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Biosensors for the determination of environmental inhibitors of enzymes

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Contents

I.	Introduction	1041
II.	Biosensors: general characteristics and functional features	1042
III.	Determination of kinetic parameters of inhibition	1050
IV.	Optimisation of operating conditions for inhibitor assays	1051
V.	Cholinesterase-based biosensors	1053
VI.	Conclusion	1061

Abstract. Characteristic features of functioning and practical application of enzyme-based biosensors for the determination of environmental pollutants as enzyme inhibitors are considered with special emphasis on the influence of the methods used for the measurement of the rates of enzymic reactions, of enzyme immobilisation procedure and of the composition of the reaction medium on the analytical characteristics of inhibitor assays. The published data on the development of biosensors for detecting pesticides and heavy metals are surveyed. Special attention is given to the use of cholinesterase-based biosensors in environmental and analytical monitoring. The approaches to the estimation of kinetic parameters of inhibition are reviewed and the factors determining the selectivity and sensitivity of inhibitor assays in environmental objects are analysed. The bibliography includes 195 references.

I. Introduction

Biochemical analytical methods have assumed great importance in the last years. They provide information about qualitative and quantitative composition of a subject under study on the basis of *in vitro* and *in vivo* studies of biochemical processes, which involve enzymes, antibodies, DNA, *etc*. The main advantage of biochemical methods is that the formation of analytical signals simulates the processes occurring in the living beings, which offers a unique possibility for rapid and selective detection of biologically active substances and elucidation of the mechanism of their effects on living organisms.[1,2]

Enzymic methods of analysis relate to biochemical methods. Enzymes have long attracted the attention of analytical chemists as highly efficient protein catalysts.[2,3] By virtue of their unique properties, such as high selectivity and sensitivity towards specific substrates and functioning in aqueous media at ambient temperatures,[4,5] enzymes appear to be ideal analytical reagents for the determination of biologically active substances by diverse kinetic methods. The development of enzymic methods of analysis was, to a certain extent, stimulated by the increasing demand for clinical and toxicological assays (*e.g.,* determination of major metabolites, diagnostics of various diseases and dysfunctions of discrete organs of an organism on the basis of changes in the composition of biological fluids, *etc*.).

The possibility of usage of enzymes for the determination of specific substrates and effectors has been at the centre of discussion since the late 1940's, *i.e*., since the beginning of the first systematic *ex vivo* studies of enzyme properties. However, the difficulties connected with the isolation of enzymes, their low stability in solution and high cost of analyses impeded the practical application of novel methods. As a result, until the early 1970's it was thought that enzymic methods ranked below routine physicochemical analyses. The situation changed radically owing to the progress in engineering enzymology and industrial biotechnology (*viz*., to large-scale production of a great variety of enzyme preparations, which sharply reduced their cost) and to the development of novel methods for stabilisation of enzymes including their fixation (immobilisation) on inert carriers.[6] Of no less importance for the development of enzymic methods of analysis was the creation of special-purpose measuring devices called biosensors.[2,3] In those, enzymes are integrated into a single system comprising a device for detecting biochemical signals (a sensor or a transducer) and a reaction medium containing essential stabilisers, effectors and other low-molecular-weight components required for their steady functioning. With the advent of biosensors, the maintenance of optimal experimental conditions and measurement of analytical signals have become routine laboratory procedures available for users having no special biochemical training or skills. It is the manufacture of biosensors that has opened the way for commercial application of enzymes for the determination of biologically active compounds. In 1996, the world's market of biosensors including those designed for clinical assays of glucose, urea, lactic acid and other essential metabolites ($\sim 70\%$ of the total sales) amounted to US \$ 508 million.[7]

The commercial success of medical biosensors is not exclusively due to their demand and considerable investment into scientific research. At a sufficiently high lower level of metabolite assays of the order of 10^{-4} mol litre^{-1}, high selectivity is the main requirement for the method. Thus, the advantages of enzymes as analytical reagents are especially well realised in biosensors.

The situation with enzymic monitoring of environmental pollutants is different in principle. Only cholinesterase-based biosensors designed for the detection of residual organophos-

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phorus pesticides in water and agricultural products [8] and immunoenzymic diagnostica for specific haptens have found commercial application. The reasons preventing practical application of enzymic methods in environmental monitoring are due to specific features of subjects under study and more strict requirements for analytical characteristics of determination of environmental pollutants in comparison with subjects of clinical analysis.[9]

As the requirements for the maximum permissible concentrations of toxic agents become more rigorous, novel procedures are required for the determination of pollutants (*e.g.*, dioxins and pesticides in drinking water and foods, radionuclides, *etc.*) at the level of 10^{-12} mol litre^{-1} (Ref. 10). Such low concentrations can be detected only in those cases where the pollutant is an effector (*viz.*, an inhibitor or an activator) of the corresponding enzymic reaction rather than its substrate.

Besides, qualitative and quantitative compositions of the main components of those items that subject to environmental and analytical monitoring (*e.g.,* natural and sewage waters, soils, sewage water sediments, *etc.*) are much more diverse than the compositions of biological media analysed with the use of medical biosensors. This imposes additional demands on the selectivity of biochemical signals and the methods used for their measurement. Many compounds, such as salts of alkaline and alkaline-earth metals, biogenic amines, phenols and surfactants, influence not only the rate but also the sensitivity of enzymic reactions with respect to inhibitors. Low temporal stability of specimens, high degree of mineralisation (which is especially apparent in the case of sewage water and silt extracts), the presence of microheterogeneities (suspensions, emulsions), coloured compounds, *etc.*, also restrict potential applications of biosensors. These limitations can be overcome through the use of special methodological approaches, since the use of biosensors for the analysis of environmental subjects differs in principle from their application for medical purposes.[11]

The present review summarises the published approaches to the development of biosensors designed for the determination of environmental pollutants as enzyme inhibitors and demonstrates the potentials of purposeful control over the selectivity and sensitivity of biosensors used for the assay of toxic substances.

II. Biosensors: general characteristics and functional features

The term biosensors refers to devices which contain a biological element (an enzyme or a cell culture, microorganisms, biological tissues, antibodies, *etc.*) and a means for measurement of the reaction (response) of this element to the alteration of the qualitative composition of the medium.[3,11] Some authors apply the term 'biosensors' to the supramolecular level of organisation of a biological component and thus separate enzyme-based or immune sensors from microbial and tissue biosensors.[12]

A general scheme of a biosensor assembly is shown in Fig. 1. Irrespective of its application, each biosensor consists of three elements, which determine its operational and analytical characteristics, *viz.*, a sensor, a biological component itself and a reaction medium. In addition to the subject to be assayed (*e.g.*, a substrate or an effector), the latter includes enzyme stabilisers, a supporting electrolyte, screening reagents, buffer components, *etc.*

The choice of a method used for detecting analytical signals determines not only the convenience of operation but also the limit of detection of the substrate. Preference is given to versatile methods and detectors of similar type for measuring the rates of different enzymic reactions. Electrochemical biosensors are represented by Clark's oxygen electrode, amperometric hydrogen peroxide sensors, pH-metric electrodes, field transistors and conductometric cells. In recent years, planar thin- and thick-filmed electrodes prepared from metals and carbonaceous materials which are photolithographed or screen-printed onto plastic or ceramic planar surfaces have received wide acceptance.[13,14]

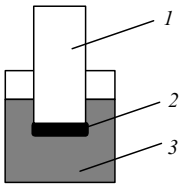


Figure 1. A schematic diagram of a biosensor; (*1*) sensor (electrode); (*2*) biological element; (*3*) working solution, *i.e.*, the reaction medium containing the analysed sample.

The selectivity of signal detection with a biosensor is provided by the selection of operation conditions for the measurement (the electrode potential, the composition of the reaction medium, *etc.*) as well as by optimisation of the biosensor design. Thus the use of electron transfer mediators makes it possible to decrease the working potential of an electrode and to exclude side oxidation (reduction) of electroreactive admixtures present in the specimen.[15] The use of additional membranes diminishes the effect of the buffer capacity of the specimen and the pH of the working solution to the response of pH-metric biosensors.[16]

The general requirements for biosensors developed for environmental and analytical monitoring were formulated by the US Environmental Protection Agency (EPA).[11,17] They are as follows: low cost of assays (US \$ 1 – 15 per assay), the possibility of detection of toxic substances at the level of 1 mg litre^{-1} – 1 μg litre^{-1} and within the range of detectable concentrations of not less than two orders of magnitude, duration of analysis of 1 – 60 min, short-term (1 – 3 h) training of laboratory personnel, application of zero or minimum supply voltage, specificity with respect to one or a group of related compounds, simplicity of the sampling procedure and portability of the device (which is designed to be easily moved by one man or transported by car).

1. The choice of an enzyme

The choice of biological elements (enzymes) to be incorporated into biosensors is usually made on the basis of a concept about the pathways of biochemical transformations of a particular compound for substrate assays or the mechanism of its toxic effect for inhibitor assays. Obviously, the great diversity of living organisms and well-characterised enzymes makes it possible in principle to detect any biologically active compound in various ways. A combination of several enzymes providing consecutive conversions of the substrate in a biosensor assembly adds new features. In each particular case, the accessibility of an enzyme (simplicity of the isolation procedure or commercial availability), its storage and immobilisation stability, efficiency of measurement procedures, detection limits and ranges of measurable substrate/inhibitor concentration are taken into account. A change in the analytical characteristics of inhibitor assays within the same enzyme – substrate system is attained by varying the sources of the enzyme, the method used for its immobilisation, the measurement conditions and the steps involved in the incubation procedure.

The data concerning the application of enzymes for assaying environmental pollutants as inhibitors [with the exception of cholinesterase (ChE) biosensors, see below] published in 1993 – 1998 are presented in Table 1. The results obtained in earlier studies are documented in several reviews.[1,9–11] It should be noted that far from every publication provides analytical characteristics of inhibitor assays, *viz.*, detection limits or the lowest limits of measured concentrations, sensitivity of assays, range of detectable concentrations, *etc.* In many cases, biosensors are used to merely demonstrate the efficiency of an idea of determination of toxic substances with immobilised enzymes (see, *e.g.*, Ref. 41) or for calculation of kinetic parameters of inhibition, usually, with reference to inhibition of free enzymes.

Table 1. Some characteristics of methods used for inhibitor assays in the environment and the experimental parameters, *viz*., concentration range (c/mol litre^{-1}), limits of detection (LD/mol litre^{-1}) and inhibitor concentrations inducing a 50% decrease in the biosensor response (I_{50}/mol litre^{-1}).

Enzyme	Method	Conditions of measurement and enzyme stabilisation	Inhibitor	Parameters of determination of the inhibitor			Ref.
				c	LD	I_{50}	
Oxidoreductases							
Cytochrome oxidase (EC 1.9.3.1)	amperometric (cyclic voltamperometry)	the enzyme was incorporated into a carbon paste together with azolectin and cytochrome *c* from horse heart; phosphate buffer, pH 7.0	cyanides	$(0.5-12)\times10^{-6}$	–	–	18
	amperometric (linear voltamperometry)	electrochemical polymerisation of pyrrol on a planar gold electrode with subsequent immobilisation with glutaraldehyde; phosphate buffer, pH 7.0	atrazine	–	5×10^{-6} (see [a]) 1×10^{-5} (see [b])	–	19
	pulse amperometry, chronocoulometry	immobilisation by cross-linking with glutaraldehyde on the surface of a glassy carbon electrode in the presence of a mediator (1,2-naphthoquinone-4-sulfonate); phosphate buffer, pH 7.0	benzoic acid	$1\times10^{-5}-5\times10^{-4}$	3×10^{-6} (see [c])	–	20
	UV/visible spectrophotometry	the enzyme, myoglobin and hemoglobin were incorporated into a sol-gel matrix	nitrogen(II) oxide and carbon monoxide	around 10^{-6}	–	–	21
Tyrosinase (EC 1.14.18.1)	amperometric (cyclic voltamperometry)	the enzyme was incorporated into a carbon paste (4%); phosphate buffer, pH 7.0; catechol was used as a substrate	cyanides	–	1×10^{-5}	–	18
	amperometric (Clark's electrode)	sorption on paper with subsequent application of a dialysis membrane; hexane saturated with phosphate buffer, pH 7.0; catechol was used as a substrate	phenylthiourea *N,N*-diphenylthiourea ethylphenylthiourea benzylthiourea isopropylthiourea allylthiourea	– – – – – –	0.013×10^{-6} 0.045×10^{-6} 0.078×10^{-6} 0.104×10^{-6} 0.137×10^{-6} 0.181×10^{-6}	0.80×10^{-6} 1.83×10^{-6} 3.08×10^{-6} 4.58×10^{-6} 5.55×10^{-6} 6.91×10^{-6}	22
	amperometric (linear voltamperometry)	the enzyme was immobilised on the electrode surface; phosphate buffer, pH 7.4; phenol was used as a substrate	benzoic acid, thiourea, propyl gallate	–	–	–	23
	amperometric (by quinone reduction current)	immobilisation in polypyrrol formed on the electrode surface by oxidation of substituted pyrrol; catechol, dopamine, L-DOPA and epinephrine were used as substrates; air-saturated phosphate buffer, pH 6.5	3,4-dichlorophenol chloroisopropyl phenylcarbamate 3-chloroaniline atrazine cyanide	– – – – –	0.4×10^{-6} 2×10^{-6} 2×10^{-6} 4×10^{-6} 0.02×10^{-6}	– – – – –	24
	amperometric (detection of stationary signals)	the enzyme was incorporated into a carbon paste prepared from mineral oil without a cross-linking reagent (immersion or planar electrodes); phenol was used as a substrate; phosphate buffer, pH 7.4	hydrazine methylhydrazine dimethylhydrazine	– – –	– – –	4.5×10^{-4} 5.5×10^{-4} 6.3×10^{-4}	25
	amperometric (cyclic and pulse amperometry, chronocoulometry)	the enzyme was immobilised on the surface of a glassy carbon electrode by cross-linking with glutaraldehyde without the carrier; phosphate buffer, pH 7.0; potassium ferricyanide is used as a mediator	cyanides	around $10^{-3}-10^{-4}$ (see [c])	–	–	26

Table 1 (continured).

Enzyme	Method	Conditions of measurement and enzyme stabilisation	Inhibitor	Parameters of determination of the inhibitor			Ref.
				c	LD	I_{50}	
Tyrosinase (EC 1.14.18.1)	amperometric (detection of stationary signals)	screen-printed carbon electrodes placed onto a ceramic mount (working area 1×5 mm); the enzyme was incorporated into a carbon paste. catechol was used as a substrate; phosphate buffer, pH 7.4	diethyl dithiocarbamate	–	2×10^{-6}	9.2×10^{-5}	27
			benzoic acid	–	5×10^{-6}	1×10^{-4}	
			4,6-dinitro-*o*-cresol	–	9×10^{-6}	2×10^{-4}	
			thiourea	–	4×10^{-6}	–	
			2,4-D	–	–	7×10^{-6}	
	amperometric (linear voltamperometry)	tyrosinase isolated from meadow mushroom (*Agaricus campestris*) carposomes was incorporated into a carbon paste; catechol was used as a substrate; phosphate buffer, pH 7.4	diethyl dithiocarbamate	–	–	4×10^{-5}	28
			benzoic acid	–	–	5.8×10^{-5}	
			thiourea	–	–	6.2×10^{-5}	
	the same	the enzyme was incorporated into a hydroxycellulose cryogel; the measurements were performed in chloroform, chlorobenzene and 1,2-dichlorobenzene containing tetraethylammonium perchlorate	benzoic acid,				29
			in chloroform,	$(1-16) \times 10^{-6}$	0.05×10^{-6}	–	
			in chlorobenzene,	$(10-85) \times 10^{-6}$	1×10^{-6}	–	
			in 1,2-dichlorobenzene	$(10-30) \times 10^{-6}$	5×10^{-6}	–	
			thiourea,				
			in chloroform,	$(1-35) \times 10^{-6}$	0.5×10^{-6}	–	
			in chlorobenzene,	$(5-100) \times 10^{-6}$	2×10^{-6}	–	
			in 1,2-dichlorobenzene	$(25-85) \times 10^{-6}$	1×10^{-5}	–	
			2-mercaptoethanol,				
			in chloroform,	$(0.5-70) \times 10^{-6}$	1×10^{-7}	–	
			in chlorobenzene,	$(0.1-35) \times 10^{-6}$	0.05×10^{-6}	–	
			in 1,2-dichlorobenzene	$(0.5-70) \times 10^{-6}$	0.5×10^{-6}	–	
	amperometric, coulometric	tyrosinase was immobilised on the surface of Clark's electrode together with a mediator (1,2-naphthoquinone-4-sulfonate)	diazinon	–	5×10^{-6}	1×10^{-3}	30
			dichlorvos	–	7.5×10^{-8}	2×10^{-5}	
Peroxidase (EC 1.11.1.7)	amperometric, photometric	the enzyme was immobilised by incorporation into a polymeric film (Eastman Kodak 55D) on the surface of a Pt electrode; the measurements were performed in acetonitrile, methanol, acetone and butan-2-ol containing 1.5% of water and butanone peroxide; phenylenediamine was used as a substrate	thiourea,				31–33
			in methanol,	–	–	1.2×10^{-4}	
			in acetone,	–	–	0.5×10^{-4}	
			in butan-2-ol,	–	–	2.1×10^{-4}	
			in acetonitrile	–	–	19.4×10^{-4}	
			ethylenethiourea,				
			in methanol,	–	–	1.3×10^{-4}	
			in acetone,	–	–	0.9×10^{-4}	
			in butan-2-ol,	–	–	4.5×10^{-4}	
			in acetonitrile	–	–	10.4×10^{-4}	
			methyl isocyanate,				
			in methanol,	–	–	1.8×10^{-4}	
			in butan-2-ol	–	–	3.7×10^{-4}	
			2-mercaptoethanol, in acetonitrile	–	–	20.2×10^{-4}	
	amperometric (cyclic voltamperometry)	the enzyme was immobilised by incorporation into a polymeric film (Eastman Kodak 55D) on the surface of a glassy carbon electrode; the measurements were performed in acetonitrile, methanol and acetone containing 1.5% of water, butanone peroxide and dimethylferrocene	thiourea,				34
			in acetonitrile,	–	–	2.4×10^{-4}	
			in methanol,	–	–	18.9×10^{-4}	
			in acetone	–	–	3.5×10^{-4}	

Table 1 (continured).

Enzyme	Method	Conditions of measurement and enzyme stabilisation	Inhibitor	Parameters of determination of the inhibitor			Ref.
				c	LD	I_{50}	
Peroxidase (EC 1.11.1.7)	amperometric (linear voltamperometry)	the enzyme was incorporated into a methyltriethoxysilane gel on the surface of a glassy carbon electrode modified with an Os-containing polymer; phosphate buffer under argon, pH 5.5, was used	methyl isocyanate [d]	$(4-40)\times10^{-6}$	5×10^{-7}	–	35
Alcohol oxidase (EC 1.1.3.13)	conductometric (planar Pt microelectrodes)	the enzyme was immobilised on the active surface of a field transistor by vapour of glutaraldehyde into a bovine serum albumin film and dextrin; Tris-buffer, pH 7.5; formaldehyde was used as a substrate	Hg(II)	$(1-100)\times10^{-6}$	–	–	36
Catalase (EC 1.11.1.6)	amperometric (detection of stationary signals)	the enzyme was immobilised in polyacrylamide gel; the membranes were fixed directly on the teflon membrane of Clark's electrode; the measurements were performed in a flow-injection regime; hydrogen peroxide was used as a substrate; phosphate buffer, pH 7.0	fluorides cyanides	10 [e] 100 [e]	1 [e] 1.5 [e]	– –	37
Aldehyde dehydrogenase (EC 1.2.1.5) and diaphorase (EC 1.8.1.4)	amperometric (linear voltamperometry)	diaphorase was immobilised on a membrane prepared from a copolymer of styrylpyridine and vinyl alcohol (PVA-SbQ); aldehyde dehydrogenase was immobilised on the same membrane or added to the working solution; phosphate buffer pH 7.5; propionaldehyde was used as a substrate	cineb and maneb cineb	0.15 [e] 7.4 [e], 1.5 [e]	– –	– –	38, 39
Glucose oxidase (EC 1.1.3.4)	potentiometric (pH-sensitive ion-selective field transistor, ISFT)	the enzyme was immobilised on the active surface of a field transistor by vapour of glutaraldehyde into a bovine serum albumin film	Cu(II) Co Sn Pb Ni Cd	4.0 – 3.0 [f] 4.2 – 3.2 [f] 3.8 – 2.0 [f] 3.5 – 2.2 [f] 3.8 – 2.0 [f] 2.0 – 1.5 [f]	– – – – – –	– – – – – –	40, 41
	the same	the enzyme was immobilised on the active surface of a field transistor by vapour of glutaraldehyde into a bovine serum albumin film and dextrin; Tris-buffer, pH 7.5	Ag(I)	$(1-100)\times10^{-6}$	–	–	37
β-Glycosidase (EC 3.2.1.21), mandelonitrile lyase (EC 1.11.1.7), peroxidase	"	the three enzymes were co-immobilised on the active surface of a field transistor by vapour of glutaraldehyde into a bovine serum albumin film; citrate buffer, pH 6.0	cyanogenic glycosides (amygdalin)	$(1-30)\times10^{-5}$	–	–	42

Table 1 (continured).

Enzyme	Method	Conditions of measurement and enzyme stabilisation	Inhibitor	Parameters of determination of the inhibitor			Ref.
				c	LD	I_{50}	
Miscellaneous oxidoreductases							
L-glycero-phosphate oxidase, pyruvate oxidase, lactate dehydro-genase	amperometric (Clark's electrode)	the enzyme was immobilised on the surface of Clark's electrode; Tris-buffer	$HgCl_2$	–	–	20×10^{-6}	43
			$HgCl_2$	–	–	0.05×10^{-6}	
			$HgCl_2$	–	–	1×10^{-6}	
			$AgNO_3$	–	–	0.1×10^{-6}	
			$CdCl_2$	–	–	10×10^{-6}	
			$ZnCl_2$	–	–	10×10^{-6}	
			$Pb(CH_3COO)_2$	–	–	50×10^{-6}	
			$CuSO_4$	–	–	25×10^{-5}	
alcohol oxidase	amperometric (detection of stationary signals)	the enzymes were immobilised by cross-linking using glutaraldehyde into a bovine serum albumin film or on activated nylon (Immobilon), on the surface of a planar ruthenium-coated (0.5%) carbon-paste electrode; Dulbecco's phosphate-buffered saline was used; incubation for 5 min	Hg(II)	–	0.05 [g]	–	44
			Cu(II)	–	0.05 [g]	–	
glycerol 3-phos-phate oxidase			Hg(II)	–	0.5 [g]	–	
choline oxidase, glutathion oxidase, sarcosine oxidase			Hg(II)	–	1.0 [g]	–	
			V(V)	–	20 [g]	–	
			Se(IV)	–	0.015 [g]	–	
			Ni	–	0.10 [g]	–	
Hydrolytic enzymes							
Liver esterase (EC 3.1.1.1)	photometric (by NADH absorption)	esterase and NAD-dependent alcohol dehydrogenase were co-immobilised on glass; the product of enzymic hydrolysis of the substrate (ethyl propionate), *i.e.*, ethanol oxidised by alcohol dehydrogenase, was assayed in a flow-injection regime	fluorides	$(0.8-8) \times 10^{-6}$	–	–	45
Acid phosphatase (EC 3.1.3.2)	amperometric (detection of stationary signals)	the enzyme (tissue sections of potato tubers) was co-immobilised with glucose oxidase on activated nylon in the presence of polyazotidine; glucose 6-phosphate was used as a substrate; phosphate buffer pH 6.6; the measurements were performed in citrate buffer pH 6.0 at 30 °C	pesticides [h],				46
			malathion,	4.4 – 15 [e] (3.0 – 2.0) [e]	3 [e] (1.5) [e]	–	
			methylparathion,	0.7 – 12 [e] (0.7 – 8.0) [e]	0.5 [e] (0.3) [e]	–	
			paraoxon,	6.2 – 18.3 [e] (3.1 – 2.0) [e]	5 [e] (1.5) [e]	–	
			aldicarb	46 – 125 [e] (46 – 125) [e]	40 [e] (40) [e]	–	
	potentiometric	the enzyme was incorporated into a polyacrylamide gel on a dialysis membrane; 4-nitrophenyl phosphate was used as a substrate	Hg(II)	–	1	–	47

Table 1 (continured).

Enzyme	Method	Conditions of measurement and enzyme stabilisation	Inhibitor	Parameters of determination of the inhibitor			Ref.
				c	LD	I_{50}	
Alkaline phosphatase (EC 3.1.3.1)	potentiometric	the enzyme was immobilised by cross-linking using glutaraldehyde into a bovine serum albumin film on the surface of a dialysis membrane; Tris-buffer pH 7.0, 34 °C; α-D-glucose 1-phosphate was used as a substrate	Hg(II)	1	–	–	47
	amperometric (detection of stationary signals)	sorption together with glucose oxidase on the surface of a planar ruthenium-coated carbon electrode; glucose 6-phosphate was used as a substrate; the rate of oxidation of hydrogen peroxide, the product of enzymic oxidation of glucose formed upon substrate hydrolysis, was measured in glycine buffer, pH 9.0; incubation for 10 min	dichlorvos	10^{-8} – 10^{-5} (see [c])	6×10^{-8} (see [c])	–	48
Urease (EC 3.5.1.5)	fluorimetric	the enzyme was immobilised on a glass surface; the measurements were performed in a flow-type detector	Hg(II)	0.5 – 100 [g]	–	–	49
	conductometric (planar Pt microelectrodes)	the enzyme was immobilised on the active surface of a field transistor by vapour of glutaraldehyde into a bovine serum albumin film in the presence of a stabilising agent (glycerol); Tris-buffer pH 7.4; urea was used as a substrate; the inhibition was studied in a kinetic regime by the rate of signal alteration after addition of the substrate	Hg(II) Cu(II) Cd Co(II) Pb Sr	$(1-50) \times 10^{-6}$ $(2-100) \times 10^{-6}$ $(5-200) \times 10^{-6}$ $(1-50) \times 10^{-5}$ $(2-500) \times 10^{-5}$ $(1-500) \times 10^{-4}$	– – – – – –	– – – – – –	50
	potentiometric (pH-ISFT)	sorption on the surface of a field transistor coated with a polymeric film (Nafion); Tris-buffer, pH 7.0; urea was used as a substrate	Ag Hg(II) Cu(I)	– – –	0.2×10^{-6} 1.5×10^{-6} 3×10^{-6}	– – –	51
	the same	the enzyme was immobilised by glutaraldehyde on the active surface of a field transistor into a nitrocellulose film; Tris-buffer, pH 7.4; urea was used as a substrate	Hg(II) Cu Co Cd Ni Pb Sn	8.7 – 3.5 [f] 6.7 – 3.3 [f] 5.8 – 2.8 [f] 5.8 – 2.2 [f] 5 – 3 [f] 4.8 – 3.2 [f] –	– – – – – – –	– – – – – – –	40
	"	sorption on the surface of a field transistor coated with a film of poly(4-vinylstyrene); Tris-buffer, pH 7.0; the activity of the enzyme recovered following inhibition with $CuCl_2$ was determined; urea was used as a substrate	EDTA	5 – 500 [e]	–	–	52

[a] Hydrodynamic method. [b] Flow-through method. [c] Calibration curve. [d] Thiourea, thiocyanates and azides do not interfere with the determination up to concentration 1×10^{-3} mol litre^{-1}. [e] μg litre^{-1}. [f] The values of $pc = -\log c$ (mol litre^{-1}) are given. [g] mg litre^{-1}. [h] The parameters for the listed pesticides were obtained using bienzyme and hybrid (in parentheses) biosensors.

This complicates comparison of experimental results obtained by different authors.

The majority of publications devoted to the practical application of analytical enzymic methods deal with the studies of biosensors including peroxidase, tyrosinase and urease. Other enzymic systems are described in only a limited number of papers, although their potentials are rather high.

Different organic compounds are determined with horseradish peroxidase, which is resistant to denaturing reagents and elevated temperatures and manifests high specific activity, and is a reliable and sensitive tool for determining the rates of enzymic reactions. The rate of peroxidase-based oxidation of different substrates can be used for determining the concentration of hydrogen peroxide; therefore, this enzyme is a common constituent of two-enzyme biosensors designed for assaying substrates or effectors of oxidoreductases.[2,3] Tyrosinase (polyphenol oxidase) fulfils similar biochemical functions by oxidising various biogenic phenols.

Peroxidase and tyrosinase inhibitors can block the active sites of enzymes (due to the complex formation with metal cofactors and uncoupling of an electron transfer chain) or manifest non-specific inactivating effects by disturbing the tertiary structures of proteins. For example, the results of Hg(II) and Fe(III) assays which employed peroxidase with photometric detection of signals[53–55] and phenylthiourea derivatives assays using an electrochemical tyrosinase biosensor[22] (both processes were characterised by low detection limits) have made it possible to recommend these methods for direct assays of toxic compounds in environmental objects without sample concentration.

The main advantage of peroxidase and tyrosinase in comparison with other enzymes consists in the possibility of using them as biosensor constituents for measurements in organic or aqueous-organic media,[56–58] including inhibitor assays.[22,29,31–33,59,60] This makes it possible to use biosensors as detectors in liquid chromatography[61] and to carry out inhibitor assays directly on organic extracts. On the whole, the stability of enzymes in organic solvents is lower than in aqueous media, however, definite approaches to their stabilisation do exist.[62] In the case of inhibitor biosensors able to function in aqueous-organic and non-aqueous media, enzymes are incorporated into hydrophilic polymers based on polyaniline,[61] a hydrophilic vinyl chloride and pyrrole copolymer Eastman AQ[31–34] and hydrogels based on hydroxycellulose[29] and methyltriethoxysilane.[35] Electrical conductivity is achieved by adding 1% – 20% of a buffer solution or a tetraalkylammonium salt to the organic solvent. Kinetic parameters of both the enzymic reaction and the enzyme – inhibitor interaction depend on the nature of the solvent and the inhibitor. Usually, the presence of polar organic solvents miscible with water decreases the sensitivity of the biosensor to reversible inhibitors of tyrosinase and peroxidase, such as thiourea and dithiocarbamate derivatives. Nonpolar solvents have insignificant effects on the inhibition kinetics.

Urease catalyses the hydrolysis of urea. High substrate specificity of the enzyme and ease of potentiometric or photometric determination of the hydrolysis product, *viz*., ammonia, provide the practical application of urease for the determination of residual heavy metal salts in environmental objects.

Thus a flow system consisting of 26 glass capillaries with a total volume of 100 μl was developed for the determination of urease inhibitors in natural and sewage waters. Urease was immobilised on the inner surface of the gelatinised capillaries by physical sorption.[63] A water sample and a substrate solution were consecutively pumped through the capillary tubes with a peristaltic pump for 15 – 240 and 20 min, respectively, and the urease activity was measured from the concentration of the ammonia formed using a potentiometric method. The activity of immobilised urease was preserved after continuous pumping of solutions for 8 h. The use of this system made it possible to detect the presence of carbaryl (1×10^{-4} mol litre^{-1}) (4 h) and copper(II) salts (1×10^{-7} and 1×10^{-9} mol litre^{-1}) (1 and 4 h). A similar indicator system based on a urease reactor (a column filled with glass beads with immobilised urease) was used to evaluate the general contamination of waters with heavy metals.[64] Ammonia concentration was measured by a photometric (with the Nessler reagent) or a potentiometric method. The detection limits of the majority of heavy metals were $(1-2) \times 10^{-6}$ mol litre^{-1}, those for iron were $10^{-8} - 10^{-9}$ mol litre^{-1}. The selectivity of individual metal assays could not be reached. This system was tested in the monitoring of pollution of drinking water sources in the Moscow Region.

Urease biosensors based on pH-sensitive field transistors possess enhanced analytical characteristics for the determination of metal ions. The progress in this area was achieved owing to both the use of ultrathin membranes contacting with the electrode and the coating of biosensors with ion-exchange polymers, which favour the concentration of cations on the electrode surface.[51,52]

Microorganisms and sections of biological tissues incorporated into biosensors may sometimes be regarded as sources of the corresponding enzymic activity. This can increase the stability of the response and reduce the cost of biosensors, since the enzymes are in a customary microenvironment in the presence of the necessary stabilising agents and effectors. The cells possess natural mechanisms, which protect enzymes against autooxidation and hydrolysis by proteolytic enzymes. Besides, inactivated enzymes are continuously reproduced owing to catabolic intracellular processes. Some applications of tissue sections of mushroom carposomes as a source of tyrosinase[28] and of potato as a source of acid phosphatase[46] are given in Table 1.

Biosensors based on the use of complete biochemical cycles in which inhibition of a particular biochemical function is measured as an analytical signal offer radically new possibilities. Special mention should first be made of the use of biosensors for the assay of herbicides (algicides) and evaluation of general toxicity in respect of the respiratory activity of microorganisms.

Biosensors designed for measuring concentrations of phytotoxic chemical compounds incorporate chloroplasts as the biological components (as whole cells of unicellular microalgae[65–67] or within thylakoid membranes isolated from these cells[68]). Illumination of chlorophyll generates a photocurrent measured as the current of oxidation of electron acceptors added to the solution instead of natural acceptors, *viz*., carbon dioxide. Ferricyanide,[68] soluble ferrocene derivatives[65,66] and diaminodurene[67] are used as mediators. The reduction of the photocurrent is a measure of the herbicide effect of the toxicants. Thus in the case of *Synechoccus sp*. cells, the detection limit of *sym*-triazine-based pesticides is 2 μg litre^{-1}, that in the case of *Chlorella vulgaris* cells is $\sim$ 1 mg litre^{-1}. When thylakoid membranes isolated from spinach cell cultures were used, a 50% reduction of the photocurrent was observed at the following concentrations (mol litre^{-1}): 1×10^{-6} (diurone), 9×10^{-6} (atrazine), 5.5×10^{-4} (sodium nitrite), 1.7×10^{-3} (sodium sulfite), 7.2×10^{-3} [Cu(II) salts], 1×10^{-3} (Pb), 1.8×10^{-3} [Hg(II)], 2×10^{-3} (Zn) and 4×10^{-3} (Cd). These data suggest that biosensors based on chloroplasts and microalgae are promising tools, especially for the determination of residual herbicides. However, these biosensors cannot compete with enzymic ones in terms of sensitivity of metal ion assays (see Table 1).

Biochemical oxygen demand (BOD) is measured with the aid of microbial biosensors designed for detection of toxic substances in aqueous media.[69–71] The magnitude of BOD can be established within 15 – 40 min (*cf*. 5 – 20 days in the titration method) by the rate of oxygen consumption in cells immobilised on the oxygen electrode membrane. The presence of toxic substances decreases the rate of oxygen consumption. In toxicity studies, a sample is introduced simultaneously with the nutrient (determination of respiratory activity) or after it (by a characteristic bend on the respiration curve).[72–74] Commercial systems for monitoring of the toxicity of sewage waters (*e.g.*, Toxiguard,[73] RODTOX[72,75]) were developed based on some microbial biosensors. Microbial biosensors for toxicity studies allow the detection of different

substances with a broad spectrum of toxic effects ranging from disturbance of transmembrane oxygen transport (surfactants, petroleum products) to uncoupling of oxidative phosphorylation and inhibition of oxidoreductases (phenols, cyanides, heavy metal ions). However, adaptive protective detoxification mechanisms decrease the sensitivity of microbial biosensors to individual groups of toxic agents in comparison with enzyme-based electrodes. Thus the detection limit of Cu(II) ions is 0.2 mg litre^{-1} with the yeast-based electrode.[1]

In addition to respiratory activity, the intensity of luminescence of luciferase microorganisms (a biosensor version of the Microtox test) can serve as the measure of toxicity.[76] The use of an electrode containing an immobilised revertant strain of *Salmonella* for the screening of chemical compounds possessing mutagenic activity has been described.[77]

2. Effect of enzyme immobilisation

Immobilisation of enzymes usually causes significant alterations in the analytical characteristics of inhibitor assays. The main reasons for the change in the sensitivity of enzymes to inhibitors upon their transition from the native to the immobilised state are as follows:[3,5] (1) diffusion-dependent retardation of the reagent (substrate, inhibitor, reaction product) transfer to the membrane including steric hindrances in the accessibility of the active site of an enzyme for high-molecular-weight inhibitors and/or substrates (*e.g.*, proteolytic enzymes), (2) changes in the enzyme microenvironment (buffer capacity, dielectric conductivity, ionic composition) and concomitant changes in electrostatic interactions and activity coefficients of the reagents.

There is no unequivocal evidence so far that the change in the enzyme affinity for a substrate or an inhibitor upon immobilisation is due to the changes in the tertiary or quaternary structure of the protein. Physical sorption or covalent cross-linking of protein globules with bifunctional reagents results in modification of very few surface functional groups. Since active and allosteric sites occupy a small part of the protein globule surface, any change in the structure of protein globules upon immobilisation is hardly likely.

Optimisation of the immobilisation procedure is aimed at retaining the activity of immobilised enzymes and implies mild, sparing treatment in order to preserve the microenvironment of the enzyme active site. This allows one to consider the enzyme-containing film on the biosensor surface as a solid solution where the native protein is uniformly distributed and 'fixed' in a homogeneous medium, which is denser and more viscous than water. Despite its obvious arbitrary character, in many cases this model describes adequately the behaviour of biosensors incorporating an immobilised enzyme.

Diffusion-dependent retardation of the reagent transfer is the main reason for discrepancy in the results of inhibitor assays employing native and immobilised enzymes. Depending on the ratio of the rates of transfer of the substrate into the membrane and its enzymic conversions, one can distinguish two regimes of biosensor functioning[3,78] which differ in stationary distribution of the substrate throughout the membrane.

Kinetic regime. The rates of transfer and diffusion of a substrate from the solution into the membrane and its diffusion into the membrane are higher than the rate of its enzymic conversion. The reactants are uniformly distributed throughout the membrane and the active sites of enzymes are saturated with the substrate. In this case, the rate of the formation of the reaction product (and, consequently, the response of the biosensor) is determined exclusively by the kinetics of the enzymic reaction. This regime is particularly suitable for detection of enzyme effectors. However, any decrease in the enzymic activity caused by desorption, spontaneous inactivation or microbial degradation influences the biosensor response. The kinetic regime is observed in the case of low specific activities of immobilised enzymes, small thickness of the membrane and lack of the substrate transfer retardation at the membrane–solution interface.

Diffusion-dependent regime. If the activity of an enzyme is high, its diffusion-controlled transfer does not fully compensate for the decrease in the substrate concentration in the enzymic reaction. As a result, the membrane incorporating the enzyme is not saturated and part of the enzyme active sites are unoccupied. The electrode response is steady and remains at this level despite the variations in the activity of the immobilised enzyme unless the reaction regime is changed. The diffusion-dependent regime is observed in the case of high specific activity of the enzyme, retardation of the substrate transfer (*e.g.,* when additional covering membranes decreasing the rate of the substrate transfer from the solution into the enzyme-containing layer are used), *etc.*[16]

If the reaction occurs in the diffusion-dependent regime, the sensitivity of inhibitor assays is lower than in the kinetic regime. A decrease in the concentration of active sites of enzymes as a result of their interaction with their inhibitors is partly compensated for by the involvement of free, nonreacted sites in the enzymic reaction which occurs independently of the enzyme–inhibitor interactions. Thus on going from the native to the immobilised enzyme, the limits of detection of Hg(II) with choline oxidase and glycerol 3-phosphate oxidase increase 2- and 2.5-fold, respectively, while the limits of detection of Hg(II) with lactate oxidase and glutamate oxidase, of cadmium with D-amino acid oxidase and of V(V) with alcohol oxidase increase more than 20-fold.[44] Moreover, many metal ions inhibit native enzymes without influencing the activities of immobilised preparations. The limit of detection of paraoxon for a luminescent biosensor containing ChE, choline oxidase and peroxidase was 0.75 μg litre^{-1} when ChE was used as a solute and the other two enzymes were immobilised in an acrylate gel and 125 μg litre^{-1} when all the three enzymes were immobilised.[79]

The diffusion-dependent and kinetic regimes represent extreme functioning regimes for biosensors and are separated by a vast transition region where the biosensor response is determined by both kinetic parameters of the enzymic reaction and the diffusion coefficients of the reagents. A comprehensive mathematical description of responses of amperometric and potentiometric biosensors has been given.[3,80–84] However, these models do not take account of the pH dependence of the rate of the enzymic reaction, which is a rough approximation, since the change in pH value of an enzyme-containing membrane can vary from 1 to 3 units during the measurements of biosensor responses.

No simulation of the effect of inhibitor on responses of biosensors including stationary and dynamic responses caused by the addition of substrates (dynamic or transition responses) have been carried out thus far. This may be due to significant complication of the mathematical model in comparison with the description of kinetics of reactions of immobilised enzymes. Besides, in the majority of cases kinetic parameters of individual stages of the inhibition reaction essential for simulation are unknown.

In the simplest case, the effect of an inhibitor can be described in terms of a mathematical model of the biosensor response in the absence of the inhibitor. In the case of uniform distribution of the inhibitor throughout the membrane, a decrease in the biosensor response can be simulated using the dependences of the biosensor response on the substrate concentration calculated for different rates of enzymic reaction. A change in the biosensor response as a result of its contact with the inhibitor will be similar to that observed in the case where a smaller concentration of the enzyme is used for the preparation of the membrane.

This approach does not take into account the effect of enzyme immobilisation on the mechanism of inhibition. In particular, on going from native to immobilised ChE the mechanism of inhibition by *N*-methyl-4-piperidinyl benzylate or tacrine (1,2,3,4-tetrahydro-9-aminoacridine)[85] is changed, which is associated with steric factors (*e.g.*, limited access of the inhibitor to the membrane or a change in the ratio of substrate/inhibitor concentrations in the membrane in comparison with that in solution) rather than with changes in the mechanism of enzyme–inhibitor

interactions. The necessity of estimating the contribution of the final rate of the inhibitor transfer into the membrane brings about additional problems.

III. Determination of kinetic parameters of inhibition

The choice of methods for quantitative evaluation of effects of inhibitors on immobilised enzymes incorporated into biosensors is determined by the type of interactions in 'enzyme–substrate–inhibitor' systems and the procedure used for measuring of analytical signals implemented in biosensors. Hereinafter it is assumed that the enzymic reaction occurs in accordance with the classical Michaelis–Menten mechanism:[4,5]

$$E + S \rightleftharpoons E{-}S \longrightarrow E, \quad E{-}S \longrightarrow P \qquad (1)$$

where E is the enzyme, S is the substrate, ES is the enzyme–substrate complex and P is the enzymic reaction product.

An important feature of the inhibition mechanism is the reversibility of interaction of the inhibitor with the active site of the enzyme. It is necessary to distinguish between reversible and irreversible enzyme inhibition upon its reaction with the inhibitor in the absence of the substrate depending on the type of dissociation of the enzyme–inhibitor complex.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{-}I \xrightarrow{k_2} (E{-}I)' \longrightarrow E, \quad E{-}I \xrightarrow{k_2} I', \quad (E{-}I)' \longrightarrow I'' \qquad (2)$$

where I is the inhibitor, E–I is the enzyme–inhibitor complex and I′ and I″ are the inhibitor decomposition products. If the dissociation of the E–I complex occurs with simultaneous decomposition of the original inhibitor, the inhibition is irreversible ($k_2 \gg k_{-1}$); if the dissociation of the complex into the original molecules prevails ($k_{-1} \gg k_2$), the inhibition is reversible.

The indication to the type of inhibition does not provide any information about the possibility of restoration of enzymic activity after the interaction of the inhibitor with the enzyme. Thus carbamates cause irreversible inhibition of ChE, but spontaneous hydrolysis of carbamoylated ChE occurs very fast and the enzyme activity is completely restored. The reactivation rate constant of carbamoylated acetyl cholinesterase (AChE) from electric eel is 0.012 min^{-1}, while that of phosphorylated AChE is 10^{-5} min^{-1} and the corresponding value for the hydrolysis of the acetylated enzyme formed upon enzymic cleavage of the substrate is 6×10^5 min^{-1} (see Ref. 86). Inhibition of urease with heavy metal ions is also expected to be reversible; however, the restoration of enzymic activity requires the use of complexones, *e.g.*, EDTA.[51,52] Toxic irreversible inhibitors, *e.g.,* contaminants-xenobiotics, are more dangerous to living organisms than reversible inhibitors because of the low rate of spontaneous reactivation and the lack of efficient detoxification mechanisms.

1. Irreversible inhibition

Irreversible inhibition is evaluated quantitatively by the level of enzymic activity before and after the contact of the enzyme with the inhibitor. If the concentration of the inhibitor is much higher than that of the active sites of enzymes, irreversible inhibition will be described by a second-order kinetic equation [87]

$$\ln \frac{v_0}{v_t} = k_{II} c_I \tau, \qquad (3)$$

where v_0 and v_t are the initial rates of the enzymic reactions before and after the contact of the enzyme with the inhibitor, respectively, k_{II} is the inhibition bimolecular rate constant, c_I is the concentration of the inhibitor and τ is the duration of the enzyme–inhibitor interaction (incubation step). The value of k_{II} depends on the nature of the enzyme and the inhibitor as well as on the reaction conditions and rate constants in separate steps of the reaction. In particular, at $\tau \to 0$:

$$k_{II} \to \frac{k_1 k_2}{k_{-1} + k_2}.$$

The value of k_{II} describes the inhibitory activity of the toxicant better than I_{50}, since it allows comparison of the results obtained under different experimental conditions, particularly, for different τ.

If the concentrations of the enzyme active sites and the inhibitor are comparable, the dependence of the relative decrease in the rate of the enzymic reaction on the inhibitor concentration for strictly irreversible inhibition is linear. However, this dependence, as applied to biosensors, is not realised, since the specific activity of immobilised enzymes should be rather high in order to provide high accuracy of measurement of the response.

In the case of irreversible inhibition (*i.e.*, at low rate of spontaneous reactivation), the inhibiting effect depends on the time of incubation and the calibration curve for the inhibitor assay is linear in the $c_I - \ln(v_0/v_t)$ coordinates and passes through the origin of the coordinates. Moreover, the relative decrease in the activity of the enzyme (the biosensor response) as a result of incubation does not depend on the concentration of the substrate, which is especially important for optimisation of conditions of inhibitor assays.

2. Reversible inhibition

A kinetic description of reversible inhibition is more complex than that of irreversible one, since the interaction of the enzyme active site with the substrate and the inhibitor may occur in different ways:

$$\begin{array}{ccccc} E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E{-}S & \xrightarrow{k_2} & E + P \\ I \;\Big\downarrow\!\Big\uparrow\; K_i & & I \;\Big\downarrow\!\Big\uparrow\; \alpha K_i & & \\ E{-}I + S & \underset{\alpha k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E{-}S{-}I & \xrightarrow{\beta k_2} & E{-}I + P \end{array} \qquad (4)$$

Here E–S–I is the enzyme–substrate–inhibitor complex, K_i and αK_i are the equilibrium constants of the enzyme–inhibitor interaction, k_1, k_{-1}, k_2, αk_{-1} and βk_2 are the rate constants of the corresponding steps of the enzymic reaction. Usually, for the inhibitors $\alpha > 1$ and $\beta < 1$, but at $\beta \to 0$ the coefficient α is < 1. For enzyme activators, the kinetic mechanism of which is described by the same equation, $\alpha < 1$ and $\beta > 1$.

Knowing the dependences of reaction rates on concentrations of the substrate and the inhibitor, one can determine the type of inhibition and the values of K_i and αK_i.[88,89] In actual practice, extreme cases may occur. Thus at $\alpha \to \infty$, complete competitive inhibition takes place. The latter also includes the interaction of the enzyme with an irreversible inhibitor in the presence of the substrate. If $\alpha \to 0$, the inhibition is referred to as non-competitive. The corresponding constants for the formation of inhibitory complexes, K_i and αK_i, are termed as competitive and non-competitive ones. Mixed type of inhibition is characterised by a combination of competitive and non-competitive inhibition, *i.e.*, in this case the inhibitor binds both to the active centre of the enzyme and the enzyme–substrate complex. Incompetitive inhibition is a particular case of mixed inhibition, if $\alpha = 1$ and $\beta = 0$.

The competitive (K) and incompetitive (K') components of mixed inhibition are calculated from the dependence of relative changes in the rate of the enzymic reaction on the concentrations of the substrate and the inhibitor:

$$\frac{v_0}{v_i} = \frac{K_M(1 + c_I/K) + c_S(1 + c_I/K')}{K_M}, \qquad (5)$$

where υ_0 and υ_i are the reaction rates in the absence and in the presence of the inhibitor and K_M is the Michaelis constant of the enzymic reaction in the absence of the inhibitor.

The total inhibiting effect is described by the reduced inhibition constant

$$\overline{K}_i = \frac{K_i K_i'}{K_i + K_i'}.$$

The constants k_{II} and K_i reflect the sensitivity of the enzyme–substrate system to the inhibitor. Their values can be used for the identification of inhibitors using a set of enzymes differing in their sensitivities to toxic agents. If the experiments are run under identical conditions (incubation time for irreversible inhibitors, substrate concentration for reversible inhibitors), it is sufficient to determine the relative changes in the rate of the enzymic reaction according to the formulae:

irreversible inhibition

$$\frac{(k_{II})_1}{(k_{II})_2} = \left(\ln\frac{\upsilon_0}{\upsilon_i}\right)_1 \Big/ \left(\ln\frac{\upsilon_0}{\upsilon_i}\right)_2; \quad (6)$$

reversible inhibition

$$\frac{(\overline{K}_i)_1}{(\overline{K}_i)_2} = \left(\frac{\upsilon_0}{\upsilon_i}\right)_1 \Big/ \left(\frac{\upsilon_0}{\upsilon_i}\right)_2.$$

The indices 1 and 2 refer to the kinetic data obtained in experiments with enzyme preparations differing in the degree of purity, the method of isolation, the source, *etc*.[90]

The relative sensitivity of enzymes to a definite group of inhibitors is established in a similar way. By illustration, the sensitivity of ChE isolated from different sources can be characterised by the relative value:

$$\exp\left(\sum_n \frac{\ln(k_{II}^i/k_{II}^0)}{n}\right),$$

where the index i relates to the enzyme the relative sensitivity of which is being calculated, the index 0 relates to the reference enzyme and n designates the number of inhibitors. The ratio of inhibition constants, k_{II}, is established for each insecticide and is then summated for all the pesticides under study.[91]

Estimation of inhibition parameters of immobilised enzymes including those incorporated into biosensors can also be used in the case of kinetic regimes. Biosensors in which the enzyme is immobilised on the electrode surface (without any carrier) or in a thin hydrophilic layer which has no substantial effect on the diffusion coefficients of the substrate and the inhibitor are best suited for this purpose. Albumin, polysiloxanes, silicates, hydrophilic polymers of the Eastman AQ 55D type, *etc*., are used as carriers.

The feasibility of formal equations for Michaelis–Menten kinetics provides indirect evidence for the lack of diffusional limitations. In particular, for tyrosinase- or peroxidase-based biosensors the current (I) of the reduction of the reaction product (quinone) or of the oxidised form of the mediator can be used as a measure of the rate of the enzymic reaction. The calibration curve for the substrate is linearised in double reciprocal coordinates $I^{-1} - c_S^{-1}$, whereas the segment intercepted on the abscissa gives the value of K_M^{-1} as well as the Lineweaver–Burk equation. Other kinds of anamorphoses of kinetic curves known in homogenous enzymic reaction kinetics can also be used for this purpose. Thus a calibration curve for determining reversible competitive or incompetitive inhibitors at a constant concentration of the substrate is linearised in the coordinates $I^{-1} - c_I^{-1}$; the inhibition constant, K_i, is determined from the segment intercepted on the abscissa.[24]

In some cases, it is more preferable to use the degree of inhibition of the enzyme (Y) instead of the biosensor response. This offers a possibility of using the data obtained for different experimental conditions for calculation. For example, the value of Y relates to the response of an amperometric biosensor:

$$Y = \frac{c_{E-I}}{c_{E-I} + c_E} = \frac{c_I}{c_I + K_i} = \frac{\Delta I}{I_0}, \quad (7)$$

where c_{E-I}, c_E and c_I are the concentrations of the enzyme–inhibitor complex, of the free centres of the enzyme and of the inhibitor, respectively, ΔI is the change in the biosensor response upon inhibition and I_0 is the biosensor response before the contact of the enzyme with the inhibitor.[29,31–34] Derivation of Eqn (7) *a priori* entails a fully competitive type of inhibition, since it is in this particular case that the decline of the response will be proportional to the concentration of the enzyme–inhibitor complex.

For practical purposes, the ratio (7) is linearised in the $Y/c_I - Y$ coordinates (the Scatchard equation) to determine K_i:[31]

$$\frac{Y}{c_I} = -\frac{Y}{K_i} + \frac{I_0}{K_i} \quad (8)$$

or in the $Y/(1-Y) - c_I$ coordinates (the Hill equation):[92]

$$\frac{Y}{1-Y} = \frac{I}{I_0 - I} = \left(\frac{c_I}{K_i}\right)^x = \left(\frac{c_I}{I_{50}}\right)^x, \quad (9)$$

where x is the empirical Hill coefficient; $x = 1$ in the case of the Michaelis–Menten mechanism, the value x for organic solvents increases to 3–4.[18,22,29] The Hill coefficient serves as a criterion of efficiency of the catalysis I_0/K_M' (K_M' is the experimental value of the Michaelis constant) and the effect of the solvent on the kinetics of the enzymic reaction.

Equations (7)–(9) were obtained assuming that the response of the biosensor is proportional to the rate of the enzymic reaction. On the whole, the dependence $\upsilon - I$ is sigmoidal and is approximated by a linear dependence in a narrow range of substrate or inhibitor concentrations.

In the case of irreversible inhibition, the inhibition bimolecular constant is calculated from Eqn (3). By expressing υ_0/υ_i through the degree of inhibition Y, we obtain the following ratio:

$$k_{II} = -\frac{\ln(1-Y)}{c_I \tau}. \quad (10)$$

The plots are linearised in the $\ln(1-Y) - c_I$ coordinates or, if the degree of inhibition is expressed on a percentage basis, $\ln(100-Y) - c_I$.[93]

If the kinetic parameters k_{II} and K_i are used for comparative characterisation of the enzyme–inhibitor interaction, it should be remembered that they do not provide any direct information about the analytical characteristics of inhibitor assays, such as limits of detection or a concentration range.

IV. Optimisation of operating conditions for inhibitor assays

1. Concentration of the substrate

A crucial role in the choice of experimental conditions belongs to the choice of working concentrations of the substrate. In irreversible inhibition, the relative decrease in the rate of enzymic reactions does not depend on the measurement conditions. If a change in the rate of an enzymic reaction by 15%–25% is taken as a statistically significant quantity, the limit of detection estimated by Eqn (3) is $(0.13-0.22)/k_{II}\tau$ In particular, the calculated values of the limit of detection of organophosphorus pesticides (τ = 2–30 min, k_{II} = 10^5–10^7 mol^{-1} litre min^{-1}), are in the range of 10^{-9}–10^{-7} mol litre^{-1}, which is consistent with the published data for enzymic assays of pesticides with free ChE.[8,86,94]

The value of v_0/v_t can be determined by analysis of any characteristics of the system which are proportional to the reagents concentration, *e.g.*, the time needed for reaching a definite (normally, not more than 30%) level of substrate conversion:

$$\frac{v_0}{v_t} = \frac{t_i}{t_0},$$

where t_0 and t_i are the times of the enzymic reactions before and after the interaction of the enzyme with the inhibitor, respectively.

The choice of small initial concentrations of substrates ($c_S \ll K_M$) has a number of advantages. If a substrate participates in several enzymic reactions (the presence of enzymes with low substrate specificity in the original preparation, significant differences in the kinetic parameters of isoenzymes) or if several substrates interact with the same enzyme, the general view of the equation describing the rate of the target enzymic reaction will be complicated:

$$v_0^1 = \frac{v_{max}^1 c_S^1}{\left[1 + \sum_{i=2}^{i=n} \frac{c_S^i}{K_M^i}\right] K_M^i + c_S^1}, \qquad (11)$$

where v_{max}^1 is the maximum rate of the enzymic reaction; the index 1 relates to the target enzymic reaction the rate of which is being determined and the index *i* relates to reactions between competing substrates/enzymes which occur in parallel. If the initial rate of the substrate c_S^i is sufficiently small ($c_S^i \ll K_M'$), the equation is simplified:

$$v_0^1 = \frac{v_{max}^1}{K_M^1} c_S^1,$$

and the effect of simultaneously occurring reactions can be neglected.[95]

For competitive and mixed–competitive ($\alpha > 1$) inhibition, the limit of detection of the inhibitor decreases with an increase in the substrate concentration. At $c_S \ll K_M$, the limit of detection of environmental pollutants ($K_i = 10^{-4}$–10^{-6} mol litre^{-1}) by the most common methods is $(0.15–0.25)K_i$ or in the range of 10^{-5}–10^{-7} mol litre^{-1}.

At $c_S \gg K_M$, the limit of detection of non-competitive inhibitors ($\alpha = 1$) does not depend on the substrate concentration. For incompetitive and mixed-non-competitive inhibition ($\alpha < 1$), the limit of detection of inhibitors decreases with an increase in the substrate concentration. In other cases, any increase in the substrate concentration will increase the limit of detection of the inhibitor.

While estimating limits of detection, one should remember that inhibitory effects are established on the basis of results of two response measurements, *viz*., before and after the contact of the enzyme with the inhibitor. Therefore, the minimum value of the degree of inhibition is equal to the double measurement accuracy of the biosensor response to the substrate. The metrological characteristics of sensors incorporated into biosensors can be used as a preliminary estimate. Thus the magnitude of steady-state oxidation (or reduction) currents on solid electrodes can be measured with ~ 1%–2% accuracy, while that of the potential of the ion-selective electrode is ~ 3%. Consequently, the minimum significant inhibition corresponds to a decrease in the amperometric and potentiometric responses by not less than 2%–4% and ~ 6%, respectively. Even in those cases where the calibration curve for the inhibitor is plotted from the absolute value of the response, it is assumed that the initial response of the biosensor (before its contact with the enzyme) is constant. In other words, the measurement error of the biosensor response after its contact with the inhibitor includes the measurement error of its response to the substrate. Therefore, whilst choosing a detection method (for irreversible, non-competitive, incompetitive and mixed–non-competitive inhibitors) preference should be given to the methods which ensure more accurate measurements of the biosensor response rather than the possibility of detection of smaller concentrations of the substrate. In other cases, sensors allowing maximum reduction of working concentrations of the substrate will be the sensors of choice.

2. Enzyme reactivation

The ability of enzymes to regain their activity after measurement of the inhibitory effect is a prerequisite for repeated application of biosensors. In the case of reversible inhibitors, this is achieved by washing of biosensors or enzyme-containing membranes with working buffer solutions which may contain stabilisers or enzyme cofactors preventing its inactivation. In some cases, natural reactivation occurs so slowly that under real experimental conditions it is necessary to use special reagents accelerating the dissociation of enzyme–inhibitor complexes. For example, heavy metal assays make use of complexing or precipitating reagents, such as EDTA, potassium iodide, sulfur-containing organic compounds (cysteine, dithiothreitol, *etc.*);[36, 51, 52, 96, 97] while antidotes (*i.e.*, substances that counteract poisons in human beings) are often used as reactivating reagents in the analysis of organic inhibitors. Thus 2-PAM (2-pyridinaldoxime methiodide) or TMB-4 [1,1′-trimethylenebis(4-hydroxyiminomethyl)pyridinium dibromide monohydrate] which accelerate hydrolysis of the phosphorylated active site of ChE are used for reactivation of the enzyme after its interaction with organophosphorus compounds.[98]

The efficiency of enzyme reactivation depends on the nature of the reactivating agent, the degree of enzyme inhibition, the nature of the carrier and the time elapsed between inhibition and reactivation. By illustration, phosphorylated ChE which is reactivated at ~ 70% inhibition restores up to 95%–100% of its initial activity, which makes it possible to use the same immobilised enzyme for 15–20 assays.[8] When heavy metal salts are used as inhibitors, the reactivation amounts to 70%–80% if the enzyme is immobilised on nitrocellulose[96] and up to 100% if gelatine is used as a carrier.[97]

The effects of reactivating reagents, similar to those of inhibitors, are strictly selective. Therefore, the former can be used for separation of inhibitory effects of individual toxic agents present in the same solution as well as for selective determination of individual inhibitors. Thus it was proposed to determine Fe(III) ions according to their reactivating effects on peroxidase inhibited with cyanides or salicylic acid,[53] and to determine EDTA according to its reactivating effect on urease inhibited with Cu(II) ions.[52] An addition of sodium iodide allows one to separate the inhibitory effects of Ag(I), Hg(II) and other heavy metal ions on urease[51] and ChE.[98]

An excess of reactivating reagents can decrease the enzyme activity down to complete irreversible inhibition as a result of elimination of cofactors from the active site or the formation of more potent inhibitors, *viz*., the products formed in the reaction of the reactivating reagent with the toxic compound.[99] However, in some cases, reactivating reagents act as allosteric activators of enzymes.

3. Acidity of the working solution

The activities of enzymes and their sensitivities with respect to inhibitors are strongly pH-dependent; therefore, a crucial role in inhibitor assays is played by the correct choice of buffer solutions and pH.

In the overwhelming majority of studies, the optimisation of the composition of reaction media is aimed at eliciting maximum response of the biosensor with respect to the substrate, but not to the inhibitor. Indeed, in many cases, especially in the case of irreversible and non-competitive reversible inhibition, the pH of the medium corresponding to the maximum inhibitory effect is close to that providing optimum functioning of the enzyme (*e.g.*, in the cyanide assay with cytochrome oxidase,[18] in dithiocarbamate and cyanide assays with tyrosinase and peroxidase[27, 59] and

in organophosphorus pesticide assays with ChE and tyrosinase).[8, 30] In all those cases where the inhibitors had competitive or irreversible inhibiting action, the maximum sensitivity of the response of amperometric biosensors with respect to inhibitors was determined by the I_0/K_i ratio and the magnitude of the biosensor response to the substrates.

In many cases, the pH of the buffer solution can strongly influence the extent of inhibition. In particular, such effects are observed if the inhibitor is involved in acid–base reactions; it is noteworthy that only one of the equilibrium forms interacts with the enzyme. For example, the pH dependence of the inhibitory effect of metal ions on ChE is due to accumulation in solutions of metal hydroxo complexes MOH^+, which competitively inhibit the enzyme.[100] As a result, the effects of Cu(II), Ni and Fe(II) ions are manifested in a very narrow range of pH which is limited from the alkaline region by the commencement of precipitation of the corresponding hydroxides. Apparently, the optimum value of pH (5.0) for Hg(II) determination with peroxidase in the presence of thiourea is associated with the hydrolytic stability of the inhibitor.[53] Colloidal solutions of heavy metal hydroxides can also inhibit enzymes, at least after long-term incubation. Thus the inhibitory effect of Hg(II) salts on urease was studied at pH 8.9;[101] the maximum inhibitory effect of beryllium ions on alkaline phosphatase is manifested at pH 9.8.[102]

Instability of inhibitors is yet another reason for the deviation of the working pH from the optimum values. Some organophosphorus pesticides are spontaneously hydrolysed in alkaline media, which markedly decreases the reproducibility of inhibition of free and immobilised ChE.[8]

Immobilisation of enzymes can significantly alter both the value of pH corresponding to the optimum enzymic activity and the sensitivity of the enzyme to the inhibitor. This is due to a change in ionic equilibrium constants caused by the differences in dielectric conductivity and ionic strength of the microenvironment of the enzyme active site and the solution. Enzyme carriers and ballast proteins being electrolytes can represent ion-exchange materials thereby increasing buffer capacity of the enzyme microenvironment and decreasing the rate of removal of hydrogen ions formed upon enzymic reactions into the solution.[78, 103] Therefore, the results of investigations of free enzymes demand verification when passing to the study of immobilised enzymes. For example, if ChE is immobilised by microencapsulation into cellulose trinitrate, the maxima of the enzymic activity and sensitivity towards organophosphorus pesticides are shifted to the alkaline region (to pH 9–10).[104] The pH maximum is not changed upon immobilisation of ChE on paper (7.5–8), but the sensitivity of the biosensor response to pH decreases in comparison with the corresponding dependence of the free enzyme.[8]

In some cases, a change in the pH dependence of inhibition due to immobilisation extends the range of compounds that can be determined. Thus hydrazinium dialkyl dithiophosphates inhibit microbial carboxyesterase at pH 3.5–4.5 but have no effect on free ChE. On the other hand, these compounds cause reversible inhibition of ChE immobilised on paper with a gently sloping maximum at pH 6.0.[105]

When immobilised enzymes are incorporated into biosensors, the working range of pH can be narrowed due to the instability of the material used for the preparation of biosensors. For example, destruction of the electrode material limits the pH value for the operation of a cholinesterase biosensor based on a carbon–epoxide composition:[106] the assays of organophosphorus pesticides were carried out at pH 7.0, although the maximum enzymic activity was observed in the pH range 8.0–9.0. In the case of electron transfer mediators, the response of oxidoreductase biosensors was also pH-dependent, which can be ascribed to the change in the redox potential of the mediator.[35, 107, 108]

4. The presence of effectors

The effect of compounds present in solution and affecting the enzymic activity is non-additive. Irreversible inhibitors used in low concentrations represent the only exception.

The presence of both a reversible effector and a substrate reduces the irreversible inhibition owing to the so-called protective effect.[109] In the course of incubation, the active site of the enzyme binds to the reversible effector to form a labile complex, which is inaccessible to an irreversible inhibitor. Following addition of the substrate, the complex dissociates, resulting in partial restoration of enzymic activity. In the majority of cases, the protective effect impairs the analytical characteristics of irreversible inhibitor assays, *viz*., any effectors present in the sample under study including such widespread compounds as alkali and alkaline-earth metal salts, can increase at definite concentrations the limit of detection of the irreversible inhibitor. Therefore, the maximally permissible concentration of salts in the sample is specified to meet the requirements inherent in the methods of enzymic analysis of environmental pollutants irrespective of whether a free enzyme or a biosensor is used in the assay.

In some cases, enzyme effectors produce a synergistic effect, resulting in a considerable decrease in the limit of detection of the inhibitor or in an increase in the selectivity of measurement. Thus peroxidase ensures a highly sensitive determination of heavy metal salts; the inhibitory effects of Hg(II), Pb, Cd and Bi(III) increase following preincubation of the enzyme with thiourea or with 1,4-dithiothreitol for Bi(III) [54, 55] bringing about nonspecific inactivation of enzymes. With *o*-dianizidine as a substrate, the limit of detection of Hg(II) is 1×10^{-5} mg litre^{-1}, that with 3,3′,5,5′-tetramethylbenzidine is 3×10^{-6} mg litre^{-1}. Cadmium and bismuth interfere with the assay when present in a 1000- or 10000-fold excess with respect to mercury. In contrast, organomercurials suppress the inhibitory effects of sulfur-containing compounds (phenylthiourea, 1,4-dithiothreitol). Thus, the selectivity and sensitivity of the method can be varied by changing the duration of incubation, the nature of the substrate and the second effector.

In the presence of magnesium ions, the sensitivity of zinc [110] and beryllium [102] ion assays with alkaline phosphatase increases, but the reproducibility of the signal decreases, as a rule. A sharp increase in the sensitivity of ChE to pesticides (chlorofos, 2,4-D, DDT) and heavy metals was observed [104] in alkaline borate buffer in the presence of calcium ions. This effect is manifested within rather narrow ranges of pH and effector concentrations. Interestingly, 2,4-D and DDT do not inhibit free ChE; therefore, in this case either a cooperative effect of calcium ions and pesticide molecules on the active site of the enzyme is manifested or indirect indication of toxic agents, *e.g.,* owing to elimination of the activating effect of calcium ions on ChE upon its incorporation into the enzyme–inhibitor complex or to spatial blockade of the access of hydrophobic pesticide molecules to the enzyme-containing membrane.

V. Cholinesterase-based biosensors

Cholinesterase is being especially widely used and thus deserves special consideration. This enzyme is the subject of up to 60% of publications devoted to biosensors. The interest in ChE of practitioners in environmental and analytical monitoring and toxicology is due to the broad range of inhibitors which involve such widespread toxicants as organophosphorus compounds, heavy metal salts, surfactants, *etc*.[8, 86, 94]

Acetylcholinesterase provides hydrolytic cleavage of a natural neurotransmitter, *viz*., acetylcholine, *i.e*., it is responsible for the discreteness of transmission of nerve pulses.[111]

$$\underset{\text{Acetylcholine}}{Me_3N^+CH_2CH_2C(O)Me} \xrightarrow{ChE} MeC(O)\text{—}ChE \xrightarrow{H_2O} MeCOOH + ChE$$

$$\text{Acetylated ChE} \rightarrow \underset{\text{Choline (alcohol)}}{Me_3N^+CH_2CH_2OH} \qquad (12)$$

Cholinesterases differ in their substrate specificities. Thus acetylcholinesterase (AChE) (EC 3.1.1.7) hydrolyses acetylcholine at the highest rate and does not hydrolyse butyrylcholine. Its activity is inhibited by an excess of a substrate, since deacylation is inhibited by the binding of the second acetylcholine molecule to the anionic site of the acyl-enzyme.[112] Butyrylcholinesterase (BChE, EC 3.1.1.8) catalyses hydrolysis of butyrylcholine with the highest efficiency but can also react with acetylcholine. Therefore, this was termed pseudo- or nonspecific cholinesterase, in contrast with the true, specific AChE.

At present, various ChE preparations for biochemical assays including the most popular AChE reagents from electric eel ('Sigma', USA, 'Boehringer', Germany) and BChE from equine blood serum (Joint-Stock Company 'Biomed', Perm, Russia; 'Sigma', USA) are manufactured on an industrial scale. These preparations are available as freeze-dried powders, AChE is also purchased as frozen aqueous solutions containing ammonium sulfate.

Numerous esters of phosphoric, phosphonic and pyrophosphoric acids, carbamates and sulfonic acid esters are irreversible and specific inhibitors of ChE. They bind covalently to the active site of the enzyme to form phosphorylated (carbamoylated, sulfonylated) ChE. Organothionophosphorus compounds (parathion, diazinon, malathion, *etc*.) are very weak reversible inhibitors of ChE *in vitro*. This is due to the inability of the sulfur atoms to form hydrogen bonds with the hydroxy group of serine-200. In the organism of insects, thionophosphorus pesticides are oxidised by nonspecific oxidases to oxygen analogues. Thus parathion gives paraoxon, malathion gives malaoxon, *etc*.[113] As a result, the inhibitory effect of pesticides increases 10 000-fold and even more. Metabolic activation of the insecticide as a result of the so-called lethal synthesis decreases the hazard of acute poisoning of humans with simultaneous preservation of high insectoacaricidal activity of the preparations. Prior to determination of thionophosphorus pesticides with ChE, they were oxidised to the corresponding oxygen analogues with bromine water[114] or 50% nitric acid.[115] An effective procedure has been also developed for the oxidation of thionophosphates with chlorine or bromine generated *in situ* by galvanostatic electrolysis.[116]

Quaternary ammonium salts, acridine derivatives and metal ions are also known as reversible inhibitors of ChE. The activity of ChE decreases also in the presence of polar organic solvents miscible with water.[117]

Cholinesterase is widely used for the determination of residual organophosphorus and carbamate-type pesticides in water, aqueous and aqueous-organic extracts from plants, soil, food products, *etc*. The published data (1993–1998) concerning cholinesterase biosensors designed for pesticide assays are listed in Table 2.

Enzyme–inhibitor specificity. The analytical parameters of determination of ChE inhibitors strongly depend on the source of the enzyme. Cholinesterases from insects manifest the highest sensitivity with respect to organophosphorus pesticides.[91, 150, 151] Thus the inhibition constant, k_{II}, of dimethyl dichlorovinyl phosphate (DDVP) is 5×10^5 mol^{-1} litre min^{-1} for AChE from *Drosophila melanogaster*, while that for AChE from bovine erythrocytes is $< 10^3$ mol^{-1} litre min^{-1} for (see Ref. 91). Cholinesterase from cockroach allows one to determine DDVP at a concentration of 8×10^{-9} mol litre^{-1}, whereas in the case of mammalian enzymes the limits of detection are 10–100 times as high.[150] Insect ChEs are also sensitive to organochloric pesticides (2,3-D, DDT, *etc*.), which seem to be reversible inhibitors.

These enzymes are difficult to isolate in sufficiently large amounts, which restricts their practical application. In future, the situation may change owing to the progress in gene engineering. The preparation of AChE from *Drosophila melanogaster* by incorporation of a mutant gene responsible for AChE synthesis into the genetic apparatus of microorganisms has been described. The yield of AChE was several milligrams per litre of the cultural fluid. This preparation is characterised by high sensitivity to organosphosphorus and carbamate-type pesticides despite its low activity in comparison with the natural AChE from the same source.[91]

Cholinesterases from blood serum[152] and brain[153] of various fish and avian[154] species as well as from the squid optical ganglion[155] are promising for analytical determination of inhibitors. The problems of low stability can be partly solved by immobilisation of the enzymes in gelatine or agar.

The majority of cholinesterase biosensors utilise only three commercial preparations of ChE, *viz*., AChE from the electric organ of eel *Electrophorus electricus*, bovine erythrocytes and BChE from equine blood serum. Comparison of analytical characteristics (see Table 2) demonstrates difficulties in establishing enzyme–inhibitor specificities for different biosensors. Thus the reported limits of detection of paraoxone using inhibition of AChE from electric eel vary from 10^{-7} (Refs 47 and 134) to 4×10^{-11} mol litre^{-1} (Ref. 136); for chlorofos (trichlorofon) and its active principle (DDVP), the scatter in the limits of detection amounts to five orders of magnitude, *viz*., from 10^{-11} (Ref. 104) to 10^{-6} mol litre^{-1} (Ref. 134).

The experimental value of k_{II} for AChE from electric eel ($< 10^3 - 4 \times 10^4$ mol litre^{-1} min^{-1}) is smaller than that for BChE from equine blood serum ($8 \times 10^4 - 2 \times 10^5$ mol litre^{-1} min^{-1}) according to different authors (Refs 86, 91 and 156). It should be mentioned that the sensitivity of chlorofos detection with AChE-based biosensors is higher and the limit of detection is lower than the corresponding analytical characteristics of butyrylcholinesterase-based biosensors. The reason, as in the case of many other similar discrepancies, may be due to diffusion-dependent inhibition of the biosensor response. Commercial preparations of AChE from electric eel normally have high specific activity, which exceeds manyfold (sometimes, hundreds times) that of BChE from equine blood serum. The latter enzyme forms denser films upon immobilisation than the immobilised AChE with high content of the ballast protein. As a result, the biosensors operate in a diffusional-kinetic regime, and the inhibitory effect of the pesticide is reduced.

Special mention should be made of early studies aimed at determining thionophosphorus pesticides without preliminary oxidation. Thus the limits of detection of malathion (carbofos) without preliminary oxidation were 0.1 (Ref. 122) and even 1.6–4.6 mg litre^{-1} (Refs 157 and 158), whereas that after oxidation with bromine water was of the order of 10^{-4} mg litre^{-1} (Ref. 159).

From the standpoint of toxicology, it is the inhibition of AChE that presents special interest. The calculated values of inhibition constants of AChE (k_{II}) correlate with the mean lethal concentrations (LC_{50}) for mice and rats as well as with hygiene and sanitary MPC standards for natural water reservoirs.[86, 113, 134] Nevertheless, the use of BChE incorporated into biosensors gives in general comparable limits of detection of pesticides which differ not more than 100-fold in both directions (see Table 2). In order to extend the range of compounds that can be determined by this method, some investigators deliberately decrease the selectivity of the biosensor response through simultaneous use of several ChE isolated from different sources.[115, 160] Tissue homogenates or biological fluids possessing esterase activity (blood, liver homogenates, *etc*.) are used for the same purpose.[161–163] By selecting substrates of different specificity, one can vary the set of esterases the activity of which is to be monitored. Thus the use of indophenyl acetate makes it possible to record the overall response of BChE and carboxylesterases. Acetyl(thio)choline is used to detect the responses of AChE and BChE, while butyryl(thio)choline is used for the BChE signal. This extends the potentialities of identification of toxic agents.

Immobilisation (stabilisation) of ChE sometimes plays a crucial role. Immobilisation decreases (or rarely increases) the sensitivity of biosensors with respect to inhibitors in comparison with the free enzyme. Covalent immobilisation by cross-linking of ChE on the electrode surface or an appropriate carrier using glutar-

Table 2. Some characteristics of methods used for the determination of pesticides with ChE-based biosensors and experimental parameters, *viz*., concentration range (c/mol litre^{-1}) and limits of detection (LD/mol litre^{-1}).

Enzyme source, substrate	Immobilisation / stabilisation	Method	Conditions	Pesticides	Parameters of inhibitor assay		Note	Ref.
					c	LD		
Potentiometric biosensors								
AChE from electric eel, BChE from equine blood serum, acetyl- and butyrylcholine	cross-linking with albumin by vapour of glutaraldehyde	pH-ISFT; conductometric (Pt electrodes)	phosphate buffer, 2.5×10^{-3} mol litre^{-1}, pH 7.4, $c_S = 8\times10^{-3}$ mol litre^{-1}, incubation for 10 min	trichlorfon	–	10^{-7} 10^{-6}	AChE BChE	118–120
				paraoxon-methyl	–	10^{-7} 10^{-6}	AChE BChE	
AChE from electric eel, acetylthiocholine	on the electrode surface, poly-ethyleneimine and glutaraldehyde	glassy carbon electrode, by a redox mediator potential	citrate buffer, 0.1 mol litre^{-1} + + $MgCl_2$, pH 7.2, $c_S = 1\times10^{-3}$ mol litre^{-1}, incubation for 10 min	DDVP	– –	2×10^{-7} 2×10^{-6}	 flow-injection regime	121
AChE from electric eel, acetylcholine	sorption on activated nylon (biodin and immunodin)	pH-ISFT	phosphate buffer, pH 7.0 and 9.0, $c_S = 2\times10^{-2}$ mol litre^{-1}	malathion	–	0.1 [a]	incubation for 1 h	122
BChE from equine blood serum, butyrylcholine	cross-linking with peroxidase and choline oxidase by poly-ethyleneimine on carbon electrodes	glassy carbon electrode	phosphate buffer, pH 7.5, $c_S = 1\times10^{-2}$ mol litre^{-1}, incubation for 10 min	trichlorfon	$10^{-9}-10^{-5}$ –	– 2×10^{-13}	native BChE immobilised BChE	123
	cross-linking by glutaraldehyde on paper, in gelatine and nitrocellulose (NC)	glass pH-ISE	phosphate buffer, pH 7.9, $c_S = 2\times10^{-2}$ mol litre^{-1}, incubation for 10 min	diazinon	0.003 – 0.3 [a] 0.002 – 0.3 [a]	– –	paper, gelatine NC	93, 124
				zolone	0.001 – 0.4 [a] 0.004 – 0.4 [a] 0.004 – 0.7 [a]	– – –	paper gelatine NC	
				DDVP	0.5 – 5.0 [a] 0.2 – 1.0 [a] 0.1 – 2.0 [a]	– – –	paper gelatine NC	
	cross-linking by glutaraldehyde on paper, nylon and nitrocellulose	the same	the same	diazinon in the presence of surfactants	–	around 10^{-9}		125
	cross-linking with albumin by glutaraldehyde on cellulose acetate	pH-ISFT	phosphate buffer, pH 7.4, incubation for 10 min	DDVP zolone	6.7 – 3.5 [b] 7.5 – 5 [b]	– –		40
Amperometric biosensors								
	BChE in a working buffer solution	choline oxidase sensor based on a Pt – H_2O_2 sensor	phosphate buffer, pH 7.0, BChE, 0.4 U ml^{-1}, 30 °C, $c_S = 3\times10^{-4}$ mol litre^{-1}	paraoxon	– –	0.5 [c] 0.2 [c]	incubation for 30 min incubation for 1 h	126
AChE from electric eel, acetylthiocholine	incorporation into a carbon paste with an epoxide resin	carbon-paste electrode	phosphate buffer, pH 7.0, $c_S = 1\times10^{-4}$ mol litre^{-1}	carbofuran carbaryl paraoxon DDVP	– – – –	2.2 [c] 20 [c] 2.7 [c] 22 [c]	without incubation	106

Table 2 (continued).

Enzyme source, substrate	Immobilisation / stabilisation	Method	Conditions	Pesticides	Parameters of inhibitor assay		Note	Ref.
					c	LD		
AChE from bovine erythrocytes, acetylthiocholine	cross-linking by carbodiimide on glassy carbon electrodes	rotating disk electrode	universal buffer, pH 8.0	trichlorfon	–	–	$K_i = 5.7 \times 10^{-7}$ (see [e]) $pI_{50} = 4.37$ [e]	127, 128
AChE from electric eel, 4-aminophenyl acetate	cross-linking with albumin by glutaraldehyde on nylon	Pt electrode	phosphate buffer, pH 8.0, $c_S = 1.2 \times 10^{-2}$ mol litre^{-1}, incubation for 5 min	carbaryl paraoxon	$5 \times 10^{-7} - 5 \times 10^{-5}$ $5 \times 10^{-5} - 1 \times 10^{-5}$	1×10^{-7} 1×10^{-7}		129, 130
AChE from electric eel, acetylcholine	cross-linking by glutaraldehyde	choline oxidase sensor based on a Pt – H_2O_2 electrode	phosphate buffer + 0.1% NaN_3, pH 7.0, $c_S = 5 \times 10^{-4}$ mol litre^{-1}, incubation for 30 min	paraoxon	1.5 – 15 [b]	1.3 [b]		131
AChE from electric eel, acetylthiocholine	incorporation into a photopolymerised gel PVA-SbQ (8%)	Pt electrode	phosphate buffer, pH 8.0, $c_S = 1.2 \times 10^{-3}$ mol litre^{-1}, 30 °C, incubation for 30 min	paraoxon	$10^{-10} - 10^{-5}$	–	flow-injection regime	132
AChE from bovine erythrocytes, acetylthiocholine	cross-linking by glutaraldehyde on a magnetic carrier	the same	phosphate buffer, pH 8.0	carbofuran paraoxon-ethyl paraoxon-methyl malaoxon	– – – –	3 [b] 3 [b] 7 [b] 7 [b]	$k_{II} = 2.6 \times 10^{-5}$ (see [d]) $k_{II} = 2.1 \times 10^{-5}$ (see [d]) $k_{II} = 1.8 \times 10^{-5}$ (see [d]) $k_{II} = 1.4 \times 10^{-5}$ (see [d])	133
AChE from electric eel, BChE from equine blood serum, acetylthiocholine	cross-linking of two enzymes with albumin by glutaraldehyde	carbon-paste electrode with cobalt phthalocyanine	phosphate buffer, pH 7.0, $c_S = 5 \times 10^{-4}$ mol litre^{-1}, 30 °C	carbaryl DDVP paraoxon	5×10^{-6} 1×10^{-6} 1×10^{-7}		without incubation	134
AChE from electric eel, acetylthiocholine	cross-linking by carbodiimide on the electrode surface	the same	phosphate buffer, pH 7.0, $c_S = 1 \times 10^{-3}$ mol litre^{-1}, 37 °C, incubation for 5 min	paraoxon	–	3×10^{-8}	extraction with heptane	135
	the same	"	phosphate buffer, pH 7.0, $c_S = 1 \times 10^{-3}$ mol litre^{-1}, 37 °C, incubation for 20 min	DDVP paraoxon	– –	6×10^{-9} 4×10^{-11}		136
AChE from bovine (be), human (he) and electric eel (ee) erythrocytes, BChE from equine blood serum (eu), acetyl- and butyrylthiocholine	cross-linking with albumin by glutaraldehyde	Pt microplanar electrode	phosphate buffer, pH 7.0, $c_S = 1 \times 10^{-3}$ mol litre^{-1}, incubation for 10 min	aldicarb	– –	430,[c] 190,[c] 3200,[c] 630 [c]	for AChE (be), AChE (ee), BChE (he), BChE (eu), respectively	137, 138
				carbaryl	–	30,[c] 0.092,[c] 1300, > 10^4 (see [c])	the same	
				carbofuran	–	3.1,[c] 0.08,[c] 150,[c] 360 [c]	"	
				methomil	–	39,[c] 0.19,[c] 0.88,[c] 1.9 [c]	"	
				propoxur	–	1.5,[c] 0.68,[c] > 10^4, 11000 [c]	"	

Table 2 (continued).

Enzyme source, substrate	Immobilisation / stabilisation	Method	Conditions	Pesticides	Parameters of inhibitor assay		Note	Ref.
					c	LD		
AChE from electric eel, acetylcholine	covalent immobilisation by carbodiimide on an affinity membrane UltraBind	choline oxidase sensor based on a Pt Clark's electrode	phosphate buffer, 0.01 mol litre^{-1}, pH 7.0, $c_S = 1 \times 10^{-2}$ mol litre^{-1}, 30 °C, incubation for 10 min	paraoxon	–	1×10^{-8}	native AChE, buffer	139
					–	1×10^{-8}	native AChE, 0.05% acetone or 5% cyclohexane	
					–	1×10^{-6}	immobilised AChE, buffer or 1% acetone	
					$5 \times 10^{-7} - 1 \times 10^{-5}$	5×10^{-7}	immobilised AChE, 1% cyclohexane	
AChE from bovine and electric eel erythrocytes, BChE from equine blood serum, acetyl- and butyrylthiocholine	incorporation of enzymes immobilised on aminated silica gel into a carbon paste	Pt electrode combined with an enzyme column	phosphate and Tris-, borate buffers, pH 6.0 – 10.0, $c_S = 1 \times 10^{-4}$ mol litre^{-1}	carbofuran	–	2.2 [c]	AChE	140
						22.1 [c]	BChE	
				carbaryl	–	2.0 [c]	AChE	
				paraoxon	–	2.8 [c]	AChE	
				chlorofe-vinphos	–	3.6 [c]	BChE	
AChE from electric eel, acetylthiocholine	incorporation into photo-polymerised gel PVA-SbQ (8%)	Pt electrode	phosphate buffer, pH 8.0, $c_S = 1.2 \times 10^{-3}$ mol litre^{-1}, 30 °C	dipterex	–	5.6×10^{-6}		141
AChE from electric eel, acetylcholine	incorporation with choline oxidase into a Langmuir – Blodgett film	Pt microelectrode	phosphate buffer, pH 7.5, $c_S = 1 \times 10^{-3}$ mol litre^{-1}	trichlorfon	–	around 10^{-6}		142
	cross-linking by carbodiimide in an agarose gel in the pores of a carbon electrode	porous carbon electrode	phosphate buffer, pH 7.0, Meldola Blue mediator, $c_S = 4 \times 10^{-4}$ mol litre^{-1}, flow-injection regime	paraoxon		1.4 [c]		143
BChE from equine blood serum, butyrylcholine	incorporation into a carrageenan gel with choline oxidase	Clark's electrode	hexane : chloroform mixture (1 : 1) saturated with water, $c_S = 2 \times 10^{-3}$ mol litre^{-1}, incubation for 5 – 20 min	paraoxon	9.4 – 56.4	4.5 [c]		144
				malathion	10.0 – 40.0	4.5 [c]		
				ethylparathion	1.0 – 10.0	0.5 [c]		
				aldicarb	10.0 – 60.0	4.5 [c]		
				carbaryl	1.0 – 10.0	0.5 [c]		
	cross-linking with albumin by glutaraldehyde on nylon, nitrocellulose and paper	carbon-paste electrode	phosphate buffer, pH 7.9, $c_S = 6 \times 10^{-5}$ mol litre^{-1}, flow-injection regime	diazinon	8.2 – 6.3 [b]	–		145

Table 2 (continued).

Enzyme source, substrate	Immobilisation / stabilisation	Method	Conditions	Pesticides	Parameters of inhibitor assay		Note	Ref.
					c	LD		
Miscellaneous methods								
AChE from electric eel, BChE from equine blood serum, acetyl- and butyrylcholine	cross-linking with albumin by glutaraldehyde	conductometric, microplanar Pt electrodes	phosphate buffer, pH 7.5, $c_S = 8 \times 10^{-3}$ mol litre^{-1}, incubation for 10–60 min	paraoxon-methyl	5×10^{-7}	–	lower assay limit	146
				paraoxon	1×10^{-8}	–		
				trichlorfon	5×10^{-7}	–		
					5×10^{-11}	–	incubation for 10 min	
ChE, indoxyl acetate	sol-gel immobilisation in tetramethyl orthosilicate	fuorescent optode	phosphate buffer, pH 7.5, $c_S = 1.63 \times 10^{-4}$ mol litre^{-1}	phenitrothion	–	17.8 [b]		147
				azinphos-ethyl	–	17.9 [b]		
				methidathion	–	37.6 [b]		
				naled	–	0.36 [b]		
				mecarbam	–	13.9 [b]		
AChE from electric eel, acetylcholine	covalent immobilisation by isothiocyanate on glass	fibre-optic sensor, change in the colouring of Bromothymol Blue	Tris-buffer, pH 7.5, $c_S = 1 \times 10^{-1}$ mol litre^{-1}	paraoxon	5×10^{-7}–5×10^{-6}	1.1×10^{-7}		148
				carbofuran	5×10^{-8}–5×10^{-7}	1.5×10^{-8}		
AChE from electric eel, indolyl-3-acetate	cross-linking by vapour of glutaraldehyde	piezocrystal sensor	phosphate buffer, incubation for 5 min	paraoxon	–	5×10^{-8}		149
				carbaryl	–	1×10^{-7}		

Note. The following abbreviation were used: pH-ISE is pH-sensitive ion-selective electrode; DDVP is dimethyl (2,2-dichlorovinyl) phosphate; [a] mg litre^{-1}; [b] the values of p$c = -\log c$/mol litre^{-1} are listed; [c] µg litre^{-1}; [d] mol^{-1} litre min^{-1}; [e] mol litre^{-1}.

aldehyde (in vapour or as an aqueous solution)[118–120, 129, 130, 133, 134, 137, 138, 149] or carbodiimide[127, 128, 135, 136, 140] are conventionally used. A decrease in enzymic activity as a result of covalent immobilisation depends on the concentration of the cross-linking reagent, the duration of treatment, the initial activity of the enzyme and the nature of the carrier. The losses are minimal (20%–40% of the initial enzyme activity) when protein films are prepared from albumin and gelatine, which are also cross-linked, and in the immobilisation of ChE on aminated carriers of the Immobilon or Biodin type. Highly reactive enzyme-containing membranes are prepared by incorporating enzymes into hydrophilic gels based on acrylamide,[79, 159] a *N*-vinylpyridinium–vinyl chloride copolymer,[132, 142] polyethyleneimine,[121, 123] tetramethyl orthosilicate[147] and polyurethane.[164] Biosensors prepared with such membranes are characterised by a rapid response to the substrate; however, the lifetime of immobilised enzymes is much less than that of enzymes obtained by covalent immobilisation on inert carriers.

Under field conditions, it is especially convenient to use biosensors with disposable membranes which can easily be replaced in the case of enzyme inactivation. Disposable membranes are prepared on the basis of inert carriers, such as nylon,[122, 125, 145, 165, 166] paper and various cellulose derivatives,[40, 93, 96, 104, 105, 124, 167, 168] aluminium hydroxide,[164, 169] industrially manufactured membranes (for dialysis and immunoblotting) or carriers for affinity chromatography.[122, 140, 141]

The largest losses (up to 99% of the original ChE activity) were observed upon cross-linking of enzyme globules with aqueous solutions of glutaraldehyde on inert carriers, which do not react with the cross-linking reagent,[124] such as cellulose trinitrate or paper. Such considerable losses are compensated for by high storage stability of the membranes (up to 6 months at room temperature) and operation lifetime (for over two weeks in a buffer solution within the biosensor).

As a rule, immobilisation of ChE does not envisage the use of additional water-soluble stabilisers. However, in some cases 0.1%–3% glycerol, glycine, non-ionogenic synthetic surfactants, 0.01%–0.05% gelatine, magnesium and calcium salts (in the case of AChE) are used to stabilise the activity of the free enzyme.

Stabilisers are necessary when ChE is used in aqueous solutions for analytical purposes, since the stability of even freshly prepared solutions is low. Thus ChEs from insects are completely inactivated within several hours,[170] AChE from electric eel is stable in a concentrated ammonium sulfate solution. Stabilisers increase the storage stability of enzymes. They form films in which the enzyme is in a solid solution. Such preparations are readily soluble and yield stable solutions the activity of which is not changed during the measurement of the inhibitory effect. In other cases, stabilisers are added directly to the enzyme solution for the same purpose. The addition of stabilisers also decreases the losses of enzymic activity in the course of immobilisation.[136] Cellulose and dextran derivatives, other polyalcohols and some non-ionogenic surfactants are used as ChE stabilisers. Probably, they inhibit nonspecific sorption of the protein on glass or a plastic, integrate inhibitors (especially, transition metal ions) into inert complexes or stabilise the tertiary structure of the protein. Incorporation of ChE into tregalose[169] and *N*-phthalylchitosane[73, 116, 155, 171] films for the development of colorimetric indicator devices designed for the determination of ChE inhibitors has been described. Stabilisers suppress the interfering effects of metal ions but do not change the kinetic characteristics of irreversible inhibition of ChE by organophosphorus pesticides.

With a decrease in the diffusion-dependent limitations of biosensor responses, *i.e.*, in the case of thinner membranes and lower specific activity of enzymes, the results of inhibitor assays obtained with free and immobilised enzymes are close. Therefore, amperometric biosensors based on membranes with lower enzymic activities than those used in potentiometric biosensors acquire certain advantage due to higher sensitivity of substrate assays.

On the other hand, comparison of efficiencies of potentiometric and amperometric biosensors under identical conditions shows that the advantages of amperometric detection are not so obvious.[94] Thus in experiments with disposable membranes with ChE immobilised on nylon, nitrocellulose or in gelatine, potentiometric biosensors manifested lower limits of detection of paraoxon and DDVP.[93, 172] This may be due to the interfering Faraday effects, particularly, to additional generation of hydrogen ions upon thiocholine oxidation on the electrode.

$$Me_3N^+(CH_2)_2SC(O)Me + H_2O \xrightarrow{ChE} Me_3N^+(CH_2)_2SH + MeCO_2H \quad (13)$$

$$2Me_3N^+(CH_2)_2SH \xrightarrow[-e]{} Me_3N^+(CH_2)_2S{-}S(CH_2)_2N^+Me + 2H^+$$

Calculation of inhibition bimolecular constants from calibration curves of pesticide assays utilising a potentiometric biosensor with disposable membranes according to Eqn (10) gave overestimated values in comparison with inhibition of the free enzyme under identical conditions. One of possible reasons for increased sensitivity of pesticide assays in the case of immobilised ChE is adsorptive accumulation of pesticides on inert carriers. In particular, this can be confirmed by the fact that non-ionogenic surfactants used in concentrations which do not influence the activity of native ChE, produce differentiating effects on the response of cholinesterase-based biosensors to various inhibitors, *viz.*, the irreversible inhibition effect of pesticides is increased, while the reversible inhibition effect of fluorides or heavy metal ions is decreased. Small concentrations of surfactants also suppress the protective effects of reversible inhibitors in organophosphoric pesticide assays.[125] This effect is due to sorption of surfactants on the membrane surface, which facilitates the transfer of hydrophobic organic pesticide molecules to the membrane, while the transfer of small hydrophilic ions (reversible ChE inhibitors) is impeded. The effect of surfactants is a maximum for hydrophilic carriers (paper, nylon) and a minimum for nitrocellulose membranes. The difference between inhibition bimolecular constants for free and immobilised enzymes changes in the same series. For hydrophobic nitrocellulose, the difference is a maximum, while that for hydrophilic paper and gelatine is virtually absent.[173, 174]

Increased sensitivity of inhibitor assays upon enzyme immobilisation is observed not only for ChE. The limit of detection of heavy metals with oxidoreductases immobilised in albumin films decreases in the following order (mg litre^{-1}): from 0.25 to 0.05 for Hg(II), from 1.00 to 0.05 for Cu(II) (alcohol oxidase), from 1.00 to 0.10 for Ni (sarcosine oxidase) and from 0.50 to 0.015 for Se(IV) (glutathione oxidase).[44] Obviously, electrostatic interactions of metal ions with charged carriers play an important role in these processes along with nonspecific sorption.

Monitoring of environmental objects. Virtually, all the data listed in Table 2 relate to model solutions containing only one ChE inhibitor. The use of cholinesterase-based biosensors for the analysis of real objects is complicated, firstly, because of the presence of considerable amounts of natural enzyme effectors (alkaline-earth metal ions, biogenic amines, *etc.*) in analysed samples and secondly, due to simultaneous presence of several inhibitors with different mechanisms of action. This fact significantly complicates the interpretation of experimental results: one has either to confine oneself to qualitative detection of anti-acetylcholinesterase compounds in analysed samples or to have recourse to their preliminary separation at the expense of simplicity and rapidity, which are the main advantages of enzymic assays.

Commercial production of test systems based on ChE and designed for monitoring residual organophosphorus and carbamate-type pesticides in environmental objects and foodstuff has already been set up. These systems, which represent indicator kits with colorimetric indication are manufactured by 'Boehringer

Mannheim' (Germany)[175] and 'Ohmicron Environmental Diagnostic Inc.' (USA)[176] and ensure qualitative detection of organophosphorus pesticides with limits of detection ranging from 0.05 to 30 μg litre^{-1}.

In the 1970's, the manufacturing company 'Midwest Research Institute' (USA) produced a family of automated indicator systems CAM-1,2 (Continuous Alarm Monitor), which represent alarm devices for detecting toxic war gases of neuroparalytic action and are adapted to civil needs.[164] These systems are supplied with pumps and aspirators and operate in a semi-automatic regime. The minimum concentrations of pesticides detectable in air and water are as follows (mg litre^{-1}): paraoxon, 0.1; DDVP, 0.2; diazinon, 0.2; dursban, 4.3; difonat, 10; malathion, 17–70 (depending on the brand); temic, 0.5; furadan, 0.75; mesuron, 12 and sevin, 20. Their service cycle includes automated substitution of enzyme-containing polyurethane reactors and electrochemical purification of electrodes, they are supplied with an acoustic signal device of contamination.

A potentiometric biosensor containing AChE immobilised in gelatine on the surface of a pH-metric glass electrode was used to control the quality of waters in the river Seine (France).[177,178] The limits of detection of pesticides with this biosensor were as follows (mg litre^{-1}): 0.003 (ethylparaoxon), 0.005 (methylparaoxon) and 0.014 (malathion, ethyl- and methylparathion). Some countries including Russia have begun the commercial production of indicator tubes which contain ChE and chromogenic substrates and are designed for standard aspirators used in air sampling.[179]

At present, the possibility to use ChE for environmental and analytical monitoring is chiefly associated with the creation of a market of cheap electrochemical sensors which can form the basis for mass-scale production of disposable cholinesterase-based biosensors that are compatible with standard measuring devices (voltamperographs, ionometers, *etc*.). Such sensors can be manufactured by photolithographic methods. Because of High-Tech production, small size (several square millimetres) and low enzyme expenditure ($\sim$ 0.1 unit per electrode), the cost of such biosensors does not exceed US \$ 1–5. The production of pH-sensitive field transistors and microplanar conductometric devices is currently under way.[118–120] A technology for the production of screen-printed metal-coated and carbon-paste electrodes with plastic mounts has been developed.[180] The cost of equipment needed for their production amounts to US \$ 30 000, which is comparable with the cost of a general-purpose voltamperograph. At present, screen-printed electrodes are manufactured in the Czech Republic,[133] Italy[180,181] and Germany.[182] All of them represent a combination of a working, auxillary electrode and a reference electrode (Ag/AgCl), which have various geometries, are arranged into a single block (sometimes, in pairs) and operate in a differential regime.

The progress in the design of biosensor elements demands adequate solution of methodological problems. As expected, the effect of the matrix of the analysed subject does not seem to be of crucial importance.[115] Biosensors are used for the determination of residual organophosphorus and carbamate-type pesticides in vegetables, fruits, juices,[137,138,183] soils and silt sediments.[134,184] The only limitation in the analysis of surface waters is their mineralisation, which should not exceed 0.1 mol litre^{-1} (Refs 122 and 177). It has to be specified, however, that the accuracy of pesticide assays with biosensors was checked using a method of additives rather than independent methods. Chromatographic methods were used for the estimation of the correctness of measurements only in the case of carbamate-type pesticide assays in vegetables,[138] of organophosphorus and carbamate-type pesticide assays in fruits and vegetables[183] and of organophosphorus pesticide assays in natural waters (freezing and subsequent thawing of standard samples).[128] The divergence of experimental results was $\sim$ 30%, which was accepted to be satisfactory.

A number of simplified sampling techniques have been developed which help elimination of laborious procedures connected with the removal of organic solvents. Thus, a 10-minute extraction with acetone followed by 10-fold dilution of extracts allows reliable determination of up to 0.1 mg kg^{-1} of pesticides (diazinon, chlorofos, tetraethyl monothiopyrophosphate) in wheat grains and combined fodders.[172] However, in this method the level of extraction is 60%–80%, which means that it is only possible to detect hazardous contamination of the products with pesticides.

If the information about the nature of toxic agents is absent, a reduced biosensor response should not necessarily be related to the concentration of the compound under study. The difference in inhibition bimolecular constants does not allow one to regard the degree of inhibition as a measure of net (molar or mass) content of pesticides even in the absence of interfering factors (diffusion-dependent inhibition of biosensor responses, protective action of effectors, *etc*.). A concept of a standard toxicant has been proposed according to which the net content of pesticides is expressed in units of concentrations of the pesticide used as a standard judging from the inhibitory effects they produce. DDVP,[164] paraoxon,[132] parathion[115] and carbaryl[180,181] were recommended as candidates for such standards. The inhibiting effects of other pesticides are calculated with the help of relative toxicity coefficients. The latter are determined in model pesticide solutions of equal concentration by estimating the degree of inhibition of ChE (*i.e*., a relative decrease in the biosensor response). Undeniably, these coefficients are not equal to the ratio of inhibition constants and depend on experimental parameters including the choice of pesticide concentrations. Similar limitations are typical of calculations of pesticide concentrations expressed as factors of excess over MPC.

Simultaneous use of several biosensors including enzyme-based ones affords additional possibilities for differentiation of biosensor responses. In contrast with the above-described determination of the ratio of inhibition constants $(k_{II})_1/(k_{II})_2$, in this case the result is obtained instantaneously, which makes it possible to use a set of biosensors[47,63,185] (or a multichannel multisensor[40,41]) for on-line or even flow measurements.

Hydrolytic enzymes, *e.g*., urease,[40,41] acid and alkaline phosphatases[47,185] and glucose oxidase[40,41] can also be used for this purpose together with ChE. Unfortunately, enzyme–inhibitor specificity of these enzymes does not allow simultaneous qualitative and quantitative determination of inhibitors. In the best case, adequate interpretation of experimental results can be achieved through the use of chemometric methods, *e.g*., by estimating individual contributions of Hg(II) ions and the overall effect of all pesticides.[185] However, in this case, too, the mutual effect of solution (sample) components is questionable, since the information about the relative sensitivity of biosensor responses is obtained for single-component solutions. Even with the use of experimental planning methods for the choice of conditions for detecting inhibitor signals, the possibility to separate the contributions of individual inhibitors was established for sufficiently high concentrations (1 mg litre^{-1}), which exceeded the existing hygiene and sanitary standards a thousand-fold.

To some extent, the selectivity of effects produced by reactivating reagents can assist in interpretation of experimental results. Thus the anti-cholinesterase effect of metal ions can be suppressed by EDTA and sodium iodide, which allows manifestation of the toxic effect of pesticides.[1,97] Contrariwise, ChE added to analysed samples can bind trace amounts of anti-cholinesterase pesticides and then the toxic effects of other pollutants can be determined with other enzymes or indicator organisms.[1]

In another approach, the very fact of ChE inhibition is considered as a proof of contamination without regard for the qualitative and quantitative composition of the analysed sample.[186] This approach is widely used in toxicological studies which make use of a special term, 'anti-cholinesterase activity' for a substance or tested subject.[187,188] The activity of AChE from blood or brain serves as a measure of chronic poisoning of living organisms with sublethal concentrations of pesticides.[189,190] The

total inhibitory effect of toxic agents (*i.e.*, the anti-cholinesterase activity of the medium) can also be used for detection of hazardous poisoning levels and for outlining the main tendencies in their variability. Studies of inhibitory effects of silt sediments in the rivers of the Morava Basin which employed biosensors based on AChE from electric eel revealed a relationship between biosensor signals (the inhibition was studied by following relative changes in the dynamic responses) and agricultural impact on the given territory.[134] The biotesting conducted by the methods accepted in biological monitoring of surface and sewage waters established a correlation between the degree of inhibition of BChE immobilised on paper or nitrocellulose and the death rate or retardation of growth of hydrobionts of the Cladocera family (crayfish *Daphnia magna Straus*) and the protococcal alga (*Chlorella vulgaris Beyer*).[191, 192] In the first place, this correlation was established for specific inhibitors of ChE. An analysis of real environmental objects revealed a certain divergence of results obtained by different methods. For example, studies of more than 200 samples of sewage waters from industrial and agricultural enterprises with the aid of potentiometric cholinesterase-based biosensors and acute toxicity tests of waters for the presence of Infusoria (*Paramecium caudatum*) have made it possible to classify all sewage waters into three groups according to the contamination source, *viz.*, (1) sewage waters from machine-building plants which are predominantly contaminated with heavy metals (high death rate among *Paramecium caudatum* with moderate levels of ChE inhibition); (2) sewage waters from petrochemical plants and enterprises of food industry which are contaminated with petroleum products, fats and other high-molecular-weight organic compounds (high death rate among *Paramecium caudatum* in the absence of ChE inhibition) and (3) sewage waters from agricultural enterprises and surface run-off wastes from rural areas (lack of toxicity for Infusoria, low and medium levels of ChE inhibition).[193]

The methods which combine biological and biochemical parameters of water quality (and, in prospect, other generalised hydrochemical parameters) seem to hold especially great promise, primarily due to the possibility to extend the range of potential toxic agents present in environmental objects.

VI. Conclusion

The explosive development of enzymic methods for monitoring of environmental objects has become possible because of a combination of fundamental and applied investigations at the boundaries between biochemistry, biotechnology, toxicology, analytical chemistry and environmental monitoring. With the advent of biosensors, purely laboratorial complex biochemical methods have been transformed into easy-to-handle measuring tools, which are comprehensible for the untrained personnel and are suitable for field use.

This does not mean, however, that the design of biosensors is exclusively confined to the selection of appropriate detectors and immobilisation of enzymes. Investigators are faced with the necessity to solve complicated problems connected with functional features of enzymes to be incorporated into biosensor membranes as well as with optimisation of measurement conditions and effects of template components. A transition from a free enzyme to a biosensor results in the narrowing of the range of experimental facilities used in the study of biologically active substances. Thus the minimum concentrations of substrates and the specific activity of enzymes are limited, while biosensors sometimes require *a priori* non-optimal conditions for their functioning, which are restricted by detectability parameters or specific applications. Optimisation of the biosensor design and the choice of service conditions always involve a compromise between the requirements for the accuracy of response detection, the sensitivity of inhibitor (substrate) assays and the limitations imposed by actual conditions of field tests.

Realisation of specific functioning of biosensors designed for evaluation of toxic effects of environmental pollutants has stimulated the research carried out for commercial purposes. In the first place, this relates to disposable biosensors based on screen-printed and solid-state sensors as well as to the simplest indicating devices with visual indication of signals. Their practical application is still limited due to complexity of interpretation of experimental results and inadequacy of the standardising basis, which bans the use of the data obtained by biochemical monitoring methods for ecological expertise. For this reason, only cholinesterase-based biosensors have found application in environmental and analytical monitoring, although biosensors allow the detection of virtually all priority-driven toxic agents with the exception of dioxins and radioactive compounds.

Nevertheless, the development of biosensors for the determination of environmental pollutants is a very promising trend, primarily because they meet demand. The methods based on the use of biosensors compete favourably with modern methods of toxicological and biological monitoring. To their obvious advantages one may relate small dimensions, ease of signal detection, possibility of integration of biosensors into automated control systems and, last but not least, possibility of quantification of toxicological effects. In our opinion, further developments in this field will proceed along three main lines.

1. Further development of the principal elements aimed at improving the metrological and operational characteristics of transducers and transition to polyenzyme sensors (*e.g.*, multisensors, sensor arrays), which help establish not only the presence of inhibitors in analysed subjects but also their nature.

2. Further extension of the range of tested compounds, mostly through optimisation of service conditions for measurement and sampling rather than by increasing of the number of enzymes; further improvement and application of biosensors designed for use in organic solvents, particularly those used as detectors in flow-injection analyses and liquid chromatography.

3. Further development of methodical and methodological aspects of applicability of biosensors in environmental and analytical monitoring; design of expert systems for estimation of biological (physiological) activity of chemical compounds, toxic effects of solid industrial and vital activity wastes; combination of biosensors with conventional methods of biological monitoring and, as a consequence, increasing the information content of biochemical monitoring; large-scale application of methods for chemometric treatment of experimental results.

The integration of different methods and approaches to environmental and toxicological monitoring of environmental objects is still in progress. Recent developments in the field of biosensors are not confined to routine application of enzymes as analytical reagents. Biosensors are used as biochemical models of living organisms and the approaches used therein are related to toxicology and biotesting.[193] First attempts have been undertaken to direct alteration of the enzyme structure with a view to changing its inhibiting specificity.[91] This approach makes use of 'artificial enzymes' (*i.e.*, oligonucleotides or polymers); their synthesis and selection were performed using molecular recognition of specific toxic agents.[194]

And, finally, the vast experience gained in the study of biosensors has served as a basis for the design of entirely new devices, *viz.*, DNA-sensors allowing direct control over mutagenic and genotoxic properties of environmental pollutants.[195] The specificity of their functioning goes far beyond the scope of this review, although their role in the development of new methods of monitoring of environmental objects is now apparent.

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