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A Novel Application of Heterocyclic Compounds for Biosensors Based on *NAD*, *FAD*, and *PQQ* Dependent Oxidoreductases

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Summary. Various five-, six-, and seven-membered heterocyclic compounds containing two nitrogen atoms as well as quinoid substances were synthesized and investigated. Electrochemical oxidation-polymerization of these heterocycles on carbon and platinum electrodes was performed in aqueous medium. Electrochemically pre-treated carbon electrodes modified with these heterocyclic compounds and were used for the electro-catalytic oxidation of *NADH* and for the design of mediated glucose biosensors.

Keywords. Benzimidazoles; 1,5-Benzodiazepines; Biosensors; Electrocatalysis.

Eine neue Anwendung organischer Heterocyklen als Mediatoren für Biosensoren auf der Basis von *NAD*-, *FAD*- und *PQQ*-abhängigen Oxidoreduktasen

Zusammenfassung. Verschiedene fünf-, sechs- und siebengliedrige heterocyclische Verbindungen mit zwei Stickstoffatomen sowie Chinone wurden hergestellt und untersucht. Elektrochemische Aktivität und elektrochemische Polymerisation der Heterocyclen an Platin- und Kohlenstoffelektroden wurden in wäßriger Lösung studiert. Durch die genannten Heterozyclen modifizierte Kohlenstoffelektroden können für die elektrochemische Oxidation von *NADH* und den Aufbau von Glucosesensoren unter Verwendung von Glucoseoxidase und *PQQ*-abhängiger Glucosedehydrogenase verwendet werden.

Introduction

Oxidoreductases are well known enzymes used for biosensor design [1, 2]. The efficiency of biosensor design considerably depends on the efficiency of electron transport from the substrate *via* the enzyme to the acceptor or/and the electrode.

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From this point of view and based on the cofactor's nature, oxidoreductases can be divided into three main groups. Flavin dependent oxidases containing tightly bound *FAD* or (*FMN*) in the active center of enzyme belong to the first group. These enzymes transfer electrons to oxygen. In some cases, one of the products is H_2O_2 which can be determined amperometrically using Pt electrodes at 0.5–0.7 V *vs*. Ag/AgCl as reference electrode. Unfortunately, at such high potential a variety of electrochemically active components existing in the sample can be also electrochemically oxidized on the surface of electrode. Artificial electron acceptors that reversibly transfer electrons from the active center of the oxidase to the electrode surface at low potentials can replace oxygen in this reaction. This replacement allows to design amperometric enzyme electrodes operating at a lower working potential and avoiding the influence of interfering substances. 2,6-Dichlorophenolindophenol [2], hexacyanoferrite(III) [3], tetrathiafulvalene [4], tetracyanoquinodimethane [5], various quinones [6–8], and ferrocene derivatives [9, 10] have been applied for this purpose. However, there still remain some problems in the long term operation of such types of biosensors. The first is competition of the mediator with oxygen for the active center of the enzyme. In the case of uncontrolled concentration of oxygen, this phenomenon results in unpredictable errors of measurement. The second is based on fact that reduced forms of the mediators are not stable in the oxygen environment and can be oxidized not only electrochemically, but by oxygen as well. The third is inactivation of enzyme molecules by H_2O_2 produced in side reactions.

NAD(*P*) dependent dehydrogenases belong to the second group. To catalyze oxidation of the substrates, these enzymes need the free diffusing cofactor *NAD*(*P*) to exert catalytic activity. Electrooxidation of *NAD*(*P*)*H* on the solid electrodes can be performed only at high potentials (0.7–1 V), and this process is complicated by side reactions: dimerization of *NAD* and oxidation of interfering substances. Various redox mediators can be used to shuttle the electrons from *NAD*(*P*)H to the electrode at substantially lower potentials [11]. Quinones [12, 13], phenazines [14], phenoxazine and phenothiazine derivatives [15–18], conducting organic salts [19], and inorganic iron salts [20, 21] can act as catalysts for the electrochemical oxidation of *NAD*(*P*)H. Biosensors based on such type of dehydrogenases, however, show poor stability due to instable mediators and/or disappearing of *NAD*(*P*)H from the surroundings of the enzyme.

The third group of enzymes consists of *PQQ* dependent dehydrogenases. These enzymes contain a tightly bound cofactor (*PQQ*) in the active center [22]. Cytochromes or ubiquinones are natural acceptors of electrons. Derivatives of ferrocene and phenazine can also serve as good mediators for *PQQ* dependent dehydrogenases [23–26]. Biosensors based on *PQQ* dependent enzymes are very promising due to their insensitivity to O_2.

We have found that modification of the surface of carbon with some heterocyclic compounds can considerably improve the electron communication between the active center of enzymes and the solid surface of the modified carbon electrode. The goal of our work was to check up a number of organic compounds, mostly insoluble in water and irreversibly adsorbed on the carbon surface, as promising agents for this purpose.

Results and Discussion

Various five-, six-, and seven-membered heterocyclic compounds containing two *o*-positioned nitrogen atoms or bearing a quinone structure were synthesized and investigated. The first group consists of benzimidazole and benzimidazolinone derivatives (**1–10**). The second group includes quinoxaline, quinoline, and naphthoquinone derivatives (**11–17**). 1,5-Benzodiazepine derivatives (**18–26**) belong to the third group. Chemical structures and electrochemical parameters of these compounds are presented in Table 1.

Electrochemical properties of mediators

It was found that all tested materials are completely and irreversibly adsorbed on carbon electrodes and show irreversible electrochemical behaviour in the potential range of 0–800 mV *vs*. saturated Ag/AgCl. A typical pattern of an electrochemical oxidation is shown in Fig. 1. The benzodiazepine derivative **22** was adsorbed on the carbon electrode. The first scan resulted in two well-pronounced oxidative peaks at 350 and 600 mV. In the following seven scans, the anodic current of the electrode constantly decreases. Almost the same electrochemical behaviour was obtained on Pt electrodes (data not shown).

The electrochemical oxidation of nitrogen containing heterocyclic compounds is not clear and depends on a number of parameters. Some speculations are based on the assumption that derivatives of 1,5-benzodiazepine are not completely aromatic structures; they can be considered as substituted aromatic *o*-diamines. Electrochemical oxidation of such compounds may be expressed as a two-electron transfer from the nitrogen atoms to the electrode (Scheme 1).

The dicationic oxidation product can undergo reorganization to a more stable system by the elimination of a cationic particle. The process can include reactions with transfer of protons, especially in the case of compounds **23–26** (Scheme 2).

Following this scheme, a first step of an electrooxidation process of benzodiazepine derivatives cannot consist of a two-electron oxidation alone; an additional one-electron transfer reaction producing a more or less stable cation radical is also required. This way can become predominant when the possibility to distribute positive charge on a conjugated electron system exists (Scheme 3).

It seems that both processes can take place depending on the potential of the electrode and the oxidative state of its surface. In any case, on the surface of electrode there will be a number of different cation radicals of the heterocyclic compound.

As mentioned above, the electrochemical oxidation process is completely irreversible. It seems that cation radicals formed in the first step of electrooxidation lead to a destruction of the seven-membered ring, and oxidative polymerization or dismutation polymerization of cations or both takes part (Scheme 4).

A similar coupling reaction occurs during the electrochemical oxidation of 2,5-dihydro-1*H*-1-benzazepines in aqueous solutions [27].

Taken into account that the surface of the carbon electrode is covered with a multi-layer of heterocyclic molecules and cycling of potential has been applied for a long time (pre-treatment of the electrode), the process of electrochemical

Table 1. Parameters of investigated compounds

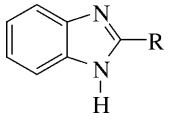
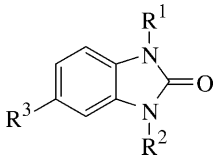
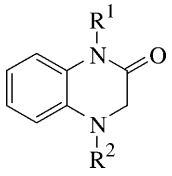
	Compounds	M.p. (°C) (solvent)	Sensitivity (μA/m*M*)		Solution in
			*NAD*H	Glucose	
	Benzimidazole derivatives				
					
1	$R = CH_3$		1.30	0.97	EtOH
2	$R = CH_2CH_3$	169–170 (Ref. [34]) (CCl_4)	0.80	0.20	EtOH
	2-Benzimidazolinone derivatives				
					
3	$R^1 = R^2 = R^3 = H$		0.85	0.21	EtOH
4	$R^1 = R^2 = H$, $R^3 = NO_2$	305–306 (Ref. [35]) (EtOH)	2.00	0.98	EtOH
5	$R^1 = R^3 = H$, $R^2 = CH_3$	190–192 (Ref. [36]) (EtOH)	4.00	0.63	Acetone
6	$R^1 = R^3 = H$, $R^2 = C(CH_3)=CH_2$	121–122 (Ref.[37]) (EtOH:H_2O = 1:1)	2.00	0.01	EtOH
7	$R^1 = H$, $R^2 = C(CH_3)=CH_2$, $R^3 = NO_2$	228–230 (Ref. [35]) (AcCN)	0.65	0.17	*DMF*:EtOH (1:1)
8	$R^1 = R^3 = H$, $R^2 = C(Ph)=CH_2$	165–167 (Ref. [38]) (AcCN)	0.62	0.28	Acetone
9	$R^1 = R^3 = H$, $R^2 = COCH_3$		0.43	0.78	Acetone
10	$R^1 = COCH_3$, $R^2 = C(CH_3)=CH_2$, $R^3 = H$	108–110 (Ref. [38]) (Et_2O)	1.10	0.38	Acetone
	1,2,3,4-Tetrahydroquinoxalinone-2 derivatives				
					
11	$R^1 = R^2 = H$	95–97 (Ref. [39]) (H_2O)	1.80	0.83	EtOH
12	$R^1 = H$, $R^2 = CH_2CH_3$		0.80	0.71	EtOH
13	$R^1 = H$, $R^2 = COCH_3$	165–166 (Ref. [39]) (EtAc)	0.62	1.68	EtOH

Table 1 (*continued*)

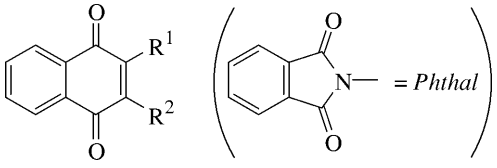
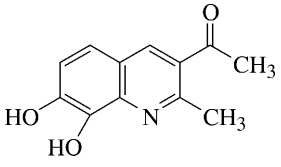
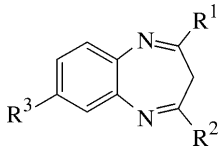
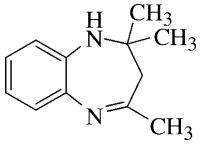
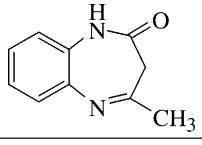
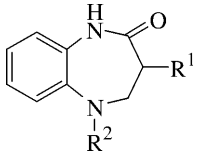
	Compounds	M.p. (°C) (solvent)	Sensitivity (μA/m*M*)		Solution in
			*NAD*H	Glucose	
	1,4-Naphthoquinone derivatives				
					
14	$R^1 = R^2 = Cl$		0.4	0.78	Acetone
15	$R^1 = Phthal$, $R^2 = Cl$	>295 dec. (Ref. [40])	0.54	3.12	Acetone
16	$R^1 = R^2 = Phthal$	no m.p. (Ref. [40])	0.4	1.14	Acetone
17	3-Acetyl-2-methyl-7,8-dihydroxyquinoline				
			1.2	1.00	H_2O
	3*H*-1,5-Benzodiazepine derivatives				
					
18	$R^1 = R^2 = CH_3$, $R^3 = H$	132–133 (Ref. [41]) (Et_2O)	0.93[a]		EtOH
19	$R^1 = R^2 = CH_3$, $R^3 = H$ (hydrochloric salt)	201–203 (Ref. [41]) (sublimation)	1.00[a]		EtOH
20	$R^1 = CH_3$, $R^2 = Ph$, $R^3 = H$ (hydrogensulfate monohydrate)	176–178 (Ref. [42]) (H_2O)	1.04 [a]		EtOH
21	$R^1 = R^2 = Ph$, $R^3 = H$	138–140 (Ref. [42]) (MeOH)	3.75[b]		EtOH
22	$R^1 = R^2 = R^3 = CH_3$ (hydrochloric salt)	208–209 (Ref. [41]) (EtOH)	1.00[c]		EtOH
23	2,2,4-Trimethyl-2,3-dihydro-1*H*-1,5-benzodiazepine				
		125–127 (Ref. [43]) (ligroin)	1.5		EtOH
24	4-Methyl-2,3-dihydro-1*H*-1,5-benzodiazepinone-2				
		146–148.5 (Ref. [35]) ($EtOH:H_2O = 1:1$)	1.10	0.25	EtOH

Table 1 (*continued*)

Compounds	M.p. (°C) (solvent)	Sensitivity (μA/m*M*)		Solution in
		*NAD*H	Glucose	
2,3,4,5-Tetrahydro-1*H*-1,5-benzodiazepinone-2 derivatives				
				
25 $R^1 = H$, $R^2 = COCH_3$	159–160 (Ref. [34]) (EtAc)	1.4	0.21	EtOH
26 $R^1 = R^2 = CH_3$	152–154 (Ref. [44]) (benzene)	0.55	0.18	EtOH

[a] Sensitivities were measured using a Pt electrode (⌀ = 0.2 mm, working potential: 0.4 V *vs*. Ag/AgCl); [b]carbon electrode (⌀ = 3 mm, 0.4 V *vs*. Ag/AgCl); [c]Pt electrode (⌀ = 0.2 mm, 0.4 V *vs*. Ag/AgCl)

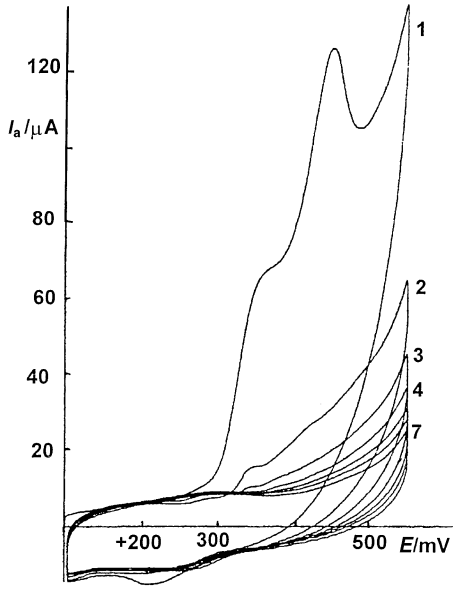
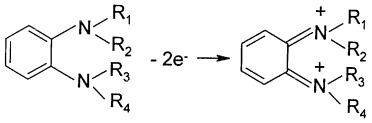
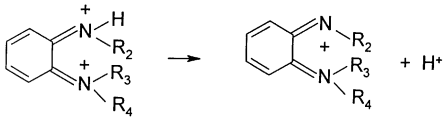


Fig. 1. Cyclic voltammograms of a carbon electrode modified with **22**; sweep rate: 50 mV/s; scanning was started at 0 V; the first seven scans are presented

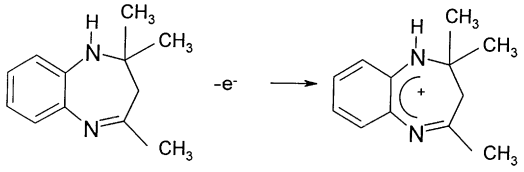
dimerization and/or polymerization can take part in an extensive range and probably will lead to the formation of an infra-structure containing semi-oxidized heterocyclic compounds. This presumption is based on the data of the electrochemical behavior of a number of heterocyclic compounds during the electrochemical cycling. The first scan showed different peaks of oxidation for the



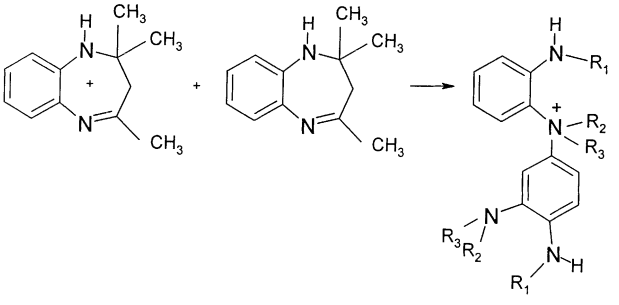
Scheme 1



Scheme 2



Scheme 3



Scheme 4

tested heterocyclic compounds. This can be expected to be due to the different structure of heterocycles and different electrochemical activity of the redox centers. Further, after several scans the surface of the electrode became very uniform (from the electrochemical point of view), without any distinctly pronounced redox peaks and with high background current (compare data in Fig. 1 and curve 1 in Fig. 2A). This means that the carbon surface is covered with a number of network-like electrochemically active structures (probably three-dimensional) possessing different electrochemical activity. However, this network can facilitate electrochemical communication between the active center of the enzyme or *NAD*H and accept electrons at much lower potential than an untreated carbon surface. Thus, the modified carbon surface can act as a mediator between the active center of the redox enzyme and can mediate electrooxidation of *NAD*H.

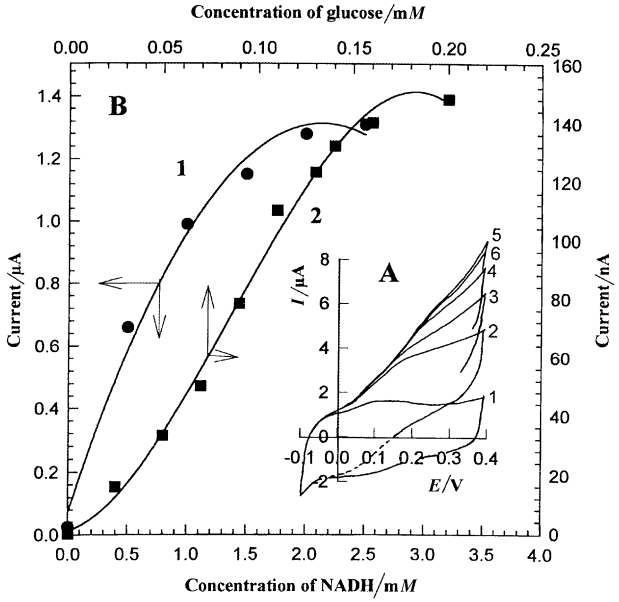


Fig. 2. A: Cyclic voltammogram of a carbon a electrode modified with **1**; sweep rate: 50 mV/s; curve 1: background, curves 2–6 have been obtained in the presence of 0.5, 1.0, 1.5, 2, and 2.5 m*M* of *NAD*H, respectively B: catalytic properties of modified carbon electrodes; curve 1: calibration curve of *NAD*H, Curve 2: calibration curve of glucose biosensor; stationary conditions, potential of the electrode: +200 mV

Catalytic properties of mediators

Electrochemical oxidation of *NAD*H on the modified carbon electrodes showed the reversible character of the mediator and was observed at potentials near 0.2 V. Typical cyclic voltammogrames are presented in Fig. 2A. As can be seen, the value of the catalytic current of electrooxidation of *NAD*H depends on the concentration of *NAD*H. Curve 1 in Fig. 2B shows the current dependence on *NAD*H concentration under stationary conditions at 200 mV. The calibration curve has a linear current-concentration dependence up to 1.1 mM of *NAD*H. Almost the same range of linearity was obtained with all 26 investigated mediators. This means that modified and electrochemically pre-treated carbon electrodes have similar electrochemical surfaces with close diffusion parameters. Calculated sensitivity of the sensor is 1.3 μA/m*M*. The sensitivities for all investigated compounds are presented in Table 1 and serv as an index of the electrochemical activity of the substance as a mediator. The sensitivity of sensors, however, was different and depends on the nature of the mediator. Molar concentrations of mediators were the same in all experiments, and differences of sensitivity can be explained by different sorption parameters of mediators and by differences in *NAD*H oxidation rates. The best mediator for *NAD*H oxidation was 1-methylbenzimidazolinone-2 (**5**).

The sensitivity of electrodes to *NAD*H oxidation was measured under potentiostatic conditions at 200 mV *vs*. Ag/AgCl (except some determinations which were carried out at higher potentials as mentioned in Table 1). The dependence of the catalytic current on the applied potential for some

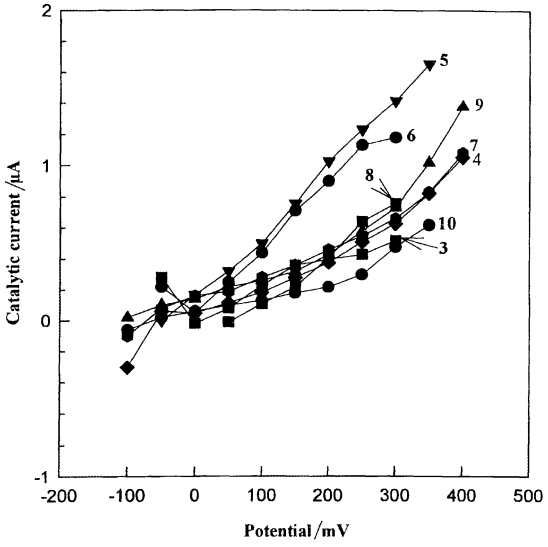


Fig. 3. Dependence of the current on the potential for different mediators; the numbers of the curves correspond to the numbers of compounds in Table 1; the data were obtained under stationary conditions; concentration of *NAD*H: 1 m*M*

benzimidazolinone derivatives is shown in Fig. 3. Introduction of electron accepting groups into the molecule of the mediator does not change considerably the sensitivity of the mediator, whereas electron donor groups such as a methyl or *iso*-propenyl significantly increase the sensitivity of *NAD*H oxidation. However, the replacement of a methyl group with ethyl (compare **1** with **2** and **11** with **12**) leads to a decrease of sensitivity. Quinones are well known mediators of *NAD*H electrooxidation [28], but the sensitivities of some carbon electrodes modified with quinone derivatives **14–17** have been found to be not higher than in the case of other investigated mediators. Probably, the limiting step of electrooxidation process is not the structure but the diffusion rate of the mediator. Benzodiazepine derivatives also show catalytic properties in the oxidation of *NAD*H, but interpretation of this phenomenon is too complicated to ensure a conclusion about the mechanism of electrochemical oxidation of *NAD*H. The absence of well-pronounced peaks of electrooxidation of *NAD*H points to a complicated multi-step process. It is possible that the sensitivity of the modified electrodes is limited by the structure of polymerized heterocycles rather than by individual electrochemical parameters of the mediators.

A number of publications and our experience show that the active site of glucose oxidase, as well as that of *PQQ* dependent glucose dehydrogenase, does not communicate directly with the carbon surface of the electrode, since *FAD* or *PQQ* are hidden inside the protein. Only small molecules like ferrocene, phenoxazines, or quinones can transfer electrons from the active centre to the surface of the electrode [9, 10, 15–18].

Adsorption of glucose oxidase on the modified carbon electrodes does not increase the background current of the electrode in the potential range from 0 to 0.5 V. Addition of glucose to the buffer solution leads to an increase of the anodic

current of the electrode. This means that the carbon surface modified with the compounds given in Table 1 and electrochemically pre-treated can communicate with *FAD* in the active center of glucose oxidase. This current-concentration relationship is shown in curve 2 of Fig. 2B. The rate of electrochemical regeneration of the active center of glucose oxidase is slower (in general) in comparison with the rate of electro-oxidation of *NAD*H, probably due to high diffusion restrictions in the globule of the enzyme and worse communication of the modified surface of the carbon electrode with the active center of the enzyme. This process is also potential dependent without strong correlation between the electrochemical parameters of mediators and sensor sensitivity. Increased sensitivity in the case of quinones was not surprising because quinones are well known mediators of glucose oxidase [29–31]. Immobilization of *PQQ* dependent glucose dehydrogenase on the surface of carbon electrodes modified with these heterocycles also leads to direct electrochemical communication between the active center of enzyme and the surface of the modified electrode [32].

It seems that enzymes are incorporated in the three-dimensional semi-oxidized polymeric structure of heterocycles on the carbon surface. This conclusion is confirmed by three facts. The calibration curve of the glucose biosensor (curve 2 of Fig. 2B) is quite short. If the K_M value of glucose ($K_M = 16$ m*M*) is taken into account, the shape of the calibration curve confirms a kinetically controlled action of the biosensor. Another fact confirming the tight incorporation of the glucose oxidase molecule into the polymeric structure is the week dependence of the sensitivity of the sensor on oxygen concentration. The competition between the natural acceptor (oxygen) and the surface of the electrode is responsible for the small lag period in the calibration curve at low concentrations of glucose. The third argument in favour of tight bonding of the enzyme globule into the polymer matrix is the stabilization of the enzyme. This phenomenon is well pronounced in the case

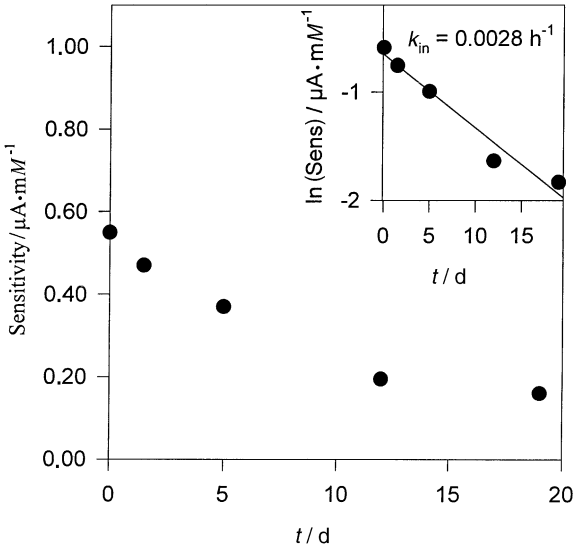


Fig. 4. Operational stability of a carbon electrode modified with **15** and *PQQ* glucose dehydrogenase at 20°C; inset: same data presented semi-logarithmically

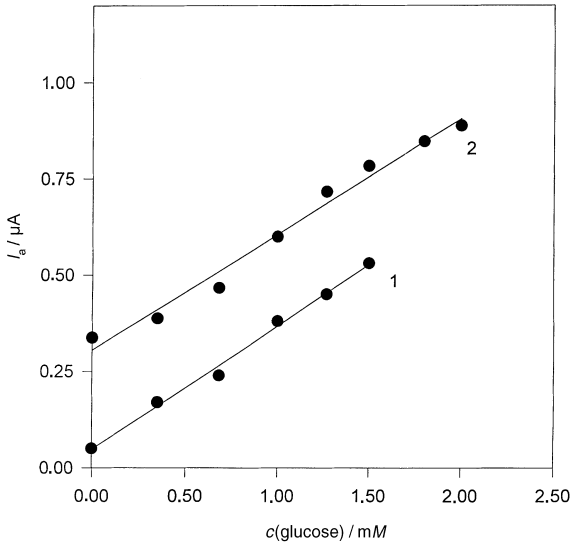


Fig. 5. Calibration curves of carbon electrode modified with **15** and *PQQ* glucose dehydrogenase *vs.* glucose obtained in phosphate buffer solution ($pH = 7.0$, 1) and in control human serum contain about 7 m*M* of glucose by consecutive addition of equal amounts of glucose (20 mm^3) to 0.5 cm^3 of serum buffer solution (1:10) (2)

of immobilization of *PQQ* glucose dehydrogenase. The activity of the enzyme incorporated into the biosensor is decreasing very slowly. This inactivation can be described as a first-order process with a rate constant of $k_{in} = 0.0028\ h^{-1}$ (Fig. 4). The inactivation of the native *PQQ* glucose dehydrogenase under the same conditions is 163 times faster ($k_{in} = 0.45\ h^{-1}$) [32].

High stabilization of the enzyme in the redox matrix and a low potential of action are essential results of this work. The shape of calibration curves obtained using buffered glucose solutions and human serum containing added glucose (Fig. 5) indicates that the surface of the biosensors is not affected by components of blood serum. The results are promising for the design of new biosensors of potential use for food industry and medicine.

Experimental

Glucose oxidase from *Aspergillus niger* and *NAD*H were purchased from Sigma. *PQQ* glucose dehydrogenase from *Erwinia sp 34-1* (30 U/cm^3) was purified in the Laboratory of Bioanalysis of the Institute of Biochemistry (Vilnius) [33]. Control human serum SERODOS was purchased from Human GmbH (Germany). Carbon rod, ultra "F" purity, 3 mm diameter electrodes were purchased from Ultra Carbon Company (USA). Compounds **1**, **14** (Merck), and **3** (Aldrich) were purchased and used without further purification. The majority of organic compounds was synthesised according to previously reported methods (Table 1). Compound **17** was kindly provided by Dr. *I. Kovalev* (MOLTECH Corp., Tucson, USA). **9** and **12** gave satisfactory elemental analyses.

All tested compounds were used as 0.1 *M* solutions in an appropriate organic solvent. For electrode modification, 3 mm^3 solution of organic compound were deposited on the tip of the carbon rod or Pt electrodes and air-dried. All electrochemical studies were carried out at 25°C using a 1 cm^3 cell equipped with a saturated Ag/AgCl reference and Pt auxiliary electrodes. Phosphate buffer

(0.05 *M*, *pH* = 7.0) containing 0.1 *M* KCl was used throughout the study. The sensitivity of modified electrodes to *NAD*H was tested under stationary conditions (working carbon electrode potential 0.2 V *vs*. Ag/AgCl. For the preparation of glucose sensors, enzyme solutions (5 U of enzyme per electrode) were deposited on the modified electrode and cross-linked by glutaraldehyde vapour. The electrodes were kept over 10% glutaraldehyde at 4°C for 2 h and washed with buffer solution. Coated electrodes were kept at 4°C until use. A polarograph OH-105 Radelkis (Hungary) was used to run the electrochemical experiments. Melting points are uncorrected. The ^{1}H NMR spectra were recorded on a Hitachi R-22 spectrometer operating at 90 MHz. Chemical shifts are reported in ppm from *HMDSO* as an internal reference and are given in δ units.

1-Acetyl-2-benzimidazolinone (**9**; $C_9H_8N_2O_2$)

A mixture of 2.1 g **10** (10 mmol), 3 cm^3 H_2SO_4 (conc.), and 5.2 cm^3 water in 40 cm^3 ethanol was kept at room temperature for 30 h. Water (50 cm^3) was added, and the reaction mixture was neutralized (*pH* = 7.0). The precipitate was collected. Recrystallisation from ethanol afforded 1.2 g (77%) of **9** which sublimates at 301–302°C without melting.

^{1}H NMR (*DMSO*-d_6): 2.53 (s, 3H, CH_3) 7.25–6.87 and 8.02–7.87 (m, 4H, aromat. H), 11.26 (br, 1H, NH) ppm.

4-Ethyl-1,2,3,4-tetrahydroquinoxalinone-2 (**12**; $C_{10}H_{12}N_2O$)

A mixture of 1.48 g **11** (10 mmol), 22 cm^3 of ethyl bromide, and 3 g of K_2CO_3 in 60 cm^3 dry methanol was refluxed for 25 h. After filtration and evaporation of the solvent *in vacuo*, the solid was crystallized from a mixture of ethyl acetate and diethyl ether (2:1) to give 1.1 g (67%) of **12**.

M.p: 120–122°C; ^{1}H NMR ($CDCl_3$): 1.17 (t, 3H, CH_3, J = 6.5 Hz), 3.19 (q, 2H, NCH_2, J = 6.5 Hz), 3.73 (s, 2H, $COCH_2$), 7.07–6.45 (m, 4H, aromat. H), 9.82 (br, 1H, NH) ppm.

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