 0.6–0.7 V relative to s.c.e.†). Analysers for hydrogen peroxide are distinguished by a short reponse time in the range 45–60 s (120 s for a continous flow-type blood analyser), but their selectivity is lower because at the H_2O_2 oxidation potentials a number of organic compounds, which may be present in the specimen, also undergo electrochemical reactions.

Apart from glucose analysers with an oxygen electrode and with a hydrogen peroxide electrode, devices have been developed in which the enzymic oxidation of glucose is effected not by oxygen but by another oxidant, for example potassium hexacyanoferrate(III):

$$CH_2OH(CHOH)_4CHO + H_2O + [Fe(CN)_6]^{3-} \longrightarrow$$

$$\longrightarrow CH_2OH(CHOH)_4COOH + [Fe(CN)_6]^{4-}. \quad (3)$$

The hexacyanoferrate(II) formed as a result of reaction (3) is oxidised at a platinum electrode:

$$[Fe(CN)_6]^{4-} \longrightarrow [Fe(CN)_6]^{3-} + e. \quad (4)$$

Reaction (4) takes place at a potential of −0.5 V relative to a silver,silver chloride reference electrode, which serves simultaneously as an auxiliary electrode.[46]

The hydrolysis of glucose takes place also with participation of quinone. The hydroquinone formed is usually detected by a platinum electrode. This principle is used in a glucose sensor with glucose oxidase (GO) immobilised on cellophane.[15] Enzyme electrodes for glucose, based on similar principles and developed previously, have been described in detail.[7,8,10,11] The use of various carbonaceous materials as the electrochemical sensor proper in similar devices has been described by Razumas et al.[21] A whole series of studies in this field have been described by Sorochinskii and Kurganov.[30]

The characteristics of various enzyme sensors, including those for the determination of glucose, are compiled in Table 1.

An enzyme electrode has been developed for the determination of glucose with the aid of which one can follow the variation of the H_2O_2 concentration in the molybdenum(VI)-catalysed reaction with iodide ions:

$$H_2O_2 + 2H^+ + 2I^- \xrightarrow{Mo(VI)} I_2 + 2H_2O. \quad (5)$$

By varying the rate of rotation of a disc electrode with a ring, on which glucose oxidase has been immobilised, one can determine the rate of mass transfer of the substrate and the rate of the catalytic reaction. The life of such an electrode is about 6 days.[47] The advantages of using rotating electrodes of the "disc with a ring" type in amperometric sensors have also been discussed by Guilbault.[20]

In the development of glucose sensors, glucose oxidase was immobilised in three ways: (1) by covalent grafting by means of a bifunctional reagent (glutaraldehyde) on a modified silica carrier Silochrome S-50; (2) with the aid of a synthetic water-soluble acrolein–vinylpyridine copolymer; (3) by oxidising the carbon components of the enzyme. The carrier with the immobilised enzyme was placed on the bottom of the cell under a porous nylon filter. With the aid of this type of enzyme sensor, it is possible to carry out in the course of two weeks more than 200 determinations of standard glucose solutions relative to a single calibration graph. The electrode response time is 10–15 s. The immobilised glucose oxidase preparation, obtained by oxidising the hydrocarbon components of the enzyme, was stored for more than two months at room temperature without loss of activity.[50] The relative standard deviation of a single determination (s_r) was about 0.03 in the substrate concentration range 0.18–0.10 g ml^{-1} and 0.13 for a substrate concentration of 0.005 mg ml^{-1}.[50] In order to increase the rate of electron transfer, various mediators are used, as mentioned above, in many biosensors. Thus a selective enzyme electrode has been proposed for the determination of glucose, the mechanism of the response of which is based on the selective oxidation of glucose to gluconic acid by $[Fe(CN)_6]^{3-}$ in the presence of glucose oxidase.[52] The $[Fe(CN)_6]^{4-}$ formed is reoxidised to $[Fe(CN)_6]^{3-}$ on the surface of the indicator enzyme electrode consisting of an aluminium rod whose end-face is coated with a graphite-containing material. A layer of gel with immobilised glucose oxidase, covered by a cellophane membrane, is deposited on the graphite. In the interval between measurements, the electrode was stored at 4 °C in phosphate buffer containing $K_3[Fe(CN)_6]$. The standard deviation s_r was 0.03–0.06 for the glucose concentration range 5×10^{-6}–5×10^{-5} M and was less than 0.03 for higher substrate concentrations.

The response time depends on the glucose concentration and amounts to 2–4 min for low concentrations. The operation of the electrode is stable for at least 3 weeks and the electrode has been used for determining glucose in blood serum and in urine relative to a calibration graph which was plotted daily. The presence of other enzymes, including catalase, does not interfere with the determination.

An enzyme electrode has been described for the determination of glucose in blood and plasma, where ferrocene and its derivatives, oxidised to ferrocenium ions, are used as the mediator between the immobilised glucose oxidase and the graphite electrode.[53] Flavin-adenine dinucleotide has been used for the same purpose.[54]

† Saturated calomel electrode (Ed. of Translation).

Table 1. Analytical possibilities of amperometric enzyme sensors.

Compound determined	Enzyme	Method of immobilisation	Amperometric detector	Range of concentrations determined	Refs.
Glucose	Glucose oxidase	Cross-linking by glutaraldehyde to the surface of the carbon paste containing added amines	Rotating disc electrode with a ring	—	[47]
	"	Cross-linking with albumin	Platinum H_2O_2 electrode	0.05 – 10 mM	[48]
	"	Entrainment in the electrode layer	Two platinum hexacyanoferrate electrodes	6.9 – 27.8 mM	[49]
	"	(a) Covalent grafting by glutaraldehyde on Silochrome S-50; (b) with the aid of a synthetic acrolein – vinylpyridine copolymer; (c) oxidation of hydrocarbon components of enzyme	Clark oxygen electrode	$3.10^{-5} – 1.10^{-3}$ M	[50]
	"	Covalent grafting to polyamide networks	Membrane O_2 electrode	0.1 – 7.0 mM	[51]
	"	Entrainment in the gel in the near-electrode layer	Aluminium rod with a graphite end face on which a layer of gel has been deposited, mediator – $K_3[Fe(CN)_6]$	$5.10^{-6} – 2.10^{-3}$ M	[52]
	"	Covalent grafting to graphite modified by 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide *p*-methyltoluene-sulphonate	Graphite electrode, mediator – ferrocene	1 – 30 mM	[53]
	"	Entrainment in near-electrode layer	Platinum disc covered by a semi-permeable membrane	0.01 – 7.00 mM	[54]
	"	Covalent grafting to collagen films	Platinum H_2O_2 electrode	$10^{-6} – 10^{-3}$ M	[55]
	"	Film with immobilised glucose oxidate	Cathode – silver, anode – platinum	0.8 mg ml^{-1}	[58]
	"	Electropolymerisation of pyrrole in the presence of the enzyme	Platinum or carbon glass electrode	$10^{-4} – 10^{-2}$ M	[57]
	"	Incorporation in a poly-*N*-methylpyrrole film	Platinum disc electrode	0.22 M	[58]
	"	Covalent grafting to a carbon glass electrode via the borate groups of 3-aminophenylboronic acid	Carbon glass electrode modified by 3-aminiphenylboronic acid	—	[59]
	"	Adsorption of glucose oxidase on a modified graphite electrode	Graphite electrode modified by the ionic sputtering of palladium and gold	$3.10^{-6} – 4.10^{-3}$ M	[60]
	"	Homogeneous membrane	Membrane O_2 electrode	1.0 – 5.0 M	[61]
	"	Glucose oxidase modified by the introduction of ferrocene derivatives	—	—	[28]
	"	Incorporation in a semipermeable membrane	Carbon paste electrode modified by *p*-benzoquinone	—	[62]
	"	Incorporation in a semipermeable membrane together with *p*-benzo-quinone	Gold electrode and platinum mesh electrode	—	[63]
	"	Attachment of *p*-benzoquinone-activated glucose oxidase to a cellulose acetate membrane with covalently bound bovine albumin	Platinum electrode	—	[64]
	"	Covalent binding to ferrocene-carboxylic acids	Carbon glass electrode	—	[65]
Uric acid	Uricase	Covalent binding to glutaraldehyde	Platinum electrode with a thin layer of immobilised enzyme	0.02 – 0.08% in serum, 0.25 – 0.75% in urine	[66]
	"	Covalent grafting to porous glass with the aid of glutaraldehyde	Platinum H_2O_2 electrode	2 – 8 mg/100 ml of serum	[67]

Table 1 (contd.).

Compound determined	Enzyme	Method of immobilisation	Amperometric detector	Range of concentrations determined	Refs.
Aromatic amines	Peroxidase	(a) Adsorption; (b) adsorption followed by treatment with glutaraldehyde	Epoxidised carbon glass electrode in a Teflon shell	0.1–3 μM	[68]
Cholesterol	Cholesterol oxidase	Incorporation in a collagen membrane	Membrane O_2 electrode	0–0.2 mM	[69]
	"	Entrainment in a 2-hydroxymethyl methacrylate gel treated with glutaraldehyde	Platinum O_2 electrode	0.4–12 mM	[70]
Ethanol	Alcohol oxidase	Entrainment in near-electrode layer under a nylon film	Platinum disc H_2O_2 electrode	10 mg in 100 g of the substance to be determined	[46]
Lactate ion (lactic acid)	Lactate monoxidase	In a gelatine gel	Clark-type O_2 probe	10^{-5}–6.10^{-4} M	[71]
	Cytochrome b_2	Cross-linking with albumin	Platinum hexacyanoferrate electrode	1.2 mM	[48]
	"	Adsorption on a current conducting matrix	Organic metals	2 mM	[72]
Galactose	Galactose oxidase	Cross-linking with albumin	Electrochemical H_2O_2 sensor	0–27.8 mM	[73]
Phenol	Polyphenol oxidase	Cross-linking with albumin	Membrane O_2 electrode	0–0.08 mM	[74]
Oxalate ion	Oxalate oxidase	Grafting to polyamide membrane	Platinum H_2O_2 electrode	10^{-7}–10^{-4} M	[75]
Pyruvate ion	Pyruvate oxidase	Immobilisation on nylon	Platinum H_2O_2 electrode, O_2 electrode	10^{-3} mM	[76]
Salicylate ion	Salicylate hydroxylase	Chemical immobilisation on the electrode surface	O_2 sensor	10^{-5}–2.10^{-3} M	[77]
Sucrose	Invertase	Adsorption and entrainment in the polymer matrix of poly(vinyl alcohol)	Pyrographite electrode	—	[78]
Acetylcholine, choline	Acetylcholinesterase, cholinesterase	Immobilisation on a nylon mesh in the near-electrode layer	Amperometric H_2O_2 sensor	1–10 μM	[79]
Butylrylthiocholine iodide (BTCI)	Cholinesterase	Incorporation in a polyacrylamide gel with the aid glutaraldehyde	Stationary amalgamated silver electrode	0.05–0.85 mg ml^{-1}	[80]
BTCI and CE inhibitors	Cholinesterase	Covalent grafting to nitrocellulose with the aid of glutaraldehyde	Stationary amalgamated silver electrode	10^{-3}–10^{-6} M 10^{-3}–$n\times10^{-11}$ M	[81] [82]
Indoxyl acetate	"	In a geletine gel on a cellulose hydrate membrane	Carbon glass rotating disc electrode	1.5×10^{-3} M -5.0×10^{-3} M	[83]
Hypoxanthine, xanthine, purine	Xanthine oxidase	Adsorption on the electrode	Conducting organic tetrathiofulvalene$^+$/tetracyanoquinodimethane$^-$ organic salts	10^{-5}–6×10^{-4} M	[84]
NADH	Glucose dehydrogenase	Adsorption on the electrode	*N*-methylphenazene$^+$/tetracyanoquinodimethane$^-$ conducting organic salts	—	[84]
α-Ascorbic acid	Ascorbic oxidase	Plate (5×5×2 mm) made of cucumber (pumpkin) skin	Clark oxygen electrode	—	[85]
Dopamine, dopaminequinone	Polyphenol oxidase	Tissue of banana fruit	Carbon paste electrode	1.3×10^{-6}– 9×10^{-5} M	[86]
Glycerophosphate	α,α-Glycerophosphate oxidase	Cross-linking with the surface of a Teflon membrane with the aid of serum albumin and glutaraldehyde	Clark oxygen electrode	—	[87]
Choline-containing lipids	Choline oxidase	Physical immobilisation—incorporation in a cellulose triacetate membrane	"	3.3×10^{-5}– 8.6×10^{-5} M	[88]
Maltose, glucose	Glucoamylase, glucose oxidase	Covalent grafting to collagen films	Platinum anode, H_2O_2 electrode	5×10^{-8} M (after two months, 2.5×10^{-7} M)	[55]
Cholesterol	Cholesterol oxidase, cholesterol hydrolase	Entrainment in a 2-hydroxymethacrylate gel treated with glutaraldehyde	Platinum oxygen electrode	4×10^{-4}– 12×10^{-3} M	[70]

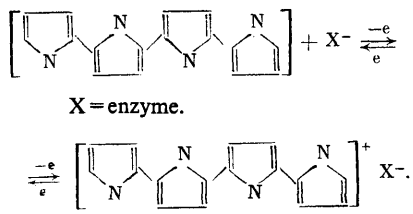
Table 1 (contd.).

Compound determined	Enzyme	Method of immobilisation	Amperometric detector	Range of concentrations determined	Refs.
Aromatic amides	Peroxidase, glucose oxidase	Covalent grafting of glutaraldehyde to the electrode surface	Carbon glass electrode	4.7×10^{-5} M	[68]
Glucose	Glucose oxidase, catalase	Entrainment in a homogeneous membrane in the near-electrode layer	Membrane oxygen electrode	1×10^{-3} M	[61]
Ethanol	Alcohol oxidase, peroxidase	Membrane in the near-electrode layer	Carbon glass electrode	—	[89]
Uric acid	Uricase, peroxidase	Covalent grafting to albumin with glutaraldehyde on a dialysis membrane	"	$(0.5-10)\times10^{-5}$ M	[90]
α-Lactate ions, pyruvate ions	Lactate oxidase, lactate dehydrogenase	Grafting to a Teflon membrane	Clark oxygen electrode	8×10^{-8}–8×10^{-6} M	[91]
Glucose, urea	Glucose oxidase and uricase	Covalent grafting by means of glutaraldehyde to graphite	Graphite electrode	$0-7\times10^{-6}$ M	[92]

Collagen films, on which the enzyme is immobilised, are subjected to preliminary activation by the acyl-azide method.[55] A platinum electrode is coated by a collagen membrane with immobilised glucose oxidase and is dried for periods ranging from 2 days to 2 weeks before measurements. Between measurements, the electrode is stored in air, since dehydration entails a denser coating of the platinum electrode by the membrane which facilitates the diffusion of H_2O_2 to the electrode. After inserting the electrode in the solution, the membrane remains tightly attached to the electrode. The resulting electrode is stable for a month or 160 days when the glucose content is measured. The response time before drying was somewhat more than 10 min, while after drying for 2 days it improved by 35% and was only 4–8 min.

In order to eliminate the interfering effect of ascorbic and uric acids, bilirubin, and aminoacids in the analysis of biological liquids for glucose, a cellulose acetate diaphragm permeable only to low-molecular-weight compounds with a small molecular size is introduced into the enzyme electrode between the membrane with the immobilised glucose oxidase and the platinum anode.[56] The standard deviation s_r in the determination of 0.20 mg ml^{-1} glucose was 2–3% (the number of determinations was 20).

One of the promising ways of obtaining enzyme electrodes involves the immobilisation of the enzyme in polymer coatings directly on the surface of the electrochemical sensor itself. The monomers, from which the polymer can be produced, are polymerised and the polymer macromolecules are cross-linked in the layer near the electrode. This new interesting method of immobilising glucose oxidase via electrochemical polymerisation has been described by Umana and Waller[57] and Bartlett and Whitaker.[58] For example, glucose oxidase is entrained by a polypyrrole film growing on the electrode surface.[57] This immobilisation procedure has a number of advantages. Polypyrrole, which is a conducting organic material, and the product of the enzymic reaction with glucose are in the immediate vicinity of the conducting material. The H_2O_2 concentration is also monitored from its reaction with the iodide anion (I^-), which is followed by the electrochemical reduction of iodine at a potential $\leqslant 0.2$ V relative to the saturated calomel electrode:



X = enzyme. (6)

The electrode obtained is stable for 7 days. The preparation of immobilised glucose oxidase in a poly-*N*-methylpyrrole film for the preparative electrochemical oxidation of *N*-methylpyrrole on a platinum disc in the presence of 0.1 M $(C_2H_5)_4NBH_4$ supporting electrolyte in an aqueous phosphate buffer (pH 7.2) containing 1 μg ml^{-1} of glucose oxidase has been described.[53] It was shown that the H_2O_2 formed is oxidised on the rear surface of the platinum and not on the polymer film. The electrode response is observed at 0.95 V (relative to the saturated calomel electrode).

Direct electron transfer also takes place between glucose oxidase and the surface of a carbon glass electrode modified with 3-aminophenylboronic acid.[59]

An interesting electrode has been proposed by Jonsson and Gorton.[60] They noted that the partial removal of oxygen from the solution analysed lowers the electrode response, but the oxygen contained in the enzyme layer on the graphite electrode is sufficient to maintain the linearity of the calibration plot in the determination of glucose at concentrations $\leqslant 1$ mM. The apparent rate constant for the Michaelis–Menten enzyme reaction is ~50 mmol. The stability of the response of the electrode obtained depends on the pH, the potential applied to the electrode, and the frequency at which samples are introduced.

A stable and fairly rapid response of the enzyme electrode is usually observed when only the glucose concentration is varied.[61] The same workers established that the response time can be reduced by diminishing the thickness of the membrane in the layer near the

electrode and by increasing the catalytic constant for the immobilised enzyme. In order to optimise the electrode response, variations in the thickness of the membrane and in the diffusion coefficient have been examined. Yet another model of the enzyme electrode for glucose has been proposed.[28] In this model, the electron-transferring agent is incorporated in the structure of glucose oxidase. Thus a modified glucose oxidase was obtained with the aid of ferrocene and its derivatives and was then deposited on the electrode. Glucose was oxidised even in the absence of oxygen directly on the electrode.

p-Benzoquinone, already mentioned, was used as the mediator.[62] It is introduced into the bulk of a carbon paste electrode whose surface is covered by a semipermeable membrane with the immobilised glucose oxidase. A similar procedure has been used also for other enzymes (gluconate dehydrogenase, ethanol dehydrogenase).

The possibility of using gold electrodes coated by a film of glucose oxidase with *p*-benzoquinone and of platinum mesh electrodes coated by a glucose oxidase film and covered by an oxygen-permeable membrane has been demonstrated.[63] One of the latest varieties of the glucose electrode has been described by Steruberg et al.[64] (Fig. 3). The immobilisation procedure proposed by the authors makes it possible to obtain membranes with a high surface activity, which are stable for a month and longer. Electrochemical monitoring and monitoring with the aid of radioactive labels demonstrated only a slight inactivation of the enzyme on immobilisation.

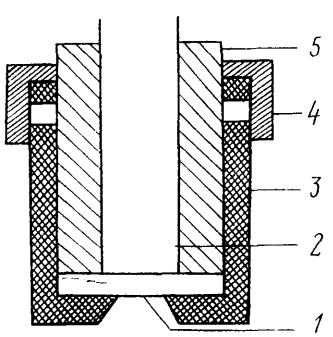


Figure 3. Schematic diagram of a rotating membrane glucose electrode:[64] *1*, membrane containing the immobilised enzyme; 2, platinum electrode (axis of rotating electrode); *3*, membrane holder; *4*, threaded lock ring; *5*, electrode shell.

The covalent binding of glucose oxidase to ferrocenecarboxylic acids has also been proposed.[65] The catalytic constant is a maximum when glucose oxidase is modified with ferroceneacetic acid.

The measurement of the response of a platinum electrode coated by a layer of immobilised glucose oxidase as a function of time and substrate concentration demonstrated a satisfactory correspondence between theoretical ideas and experiment.[93] The variation of the concentration profiles in the mathematical model of a multilayer enzyme sensor also agrees satisfactorily with the experimental findings for a commercial sensor for the determination of glucose.[94] These calculated concentration profiles describe the operation of the sensor more satisfactorily than the single-layer model.

2. The use of various enzymic reactions in biosensors

The glucose electrodes constitute the largest group of amperometric enzyme sensors. Other enzymes and enzymic reactions have so far been used much more rarely for such purposes (Table 1).

For example, uric acid is determined with the aid of enzyme sensors based on immobilised uricase:[66,67]

$$\text{uric acid} + O_2 + H_2O_2 \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2. \quad (7)$$

A decrease in the amount of dissolved oxygen during the oxidation of the substrate has been detected at −0.6 V (relative to the saturated calomel electrode) by a platinum electrode with a thin layer of immobilised enzyme.[66] The error of the determination does not exceed 4% in the determination of uric acid in urine and 6% in its determination in blood serum. When the electrode is used several times per week, its activity diminishes by only 29% in the course of 100 days.

The use of covalent grafting of the enzyme to the porous glass of the electrode by glutaraldehyde has been described.[67] Such an electrode proved to be very stable during operation (~10 months), which permitted its employment under flow conditions.

The operation of electrodes with peroxidase is based on the oxidation of the hexacyanoferrate(II) ion by hydrogen peroxide catalysed by immobilised peroxidase:

$$H_2O_2 + 2[Fe(CN)_6]^{4-} + 2H^+ \xrightarrow{\text{peroxidase}} 2H_2O + 2[Fe(CN)_6]^{3-}. \quad (8)$$

The hexacyanoferrate(III) ion formed is reduced at a carbon glass electrode at 0 V (relative to the silver,silver chloride electrode).[68] In the presence of aromatic compounds, which activate the peroxidase oxidation of hexacyanoferrate(II), the rate of the enzymic reaction and the cathodic current increase, which makes it possible to carry out the determination of, for example, a series of aromatic amines.[68] The range of the measured concentrations varies as a function of the type or substrate. The lower limits of the measurable concentrations of *o*-dianisidine and benzidine are 0.055 and 0.065 μmol, while for the remaining compounds investigated the limit is higher. The coefficient of the sensitivity of the above enzyme electrodes varies from 0.215 to 0.0022 μA μmol^{-1} on passing from *o*-dianisidine to 1,2-diaminobenzene. For electrodes of the second type (Table 1), this coefficient is higher, as a result of which the lower limit of the measurable contents of aromatic amines is appreciably reduced.

Cholesterol is one of the physiologically important components of blood. Under the influence of cholesterol oxidase, cholesterol is oxidised:

$$\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-3-one} + H_2O_2. \quad (9)$$

A series of enzyme electrodes have also been developed for its determination.[46,69,70] The readings are based on the H_2O_2 reduction current at a platinum oxygen electrode on the surface of which a gel with the immobilised enzyme is deposited.[70] The gel is retained by two membranes: the inner membrane made of cellulose acetate and the outer membrane made of silk. The electrode response time is 0.5 min in the flow regime and 2 min in the static regime. The standard deviations s_r are 0.027 and 0.045 in each regime respectively.

Enzyme electrodes have been developed for the determination of ethanol in blood.[46] Alcohol oxidase is immobilised on the surface of a platinum disc electrode. At a potential of −0.6 V, the loss of oxygen, which is proportional to the ethanol content, is measured:

$$RCH_2OH + O_2 \xrightarrow{\text{alcohol oxidase}} RCHO + H_2O_2. \quad (10)$$

Alcohol dehydrogenase is used for the same purposes,[46] nicotinamide-adenine dinucleotide (NADH) and flavin mononucleotide (FMN) participating in the enzyme reaction and the chemical reactions associated with it. Reduced FMN is oxidised at a platinum anode, generating a current proportional to the ethanol concentration in the test solution. The enzyme–electrode processes occurring in the amperometric sensors based on membranes with immobilised lactate dehydrogenase are similar to the processes observed in the determination of ethanol with participation of FMN, but are used to determine lactic acid.[46]

The lactate sensor makes it possible to avoid the use of NADH and FMN. The following enzyme reaction takes place in it:

$$CH_3CHOHCOO^- + 2[Fe(CN)_6]^{3-} \xrightarrow{\text{lactate dehydrogenase}} CH_3COCOO^- + 2[Fe(CN)_6]^{4-} + 2H^+. \quad (11)$$

The choice of the optimum parameters, especially the thickness of the membrane, makes it possible to determine the amount of lactate in blood in 1 min without preliminary preparation. Apart from the platinum electrode, gold or carbon working electrodes can be used.[46]

An automatic analyser with an enzyme sensor has been described for the determination of the concentration of lactate ions and the measurement of the activity of lactate dehydrogenase.[71] The enzyme sensor consists of the so called oxygen probe of the type of the Clark electrode on which is fixed a layer of gelatin where lactate monoxidase is immobilised. The standard deviation s_r is less than 0.01. The enzyme sensor operates stably for 55 days. After the addition of NADH and pyruvate ions to the sample, it is possible to carry out in succession a determination of the lactate ions and the estimation of the activity of lactate dehydrogenase, for which the calibration plot is linear in the range 2.7–54.0 units of biological activity per litre.

Apart from lactate dehydrogenase, the enzyme cytochrome b_2, which catalyses electron transfer to mediators, i.e. $[Fe(CN)_6]^{3-}$, can also be used for the determination of lactic acid in such analyses.[48,72]

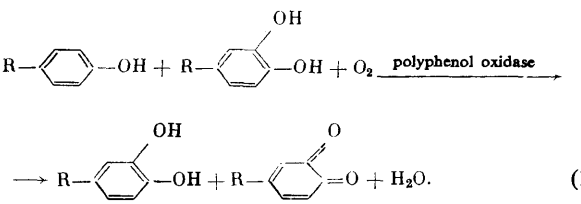
Certain improvements to enzyme electrodes have been proposed.[95] The essential feature consists in the fact that ionic compounds (C_{10}–C_{30} carboxylic acids, etc.) or non-ionic compounds [4,4′-bipyridyl, 1,2-bis-(4-pyridyl)ethylene, etc.] are introduced into the electrically conducting support made from powdered graphite, pyrographite, and metals. Functional groups for the orientation and fixation of electroactive enzymes such as cytochrome b_2, cofactors, or other biocatalysts are then formed on the surfaces of such electrodes. The surfaces of the carbon-containing supports can be coated by functional oxygen-containing groups by simple chemical oxidation. The advantage of the proposed "functionalised" electrodes consists particularly in the fact that the biocatalysts are in direct contact with the support, i.e. with the electrode itself. The oxidation or reduction of organic substrates therefore takes place under the influence of enzymes without the additional introduction of oxidants, reductants, or mediators into the solution. Such electrodes can be used not only for electroanalytical purposes but also for specific electroorganic synthesis. Enzyme electrodes are also employed for the determination of aminoacids.[8,46] Here the membrane with the covalently immobilised amino-oxidase catalyses the reaction

$$RCHNH_2COOH + O_2 + H_2O \xrightarrow{\text{amino-oxidose}} RCOCOOH + NH_3 + H_2O_2. \quad (12)$$

The enzyme exhibits group specificity and catalyses the oxidation of all the *L*-aminoacids present in the liquids analysed.

For the determination of galactose[73] and phenols,[74] enzyme electrodes have been developed whose operation is based on the reactions

$$\text{galactose} + O_2 \xrightarrow{\text{galacto-oxidase}} \text{galactosoaldehyde} + H_2O_2, \quad (13)$$



(14)

A simple and rapid method for the determination of oxalate ions in food products with a minimum of auxiliary operations has been developed. It is based on the use of a new voltammetric enzyme sensor.[75] Oxalate oxidase is immobilised on a commercial polyamide membrane. The response time of the sensor ranges from 20 to 60 s depending on the concentration of $C_2O_4^{2-}$ ions. The analytical signal is stable for almost 150 days.

A sensor, whose operation is based on the oxidation of pyruvate by dissolved atmospheric oxygen in the presence of HPO_4^{2-} ions with formation of H_2O_2, is used in clinical analysis for the determination of pyruvate ions. The process is monitored with the aid of an oxygen or H_2O_2 electrode.[76]

An enzyme electrode for the determination of salicylates in blood serum has been developed on the basis of salicylate hydroxylase immobilised on the surface of an oxygen electrode. In the determination, the enzyme sensor is immersed in a solution of a mixture of phosphate buffer with Complexon III (pH 6.75). Next the coenzyme β-NADH is added, after 5–10 min the substrate is introduced, and after 1–6 min the current is measured as a function of the salicylate concentration with the aid of the enzyme electrode[77] (s_r = 0.013–0.106).

Invertase is an enzyme which cleaves sucrose catalytically into *D*-glucose and *D*-fructose. It was immobilised by being incorporated in polymeric coatings at the instant of their formation directly on the surface of the electrode.[78] Polymeric coatings on pyrographite and aluminium electrodes can be obtained by cathodic polarisation in solution in poly(vinyl alcohol) containing boric acid.

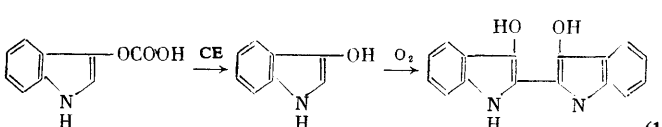
Selective biosensors based on immobilised cholinesterase (CE) and the determination of acetylcholine and choline in a 0.1–1 ml sample have been described.[79] The response time is about 1–2 min. The possibility of the simultaneous determination of these compounds in a single sample with the aid of two sensors under both stationary conditions and in a flow system has been demonstrated.

An enzyme electrode has also been proposed for the determination of the concentration of thiocholine esters.[80] The authors used, as the cholinesterase substrate, butyrylthiocholine iodide, which hydrolyses in the presence of cholinesterase:

$$C_3H_7COSCH_2{-}CH_2{-}\overset{+}{N}(CH_3)_3I + H_2O \xrightarrow{CE} C_3H_7COOH + HSCH_2{-}\overset{+}{N}(CH_3)_3I. \quad (15)$$

The determination is based on the electrochemical activity of the hydrolysis product—a thiol. the volume of the solution analysed is 4–5 ml. The error of the determination does not exceed 9%. With the aid of this electrode, it is possible to determine also other thiocholine esters as well as cholinesterase inhibitors, for example, phosphorus-containing pesticides, which is extremely important for the solution of the problem of monitoring the environment. Various procedures for the immobilisation of cholinesterase have been assessed: incorporation in a polyacrylamide gel accompanied by treatment with glutaraldehyde and adsorption on carriers—silica gel, activated charcoal, and various films.[80] The advantages and disadvantages of this procedure as regards use in enzyme sensors have been indicated. An improved model of such a sensor makes it possible to determine phosphorus-containing pesticides and certain physiologically active substances down to very low concentrations of the order of $n \times 10^{-11}$–$n \times 10^{-12}$ M.[81,82]

A number of synthetic compounds, including indoxyl acetate, are also hydrolysed in the presence of cholinesterase. Indoxyl, the product of the hydrolysis of indoxyl acetate, dimerises in the presence of atmospheric oxygen in an alkaline medium (pH 7.5–8.0). The rate of oxidation of indoxyl acetate has been studied with the aid of a carbon glass rotating disc electrode with immobilised cholinesterase:[83]



(16)

Enzyme sensors with conducting organic salts, functioning as the electrode material, have been developed. The possibility of determining hypoxanthine, xanthine, and purine in blood plasma with the aid of xanthine oxidase immobilised on the tetrathiofulvalene$^+$/tetracyanoquinodimethane$^-$ electrode has been demonstrated. The response and stability of the sensor are influenced by temperature, pH, and traces of oxygen. Glucose dehydrogenase, immobilised on the *N*-methylphenazine$^+$/tetracyanoquinodimethane$^-$ electrode, is used to determine NADH.[84]

Various enzyme-containing plant materials, rather than preparations of the enzymes themselves have recently come to be used increasingly in the development of the enzyme electrodes. Plant tissues in biosensors function as sources of catalytic enzyme activity. For example, an enzyme sensor consisting of a Clark electrode and a plate comprising a cucumber or pumpkin skin, which serve as sources of ascorbic oxidase, has been devised for the amperometric determination of α-ascorbic acid.[85] The determinations are carried out in a buffer solution (pH 6.0) with 0.5 mM EDTA. The enzyme activity is sufficient to ensure 50–80 determinations. The plates can be stored, without loss of activity, in 50% glycerol for a year. Phenols, acids, and vitamins B do not interfere with the determination. Ascorbic acid has been determined with the aid of this electrode in juices and pharmaceutical preparations with $s_r = 0.03$.

A similar principle has been used in another study.[86] The tissue of the banana fruit is introduced into a carbon paste electrode and dopamine is determined. The response time of this biosensor is not more than 12 s. The catalytic reaction current increases with increase in the content of the plant tissue in the paste. The optimum content of the tissue is 5.5%. Catechol and hydroquinone interfere with the determination.

Among the latest designs of amperometric sensors based on the Clark electrode, mention may be made of sensor systems for glycerophosphate and choline-containing lipids.[87,88]

New attempts have been made to improve the components of the enzyme oxygen electrode. A film permeable to H_2O_2 and impermeable to substrates has been proposed for this purpose. It is made from polyamides, polyurethane, cellulose acetate, and other polymers or copolymers.[96] Enzymes whose reaction with substrates proceeds with formation of H_2O_2 are immobilised on the porous surface of such films. They include, for example, glucose oxidase, uricase, xanthine oxidase, *D*- and *L*-aminoacid oxidases, as well as enzymes producing substrates for enzymic reactions with liberation of H_2O_2, for example, lipase, mutarotase, and phosphorylase. The smooth side of the film is applied to the surface of a silver anode (other metals are also employed). Such films are very strong, are satisfactorily joined to the electrode, and ensure an effective contact between the enzyme and the substrate, selective permeability, and a high accuracy of the measurements.

3. Polyenzyme biosensors

In those cases where the products or substrates of an enzymic reaction are electrochemically inactive, successive reactions of the substrates with the aid of several immobilised biocatalysts are used in the construction of amperometric sensors. Dienzyme reactions are most often employed. For example, the operation of electrodes sensitive to disaccharides is based on this principle.[10] Two enzymes are usually employed for the determination of lactose and maltose: glucose oxidase, which oxidises glucose via reaction (1), and β-galactosidase or maltase. The disaccharide, diffusing through the membrane, splits off glucose under the influence of the corresponding enzyme and the glucose is then oxidised by glucose oxidase to gluconic acid. The consumption of O_2, which is proportional to the content of the disaccharide in the solution analysed, is measured with an oxygen electrode:

$$\text{lactose} + H_2O \xrightarrow{\text{β-galactosidase}} \text{glucose} + \text{galactose}, \quad (17)$$

$$\text{maltose} + H_2O \xrightarrow{\text{maltase}} \text{glucose}. \quad (18)$$

In order to determine sucrose, it is necessary to have an additional enzyme which epimerises α-glucose—a sucrose hydrolysis product—to the β-form, which is oxidised by glucose oxidase:

$$\text{sucrose} + H_2O \xrightarrow{\text{invertase}} \text{α-D-glucose} + \text{fructose} \quad (19)$$

$$\text{α-D-glucose} \xrightarrow{\text{mutarotase}} \text{β-D-glucose}. \quad (20)$$

The rate of the non-enzymic mutarotation is high and it is therefore possible to abandon the use of mutarotase. The sensitivity of the electrode to the disaccharide is then greatly reduced.[10]

The joint immobilisation of glucoamylase and glucose oxidase is also employed for the determination of maltose:[55]

$$\text{maltose} + H_2O \xrightarrow{\text{glucoamylase}} 2\beta\text{-D-glucose}. \quad (21)$$

It has been suggested that a system comprising two immobilised enzymes, cholesterol oxidase and cholesteric ester hydrolase, be used for the determination of cholesterol esters.[70] The solution analysed is initally acted upon by cholesterol hydrolase and then cholesterol oxidase, after which the total amount of cholesterol is found.

For the determination of aromatic amines, the enzyme electrodes are prepared using glucose oxidase in order to replace the unstable substrate H_2O_2. A mixture of peroxidase and glucose oxidase in 0.02 ml of the basis buffer solution is deposited on a carbon glass electrode and treated with a 2.5% solution of glutaraldehyde (12–14 h, 4 °C). In the presence of 0.1 mM glucose and 1.0 mM potassium hexacyanoferrate(III) in the buffer solution, the parameters of these electrodes are close to those of the corresponding monoenzyme electrodes. They exhibit the highest stability, retaining 45–62% of their sensitivity for 40 days at 18–22 °C. During the functioning of these dienzyme electrodes, hydrogen peroxide is formed under the influence of glucose oxidase and hexacyanoferrate(III) is generated by the peroxidase. Under conditions where the peroxidase reaction is rate-limiting (at a glucose concentration about 0.1 mM), the activators (aromatic compounds) increase the rate of the process and the current in the system increases. At a lower glucose concentration, the glucoside reaction becomes rate-limiting and activators do not affect the electrode current. The dienzyme electrodes obtained are more convenient for practical use in view of their higher stability. They are used to determine aromatic amines, for example, *o*-dianisidine,[68] glucose,[61] ethanol,[89] uric acid,[90] and α-lactate and pyruvate ions.[91]

In order to determine ethanol, the electrolysis is carried out in a buffer solution (pH 7–8) with subsequent measurement of the reduction current.[89] Potassium hexacyanoferrate(III) at a concentration of 1.0–1.5 mM is introduced into the test solution before electrolysis to increase the selectivity of the determination. Uric acid is determined in the diagnosis of haematological diseases with the aid of an electrode based on immobilised microbial urease and peroxidase.[90] In a borate buffer solution containing 1 mM potassium hexacyanoferrate(III), the residual current does not exceed 1–2 mA when an enzyme electrode is used. After the addition of uric acid to the solution, the current increases appreciably. The dependence of the stationary current on the substrate concentration is linear up to 0.30–0.35 mM. The duration of the determination is 2–3 min.

These dienzyme electrodes exhibit a high specificity and a satisfactory reproducibility in the determination of uric acid in urine, blood, and serum. The electrodes are also very stable, retaining their initial sensitivity at the level of 50% under the conditions of daily 5 h use and on storage in buffer solution at 20–22 °C for 70 days.

The introduction of α-lactate or pyruvate ions into a phosphate buffer solution (pH 7.4) containing 10^{-3} M NADH induces an appreciable decrease of the dienzyme electrode current (ΔI = 0.5 μA), whereas in the absence of NADH in the solution ΔI < 8 mA.[91]

The mechanisms of the enzyme-catalysed reactions in the layer near the electrode are presented below:

$$O_2 + 2e \xrightarrow{\text{lactate oxidase}} H_2O_2, \quad \alpha\text{-lactate} \xrightarrow{-2e} \text{pyruvate}, \quad (22)$$

$$H^+ \xrightarrow{\text{lactate dehydrogenase}} NAD^+ + 2e, \quad \text{pyruvate} + 2e \rightarrow \alpha\text{-lactate}. \quad (23)$$

This enzyme electrode "does not distinguish" α-lactate and pyruvate ions and can be used for the quantitative determination of lactate only in those specimens where the concentration of pyruvate ions is much lower than that of lactate. The duration of the determination is 3–5 min and the electrode is stable for more than 10 days with a reproducibility of the determination of ±10%.

A dienzyme electrode with glucose oxidase and uricase, the latter being necessary to suppress the interfering influence of urea, can be employed to determine glucose and urea.[92]

An enzyme electrode with trypsin and glucose oxidase, adsorbed on the surface on a graphite electrode modified by aluminium, titanium, and zirconium oxides, has also been described.[97]

III. Flow analysers based on immobilised enzymes

The concept of monitoring, continuous *on line* and *in line* type analysis with the aid of physicochemical and biological procedures, which can ensure mathematical checks on a real time scale, has acquired very great importance in the methodology of analytical chemistry.

Studies on the development of the so called enzyme reactors or analysers with immobilised enzymes have become important for the continuous analysis of various substances to determine their content of enzyme substrates, inhibitors, and activators, for making possible the automatic monitoring of the composition of air, water, and other objects in the environment, and for the determination of components in a flow. They usually contain a fairly large amount of an enzyme immobilised on a carrier, in the form of layers or rings, which makes it possible to convert the entire substrate in the specimen into the product during the relatively short time interval when the specimen is in the reactor.

Three types of reactors, differing in form and in the disposition of the carrier, the immobilised enzyme, and the specimen, have been differentiated: a tubular reactor for gaseous specimens; a reactor filled with layers of spherical beads containing the immobilised enzymes; a tubular reactor filled with solid spherical beads made up of fibres with the immobilised enzyme.[24] Reactors for the determination of glucose and lactose with amperometric detection have been developed on the basis of such principles.

Reviews have been devoted to the study of the structure and properties of enzyme membranes for bioreactors obtained by the immobilisation of one or several enzymes on natural or synthetic carriers.[24,98] Flow enzyme reactors can be used also for the continuous recording of the kinetics of enzymic reactions.[7] Industrial specimens of enzyme reactors and the mechanisms of their functioning have been described in a review devoted to the use of immobilised enzymes in analytical chemistry.[99]

The employment of enzyme reactors and various enzyme electrodes in a version of the flow-injection analysis is extremely promising.[100] Mathematical models of the functioning of such analysers have been developed in relation to the determination of glucose with the aid of an indicator oxygen electrode consisting of a platinum disc to the surface of which two cellulose membranes were attached. Glucose oxidase in a polyurethane layer is placed between the membranes. The proposed mathematical model has been almost fully confirmed experimentally.[101] With the aid of this system, it is possible to determine glucose in almost 300 specimens (sample volume 1.5 ml) in the course of 1 h and the linear relation between the current and glucose concentration holds in the range 0.01–100 mM.

Platinum macroelectrodes and microelectrodes with glucose oxidase immobilised on a nylon mesh, placed tightly against the platinum electrode or immobilised directly on the surface of the platinum wire, have also been used.[102] The calibration plots are linear in the glucose concentration ranges $1 \times 10^{-5}-3 \times 10^{-3}$ and $1 \times 10^{-4}-2 \times 10^{-2}$ M respectively.

In order to determine glucose in the flow-injection version of analysis, use is also made of graphite electrodes, both a glass mesh electrode[103] and an electrode modified by the ionic sputtering of palladium and gold[60] (Table 1), with covalently immobilised or adsorbed glucose oxidase. The productivity of the reactor varies from 30 to 77 samples per hour with $s_r = 0.02$ as the flow rate varies from 0.5 to 2 ml min^{-1}. In the glucose concentration range 2.5–10 mM, a linear calibration graph is observed.[103] The electrode retains 30% of its initial activity for 40 days during storage at 2 °C.

There exists an example of the use of a flow-injection system based on a spherical enzyme reactor for the study of the kinetics of the mutarotation of *D*-glucose in the presence of phosphate ions and *D*-galactose.[104] Glucose, fructose, and sucrose present jointly in the given system can be determined with the aid of a series of enzyme reactors arranged in parallel and a multichannel potentiostat.[105] In the determination of glucose, a reactor with immobilised glucose oxidase was placed ahead of the corresponding reactor in order to eliminate the interfering effect of glucose. In this case, hydrogen peroxide was detected amperometrically. The interfering effect of ascorbic acid was fully eliminated by using a reactor with immobilised ascorbate oxidase. In the analysis of fructose, potassium hexacyanoferrate(II) was detected. The reproducibility of the system was 1.6–1.8%.

One of the latest devices of this type for the determination of free and total cholesterol (ChL) in blood serum is of great practical importance.[106] After the introduction of the microsample into the carrier stream containing a phosphate buffer solution, $K_4[Fe(CN)_6]$, and Triton X-100, the stream is divided into two components, one of which is passed through a column with immobilised cholesterol esterase, after which the two streams are combined and are passed through a column with immobilised cholesterol oxidase.

The amperometric detector with two polarised electrodes records two peaks: the first corresponds to the content of free cholesterol and the second corresponds to the total cholesterol in the sample ($c_L = 8 \times 10^{-7}$ and 3×10^{-6} mM respectively‡).

Apart from the flow-injection systems enumerated, there also exist a whole series of examples of the use of enzyme electrodes in a continuous regime. Thus it has been suggested that a platinum mesh electrode subjected to a preliminary treatment with 3-aminopropyltriethoxysilane and containing an immobilised mixture of enzymes—glucose oxidase and catalase or glucose oxidase and peroxidase with bovine albumin and glutaraldehyde—be used for the determination of glucose in blood serum and urine.[107] The calibration graph is linear in the range 20–800 mg $litre^{-1}$ for a sample volume of 5 μl (glucose oxidase–catalase) and 30–40 mg $litre^{-1}$ for a sample volume of 1 μl (glucose oxidase–peroxidase).

The possibility of using sensors for glucose, lactate, and urea in systems with constant sampling of blood for the continuous express monitoring *in vivo* of blood metabolites has been examined.[27,28] The aim of the authors of the above investigations was in essence to create, on the basis of such systems, sensors implanted in the patient's organs.

‡ c_L represents the lower limit of the measured concentrations.

Under flow conditions at a flow rate of 7 ml min^{-1}, an enzyme electrode with uricase immobilised on porous glass was used to determine uric acid.[67] With the aid of this device, it is possible to analyse up to 100 specimens per hour with an accuracy to within 2–4%. The enzyme then remains active for a long time and loses 70% of its initial activity only after 10 months.

Flow analysers based on alcohol, lactate, and glutamate dehydrogenases with regeneration of the NADH cofactor, capable of being used in repeated measurements, have been proposed.[108] The enzyme with immobilised NADH is retained between a platinum electrode and a semipermeable membrane. NADH is regenerated either by a direct electrochemical procedure or with the aid of the electrochemical oxidation of phenazine methosulphate. The linearity of the calibration plot is observed up to a concentration of 1.5 mM for lactate, 0.5 mM for glutamate, and 40 mM for ethanol. The enzyme flow analyser based on dehydrogenases is illustrated schematically in Fig. 4. A reactor based on immobilised cholinesterase has been used for the continuous monitoring of air and running water for the content of cholinesterase inhibitors—insecticides based on organophosphorus compounds and carbamates. Cholinesterase is immobilised by being entrained in a starch gel and is fixed on the surface of a mixed polyurethane carrier placed in the electrochemical cell (Fig. 5).[99] A theoretical description of the inhibitor analytical method using an enzyme flow system, which has recently acquired increasing importance in connection with the need to monitor an increasing number of toxic compounds, has been published.[109]

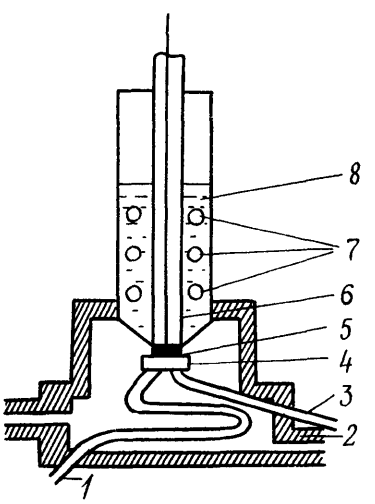


Figure 4. Schematic diagram of an enzyme flow electrode based on dehydrogenases: *1*, inlet for the flow of the test solution; *2*, heat exchanger; *3*, outlet for the products of the enzymic reaction and the stream containing the test substance; *4*, microchamber; *5*, semipermeable membrane; *6*, platinum electrode (disc); 7, auxiliary electrode; *8*, buffer solution.

One of the latest versions of the reactor for enzymic reactions has been proposed by Cerkasov and Linek.[43] The enzyme preparation is fixed on the surface of the stirrer or on the surface of a readily removable stationary component placed within the reactor.

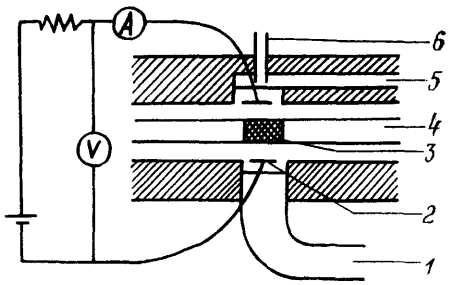


Figure 5. Schematic diagram of an electrochemical cell with an enzyme electrode based on immobilised cholinesterase: *1*, outlet for the products of the enzymic reaction; *2*, platinum electrode; *3*, immobilised cholinesterase; *4*, compartment (holder) for the immobilised cholinesterase; *5*, inlet for the stream of water or air supplied; *6*, inlet for the stream of substrate supplied.

IV. Amperometric sensors in immunoenzyme analysis

The ability of antibodies to interact highly specifically with various compounds and the ability of enzymes to accelerate chemical reactions are widely used in immunoenzymology.[7] As an alternative to radioimmunological analysis, approximately 15 years ago enzyme-markers were used in a quantitative immunological method,[110–112] which was called immunoenzyme analysis (IEA). This method is safe as regards health and the useful life of enzyme-markers is several years. It is applicable to all antigen (hapten)–antibody (AG–AB) systems and is extremely sensitive. Immunoenzyme analysis (IEA) makes it possible to detect antigens, antibodies, and other substances in biological systems at negligibly low concentrations of $10^{-14}-10^{-12}$ M.[113–118] There are two varieties of IEA: the heterogeneous solid-phase variety and the homogeneous variety. In the homogeneous IEA, the activity of the enzyme-marker changes during the immunological response. In this case, the stage involving the physical separation of the AG–AB complexes, required in the heterogeneous version, is unnecessary. With the aid of the homogeneous version of the analysis, it is possible to determine only substances with a relatively low molecular weight, for example, medicinal drugs and hormones.[113] A more clear-cut classification of IEA procedures has been given in a review.[117] It is based on the fact that, in any version of the IEA, it is possible to measure the enzyme activity of the labelled compound present either in a specific immune complex or in a complex with free binding centres which do not enter into the reaction. The majority of the immunoenzyme tests are based on spectrophotometric and fluorimetric methods for the determination of enzyme activity.[117] The use in IEA of electrochemical sensors, combining the principles of the action of analytical systems based on immobilised enzymes and immunoenzyme analytical methods, merit attention from the practical point of view. Such systems, which have been called immunoenzyme electrodes, are extremely promising because of the possibility of automating immunoenzyme methods and creating compact immunoenzyme analysers. However, one should note that the concept of "immunoenzyme electrodes" is not used quite correctly in our view. For example, the electrodes described in a number of studies[113–117] do not always contain a layer of immobilised enzyme and it would therefore be more correct to classify this procedure as immunoenzyme analysis with amperometric (voltammetric) indication of the analytical signal. Such systems usually contain an immobilised antigen or antibody.

The oxygen sensor described above was one of the first to be used in competitive solid-phase IEA.[118] A capron mesh, containing immobilised antigens against human serum albumin (anti-HSA) or insulin, was fixed on the surface of an oxygen electrode. In a solution containing HSA or insulin, the immobilised antibodies react with the antigen. If an enzyme is grafted beforehand to HSA or insulin (catalase to HSA and glucose oxidase to insulin), then an anti-HSA.HSA-E or an anti-insulin.insulin-E, where E is the enzyme, is formed on the capron mesh. When the solution contains, together with the labelled antigen molecules, also the antigen without enzymes, then mixed complexes are formed where the amount of enzyme is higher the lower the concentration of the unlabelled antigens. Under conditions where the response of the oxygen electrode depends on the enzyme concentration, the readings of the electrode are proportional to the antigen concentration in solution. The response time for such a system is 2 min. The overall duration of the determination, including the regeneration of the membrane by treatment with a glycine buffer solution, is 10 min. The antibody immobilised on the capron mesh retains its activity for several days.

It has been suggested that one should use alkaline phosphatase as the marker of the α-acid glycoprotein antigen fixed by glutaraldehyde in a competitive IEA method involving the binding of the excess antigen and the enzyme-labelled antigen to a limited number of highly specific antibodies:[119]

$$\begin{array}{lll} \mathrm{AG} & \nearrow \mathrm{AG:AB} & \\ & +2\mathrm{AB} & \\ \mathrm{AG^*} & \searrow \mathrm{AG^*:AB} & \end{array} \qquad (24)$$

The "bound" and "free" labelled antigens are separated under the conditions of heterogeneous IEA and a calibration plot of the ratio of the "bound" and "free" antigens against the antigen concentration is plotted (the standard deviation $s_r = 0.03-0.05$). A carbon paste electrode is used as the electrochemical sensor.

The heterogeneous version of IEA has also been applied to the determination of immunoglobulin G.[120] In this case the determination is carried out directly on the surface of the carbon glass electrode on which the antibodies had been adsorbed. The surface of the electrode then functions as the immunological solid phase and the electrochemical detector proper. The enzyme-marker is glucose oxidase with benzoquinone as the mediator. Procedures have been developed for the regeneration of the electrode surface after the determination, which is important for the automation of the IEA procedure.

The current arising in the electrochemical cell with serum as the electrolyte and a carbon glass disc working electrode, coated by the immobilised immunoglobulin or anti-immunoglobulin combined in a complex with immunoglobulin, increases following the addition of the serum containing immunoglobulin (the serum–immunoglobulin system) or anti-immunoglobulin (the serum–immunoglobulin–anti-immunoglobulin system).[121] It has been suggested that the complexes formed have a double-layer structure, which induces an increase in the current.

A flow immunoreactor with a platinum electrode and a flow rate of 0.5 ml min^{-1} has also been proposed for the determination of immunoglobulin G. In this case, the immunoglobulin was

immobilised on a carrier and the marker was glucose oxidase attached with the aid of *p*-benzoquinone. The analyser works stably for almost three months with an error in the determination of $\pm 3\%$.[122]

An amperometric immunodetector, based on the oxidation of glucose by immobilised glucose oxidase with ferrocenium ions playing the role of a mediator and an antigen marker simultaneously, has been used to determine the medicinal drug lidocaine (α-diethylamino-2,6-dimethylacetanilide), whose concentration in plasma must be carefully monitored.[123] Under the conditions of homogeneous competitive IEA, the antigen–ferrocenium ion complex then plays the role of an electron acceptor for glucose oxidase. The catalytic current arising in the enzymic oxidation of glucose diminishes after the binding of the antigen–ferrocene complex to the antibody. This inhibition is reversible when the free antigen is added. The duration of the determination does not exceed 15 min ($s_r = 0.03-0.06$).

In another case, in the study of the same immune complex (lidocaine–anti-lidocaine), glucose-6-phosphate dehydrogenase was used as the antigen marker. Its activity was determined from the rate of oxidation of NADH at a platinum electrode.[124]

An amperometric sensor of the flow type has been developed[125] for the determination of α-asparaginase. The enzyme-marker was horseradish peroxidase. Its enzyme activity was estimated with the aid of a carbon electrode and an I_3^- depolariser. The concentration c_L for α-asparaginase is in the case 1.8×10^{-10} M, which corresponds to clinically significant enzyme concentrations. The analyser was tested on a wide range of objects: immunoglobulin, α-amylase, the antibody to the HIV virus and the X virus of the potato, egg albumin, and cell suspensions.[126] The results of the determination are consistent with the results of spectrophotometric analysis. The productivity of the sensor (more than 350 tests per hour) makes it possible to use it to estimate the influence of biotechnical manufacturing processes on the environment and man.

The analytical possibilities and advantages of the flow-injection immunological analysis, which consists in injecting the substrate (10–20 μl) into a stream of liquid flowing through a microreactor with an immobilised enzyme covalently bound to the antigen (antibody) have been examined in two communications.[127,128] The degree of the immunological reaction is estimated, for example, from the oxidation current of the products of the enzymic reaction at a platinum electrode.[127] Under these conditons, c_L for the substrate is 10^{-15} M. After suitable treatment of the immunosorbent, it is possible to carry out 500–600 determinations for almost three months with high metrological characteristics.[128]

An analyser based on glucose oxidase immobilised on a gel support as a result of an immunlogical reaction has also been used in the flow-injection version of analysis.[129] Such indirect immobilisation makes it possible to replace the carrier readily and to regenerate easily the system after the determination with only a 5% loss of the initial activity. The entire procedure takes less than an hour. The reversible immobilisation of the enzyme is achieved by a different sequence of reactions including the antigen–antibody interaction.[129] The ranges corresponding to the linearity of the calibration plots are $1.1 \times 10^{-9}-1.1 \times 10^{-5}$ and $1.1 \times 10^{-10}-1 \times 10^{-6}$ M. Three principles are combined in such devices: an enzyme electrode, a flow system, and an immunological reaction.

In all the versions of the IEA examined, the enzyme is used as an antigen or antibody marker, whose redistribution between the solid and liquid phases depends on the concentration of the test compound. New IEA systems, based on the fact that the appearance of the enzyme in solution is a consequence of a specific antigen–antibody interaction, are being developed nowadays.[130] One of the ways of creating such systems involves the incorporation of the enzyme within liposomes on the surfaces of which the antigen is attached chemically. The addition to the system of an antibody and of a complement- (set of enzymes) containing solution leads to the formation of the antigen–antibody complex on the liposome and its lysis by the action of the complement bound to the antibody fragments. The lysis is accompanied by the transfer of the enzyme to the solution. The addition to the system of the antigen to be determined inhibits the lysis as a consequence of the attachment to the liposome of a smaller number of antibody and complement molecules. The rate of appearance of enzyme activity in the solution serves as a characteristic of the initial antigen concentration.[131] The duration of the analysis of theophylline (liposomes with peroxidase) is 15 min and the sensitivity is 4×10^{-9} M for the electrochemical recording of the peroxidase activity in the oxidation of NADH.[132] Apart from enzymes, electrochemically active marker species (ions, substrates) can be incorporated in liposomes.

The principal data characterising the properties and the design of the immunoenzyme electrodes described above are presented in Table 2. It is noteworthy that the chemical amplification of the signal, which occurs under the influence of enzymes, can be used also for the determination of hormones, certain medicinal drugs, neuromediators, cyclic nucleotides, and certain biologically important compounds, whose concentration in the organism is less than 10^{-6} M.[131] A series of reviews has also been devoted to these and other problems concerning the use of immunoenzyme sensors.[20,21,25,26,114,119,133,134]

In conclusion one should note that the problem under discussion is of extremely great current interest. At the present time there exist very many devices (biosensors) of different designs with amperometric indication of the analytical signal and based on immobilised enzymes. In many instances, both potentiometric and amperometric enzyme sensors are constructed for such purposes, the latter finding more extensive applications because they usually exhibit a greater range corresponding to the linearity of the analytical signal and a lower limit of the concentrations which can be determined. Furthermore, in the amperometric version of the biosensor, in contrast to its potentiometric analogue, it is possible to immobilise the enzyme directly on the electrode surface by incorporating it in a polymer film which can be produced in the course of electropolymerisation.

The present review concerning the state of the problem of amperometric biosensors is naturally far from being able to cover exhaustively the entire literature published at the present time. There is no need to compare in it the advantages and disadvantages of a particular biosensor, because each biosensor is usually constructed for the solution of some kind of practical problem, normally operates under specific conditions, and is usually applicable to the determination of specific compounds in a specific medium. One may say with much confidence that detectors with immobilised enzymes, a wide variety of which exist, supplement one another. Only practice can provide an answer to the question of which biosensor is best or worst in each specific case depending on the operating conditions and the nature of the object to be analysed.

The need for the biosensors under consideration is rapidly and continuously increasing and the requirements as regards their operating properties are becoming more stringent. Further development of theoretical and practical aspects of their application involves the expansion of the assortment of the industrially produced enzymes and the improvement of the methods for their

Table 2. Characteristics of immunoenzyme electrodes.

Substance determined	Compounds used		Immobilisation procedure	Electrochemical sensor	Range of concentrations determined	Refs
	AG(AB)	Eznyme (marker)				
Human serum albumin (HSA), insulin	AB (anti-HSA), anti-insulin	Catalase for HSA, glucose oxidase for insulin	AB on capron mesh	Membrane oxygen electrode	Less than 10^{-8} M HSA; Less than 10^{-6} M insulin	[118]
α-Acid glycoprotein	AG	Alkaline phosphatase	Treatment with glutaraldehyde	Carbon paste electrode	1.0–10.0 ng ml^{-1}	[119]
Rabbit immuno-globulin G	Anti-immunoglobulin G	Glucose oxidase—AG marker	AB adsorbed on the surface of a carbon glass electrode	Carbon glass electrode	4×10^{-16} mol cm^{-2} of electrode surface	[120]
Immunoglobulin G	"	Glucose oxidase—AG marker (attached with the aid of *p*-benzo-quinone)	Immobilisation on a carrier	Platinum electrode in a flow immunoreactor		[122]
Lidocaine	Antilidocaine	Glucose oxidase with ferrocenium ion—AG marker and mediator simultaneously	Covalent grafting to a carrier	Gold disc working electrode, platinum gas counterelectrode	5–50 mM	[123]
α-Asparaginase	Anti-asparaginase	Horseradish peroxidase—AG marker	Immobilisation of AB to asparaginase in hollows on polystyrene plates	Graphite electrode in a flow analyser	1×10^{-11}–4×10^{-10} M	[125]

immobilisation, an increase in the sensitivity and stability of the electrochemical sensors themselves, and improvement of the materials used for their preparation. Various modifications of the electrode surface play a major role in this connection.[135–136]

It has been suggested that biosensors constitute the future of analytical chemistry and the present revolution in this field of science has been expected for some time. One of the reasons for this view is apparently the large volume of research into biosensors.

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