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The Applications and Prospects for Further Applications of Biocatalysts in Electrochemical Systems

A.I.Yaropolov and I.V.Berezin

A new field involving the use of biologically active substances for the acceleration of electrode processes is examined. The coupling of enzymic and electrochemical reactions can be achieved in two ways: by mediated and direct electron exchange between the active centre of the enzyme and the electrode. The mechanism of the electrochemical oxidation of hydrogen with participation of immobilised hydrogenases is analysed in detail. Data indicating a tunnel mechanism of electron transport in the enzyme catalysis of electrode processes are presented. The principal applications of bioelectrochemical systems are indicated: as analytical sensors, as bioelectrochemical energy converters, and in bioelectrosynthesis.
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I. INTRODUCTION

The field of bioelectrochemical research has two main components. The first involves the study of membrane phenomena, which are extremely common in living nature. Its main feature is the study of the properties of membranes, including membranes with incorporated enzyme systems. This is of considerable interest for the understanding of the energy conversion process and electron transport in living organisms. The second component in bioelectrochemistry is the use of biological systems as catalysts of electrode reactions. Its development is due to the high specificity and efficiency of enzymic reactions and also the possibility of their occurrence under mild conditions.

Biocatalysts can be employed to create electrochemical analytical devices, in the development of electrochemical energy converters, in the specific electrosynthesis of complex organic compounds, and also for the elucidation of the mechanisms of enzyme reactions by electrochemical methods.

At the present time one can speak of bioelectrocatalysis as a phenomenon involving the acceleration of electrode processes under the influence of biocatalysts.[1] The study of bioelectrocatalysis implies in the first place the study of the methods of coupling enzymic and electrochemical reactions and also of the mechanism of electron transport in the enzyme–electrode system.

The aim of the present study was to demonstrate the advances and prospects in this new branch of chemical research.

II. METHODS FOR THE COUPLING OF ENZYMIC AND ELECTROCHEMICAL REACTIONS IN BIOELECTROCATALYSIS

1. Electron Transport Using a Mediator

The coupling of enzymic and electrochemical reactions by means of a mediator can be achieved mainly in three ways, which differ in the mobility of the mediator and enzyme in relation to the electrode surface (Fig.1): (a) the enzyme and the mediator are in the bulk of the solution and the mediator diffuses to the electrode surface; (b) the enzyme is immobilised on the electrode surface and the mediator is in the bulk of the solution; (c) the enzyme and the mediator are immobilised on the electrode surface.

The mediator is as a rule either an electrochemically active product of the enzymic reaction or a transferring agent specially selected for the given system. The use of chemical compounds as mediators for the coupling of specific enzymic and electrochemical reactions imposes specific requirements which must be met by such transferring agents and which have been formulated.[2,3]

1. The stage involving the interaction of the mediator with the active centre of the enzyme must be rapid, i.e. the mediator must be a specific substrate of the enzyme.

2. The normal oxidation-reduction potential of the mediator must be close to that of the fuel of the oxidant. It is especially important that this condition be fulfilled in the development of electrochemical energy generators, since otherwise the efficiency of such systems diminishes. On the other hand, a transferring agent with a low oxidation-reduction potential is readily oxidised by oxygen, which is particularly undesirable when enzymes are used in electrochemical analytical devices.

3. The mediator must be electrochemically oxidised or reduced at the electrode of the given material under conditions close to reversibility.

4. The mediator system must be stable.

One of the first studies on bioelectrocatalysis was devoted to the catalytic properties of the enzyme hydrogenase in the electrochemical oxidation of hydrogen. The aim of these studies was to create the hydrogen anode of a fuel cell,[4-7] to determine the hydrogenase activity,[8,9] or to investigate the possibility of the electrochemical evolution of hydrogen in the presence of the enzyme.[10]

The possibility of creating a fuel cell anode on the basis of the *E.coli* hydrogenase has been examined.[4] The methylene blue–leuco-methylene blue system, which has an oxidation-reduction potential of +0.01 V relative to the hydrogen electrode (at pH 7.0), was used as the diffusionally mobile transferring agent. In this system methylene blue is enzymatically reduced by hydrogen to the leuco-form, which is then reoxidised at the platinum electrode. The maximum current density at the anode is 0.16 mA cm^{-2}.

The formation of hydrogen from the protons by the reduction of methylviologen in the presence of hydrogenase has been investigated by Yagi.[10] The reversibility of this system

with respect to hydrogen was demonstrated in this way.

It has been shown[1,2,3,11] that the optimum transferring agent for the hydrogen–hydrogenase–electrode system is methylviologen, whose oxidation-reduction potential is close to the reversible hydrogen electrode potential at a pH close to neutrality. It was established that, when the hydrogenases from *Thiocapsa roseopersicina* and *Pseudomonas aestuarii* are used for the anodic oxidation of hydrogen in the H_2–enzyme–methylviologen–carbon electrode system, it is possible to achieve this reaction under conditions close to reversibility.

The systems examined above contain the enzyme and the mediator in solution. From the practical point of view a system in which at least one of the components is on the electrode surface are more convenient.

The system for the oxidation of hydrogen based on hydrogenase and methylviologen as the mediator can serve as an example of the systems where the enzyme is immobilised on the electrode surface and the mediator is diffusionally mobile.[12] The authors developed a method for the adsorption immobilisation of hydrogenase on the surface of a carbon black electrode with a specific surface of ~100 $m^2\ g^{-1}$. The hydrogenase is adsorbed on the carbon black irreversibly and retains approximately 90% of the activity of the native enzyme. The enzymic reaction occurring in the immediate vicinity of the electrode surface in the presence of methylviologen and hydrogen results in the formation of the reduced form of the mediator, which can be reoxidised at the electrode or can diffuse into the bulk of the solution, the relative contributions of these processes depending on the electrode potential. The studies were carried out by the method involving the use of semi-immersed and rotating disc electrodes. The results obtained show that, in the presence of a sufficiently active enzyme, the reaction is controlled not only by the kinetics of the oxidation of hydrogen but also by the diffusion of the latter to the electrode surface. At a potential of −0.15 V and a methylviologen concentration of 8×10^{-3} M the current density at the electrodes was 1.1–1.2 mA cm^{-2}.

A number of studies have been devoted to the development of a biocathode for the fuel cell. The hydrogen electrochemical reduction reaction on carbonaceous materials in neutral solutions proceeds with a high overpotential. It has been suggested that a mediator enzyme system be used to activate this reaction.[13–16] The oxidation-reduction potential of the transferring agents in such systems is more positive than the range of potentials in which oxygen is electrochemically reduced on the carbon electrode. The cytochrome *c* oxidase–*NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) system has been used,[18] TMPD being oxidised by the enzyme to a radical (Wurster's Blue) and the latter being reduced at the cathode. This mediator can be oxidised by oxygen also in the absence of a catalyst, but in the presence of cytochrome *c* oxidase the rate of this reaction greatly increases. It has been shown that TMPD–Wurster's Blue system is electrochemically reversible at the platinum and graphite electrodes and that its redox potential is 0.7 V.

The transferring system incorporating 4,4'-dihydroxybiphenyl and its oxidised form has also been used in the cathodic reduction of oxygen in the presence of laccase.[17] These mediators were resorted to in connection with the search for a system of transferring agents whose oxidation-reduction potential is close to the equilibrium potential of the oxygen electrode. Potato *o*-diphenol oxidase has been used to accelerate the oxygen electrochemical reduction reaction.[18] The mediators were catechol and caffeic and chlorogenic acids. The authors were able to shift the oxygen electrochemical reduction potential into the positive region by 300–350 mV.

NAD- and NADP-dependent enzymes play a major role in metabolic processes. Bioanodes employing dehydrogenases and the NAD–NADH redox transferring system have been described.[18–20] A disadvantage of these systems is the occurence of irreversible processes: frequent repetition of the electrochemical reaction lead, in particular, to the dimerisation of the transferring agent. In addition, the electrochemical NADH oxidation and NAD reduction processes proceed with a high overpotential. It has been shown[20] that the electrochemical oxidation of NADH on smooth and platinised platinum and also on pyrographite proceeds only at a potential above +1.3 V (relative to the normal hydrogen electrode). In the presence of phenazine metasulphate, the NADH oxidation potential at the pyrographite electrode falls to +0.4 V. The authors[20] describe the electrochemical oxidation of sodium formate at the pyrographite electrode in a system with the NAD-dependent formate dehydrogenase using phenazine methosulphate as the mediator and also using a two-enzyme system with an NAD-dependent formate dehydrogenase and an NADH-dependent dehydrogenase, where methylviologen is the electron transferring agent. The sodium formate oxidation currents in the given system at a potential above +0.2 V were 15 mA cm^{-2}. Systems in which the enzyme and the mediator are simultaneously immobilised

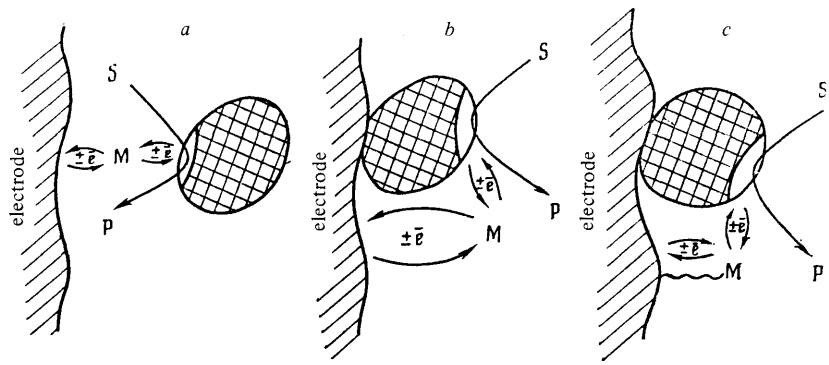


Figure 1. Schematic illustration of the possible ways of coupling enzymic and electrochemical reactions with the aid of a mediator; S = substrate, P = product of enzymic reaction; M = electron transferring agent (for the description of the schemes, see text).

on the electrode surface are superior to those described above. The possibility of creating a model of the enzyme fuel cell, using alcohol as the fuel, and also of devising an analytical electrochemical sensor for alcohol has been investigated.[21,22]. The alcohol oxidation catalyst was horse liver alcohol dehydrogenase and the mediator was nicotinamide-adenine dinucleotide. The enzyme and the cofactor were immobilised on the electrode surface in two ways: by adsorption via the hydrophobic and electrostatic interactions of the enzyme–cofactor system with the electrically conducting carrier and covalently by grafting NAD to the carrier and by sorbing the apoenzyme on the cofactor. It has been shown that the cofactor retains its physiological activity only when it is separated from the carrier by a distance of ~10 Å. When the enzyme and NAD were introduced into the cell, a stable current and e.m.f. were generated. The introduction of a small amount of potassium cyanide, which is an inhibitor of the enzyme activity of alcohol dehydrogenase (ADH), into the electrolyte reduced the current. The current was proportional to the substrate concentration and reached a maximum of 0.6 mA at a potential of 0.2 V.

Yastrebova et al.[23] also used the ADH–NAD enzyme–cofactor complex immobilised on the surface of a carbon black electrode as the mediator in the electrochemical oxidation of ethanol. The half-wave potential $E_{1/2}$ in the oxidation of NADH in the enzyme–cofactor complex is then lower by 0.22 V than $E_{1/2}$ for the oxidation of NADH adsorbed on the carbon black. The direct involvement of the enzyme in the electrochemical oxidation of NADH was confirmed by adding an ADH inhibitor (phenanthroline) to the adsorbed complex, which resulted in the disappearance of the catalytic effect. A mechanism for the oxidation of ethanol at the enzyme electrode has been proposed. The method suggested makes it possible not only to determine ethanol in the concentration range 3×10^{-5}–5×10^{-4} M but also to regenerate the NAD coenzyme using ADH. ADH and the following mediators have been employed[24] for the oxidation of ethanol: methylene blue and the *N*-methylphenazine ion, which oxidise NADH, are produced by the reduction of NAD due to the occurrence of the enzymic reaction and are themselves oxidised at the electrode. The anodic compartment of the electrochemical cell employed contained the enzyme methanol dehydrogenase, which oxidises methanol, according to the data of Varfolomeev et al.[2] in the presence of phenazine methosulphate and phenazine ethosulphate as mediators. The maximum current in the system reached 3.7 mA.

The FAD-dependent enzymes glucose oxidase and amino-acid oxidases belong to the group of enzymes which play a significant role in oxidation-reduction processes in living organisms. L-Aminoacid oxidase, bound covalently to the surface of a carbon black electrode modified with 2,4,6-trichlorotriazine, has been used[26] to determine L-aminoacids. The electrode potential varies linearly with the aminoacid concentration in the range 10^{-5}–10^{-2} M.

The study of the electrochemical oxidation of glucose in the presence of bio-organic catalysts is of great interest because this reaction takes place on platinum with a high overpotential. A carbon electrode modified with 2,4,6-trichlorotriazine with the covalently attached enzyme has been used[27] in the study of the reduction of glucose oxidase, which occurred at a potential of −0.51 V (relative to the Ag/AgCl electrode), which is more positive by 100 mV than in the reduction of the apoenzyme. The enzymic oxidation of glucose on a vitreous carbon electrode with adsorbed mediators has been investigated.[28] Glucose oxidase was deposited on the modified electrode and was separated from the bulk of the solution by a dialysis membrane. The transferring agents were tetracyano-*p*-quinodimethane and the potassium salt of its anion, pyocyanin, 9,10-phenanthrenequinone, dichlorophenol-indophenol, and dextran-dopamine. These compounds are substrates of glucose oxidase. The maximum current in the system depended on the concentrations of the components in the layer near the electrode and on the nature of the transferring agent, reaching 67 μA cm^{-2} under anaerobic conditions. The electrochemical oxidation of glucose has been investigated[29,30] in the presence of glucose oxidase immobilised on organic metals—the complexes of *N*-methylphenazinium (HMP^+) and *N*-methylacridinium (HMA^+) ions with the tetracyanoquinodimethane radical-ion. The biocatalytic effect occurred at potentials in excess of −0.05 V (Ag/AgCl). The dependence of the current on the glucose concentration was described by a curve with saturation, the limiting current depending on the electrode potential. The electrode with the immobilised enzyme retains its activity for more than 100 days.

Metalloenzymes, containing iron and copper ions in their molecule, play a major role in electron transport in biological systems. The cytochrome b_2–potassium hexacyanoferrate(III)/hexacyanoferrate(II) system has been used to activate the lactate oxidation reaction on the platinum electrode.[31,32] Studies were performed in a two-electrode cell whose anodic and cathodic compartments were separated by an electrically conducting bridge. A solution of the lactate, potassium hexacyanoferrate(III), and the enzyme was placed in the anodic compartment, while a solution of potassium hexacyanoferrate(III) was placed in the cathodic compartment. The enzymic reaction resulted in the formation of hexacyanoferrate(II), which was then electrochemically oxidised at the anode. The hexacyanoferrate(III) was electrochemically reduced at the cathode. The current in the external circuit when the cell was switched on varied linearly with the lactate concentration in the range from 5×10^{-6} to 4×10^{-3} M. 2,6-Dichlorophenol-indophenol, phenazine methosulphate, methylene blue, and indigocarmine have also been used as transferring agents. Johnson and co-workers[33,34] employed a mediator system to control the potential in the layer containing the enzyme galactose oxidase. In the course of the enzymic reaction, the active centre is reduced and then reversibly reoxidised by the mediator. The choice of the transferring agent is of decisive importance for the establishment of the appropriate potential in the system. Hexacyanoferrate(III), 2,6-dichlorophenol-indophenol, tetracyanomonophenanthroline ferrate, and tripyridinecobalt were used as the mediators.

As can be seen from the data presented, the mediator method of coupling the enzymic and electrochemical reactions using soluble mediators has been studied in fair detail. A new superior procedure for mediated transport, using transferring agents and enzymes in an immobilised state on electrodes as well as the direct electron exchange between the active centres of enzymes and the electrode, are of interest.

2. Electrochemical Reactions of Proteins on Electrodes

In connection with the possibility of using enzymes in electrocatalytic processes, the study of the electrochemical properties of proteins is of interest. The application of electrochemical research methods can yield important information about the mechanism of electron transfer in protein molecules and about the relation between the rate of electron transfer (or the rate of change of potential) and the conformational rearrangements of proteins.

The adsorption of proteins on the surfaces of electrodes plays a major role in the study of their electrochemical reactions. The adsorption of proteins on mercury electrodes and

the mechanism of electron transfer in such systems have been investigated by Kuznetsov and co-workers.[35-37] It has been shown that protein is rapidly adsorbed on the surface of mercury with formation of a monolayer of the denatured protein 8–10 Å thick. This protein layer is adsorbed irreversibly and covers approximately 80% of the mercury surface, forming a porous structure. The active centre of the denatured protein does not participate in electron exchange with the protein molecules in solution. The latter exchange electrons with the electrode through the pores in the irreversibly sorbed protein.

It is believed,[38] however, that, owing to the high surface pressure, the adsorbed molecules in the first monolayer on the mercury electrode are not denatured.

It has been shown that horse heart cytochrome *c* is reduced irreversibly on mercury.[39-42] The anodic potential scan does not show oxidation of the reduced protein. Cytochrome *c* is reduced with an overpotential[42] and the reduction process depends on the pH, the ionic strength of the solution, and the protein concentration.

Study of the behaviour of cytochrome *c* on a gold electrode modified with γγ-bipyridyl and 1,2-bis(4-pyridyl)ethylene showed that the prosthetic group of the protein undergoes reversible oxidation-reduction in the given system. It is of interest that modification of the lysine residues of cytochrome *c* causes cytochrome *c* to cease being a substrate of cytochrome *c* oxidases and at the same time the reversible electrochemical oxidation-reduction of the protein on the electrode stops.[43-48] The reversible oxidation-reduction of cytochrome *c* at the equilibrium potential has also been observed on a tin-doped indium oxide electrode[49] and a gold electrode modified with 1,2-bis(4-pyridyl)ethylene.[47] The oxidation-reduction rate constant on electrodes of this type is 0.014–0.019 cm s^{-1}.

Saponeva et al.[50] and Czochralska et al.[51] investigated the reduction of bacteriorhodopsin on mercury and platinum electrodes. The electrochemical behaviour of proteins has been examined in detail in a review.[52]

The proteins investigated[35-51] do not exhibit enzyme activity and behave solely as electron or oxygen transferring agents. The possibility of direct electron transfer to the active centres of enzymes in the absence of substrates has been investigated for xanthine oxidase,[53] lactate dehydrogenase,[54] catalase and superoxide dimutase,[55] peroxidase,[56] cholesterol oxidase,[57] and glucose oxidase.[58]

The haemin in the peroxidase molecule is electrochemically inactive on an amalgamated gold electrode and on gold and pyrographite electrodes. The reversible oxidation-reduction process observed on the gold amalgam corresponds to a reaction involving the S–S bonds.[56]

The electrochemical properties of cytochrome P-450 have been investigated[39] and it has been shown that cytochrome P-450 is reduced by mercury irreversibly at a potential of −1.4 V. Flavin-adenine dinucleotide (FAD), which forms part of the composition of the active centre of xanthine oxidase and D-aminoacid oxidase, is reduced on mercury in the range of potentials corresponding to free FAD.[36] The prosthetic group of another FAD-dependent enzyme, namely glucose oxidase, is reduced at the equilibrium oxidation-reduction potential of the enzyme.[58] The reversible oxidation-reduction on the mercury electrode of the prosthetic group of cholesterol oxidase, in which FAD is bound covalently to the apoenzyme has been observed, but it takes place with an overpotential.[57] One must note that the oxidation-reduction processes associated with the prosthetic groups of enzymes cannot indicate unambiguously the possibility of the effective electrocatalysis of the corresponding reactions by these enzymes, i.e. the redox reactions of the prosthetic groups in homogeneous enzyme catalysis differ from the corresponding redox reactions of the same groups in electrochemical systems. For example, peroxidase, in which the reduction of haemin has not been observed under anaerobic conditions,[56] is an effective catalyst of the electrochemical reduction of hydrogen peroxide. This effect will be discussed in detail below.

3. Direct Electron Transfer Between the Active Centre of the Enzyme and the Electrode

A fundamental problem in bioelectrocatalysis is that of the direct coupling of the enzymic and electrode reactions. The possibility of the direct electron transfer from the electrode to the active centre of the enzyme (or conversely) has been discussed.[11]

The preparation of effective catalysts of the electrochemical reduction of oxygen is of great practical importance. It is known that a steady-state potential established in an oxygen atmosphere for electrodes made of carbonaceous materials is far from the equilibrium oxygen potential and amounts to ~0.6–0.7 V. Cytochrome *c* has been used[59,60] as the catalyst of this reaction. It has been shown that the catalysis by cytochrome *c* occurs only in the adsorbed state over the entire experimental range of potentials (from −0.18 to +0.6 V). It has been established that in the presence of the biocatalyst not only does the rate of reduction of oxygen to hydrogen peroxide, i.e.

$$O_2 + 2\bar{e} + 2H^+ \rightarrow H_2O_2,$$

increase but also the parallel reaction involving its reduction to water occurs

$$O_2 + 4\bar{e} + 4H^+ \rightarrow 2H_2O.$$

The second reaction is associated with the rupture of the oxygen–oxygen bond and apparently proceeds via the adsorption of the oxygen molecules on the central iron atom in the active centre of cytochrome *c*.

A group of Japanese workers[61] established that cytochrome c_3 from *Desulfovibrio vulgaris* catalyses the reduction of molecular oxygen in the adsorbed state on the mercury electrode. In this case oxygen was fully reduced to water, i.e. via a four-electron mechanism without the formation of hydrogen peroxide. The maximum acceleration of the reaction was observed when the mercury electrode surface was 50% filled by the adsorbed cytochrome c_3.

The electrochemical reduction of oxygen and the direct electron transfer in the oxygen–*Polyporus versicolor* laccase–carbon electrode system has been studied in detail.[62,63] The enzyme was present on the surface of electrodes in the adsorbed state. The introduction of laccase into the system shifts the steady-state electrode potential towards positive values and accelerates the electrochemical reduction of O_2 in the potential range 1.2–0.6 V, the potentials and currents at the given potentials increasing with increase of the amount of enzyme adsorbed on the electrode. Gold, pyrographite, vitreous carbon, and carbon black were used as the electrode materials. The studies were performed under aerobic and anaerobic conditions. The maximum potential of +1.207 V was established in an oxygen atmosphere for electrodes which had been kept beforehand in a solution of laccase at a concentration of ~10^{-5} M for 24 h. The introduction of a laccase inhibitor into the system (F^- or N_3^- ions) virtually stopped the acceleration of the electrochemical reduction of oxygen (Fig.2). The stable and reproducible potential of +1.20 V permitted a detailed investigation of the mechanism of the electrochemical reduction of oxygen in the vicinity of the

equilibrium potential. The linear relation between the current and the overpotential is maintained in the potential range 1.20–1.15 V. In the potential range 1.18–1.15 V the slope of the polarisation curve increases up to $\partial E/\partial \lg i \cong 0.03$ V. At potentials more negative than 1.15 V, we have $\partial E/\partial \lg i \cong 0.06$ V. Along the 1.20–1.15 V section of the polarisation curve, $\partial E/\partial \lg i \cong \partial E/\partial \lg p_{O_2}$, i.e. the reaction is of first order with respect to oxygen and the dependence on pH is given by $\partial E/\partial \mathrm{pH} \cong 0.06$ V. The calculated stoichiometric number was found to range from 0.75 to 1.8 with an average of 1.2. Consequently, the slow stage in the electrochemical reduction of oxygen is repeated once. On the basis of the experimental data, the authors proposed a mechanism for the electrochemical reduction of oxygen in the presence of laccase:

$$\begin{gathered}
E + O_2 \rightleftharpoons EO_2, \\
EO_2 + \bar{e} \rightleftharpoons EO_2^-, \\
EO_2^- + H^+ \rightleftharpoons EO_2H, \\
EO_2H + 2\bar{e} + H^+ \rightarrow EO^- + H_2O \ (E = 1{,}2 \div 1.15\mathrm{V}), \\
EO^- + \bar{e} + H^+ \rightleftharpoons EOH^-, \\
EOH^- + H^+ \rightleftharpoons E + H_2O, \\
\hline
O_2 + 4\bar{e} + 4H \rightleftharpoons 2H_2O,
\end{gathered}$$

where E is the enzyme.

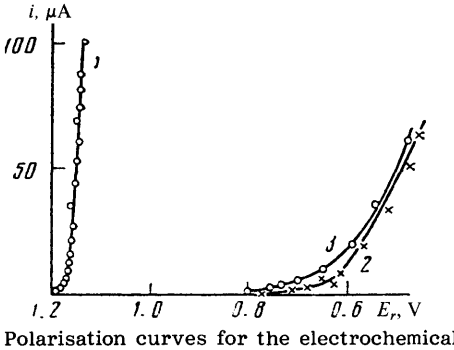


Figure 2. Polarisation curves for the electrochemical reduction of oxygen at an electrode with immobilised laccase: 1) in an oxygen atmosphere; 2) in the presence of the N_3^- inhibitor; 3) background curve in the absence of enzyme.

The synchronous transfer of two electrons in the slow stage is unusual for an electrochemical reaction. However, the authors postulate that the presence of a two-electron acceptor in the laccase molecule makes it possible to achieve this reaction.

The non-mediated catalysis of the electrochemical reduction of oxygen using potato *o*-diphenol : oxidoreductase has been investigated.[16] In the presence of the enzyme adsorbed on the carbon black electrode, a potential of +1.13–1.16 V is established in an oxygen atmosphere. It has been shown using a disc electrode with a ring that, as for the electrode with laccase, oxygen is reduced directly to water in the given system without the formation of H_2O_2.

The electrochemical oxidation of hydrogen in the presence of hydrogenase is of considerable interest in connection with the search for new non-metallic catalysts for the hydrogen electrode of the fuel cell. Results indicating the possibility of the direct oxidation of H_2 at carbon black electrodes by the hydrogenase from *Thiocapsa roseopersicina* immobilised by adsorption have been obtained.[64] The equilibrium hydrogen potential has been realised at these electrodes. Analysis of the kinetic parameters of the system led to the following mechanism of the electrochemical oxidation of hydrogen:

$$\begin{gathered}
E + H_2 \rightleftharpoons EH_2, \\
EH_2 \rightleftharpoons EH^- + H^+, \\
EH^- \rightleftharpoons EH + \bar{e}, \\
EH^- \rightarrow EH^+ + \bar{e} \ (E = 0.03 \div 0.05\mathrm{V}), \\
EH^+ \rightleftarrows E + H^+.
\end{gathered}$$

The rate-limiting stage is the transfer of the second electron on the assumption that all the remaining stages are reversible.

Study of the possibility of direct electron transfer in electrochemical systems containing the enzyme glucose oxidase and glucose is of interest for the creation of a reagent-free glucose sensor also in biochemical fuel cells. The electrochemical oxidation of glucose at a platinum electrode with immobilised glucose oxidase has been studied.[65–68] The enzyme was fixed with the aid of glutaraldehyde or polyacrylamide gel on the surface of the electrode which had been charged beforehand or with glutaraldehyde on the uncharged electrode. The method used to modify the platinum surface has been described.[69] Positive results have been obtained only for the first method of immobilising the enzyme on the charged electrode. The steady-state currents exceed in this instance by a factor of 2–3 the currents obtained using the platinum electrode without the enzyme and reach 10 μA cm^{-2}. In the remaining cases of enzyme immobilisation on platinum catalytic effects were not observed at all. The authors explained these overall negative results by the poor contact between the enzyme and the electrode.[65] The influence of the Ag^+ and Hg^{2+} ions, which inhibit glucose oxidase, on the electrocatalytic activity of the enzyme immobilised on the surface of platinum mesh has been investigated.[70] The electrode potential established in the presence of glucose and the enzyme decreased linearly with increasing inhibitor concentration. This can serve as one of the pieces of evidence showing that in this system there is direct electron transfer between the electrode and the active centre of glucose oxidase, although other data concerning this hypothesis are not quoted.

In all the investigations enumerated above the bioelectrocatalysts were present either in a dissolved form or in an immobilised state on the surfaces of metallic and carbon electrodes. However, direct electron transfer in the enzyme–electrode system cannot be achieved in all cases using the above procedures for the immobilisation of the enzyme on the electrode.

Studies involving the use of conducting complexes of tetracyanoquinodimethane and low-molecular-weight or polymeric organic compounds as carriers for the immobilisation of enzymes are of great interest for the development of a general approach to the creation of non-mediated bioelectrocatalytic systems.[71,72] A method has been developed[73–75] for the application of polymeric electrically conducting carriers in order to achieve non-mediated electron transport in the electrode–enzyme active centre system. The inclusion of the enzyme in a current conducting matrix improves contact between the active centre of the catalyst and the carrier. Immobilisation of enzymes and the formation of matrices with a high electrical conductivity (up to 10^{-2} Ω^{-1} cm^{-1} [76]) is achieved by the coprecipitation of polymeric polycations and enzymes by means of a half-reduced tetracyanoquinodimethane (TCQDM) salt. This immobilisation procedure is also distinguished by a high capacity of the carrier with respect to the protein. The radical-ion TCQDM salts and heat-treated

polyacrylonitriles (HTPAN) have been used[73–75] to immobilise hydrogenase. Data concerning the composition, capacity with respect to the protein, electrical conductivity, and the percentage retention of enzyme activity for the polymeric carriers of hydrogenase investigated are listed in the Table. Immobilisation activates hydrogenase with simultaneous increase of the stability of the enzyme with respect to denaturation in air. It is interesting to note that one of the polymers employed, namely poly-1-propargyl-1-methyl-4,4-bipyridylium (PPMB) is a substrate of hydrogenase. The electrocatalytic properties of the hydrogenase immobilised on polymer–TCQDM complexes were investigated in relation to the formation of H_2 on a polymeric enzyme electrode, which occurs only in the presence of hydrogenase in the system. It was shown that the electron transfer to the active centre of the enzyme in the course of the study of the hydrogen formation process up to the preliminary electrochemical reduction of the carrier with hydrogenase can be achieved from any section of the polymer matrix. The poly-1-propargylpyridinium (PPP)–TCQDM polymeric conductors with immobilised glucose oxidase have been used for the electrochemical oxidation of glucose.[72] A significant increase of the current was observed only in the presence of the enzyme on the electrode and of glucose in solution. The linear dependence of the currents at a specified potential on the glucose concentration in solution was maintained in the concentration range 0.15–2.0 mM. The experimental data were described by a parabolic function and were interpreted in terms of the Michaelis–Menten equation. A linear plot obtained employing the reciprocals of the variables yielded $i_{max} = 67$ μA cm^{-2} and a Michaelis constant of $4.0 \pm 0.5 \times 10^{-3}$ M at a potential of 140 mV (Ag/AgCl).

After immobilising cytochrome b_2 on the complex of TCQDM with *N*-methylphenazine (NMP), Kulis and Svirmickas[77] observed the acceleration of the electrochemical oxidation of lactate with electron transfer from the active centre of the enzyme to the electrode without the involvement of a low-molecular-weight mediator. On the basis of this system, the same workers[78] developed a reagent-free analytical sensor for lactate.

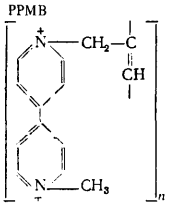
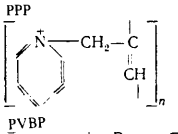
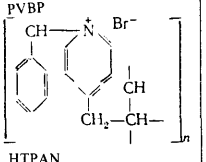
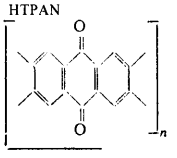
The reduction of hydrogen peroxide by peroxidase immobilised by adsorption on the NMP–TCQDM and NMA–TCQDM electrodes has been described.[79] Hydrogen peroxide was reduced at potentials of ~0.25 V relative to the saturated silver/silver chloride electrode. In the absence of peroxidase this reaction proceeds only at potentials below −0.2 V. The above studies confirm the possibility of the successful employment of radical-ion matrices ("organic metals") as electrode materials for the catalysis of electrochemical reactions by immobilised enzymes.

It is seen from the literature data quoted that there has been a growth of interest recently in the study of the direct electron transfer between the active centre of the enzyme and the electrode. However, all the studies listed above were designed to investigate the direct transfer in a specific system and were not concerned with problems of the characteristics of the electron transfer in bioelectrochemical systems as a whole

4. The Mechanism of Electron Transfer in the Enzyme Catalysis of Electrode Processes

The main problem in systems for the non-mediated oxidation or reduction of the active centre of the enzyme at the electrode involves the study of the mechanism of electron transfer from the enzyme to the electrode (or the converse process). The kinetic model of bioelectrocatalysis proposed by Varfolomeev and Berezin[80,81] cannot naturally provide an exhaustive answer. Probably the direct electron transfer requires a specific orientation of the enzyme active centre relative to the electrode and the shorter the distance from the active centre to the electrode the greater the probability of the transfer. However, the active centres of enzymes are apparently localised within the protein globule and after immobilisation can be located at an appreciable distance from the carrier surface.

Composition and characteristics of polymeric carriers.

Polymer	Probable composition of carrier	*A*, mg protein/g carrier	ρ, Ω cm	Retention of activity, %
PPMB 	$[(PPMB^{2+})(TCQDM)^{-}_{2} \cdot (TCQDM^{0}_{2})]_n$	200±20	$3.6 \cdot 10^4$	200
PPP 	$[(PPP^{+})(TCQDM)^{-}_{n}(TCQDM^{0})]_m$ $m/n = 0.25$	200±20	$1.2 \cdot 10^4$	400
PVBP 	$[(PVBP^{+})(TCQDM)^{-}(TCQDM^{0})]_n$	70±10	10^8–10^9	—
HTPAN 	HTPAN	25±5	$2.0 \cdot 10^3$	30

Notation: *A* = capacity of the carrier with respect to protein; ρ = resistivity of the carrier; PVBP = poly-1-vinyl-1-benzylpyridinium chloride.

The dependence of the efficiency of electron transfer on the distance between the active centre of laccase and the electrode has been studied[82] in relation to the electrochemical reduction of oxygen. Initially cholesterol was adsorbed on the surface of a carbon black electrode from alcoholic or heptane solutions or lecithin was adsorbed from a solution of benzene and the enzyme was then sorbed on top of the lipids. Thus the distance between the electrode and the laccase molecule increased to 6, 17, or 20 Å respectively (Fig.3). The oxygen electrochemical reduction currents at the appropriate potential were normalised with respect to the maximum rate of the enzymic reaction measured in independent experiments.

With increase of the distance from 6 to 17 Å, the authors observed a decrease of the rate of electron transfer through the cholesterol layer approximately by a factor of 4.5, while the decrease following the increase of the distance to 20 Å

(through the lecithin layer) was by a factor of ~200. The authors assume that the idea of the tunnel mechanism of electron transfer between the active centre of the enzyme and the electrode using the simple kinetic scheme

$$E + \bar{e} \rightleftharpoons E^{\neq} \xrightarrow[O_2]{} E + H_2O,$$

where E is laccase in the initial state and $E^{\neq}$ the enzyme in the reduced state, is sufficient to account for this phenomenon and also for the bioelectrocatalytic effect.

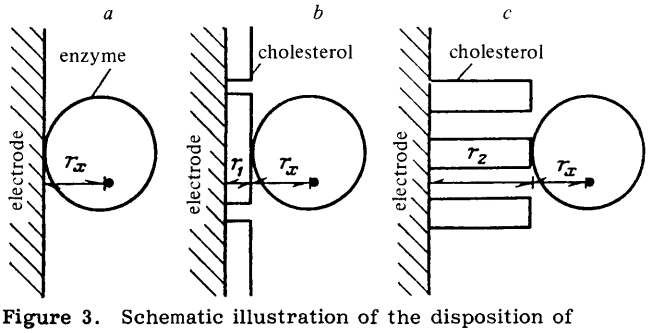


Figure 3. Schematic illustration of the disposition of the enzyme relative to the electrode surface: *a*) laccase adsorbed on the electrode surface; *b*) enzyme adsorbed on top of the liquid with a planar orientation relative to the electrode; *c*) enzyme adsorbed on top of the lipid with a vertical orientation relative to the electrode; r_1 = thickness of lipid layer with a planar orientation of cholesterol, r_2 = ditto with a vertical orientation, r_x = distance from the surface to the electron-accepting site in the enzyme.

The second stage is enzymic and limits the rate of the reaction. The first stage is electrochemical and proceeds reversibly and via the tunnel mechanism. These conclusions have been confirmed by potentiodynamic experiments under anaerobic conditions with laccase adsorbed on the electrode surface. Reversible maxima corresponding to the oxidation-reduction potential of copper of the first type, which is a primary electron acceptor in the active centre of laccase, are observed at potentials of ~+0.75 V. The tunnelling stage can be described by the equation:[83]

$$k_t(r) = A \exp(-2r/a),$$

where A is the frequency of the collisions of the electron with the potential barrier, U the height of the barrier, $a = \hbar[2m(U - E)]^{1/2}$, $\hbar$ the Planck constant, m the mass of the electron, and E the energy of the electron.

Thus in the system considered the probability of the tunnel electron transfer over a distance greater than 20 Å is sharply reduced, which has also been observed experimentally.

III. PRINCIPAL APPLICATIONS OF BIOELECTROCATALYTIC SYSTEMS

The enzymes which are effectively involved in the catalysis of electrochemical reactions can find applications in the conversion of chemical energy into electrical energy in the so-called biofuel cells.[84–86]

Using the high substrate specificity and efficiency of the catalytic action of immobilised enzymes, it appears possible to achieve the specific electrode synthesis of many organic compounds.

The application of enzymes in different kinds of electroanalytical sensors, which has been discussed by Bauman et al.[87] and by Updike and Hicks,[88] makes it possible to extend the range of compounds capable of being determined and to simplify the technique of the measurements.

1. Enzyme Electrodes

The procedure for the coupling of the enzymic and electrochemical reactions with mediation is most often used in electroanalytical enzyme sensors. The employment of these analytical devices significantly simplifies the analytical technique, makes it possible to extend the range of compounds which can be determined, and in many cases increases the sensitivity of the measurements.

The required compound, which may be electrochemically inactive, is determined from the change in the concentration of other components of the enzymic reaction (substrates or products). For example, the concentration of glucose, which is relatively inactive electrochemically, can be determined amperometrically by recording the electrochemical oxidation of the reaction product—hydrogen peroxide,[89] or the electrochemical reduction of the substrate—oxygen,[90] in reaction (1). It is also possible to follow the variation of the pH of the solution as a result of the formation of the product—gluconic acid.[91]

$$\text{Glucose} + O_2 \xrightarrow[\text{glucose oxidase}]{\text{immobilised}} \text{gluconic acid} + H_2O_2, \qquad (1)$$

$$H_2O_2 + 2I^- + 2H^+ \xrightarrow[\text{peroxidase}]{\text{immobilised}} I_2 + 2H_2O, \qquad (2)$$

$$H_2O_2 + 2\,Fe(CN)_6^{4-} + 2H^+ \xrightarrow{\text{peroxidase}} 2Fe(CN)_6^{3-} + 2H_2O. \qquad (3)$$

Another approach to the determination of glucose concentration involves the use of two-enzyme systems in which one of the products of the first enzymic reaction is the substrate of the second reaction. In this case the concentration of glucose is determined potentiometrically with the aid of an electrode sensitive to iodide ions[92] [reaction (2)] or amperometrically by recording the hexacyanoferrate(II) reduction currents [reaction (3)] at platinum or carbon electrodes.[93] A two-enzyme system employing glucose oxidase and cytochrome P-450, catalysing the conversion of aniline, by the hydrogen peroxide formed on oxidation of glucose, into *p*-aminophenol, which then undergoes anodic oxidation to the quinone, is also known. The limiting currents are then proportional to the glucose concentration.[94]

Instead of oxygen, artificial electron acceptors (AFA) may be used for the enzymic oxidation of glucose:[95]

$$\text{Glucose} + \text{oxidised AEA} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{reduced AEA}. \qquad (4)$$

Artificial electron acceptors are electrochemically active compounds and their reduced forms can be readily determined amperometrically. The possibility of determining glucose using polyviologens as AEA in the presence of oxygen has been described.[96] Measurements were made at pH 8.0, i.e. under conditions where glucose is not oxidised by oxygen in the presence of glucose oxidase. The AEA were separated from the bulk of the solution by a dialysis membrane.

Numerous methods for the determination of various organic and inorganic compounds with the aid of enzyme electrodes have now been described in the literature. These procedures as well as the construction of enzyme sensors have been discussed in detail in a number of reviews.[97-99]

Apart from being used in sensors, enzyme electrodes have been recently widely employed for medical purposes. The so-called implanted electrodes can serve as a source of energy and can transmit information about the changes in the organism. The applications and the principle of the operation of the implanted electrodes have been discussed in a review.[100]

A new type of biosensor, in which a particular effect of the enzyme reaction is converted into an electrical response by means of thermistors, transistors, or piezoelectric crystals, has appeared during the last decade.[101-103] Such analytical devices differ from those known previously by a rapid response, a high sensitivity, and universality. For example, the principle of the operation of the biothermistor is as follows. When heat is evolved as a result of an enzymic reaction, the temperature of the surrounding medium is altered and the thermistor responds to it. Over a certain range, the temperature change and hence the electrical response are proportional to the concentration of the component determined.

The new biosensors have found extensive applications in the critical analyses of a wide variety of metabolites, such as ascorbic acid, urea, glucose, creatine, cholesterol, ATP, lactate, etc., and also for the monitoring of enzymic processes. The combination of solid-phase immunoenzyme analysis with calorimetric monitoring made it possible to determine the concentrations of certain compounds below 10^{-13} M.

2. The Creation of Highly Effective Energy Converters Based on Biocatalysts

The characteristic features of enzyme catalysts make it possible to use biochemical reactions in electrochemical energy generators. There is a possibility of creating fuel cells which can use organic compounds as fuel—alcohol and carbohydrates. On the other hand, the high catalytic activity of biocatalysts makes it possible to devise fuel cells with an enhanced power, for example, in systems for the biophotolysis of water.[104]

As early as the beginning of the 1960s, the possibility of creating biochemical fuel cells was discussed.[84] Systems which can be divided into two classes exist at the present time. The first class comprises systems using biocatalysts (in the form of microorganisms, cells or enzymes) for the conversion of a particular material into an electrochemically active compound.[105-107] For example, phosphate esters and catechol and *p*-aminophenol ethers were hydrolysed[106,107] by hydrolases and the products of this reaction were then oxidised electrochemically at the electrode as the fuel. It has been shown that, when alkaline phosphatase is used, the oxidation reaction of the products of the enzymic reaction proceeds at potentials more negative by 0.3–0.4 V than the electrochemical oxidation of the initial substrates.

Methods for the conversion of complex organic substances into the simplest fuel as a result of the action of microorganisms have been described.[108-111] The primary substances employed were saccharides, urea, lactate, formic acid, and hexadecane. Ammonia, hydrogen, and methane were converted by the action of biocatalysts into a fuel capable of being oxidised at the electrode. A characteristic feature of such fuel cells is the presence of one or several stages which are not oxidation-reduction reactions and are used to convert the secondary fuel into the electrochemically active primary fuel.

The second class includes systems based on the direct utilisation of enzyme catalysts in bioelectrochemical reactions.[17] Evidently, such a function can be fulfilled only by oxidation-reduction enzymes. It is therefore promising to investigate the electrocatalytic properties of hydrogenases, glucose oxidase, acid dehydrogenases, methanol, and methane oxidases, as well as enzymes which activate the electrochemical reduction of oxygen. Fuel cells employing the enzyme glucose oxidase have been described.[66,112] The biofuel cell based on a hyaluronidase immobilised on Sepharose-CNBr reached a power output of 2.8 $\mu W\ cm^{-2}$.[113]

Electrodes for biofuel cells operating via a mechanism involving direct electron transfer and employing the enzymes laccase[62] and hydrogenase[114] have been described in the literature.

The so-called "cofactor-modified electrodes" can also find applications in fuel cells. The possibility of employing cofactors in electrochemical processes have been discussed in two investigations.[115,116]

A number of studies whose aim was to devise an anode for a fuel cell employing methanol as the fuel have been published in recent years.[117,118] The catalyst in this system was an NAD-independent methanol dehydrogenase and the electron acceptor was phenazine ethosulphate or phenazine methosulphate. The reduced mediator was oxidised at a platinum electrode. The cathodic reaction employed involved the reduction of oxygen on platinum. The maximum power output of the cell was 20 $mW\ mg^{-1}$.

3. Specific Electrosynthesis Under the Influence of Immobilised Enzymes

When biocatalysts are employed, it is possible to synthesise effectively compounds whose synthesis by other methods is difficult.[119,120] On the basis of the data of Wollenberger et al.,[121] it is possible to put forward a method of synthesis employing the oxidation reaction of ketosteroids catalysed by the enzyme steroid Δ^1-dehydrogenase. Wingard et al.[122] discussed the possibility of the electrosynthesis of ATP in the presence of ATP phosphatase from ADP and orthophosphate. Scheller et al.[123] proposed a method for the demethylation of benzphetamine, *p*-nitroanisole and aminopyridine in the cathodic reduction of oxygen to hydrogen peroxide in the presence of cytochrome P-450.

Methods whereby it is possible to regenerate enzyme cofactors have been recently discussed fairly frequently in the literature.[124-126] Torstensson and Johansson[127] proposed a method for the regeneration of nicotinamide-adenine dinucleotide (NAD) in a complex with alcohol dehydrogenase immobilised on the surface of a vitreous carbon electrode. The complex was obtained by employing an NAD analogue, namely $NH_2-(CH_2)_6-NHCOCH_2-NAD^+$. The electrode surface was modified with 2,4,6-trichlorotriazine. An enzymic reaction occurred in the presence of ethanol with formation of the NADH-analogue, which was then reoxidised electrochemically to the NAD-analogue. A deficiency of this method is

the occurrence of electrochemical side processes which lead to the degradation of the NAD-analogue and the formation of enzymically inactive dimeric compounds.

An NAD regenerating system employing PMS^+† as the mediator has been proposed.[128,129] The cofactor analogue NAD-dextran is reduced to NADH-dextran in the presence of substrates of the enzymes alcohol dehydrogenase and lactate dehydrogenase. NADH-dextran was oxidised chemically in the presence of PMS^+ to NAD-dextran. The PMSH formed is oxidised reversibly to PMS^+ at the electrode. The proposed method also makes it possible to regenerate NADP in the presence of glutamate dehydrogenase. The efficiency of the regeneration of the cofactor in such systems reaches 99.85%.

Reactions involving the electrosynthesis of a number of carbohydrate derivatives using methane monoxygenase have been described.[48,119]

It is to be expected that interest in the preparation of organic substances of this kind with the aid of the bioelectrosynthetic method will steadily grow. This is associated with the necessity for the large-scale manufacture of certain substances, for example, for the pharmaceutical industry, whose synthesis by the known organic synthetic methods is difficult.

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† PMS^+ and PMSH are the oxidised and reduced forms of phenazine methosulphate respectively (Ed.of Translation).

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Bakh Institute of Biochemistry,
USSR Academy of Sciences