Enzymatic methods of analysis: novel approaches and applications

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The results of a series of investigations dealing with the development of enzymatic methods for determination of biologically active compounds, *viz.*, inhibitors, activators, and substrates of native and immobilized enzymes of the oxidoreductase (peroxidases, alcohol dehydrogenases) and hydrolase (alkaline and acid phosphatases) classes isolated from diverse sources are summarized. Novel original approaches, proposed by the authors, for improving the sensitivity, selectivity, and rapidity of the methods are discussed. Numerous examples of application of the developed enzymatic procedures for the analysis of a wide range of samples are given.

Key words: enzymatic methods of analysis, inhibitors, activators, substrates, immobilized enzymes, test methods, environmental samples, biological fluids, pharmaceuticals, foodstuffs.

1. Introduction

In recent years, determination of traces of biologically active compounds in environmental samples, foodstuffs, microbiological and pharmaceutical products, and solution of a number of medical and biochemical problems have been carried out more and more often using enzymatic methods of analysis based on the dependence of the rate of an enzyme-catalyzed chemical reaction on the reactant and biocatalyst concentrations.[1] The rate of an enzymatic reaction is monitored by various instrumental methods or by sight. Enzymatic methods develop at the boundary of analytical chemistry and biochemistry and refer to a promising field of modern chemical analysis. Owing to the high catalytic activity and specificity of biocatalysts, enzymatic methods are highly sensitive and selective. Additional advantages of these methods include rapidity, simplicity of the equipment and the experimental procedure, and relatively low cost. These features account for the wide introduction of enzymatic methods into practice of clinical, biochemical, and agrochemical laboratories, research centers, or environmental services. Enzymatic methods are suitable for determination of enzymes, their substrates, and compounds affecting the catalytic activity of enzymes, *i.e.*, their effectors, activators, or inhibitors.

For more than 25 years, the Chair of Analytical Chemistry at the Department of Chemistry of the M. V. Lomonosov Moscow State University has been engaged in the development of the fundamental grounds and practical applications of enzymatic methods of analysis. During these years, inorganic and organic inhibitors and activators of enzymes of the oxidoreductase type (peroxidases, alcohol dehydrogenases, alcohol oxidases, superoxide dismutases) and hydrolase type (alkaline and acid phosphatases and pyrophosphatases) were identified; the mechanisms of action of effectors were studied; and numerous procedures for their determination were developed. The search for new approaches to improvement of analytical characteristics of enzymatic methods and extension of the range of analytes and samples has always received attention.[1—3]

Of particular interest are approaches that open up the way for highly sensitive, selective, and simultaneously rapid and simple enzymatic determination of biotics (zinc, magnesium, fluorine); xenobiotics (mercury, organomercurials, lead, cadmium, bismuth, phenols); and aliphatic fatty acids. In this review these approaches are discussed in relation to peroxidases, alcohol dehydrogenases, alkaline and acid phosphatases, *i.e.*, highly active, stable, and readily commercially available enzymes both native and immobilized on various supports. It is noteworthy that in all the studies described and procedures we developed, the catalytic activity of enzymes was characterized by the rate of indicator reactions by spectrophotometric monitoring of absorbance of the reaction products *vs.* time. The absolute initial rates of various indicator reactions (υ_0/μmol L^{-1} min^{-1}) were calculated from the relation:

$$\upsilon_0 = \Delta c/\Delta t = \Delta A/\Delta t \cdot 1/l\varepsilon = \tan\alpha/l\varepsilon,$$

where Δc is the concentration increment (mol L^{-1}) of the indicator reaction product over time (Δt); A is the absorbance of the reaction solution; l is the cell thickness (cm);

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ε is the molar extinction coefficient of the enzymatic reaction product; tanα is the slope of the kinetic curve plotted in the A—t (t/s) coordinates.

When working with immobilized enzyme preparations, the enzymatic reaction rate was monitored visually by detecting the instant of appearance of the required color on the support.

The degrees of inhibition (I (%)) and activation (A (%)) of the enzyme by metal ions and organic compounds were calculated from the formulas:

$$I\ (\%) = 100 \cdot (\upsilon_0 - \upsilon_I)/\upsilon_0;$$

$$A\ (\%) = 100 \cdot (\upsilon - \upsilon_0)/\upsilon_0,$$

where υ_0 and υ_I (or υ) are the initial rates of a particular indicator reaction in the absence and in the presence of the metal ion (and/or organic compound), respectively.

2. Combined action of an analyte, *i.e.*, a metal ion (or organometallic compound), and an organic compound

Heavy metal ions are known as effective inhibitors of the catalytic activities of urease,[4] glucose oxidase,[5] invertase,[6,7] choline esterase,[8] and alcohol dehydrogenase;[9,10] however, their inhibitory action is manifested at concentrations exceeding their maximum permissible concentration (MPC) in potable and natural waters.[11] We showed that Hg^{II}, Cd^{II}, Pb^{II}, and Bi^{III} ions inhibit only slightly the catalytic activity of horseradish peroxidase in the oxidation of *o*-dianisidine with hydrogen peroxide (Table 1), the degree of inhibition remaining virtually the same after their preincubation with the enzyme.[12,13] Thus, sensitive and selective enzymatic determination of individual heavy metal ions is impossible despite the different types of their inhibitory action on peroxidase: mercury and lead ions are reversible competitive inhibitors, cadmium ions are reversible noncompetitive inhibitors, and bismuth ions are irreversible inhibitors of the enzyme.

2.1. Use of an organic compound as an enzyme inhibitor. Sulfur-containing organic compounds (in particular, dithiothreitol) possessing rather strong reducing properties are known to decrease the catalytic activity of enzymes by disturbing their structures.[14] We showed that thiourea not only inhibits peroxidase itself, but also markedly enhances the inhibitory action of metal ions introduced into the indicator reaction after preincubation of the enzyme with thiourea for at least 2—4 h. This is due to the fact that thiourea reduces the S—S bonds in the peroxidase molecule and, as a result, enzyme loses partly the catalytic activity and facilitates the access of metal ions to the thiol groups formed and to the carboxy groups of the enzyme.[15] In other words, thiourea "predisposes" peroxidase for the action of heavy metal ions. The degree of the inhibitory action of metals in the presence of thiourea increases in the series $Cd^{II} \approx Pb^{II} < Bi^{III} < Hg^{II}$ and correlates with the thermodynamic solubility constants of their sulfides K^0_S (see Table 1). Mercury ions proved to be the most effective inhibitors of peroxidase activity in the presence of thiourea; this was used to develop one of the most sensitive, selective, and precise (relative standard deviation $s_r = 0.03$, the number of measurements $n = 3$) procedures for mercury(II) determination.[16]

For sensitive determination of Bi^{III} in the concentration range of 0.2—1 ng mL^{-1}, it is more appropriate to replace thiourea by 1,3-dithiothreitol, which, according to published data,[15] reduces rather easily (without preincubation) the S—S bonds in proteins upon the thiol—disulfide exchange. As a result, the inhibitory action of the metal on the catalytic activity of peroxidase in the oxidation of *o*-dianisidine is enhanced. Determination of 0.2 ng mL^{-1} of Bi^{III} ($s_r = 0.04$, $n = 3$) is not interfered with by Ca^{II}, Mg^{II}, Zn^{II}, Ni^{II}, Fe^{III}, and Al^{III} in 1000-fold amounts, Pb^{II} and Ag^{I} in 100-fold amounts, and Cd^{II} in tenfold amounts; Hg^{II} interferes with the determination when present in commensurable amounts.[17]

The enzymatic methods for determination of organomercurials, especially highly toxic and volatile methylmercury are of considerable interest. We found that organomercury compounds (OMC) are weak peroxidase inhibitors: the inhibitory action of methylmercury and

Table 1. Analytical characteristics of the determination procedures of metal ions based on their inhibitory action on the catalytic activity of horseradish peroxidase in *o*-dianisidine oxidation with hydrogen peroxide depending on the solubility constants of their sulfides (0.1 *M* phthalate buffer solution, pH 5.0)

Metal ion	Quantifiable concentration range/ng mL^{-1}		c_{min}* /ng mL^{-1}	K^0_S
	in the absence of thiourea	in the presence of thiourea		
Hg^{2+}	25—100	$(1—10) \cdot 10^{-2}$	$8 \cdot 10^{-3}$	$1.3 \cdot 10^{-26}$
Bi^{3+}	$\geq 10^3$	2—10	1	$1.0 \cdot 10^{-19}$
Cd^{2+}	$\geq 10^3$	2—50	1	$5.0 \cdot 10^{-14}$
Pb^{2+}	$\geq 10^3$	20—150	1	$1.3 \cdot 10^{-14}$

* Metal detection limit in the presence of thiourea calculated using the 3S-criterion.

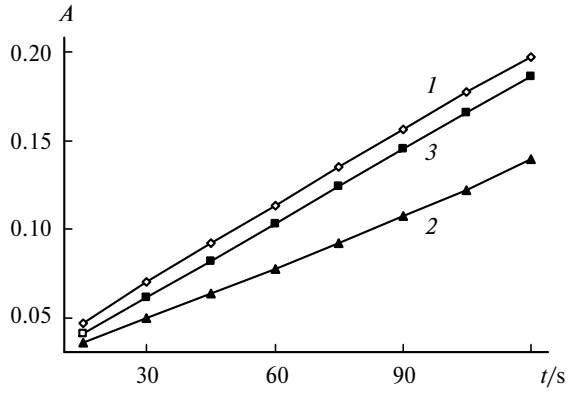

Fig. 1. Kinetic curves for peroxidase-catalyzed oxidation of *o*-dianisidine in the absence (*1*) and in the presence of phenylthiourea (*2*), phenylthiourea and methylmercury (*3*).

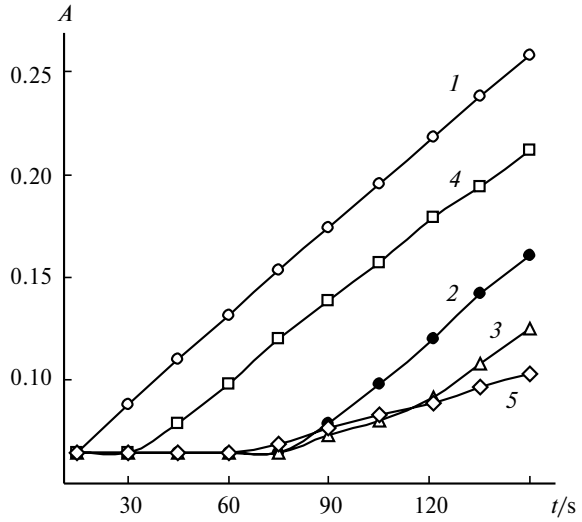

Fig. 2. Kinetic curves for horseradish peroxidase-catalyzed oxidation of *o*-dianisidine with hydrogen peroxide in the absence (*1*) and in the presence of DEDTC (*2*), DEDTC and cadmium(II) (*3*), DEDTC and bismuth(III) (*4*), DEDTC and lead(II) (*5*).

ethyl- and phenylmercury on the catalytic activity of horseradish peroxidase in the oxidation of *o*-dianisidine is observed at concentrations of ≥ 0.1 mmol L^{-1} and does not depend on the incubation time with the enzyme. However, the combined action of methylmercury with phenylthiourea or with 1,3-dithiothreitiol as peroxidase inhibitors increases the rate of the indicator reaction with respect to that in the absence of methylmercury but in the presence of a sulfur-containing compound (Fig. 1). The situation where a compound having a slight if any effect on the enzymatic activity can reduce or suppress the inhibitory effect of another compound is called liberating effect in enzymatic catalysis.[18] On the basis of the discovered liberating effect of methylmercury, we developed a procedure for its determination at a level of 0.1 μmol L^{-1}. This approach, *i.e.*, the addition of a sulfur-containing compound, resulted in a 1000-fold increase in the sensitivity of enzymatic determination of methylmercury.

2.2. The use of organic compound as a second enzyme substrate. Interesting results were obtained in our study of the effect of heavy metal ions and OMC on the kinetics of peroxidase oxidation of *o*-dianisidine in the presence of sodium diethyldithiocarbamate (DEDTC). This organic sulfur-containing compound functions as a second peroxidase substrate, being oxidized more easily and more early than *o*-dianisidine. This results in an induction period in the kinetic curves (Fig. 2), which becomes longer as the DEDTC concentration increases. The slope of the second section of the kinetic curves, which characterizes the rate of enzymatic oxidation of *o*-dianisidine, decreases.[19]

The introduction of heavy metal ions or OMC into the indicator system, together with DEDTC, results in different changes in the kinetic parameters of the enzymatic reaction (Table 2). It can be seen from Table 2 that in the presence of Bi^{III}, the induction period (τ_{ind}) becomes shorter and Cd^{II} decreases the rate of peroxidase-catalyzed *o*-dianisidine oxidation, this decrease becoming more pronounced with an increase in the Cd^{II} concentration. The Pb^{II} and OMC change both kinetic parameters. The cause of this effect of metal ions and OMC was described in detail in our previous publications.[13,19]

The presence of inverse proportionality between the induction period and the Bi^{III}, Pb^{II}, and OMC (methyl-, ethyl-, and phenylmercury) concentration was used to develop enzymatic procedures for their determination; the metrological parameters of the procedure are presented in Table 2. The procedure for Cd^{II} determination is based on the linear dependence of the rate of peroxidase-catalyzed *o*-dianisidine oxidation on the metal concentration in the range of 0.05—0.5 μg mL^{-1} (see Table 2). The Cd^{II} determination is not interfered with by a fivefold amount of Bi^{III} present in the reaction system.[13]

Thus, these studies demonstrated the expedience of studying not only separate, but also combined action of a metal ion (or OMC) and an organic sulfur-containing compound, because the action of the former depends on the nature of the latter.

3. The use of various analytical signals

In the study of the effect of phenol and its derivatives, which are priority pollutants of the environment, on the catalytic activity of horseradish peroxidase, we found that phenolic derivatives have different effects on the rates of plant peroxidase-catalyzed oxidation of aromatic diamines: benzidine, *o*-dianisidine, and 3,3′5,5′-tetramethylbenzidine (TMB).[21—24] In terms of the type of their influence, they were divided into two groups. The first group includes phenolic compounds whose redox

Table 2. Analytical characteristics of the action of metal ions (M) and OMC on peroxidase-catalyzed oxidation of *o*-dianisidine in the presence of DEDTC (L) (0.1 *M* phthalate buffer solution, pH 5.0)

Ion	$\log\beta_{ML_m}$[a] (*m*)	c_M/μg mL^{-1}, c_{RHg^+}/μmol L^{-1}	Analytical signal	Type of action[b]		c_L(M)[c]/μg mL^{-1}; $c_{min}(RHg^+)$/μmol L^{-1}
				τ_{ind}	υ	
Hg^{2+}	44.56 (2)[20]	< 0.0001	—	—	—	—
$MeHg^+$	—[d]	0.2—10	τ_{ind}	↓	↓	0.06
$EtHg^+$	—[d]	0.6—5	τ_{ind}	↓	↓	0.4
$PhHg^+$	—[d]	0.6—5	τ_{ind}	↓	↓	0.9
Bi^{3+}	35.42 (3)[20]	0.05—1	τ_{ind}	↓	—	0.05
Cd^{2+}	17.83 (2)[20]	0.05—0.5	υ	—	↓	0.05
Pb^{2+}	20.19 (2)[20]	0.0002—0.001	τ_{ind}	↓	↓	0.01

[a] Stability constant of the complex.
[b] Decrease in the signal (↓) or no effect (—); υ is the initial rate of the indicator reaction in the presence of an organic compound.
[c] Lower quantifiable concentration limit (c_L).
[d] No published data are available.

potential is higher than that of the peroxidase-reducing substrate (Fig. 3). In the presence of these compounds, the rate of the indicator reaction markedly decreases in direct proportion to the logarithm of their concentration. These phenols are enzyme inhibitors.

The kinetic curves for the indicator reaction in the presence of second-group phenols with redox potentials lower than that of the key peroxidase-reducing substrate (see Fig. 3) have an induction period whose length is directly proportional to the phenol concentration. After completion of the induction period, the reaction proceeds at a somewhat lower rate than in the absence of phenols. Thus, the shape of kinetic curve is similar to that for *o*-dianisidine oxidation in the presence of DEDTC and Pb^{II} (see Fig. 2, curve *5*). The kinetic and spectrophotometric data that we obtained[21,23] indicate that the introduction of such compounds into an indicator enzymatic reaction induces combined oxidation of the key substrate (benzidine, *o*-dianisidine, or TMB) and the corresponding phenol, *i.e.*, peroxidase catalyzes the oxidation of two substrates. Hydrogen peroxide oxidizes first the phenol, which has a higher reducing ability, and only after that, does the oxidation of the key substrate start, as indicated by increase in the absorbance of the oxidation product.

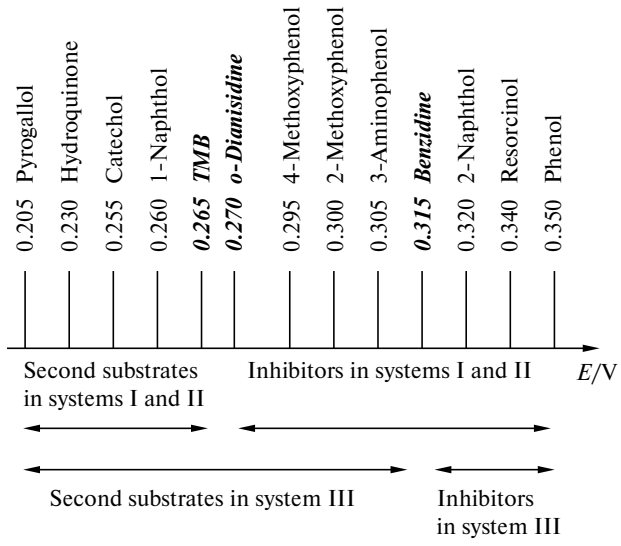

Fig. 3. Type of influence of phenolic compounds in the presence of horseradish peroxidase *vs.* their redox properties in indicator systems: TMB—H_2O_2 (I), *o*-dianisidine—H_2O_2 (II), benzidine—H_2O_2 (III) (real redox potential (*E*) was measured *vs.* silver chloride electrode, t = 25 °C).

We showed[21,23] that the type of influence of phenols does not depend on their acid-base properties, but is mainly determined by the ratio of the redox potentials of the phenol and the key substrate; the structure and the size of the phenol molecule and the oxidation mechanism of the key peroxidase substrate are other essential factors.

The possibility to quantify isomeric phenols (for example, 1- and 2-naphthols as the second substrate and the inhibitor, respectively) when present simultaneously using different analytical signals can be regarded an important outcome of this study.[25]

An induction period in the kinetic curves for peroxidase-catalyzed oxidation of *o*-dianisidine and TMB appeared also in the presence of ascorbic acid in concentration of 0.1—10 μmol L^{-1}. The direct proportionality between the length of the induction period and the concentration of ascorbic acid underlies the procedure for its enzymatic determination.[26]

Thus, the use of two different analytical signals, *viz.*, the indicator reaction rate and the length of induction period, opens up the way for determination of organic

compounds that perform different functions in the indicator reaction, being either inhibitors or second peroxidase substrates. This approach can be recommended for determination of other classes of organic compounds with different redox potentials in peroxidase-catalyzed reactions.

4. Variation of the nature of the substrate

The use of oxidoreductases and hydrolases with a broad substrate specificity (which refers to all enzymes considered in this review) opens up one more opportunity for improving the analytical characteristics of enzymatic determination of effectors, namely, variation of the nature of substrates. Good prospects of this approach were demonstrated in relation to enzymatic determination of metal ions and OMC. The replacement of *o*-dianisidine (*o*-D) by other aromatic diamines, *o*-phenylenediamine (PDA) or TMB, resulted in a substantial increase in the sensitivity of determination of Hg^{II} and Cd^{II} based on their inhibitory action on the catalytic activity of horseradish peroxidase[12,27,28] and methylmercury determination based on its influence on the induction period in the oxidation of an aromatic diamine in the presence of DEDTC (Table 3).[19] In some cases, the variation of the nature of the reducing substrate increases the selectivity of determination of metal ions (see Table 3).

5. The use of enzyme preparations isolated from different sources

A highly promising way of directed change in the analytical characteristics of enzymatic determination of substrates and effectors is the use of specimens of the same enzymes isolated from different sources. It was found that the degree and, in some cases, the type of influence of the same effectors on enzymes of different origin are different. This is obviously due to different structures of enzyme active sites and their surrounding, different amino acid sequences and their mutual positions, *etc.*

A comparison of the type and degree of action of mercury(II) on the activity of peroxidases isolated from horseradish and peanuts has shown that mercury(II) starts to exhibit individual (in the absence of thiourea) inhibitory action on peanut peroxidase at concentrations 250 times lower (0.1 ng mL^{-1}) than on horseradish peroxidase (see Table 1).[29] In addition, thiourea decreases the detection limit of mercury(II) in horseradish peroxidase-catalyzed reaction by more than four orders of magnitude, while in the presence of peanut peroxidase, thiourea almost does not influence the degree of inhibitory effect of mercury. Obviously, mercury(II) reacts with peanut and horseradish peroxidases by different mechanisms, which may be due to some differences in their structures. The procedure developed using peanut peroxidase allows one to determine mercury at the MPC level (c_{min} = 0.1 ng mL^{-1}) but it is inferior in sensitivity and selectivity to the mercury(II) determination procedure using horseradish peroxidase in the presence of thiourea. Determination of 0.2 ng mL^{-1} of Hg^{II} with peanut peroxidase is not interfered with by 50- and 100-fold amounts of Pb^{II} and Cd^{II}, while in the case of horseradish peroxidase, by 1000-fold amounts. However, if there is no need to introduce thiourea into the peanut peroxidase-catalyzed reaction, the incubation stage of mercury(II) with the enzyme is eliminated and, hence, the procedure becomes simpler and the time of analysis appreciably decreases (from 30 to 5 min).[29]

Table 3. Analytical characteristics of the determination procedures of mercury, methylmercury, and cadmium using native horseradish peroxidase in the oxidation of various substrates[12,19,27,28]

Reducing substrate	Concentration range (s_r for c_l)	c_{min}	Limitations[a]
		Mercury(II)/pg mL^{-1}	
o-D	10—1000 (0.03)	8	10^3-fold excess of Cd^{II}, Pb^{II}, Bi^{III}, 10^5-fold excess of Fe^{III}
PDA	2—5000 (0.05)	0.8	100-fold excess of Cd^{II}, Bi^{III}
TMB	0.6—5000 (0.05)	0.3	10^3-fold excess of Cd^{II}, 10^5-fold excess of Bi^{III}, Pb^{II}
		Cadmium(II)/μg mL^{-1}	
o-D	0.05—1 (0.06)	0.03	Commensurable amounts of Hg^{II}, Bi^{III}; 100-fold excess of Ag^{I}, Zn^{II}, Pb^{II}
TMB	0.01—1 (0.05)	0.008	—[b]
		Methylmercury/μmol L^{-1}	
o-D	0.2—10 (0.03)	0.06	—[b]
PDA	0.2—10 (0.03)	0.06	—[b]
TMB	0.05—5 (0.03)	0.03	Hg^{II} at a 100 MPC level, 100-fold excess of $EtHg^+$ и $PhHg^+$ [c]

[a] Interfere with the determination at the c_l level.
[b] No data.
[c] The influence of Pb^{II}, Bi^{III}, Fe^{III}, Zn^{II}, Cu^{II}, and Cd^{II} at the MPC level was eliminated by adding EDTA.

Table 4. Characteristics of the action of a number of inhibitors (L) of the catalytic activity of horseradish peroxidase (I) and peanut peroxidase (II) in the *o*-dianisidine oxidation (β_n is the stability constant of the inhibitor complex with iron(III) FeL_n)

L[a]	$\log\beta_n(n)$	C_L^{inh}/μmol L^{-1} [b]		τ_{inc}/min[c] (τ_{inc}/h)	
		I	II	I	II
HF	16.1 (5)	0.5—100	1—100	5	0
HCN	43.9 (6)	0.0008—0.01	—[d]	0	—[d]
Tar	11.9 (2)	20—100	10—100	60	15
EDTA	14.6 (1)	0.01—0.1	0.5—100	(12)	0
Ox	20.2 (3)	1—10	1—100	60	15
SSal	33.1 (3)	0.0008—0.01	100—1000	30	(18)
Sal	36.3 (3)	0.00002—0.0001	100—1000	30	(18)

[a] Notation: *Tar*, *Ox*, *SSal*, *Sal* are tartaric, oxalic, sulfosalicylic, and salicylic acids, respectively.
[b] Range of L concentrations in which the inhibitory action is manifested.
[c] Duration of enzyme incubation with the inhibitor (τ_{inc}).
[d] No data.

Horseradish and peanut peroxidases also have different sensitivities with respect to inhibitors that decrease the catalytic activity of these enzymes upon the formation of strong iron(III) (peroxidase cofactor) complexes.[30,31] The inhibitory action of inorganic (HF and HCN) and organic ligands (tartaric, oxalic, salicylic, and sulfosalicylic acids and EDTA) on the peroxidases increases with an increase in the stability constants of their iron(III) complexes, but the extents of this influence are different for the two plant peroxidases (Table 4). It is evident that the use of horseradish peroxidase is preferred for the determination of these compounds.[31]

The above-described established different (either inhibitor or a second substrate) functions of phenols in the horseradish peroxidase-catalyzed oxidation of aromatic diamines was verified in the same reactions in the presence of other plant peroxidases isolated from peanut cells, the xylotropic fungus *Phellinus igniarius*, the alfalfa cell culture *Medicago sativa*, and soya hulls. It was shown that different peroxidases exhibit different sensitivities with respect to the action of these organic compounds (which is of the same type in all cases) (Fig. 4).[21—24]

Different sensitivities of enzymes isolated from different sources with respect to the same inhibitors were also observed for alcohol dehydrogenases. A study of the influence of a number of metal ions on the catalytic activity of alcohol dehydrogenases from baker´s yeast (ADH-I) and horse liver (ADH-II) in the oxidation of ethanol with nicotinamide adenine dinucleotide (NAD^+) has shown that Hg^{II}, Ag^{I}, Cd^{II}, Cu^{II}, and Zn^{II} inhibit the catalytic activity of both ADHs in different concentration ranges (Table 5).[2,32,33] The efficiency of the inhibitory action of

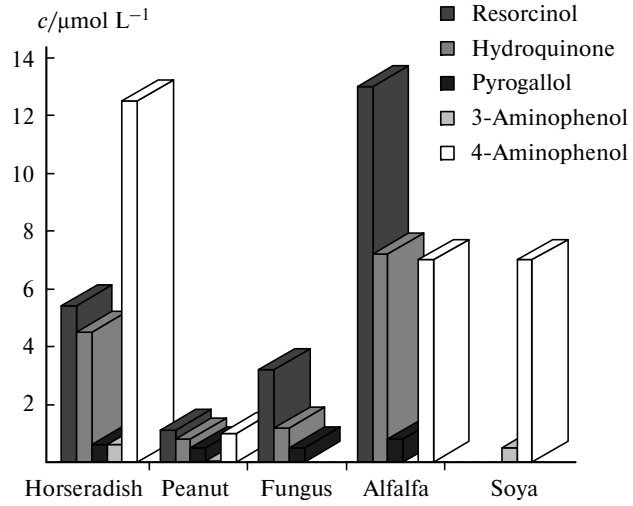

Fig. 4. Comparison of the lower limits of determined concentrations of phenolic compounds with peroxidases of different origin.

metal ions on the catalytic activity of ADH-I and ADH-II decreases along the following sequences, respectively:

$$Hg^{II} > Ag^{I} > Cd^{II} \approx Pb^{II} > Zn^{II} > Cu^{II} \gg Bi^{III};$$

$$Hg^{II} > Cd^{II} > Zn^{II} \gg Cu^{II} > Co^{II}.$$

An interesting feature of ADH-II, unlike ADH-I, is that it is inhibited by Co^{II}. The degree of inhibitory action of metal ions on the ADH catalytic activity is determined by the number of thiol groups in the enzyme molecule (28 in ADH-I and 14 in ADH-II (see Ref. 34)); cations that have affinity to these group exhibit the highest inhibitory action on the enzyme.

Mercury(II) is the most effective inhibitor of alcohol dehydrogenases. The different accessibilities of reactive sulfhydryl groups in the ADH molecules from baker´s yeast and the horse liver and the different structures of their active forms (tetramer and dimer, respectively) allow one to determine Hg^{II} using ADH-I in considerably lower concentrations than using ADH-II (see Ref. 2).

Table 5. Characteristics of the inhibitory action of metal ions (M) on alcohol dehydrogenases

M	C_M^{inh}/μg mL^{-1}*		τ_{inc}/min	
	ADH-I	ADH-II	ADH-I	ADH-II
Hg^{II}	$1\cdot10^{-6}$—$1\cdot10^{-2}$	0.01—1.0	0	0
Ag^{I}	$1\cdot10^{-6}$—$1\cdot10^{-3}$	0.05—10	5	0
Cd^{II}	$1\cdot10^{-6}$—$1\cdot10^{-2}$	1—100	0	0
Zn^{II}	$1\cdot10^{-4}$—0.1	0.1—1.0	5	5
Pb^{II}	$5\cdot10^{-3}$—$1\cdot10^{-2}$	10—100	15	0
Cu^{II}	0.01—1.0	1—100	0	0

* Concentration of the metal at which the inhibition is manifested.

Methylmercury, like Hg^{II}, has a substantial inhibitory effect on alcohol dehydrogenases; however, this is manifested at higher concentrations in the case of ADH-II (1—100 μmol L^{-1}) than in the case of ADH-I (0.001—10 μmol L^{-1}).[2,35] The inhibition of both ADHs by methylmercury is of the same, noncompetitive type. The degree of inhibition (I (%)) by these effectors is much higher for ADH-I than for ADH-II, in particular, 85 and 10% for mercury(II) ($c_{Hg^{II}} = 0.1$ μmol L^{-1}) and 68 and 7% for methylmercury ($c_{MeHg^+} = 1$ μmol L^{-1}), respectively.

A study of the influence of a broad range of mono- and dibasic saturated nonbranched carboxylic acids on alcohol dehydrogenases showed that pentanoic, decanoic, succinic, and decanedioic acids are most efficient inhibitors of ADH-II. These acids present in concentrations of 0.1 mmol L^{-1} inhibit the catalytic activity of ADH-II by 15, 25, 37, and 45%, respectively. Meanwile, decanoic, succinic, and decanedioic acids almost do not influence the activity of ADH-I ($I \leq 8\%$), while pentanoic acid inhibits both ADHs to the same extent. The inhibitory action of these acids on the catalytic activity of ADH-II forms the basis of enzymatic procedures for their determination with detection limits of 20—0.6 μmol L^{-1}, respectively ($s_r = 0.02$—0.03 for the concentration c_l).[36]

Alkaline phosphatases belong to the class of hydrolases and catalyze hydrolysis of phosphates. The specificity of action and catalytic activity of alkaline phosphatases depend on the source of the enzyme. The nature of the organism tissue from which the enzyme was isolated is significant, especially in the case of alkaline phosphatases.[37]

Alkaline phosphatases are metal-dependent enzymes containing zinc ions needed for their catalytic activity in the active site and magnesium ions in allosteric sites. The content of zinc in the alkaline phosphatase molecule is determined by its spatial structure and varies from two to four atoms per enzyme subunit for the dimer or reaches 16 atoms for the tetramer. We studied the properties and effectors of alkaline phosphatases from *Escherichia coli* and chicken intestine (these are dimers) and the phosphatase from the seal small intestine (tetramer).

Hydrolysis of *p*-nitrophenyl phosphate giving colored *p*-nitrophenolate ion at pH > 7 was chosen as the indicator reaction for measuring the catalytic activity of alkaline phosphatases. In the concentration range of 0.01—10 μg mL^{-1}, zinc was found to inhibit all three alkaline phosphatases (pH 9.8).[38] The sensitivity to zinc ions (I (%) for $C_{Zn} = 2.5$ μg mL^{-1}) decreases in the following sequence: seal intestine alkaline phosphatase (60%) > chicken intestine phosphatase (35%) > > *E. coli* phosphatase (15%). This may indicate that the accessibility of allosteric centers of enzymes decreases in this series and the displacement of magnesium ions becomes more difficult. In addition, the degree of inhibition of these enzymes by zinc ions is virtually independent of the incubation time unless it exceeds 30 min. The inhibitory action of zinc(II) is selective and can be observed at rather low concentrations only for the alkaline phosphatase from seal intestine. This inhibitory effect was used to develop the procedure of zinc determination in the concentration range of 1—10 μg mL^{-1} ($s_r = 0.03$ at the concentration c_l).

Study of the effect of magnesium (in the concentration range of 2—0.2 mg mL^{-1}) on the catalytic activity of three alkaline phosphatases has shown that magnesium efficiently activates alkaline phosphatase from chicken intestine (the activation degree $A = 600\%$ for $c_{Mg} = 0.02$ mg mL^{-1}), weakly activates the *E. coli* enzyme ($A = 20\%$ for $c_{Mg} = 0.2$ mg mL^{-1}), and does not affect the catalytic activity of alkaline phosphatase from seal intestine.[39] The activating effect of Mg^{II} on the alkaline phosphatase from chicken intestine underlies the selective enzymatic procedure for its determination in the concentration range of 0.6—6 ng mL^{-1} ($s_r = 0.04$ for the concentration c_l).

6. Variation of the nature of buffer solution

Buffer solution is a necessary component of any biochemical reaction sensitive to even minor changes in the pH of the reaction medium. The components of buffer systems can interact with the enzyme, substrate, or biocatalyst effectors. Our systematic research of the catalytic activity of some metalloenzymes, acid and alkaline phosphatases, and peroxidases in inorganic and organic buffer solutions has shown that by varying the nature and the concentration of the buffer solution one can modify deliberately the sensitivity and selectivity of determination of not only enzymes, but also their inhibitors, especially metal ions. The validity of this statement will be demonstrated below using particular examples.

6.1. Increase in the sensitivity of enzyme determination. A study of the effect of the nature of buffer solution on the kinetics of peroxidase-catalyzed oxidation of PDA (pH 5.0) and TMB (pH 7.0) has shown that the rate of enzymatic oxidation of PDA decreases in the following series of 0.1 *M* buffer solutions: phthalate > borate—succinate > citrate > acetate; the rate of TMB oxidation in buffer mixtures containing phosphate ions (borate—phosphate, phosphate, and phosphate alkaline) is higher than in borate or borate—phthalate buffer solutions.[28] Phosphate ions accelerate the oxidation of TMB, this acceleration being directly proportional to the concentration of phosphate ions in the buffer solution. Thus, it is recommended to use borate—phosphate and phthalate buffer solutions to ensure high catalytic activity of horseradish peroxidase in the oxidation of TMB and PDA, respectively.

The catalytic activity of alkaline phosphatase from *E. coli*, chicken intestine, and seal small intestine is maxi-

mum at pH 9.8 to 10.0 and depends on the nature and concentration of the buffer solution.[38,40] Different alkaline phosphatases exhibit the highest catalytic activity in different buffer solutions: that from *E. coli*, in 0.1 *M* carbonate solution (this may point indirectly to a greater number of non-ionogenic amino acid residues stabilized by carbonate and hydrocarbonate ions and/or a denser packing of the enzyme); that from chicken intestine, in a 0.025 *M* glycine solution (this is probably caused by incorporation of glycinate ions ($CH_2(NH_2)COO^-$) into the phosphatase molecule and interaction with the positively charged amino acid residues in its protein globule, which stabilizes the enzyme); and alkaline phosphatase from seal intestine, in a 0.05 *M* tris-HCl buffer solution. All three alkaline phosphatases are least active in the borate buffer solution. The optimal concentrations of the buffer solutions where the catalytic activity of the phosphatases is maximum are also different.

6.2. Increase in the sensitivity of determination of inhibitors. Study of the effect of zinc ions on the catalytic activity of alkaline phosphatase from seal intestine in various buffer solutions (borate, carbonate, Tris-HCl, glycine) under the optimal conditions for hydrolysis of *p*-nitrophenyl phosphate showed that zinc inhibits the enzyme in any buffer system but at different concentrations (c_{Zn}/μg mL^{-1} for $I = 50\%$), increasing in the following series of buffer solutions: borate (0.04) < carbonate (0.25) < Tris-HCl (1.5) < glycine (2.2).[41] Obviously, the reason is that the equilibrium concentration of zinc ions in inorganic buffer solutions is higher than in organic ones whose components can form stable complexes with zinc. When zinc concentration is ≥ 5 μg mL^{-1}, the degree of phosphatase inhibition does not change in any of the buffer solutions, apparently, because of saturation of the zinc-binding enzyme sites by metal ions at high contents of the metal in the reaction mixture.[41]

The concentration of the buffer solution is equally important in determining the degree of inhibition of alkaline phosphatase by metal ions. For example, in the case of alkaline phosphatase from *E. coli*, the concentration of metal ions required to attain the same inhibition degree increases by two orders of magnitude on going from 0.05 to 0.1 *M* Tris-HCl buffer solution.

The replacement of 0.05 *M* Tris-HCl buffer solution by a 5 *M* borate buffer (pH 9.8) markedly increased the sensitivity of zinc determination based on its inhibitory action on alkaline phosphatase from seal intestine in the hydrolysis of *p*-nitrophenyl phosphate: the ranges of zinc concentrations determined were 1—10 and 0.01—0.1 μg mL^{-1}, respectively.[40]

Interesting results have been obtained in our study of the inhibitory effect of fluoride ions on the catalytic activity of Fe-dependent acid phosphatase from potato tubers in the hydrolysis of *p*-nitrophenyl phosphate carried out 0.1 *M* acetate and citrate buffer solutions at pH 5.0 (see Ref. 42). The replacement of the citrate buffer solution by the acetate buffer with the same concentration increased tenfold the sensitivity of fluoride determination (c_{min} 0.5 and 0.05 μg mL^{-1}, respectively). The increase in the enzyme sensitivity to fluoride ions in the acetate buffer solution is apparently due to the higher accessibility of the acid phosphatase active site caused by lower stability of iron(III) acetate complexes compared to citrate complexes.

Thus, it was shown that variation of the nature of the buffer solution in enzymatic processes allowed one to increase the sensitivity of determination procedures for metal ion inhibitors.

6.3. Increase in the selectivity of determination of inhibitors. The variation of the nature of the buffer solution allows one to control not only the sensitivity, but also the selectivity of inhibitor determination. Thus when the hydrolysis of *p*-nitrophenyl phosphate in the presence of alkaline phosphatase from seal intestine is carried out in the borate buffer solution, zinc determination at the c_l level (corresponding to its MPC in water) is interfered with by only $2.5 \cdot 10^5$- and $7 \cdot 10^5$-fold excess of potassium and cesium, respectively.

The results of studying the influence of the nature of the buffer solution on the degree of inhibition of horseradish peroxidase by transition and heavy metal ions in the oxidation of *o*-dianisidine (Fig. 5) show that Fe^{III}, Bi^{III}, and Zn^{II} ions have the most pronounced inhibitory effect on the enzyme in any of the buffer systems. The Mn^{II}, Pb^{II}, Cu^{II}, and Cd^{II} ions virtually do not influence the peroxidase catalytic activity ($I < 20\%$). Meanwhile, Hg^{II} ions slightly activate the enzyme through the formation of mixed soluble hydroxo complexes.

It can be seen from the histogram that the inhibitory action of zinc ions is maximum in the acetate buffer solution where almost all ions are free (not complexed with the acetate ion). The cadmium ions do not inhibit peroxidase in any of the buffer systems studied. Thus, the peroxidase inhibition by zinc in the presence of cadmium is selective in all buffer mixtures; however, the highest selectivity of the enzyme with respect to other metals was observed in the citrate buffer.

A comparison of the mole fractions (α-coefficients) of free metal ions in different buffer mixtures with the degrees of inhibition of the peroxidase activity by these ions revealed a correlation between these values for copper and zinc ions. The greater the $p\alpha_M$ value the lower the concentration of free metal ions in the buffer solution and the less pronounced their inhibitory action on the enzyme (see Fig. 5).

Thus, the correct choice of the buffer solution for the enzymatic reaction is favorable for increasing not only the sensitivity but also the selectivity of action of effectors on the biocatalyst. The developed approach to investigation of various buffer systems whose components are complexed,

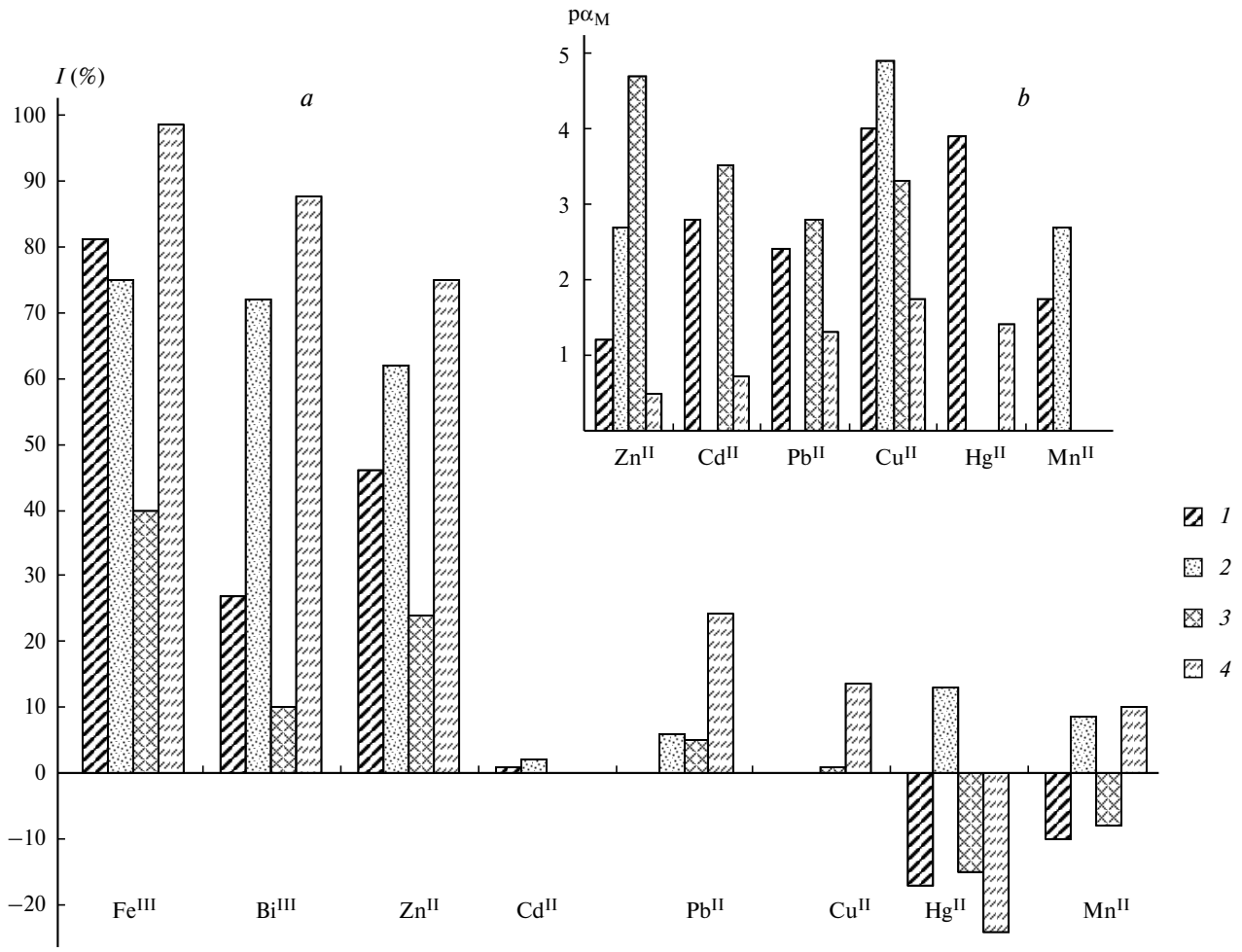

Fig. 5. Degree of inhibition (I (%)) of horseradish peroxidase by metal ions in *o*-dianisidine oxidation (*a*) and logarithms of the mole fraction of free metal ions ($p\alpha_M = -\log\alpha_M$) (*b*) depending on the nature of the metal and buffer solution: (*1*) phthalate (*Pht*), pH 4.2; (*2*) citrate (*Cit*), pH 3.5; (*3*) tartrate (*Tar*), pH 4.5; (*4*) acetate (*Ac*), pH 4.0 (buffer solution concentration 0.1 mol L^{-1}).

more or less strongly, with analyte metal ions can be extended to other enzymes.

7. Use of enzyme preparations immobilized on supports with different properties

In recent years, immobilized enzymes have been used more and more often for the design of test devices and test methods for highly sensitive, selective, and rapid determination of biologically active compounds in the environmental samples and for medicine.[43] Using peroxidase and alkaline phosphatase, we demonstrated that a rational selection of the support, the modifier, and the method for biocatalyst immobilization may not only give highly active and stable enzyme preparations, but also allows one to control the sensitivity of these preparations with respect to substrates and effectors.

7.1. Selection of the supports and the methods for peroxidase and alkaline phosphatase immobilization. For successful development and practical analytical application of test methods based on immobilized enzymes, in particular, horseradish peroxidase and alkaline phosphatase from chicken intestine, it is significant to choose the supports and enzyme immobilization conditions that ensure their high catalytic activity with minimum consumption of the native preparations.

For physical (sorptional) immobilization of peroxidase and alkaline phosphatase, we chose their incorporation into the network of natural polysaccharide gels, because this method of immobilization usually disturbs the enzyme structure to a lesser extent and the properties of enzymes immobilized in this way differ little from the properties of native enzymes in solution. To this end, we selected the most widely used and readily available polymers, namely, chitosans with different molecular masses, carboxymethylcellulose, agarose, dextran, starch, alginic acid, and β-cyclodextrin.

Silica gel, microcrystalline cellulose (MCC), chromatographic paper, and synthetic polymers, *viz.*, polystyrene (plate for immunochemical analysis) and polyurerthane foam (PUF), were used as supports for peroxidase and alkaline phosphatase immobilization for the development of test methods.[44]

Each of the above-listed polysaccharides was found to increase the activity or stability of immobilized peroxidase and alkaline phosphatase. For example, for PUF the activity of immobilized peroxidase preparations increases in the following series: chitosan (molecular mass (MM) 120 kDa) < carboxymethylcellulose < agarose = calcium alginate = dextran < starch < chitosan (MM 10 kDa), while their stability increases in the series carboxymethylcellulose < chitosan (MM 10 kDa) = starch = calcium alginate < β-cyclodextrin < chitosan (MM 120 kDa). Thus, chitosan with MM 10 kDa has the highest activating effect with respect to peroxidase and chitosan with MM 120 kDa has the most pronounced stabilizing action at a minimum content on the support.

The highest activating effect on alkaline phosphatase was found for calcium alginate, while the highest stabilizing effect at a minimum content on PUF was found for *N*-phthalylchitosan.

Apart from the activity and stability of the immobilized enzyme, the reproducibility of the time of appearance of the color of the indicator reaction product on the support surface is also significant in the case of visual detection. On a PUF tablet, a clear-cut easily detectable and reproducible (the s_r value not higher than 0.09) green-to-red color change caused by the indicator reaction of *o*-dianisidine oxidation was observed only upon peroxidase incorporation into chitosans, agarose, and calcium alginate.[45] When the indicator reaction of *p*-nitrophenyl phosphate hydrolysis is carried out in the presence of ammonium molybdate and Malachite green on PUF containing alkaline phosphatase immobilized in calcium alginate and *N*-phthalylchitosan, a clear-cut sharp colorless-to-green color change takes place, the time of this change being highly reproducible (the s_r values did not exceed 0.08 and 0.06, respectively).[46]

For the development of the test procedures for determination of peroxidase and alkaline phosphatase effectors, enzyme preparations immobilized in chitosan with MM 120 kDa and *N*-phthalylchitosan, respectively, were used. Characteristics of these preparations are summarized in Table 6.

As a result of the study, PUF was recognized to be the best support, as on this support biocatalysts exhibit the highest catalytic activity at a minimum consumption of the native enzyme; immobilized preparations are stable for the longest period of time (see Table 6); color transitions on the PUF are bright, sharp, and easily detectable, which ensures good reproducibility of recording of the time they appear and simplicity of visual detection.[45]

7.2. Comparison of the analytical characteristics of test procedures for determination of a number of heavy metal ions and organomercury compounds using enzymes immobilized on various supports. The activity and stability of peroxidase immobilized on various supports was evaluated by means of indicator reactions of *o*-dianisidine, PDA, and TMB oxidation in which color changes on the support were most clear-cut and sharp, which is necessary for visual monitoring of the rate of the enzymatic process (Table 7).

On the basis of the inhibitory action of mercury(II) (which is enhanced in the presence of thiourea, as in the case of native enzyme) on the catalytic activity of peroxidase immobilized on a polystyrene plate,[47,48] on silica gel,[49] chromatographic paper,[50,51] microcrystalline cellulose,[49] and PUF[52] in the oxidation of aromatic diamines, test procedures for Hg^{II} determination were developed.

Table 6. Characteristics of the horseradish peroxidase and alkaline phosphatase preparations immobilized on various supports

Enzyme (polysaccharide)	Support	$c_E{}^a$/μ*M*	$\tau_{so}{}^b$/days	$s_r{}^c$ ($n = 3$)
Peroxidase (chitosan)	Silica gel	1	180	0.04
	Paper	10	30	0.12
	Microcrystalline cellulose	0.1	150	0.08
	Polyurethane foam	0.01	550	0.06
Alkaline phosphatase (*N*-phthalylchitosan)	Silica gel	1	120	0.12
	Microcrystalline cellulose	0.1	90	0.10
	Polyurethane foam	0.1	400	0.04

[a] Lowest concentration of the native enzyme needed to obtain highly active immobilized preparation.
[b] Storage time of the immobilized enzyme preparation during which it retains at least 50% of the initial activity.
[c] Reproducibility of the results of measurement of the indicator reaction rate based on the instant of appearance of the color of the final product.

Table 7. Analytical characteristics of test procedures for determination of metal ions inhibiting enzymes immobilized on various supports[38,47—54]

Support	M^{II}	Indicator reaction	Detected color change	c_L/ng mL^{-1}
		Horseradish peroxidase		
Polystyrene plate	Hg	*o*-D—H_2O_2	Green → red	0.01
		TMB—H_2O_2	Light blue → cherry	0.001
Chromatographic paper		PDA—H_2O_2	Brown → green	0.04
		TMB—H_2O_2	Light blue → brown	0.01
Silica gel		TMB—H_2O_2	Light blue → yellow	0.01
MCC		TMB—H_2O_2	Light blue → yellow	0.005
PUF		*o*-D—H_2O_2	Green → red	0.001
PUF	Cd	*o*-D—H_2O_2	Green → red	0.1
PUF	Pb	*o*-D—H_2O_2	Green → red	0.5
		Alkaline phosphatase from chicken intestine (I) and seal small intestine (II)		
Polystyrene plate	**I**—Pb	Hydrolysis of *p*-nitrophenyl phosphate	Colorless → green	1
Silica gel				1
MCC				0.5
PUF				0.05
PUF	**II**—Zn			0.1

The characteristics of the procedures are summarized in Table 7.

It is noteworthy that thiourea markedly (by 40—50%) inhibits native peroxidase only after their preincubation for 4 h, while peroxidase immobilized on a polystyrene plate, silica gel, chromatographic paper, or microcrystalline cellulose is inhibited upon 5-min incubation; when the indicator reaction is carried out on PUF, no preincubation is needed.[52]

A study of the effect of cadmium(II) and lead(II) on the catalytic activity of PUF-immobilized peroxidase showed that these ions inhibit (after 15-min incubation with the biocatalyst) the immobilized enzyme to a greater extent than the native enzyme (see Table 7).[46]

Test procedures for the determination of organomercury compounds (methyl-, ethyl-, and phenylmercury) based on their liberating action on the catalytic activity of PUF-immobilized peroxidase pre-inhibited by phenylthiourea in the oxidation of *o*-dianisidine ($n = 3$):

c_l/ng mL^{-1}	0.2	2	50
s_r	0.11	0.12	0.12

and based on their effect on the length of the induction period arising in the kinetic curves of TMB oxidation in the presence of diethyldithiocarbamate ($n = 3$) were developed:[53,54]

c_l/ng mL^{-1}	2	20	100
s_r	0.09	0.11	0.12

Hydrolysis of *p*-nitrophenyl phosphate was chosen as the indicator reaction for assaying the activity and stability of alkaline phosphatase preparations immobilized on silica gel, MCC, and PUF. Lead(II) was found to be an efficient inhibitor of alkaline phosphatase immobilized on various supports, which served as the basis for the development of sensitive test procedures for its determination (see Table 7).[55]

An important conclusion is that the nature of the support has an essential influence on the analytical characteristics of the enzymatic test procedures.[46] The test procedures we proposed for determination of mercury(II), cadmium(II), lead(II), and organomercury compounds using PUF-immobilized peroxidase and alkaline phosphatase with visual detection are distinguished by simplicity, rapidity (duration of analysis does not exceed 5 min); they are more sensitive than the previously developed procedures that use native enzymes and other known methods and comparable only with atomic absorption determination methods.[2]

All the test procedures we developed are fairly selective. For example, a study of the interfering influence of metal ions and anions (Cd^{II}, Bi^{III}, Pb^{II}, Cu^{II}, Zn^{II}, $Fe^{II,III}$, Ni^{II}, Co^{II}, Ca^{II}, Mg^{II}, Cl^-, Br^-, I^-, F^-, S^{2-}, PO_4^{3-}, SO_4^{2-}, CO_3^{2-}) on the determination of mercury(II) using PUF-immobilized peroxidase showed that only Cd^{II}, Bi^{III}, and Fe^{III} interfere with the determination of 1 ng mL^{-1} of Hg^{II} when present in 10^5-, 10^5-, and 10^6-fold amounts, respectively.[52]

7.3. Use of immobilized enzymes isolated from different sources. As in the case of native biocatalysts, an efficient approach for controlling the sensitivity and selectivity of the test procedures for determination of effectors is the use of immobilized enzymes isolated from different sources. We demonstrated this most clearly in the devel-

opment of enzymatic test procedures for determination of zinc based on its inhibitory action on alkaline phosphatases.[38] First of all, it should be noted that highly active immobilized alkaline phosphatase preparations from *E. coli*, chicken intestine, and seal small intestine were obtained using different concentrations of the native enzymes: 1, 0.1, and 0.01 μmol L^{-1}, respectively.[44] This may be due to differences in the enzyme structure and molecular mass.

The immobilized alkaline phosphatases from *E. coli* and chicken intestine retained more than 50% of the initial activity for 400 days, while the alkaline phosphatase from seal small intestine retained 92% of the initial activity during the same period.

It was found that zinc(II) has almost no influence on the catalytic activity of immobilized alkaline phosphatase from chicken intestine but inhibits the phosphatases from *E. coli* and seal intestine in the concentration ranges of 2.5—50 μg mL^{-1} and 0.1—50 μg mL^{-1}, respectively. As in the case of native enzymes, the degree of the inhibitory action of zinc(II) on alkaline phosphatase from *E. coli* is much lower than that on the seal intestine phosphatase. In addition, zinc(II) inhibits the immobilized alkaline phosphatase from seal small intestine at concentrations 500 times lower and over a wider concentration range than it inhibits native phosphatase from the same source. Apparently, this significant difference in the degree of zinc(II) inhibitory action on the native and immobilized phosphatase from seal intestine is due to the zinc(II) microconcentration effect caused by the high sorption capacity of the support (PUF).[38]

Thus, as in the case of native enzymes, only alkaline phosphatase from seal intestine is suitable for the development of a sensitive zinc determination procedure (see Table 7). Zinc(II) determination using this phosphatase immobilized on PUF is interfered with only by magnesium(II) present in a $5 \cdot 10^5$-fold amount, while lead(II), which was noted above to be an efficient inhibitor of alkaline phosphatase from chicken intestine, does not affect the activity of the enzyme isolated from the seal intestine.

The developed zinc(II) determination test procedure is rather selective and is 10 times as sensitive as the determination procedure using native alkaline phosphatase from seal intestine.

Thus, we demonstrated that both native and immobilized alkaline phosphatases isolated from different sources have different sensitivities and selectivities with respect to the ion cofactor metal, zinc(II). The alkaline phosphatase from seal small intestine is most promising for chemical analysis for selective and sensitive determination of zinc(II), while alkaline phosphatase from chicken intestine is preferred for lead(II) determination.

8. Implementation of the developed approaches in enzymatic procedures for analysis of particular samples

The new original approaches to the targeted increase in the sensitivity, selectivity, and rapidity of the enzymatic methods were applied to analysis of a broad range of samples including environmental samples (sea,[2,16,19] river,[29,32] underground,[30] and tap waters;[2] soils of various origin,[29,53] and grasses), pharmaceuticals,[41] biological fluids (blood, urine),[16,39] and foodstuffs.[2,26] Prior to analysis of each material, the effects of its properties and composition (pH, ionic strength, salinity (for sea water), the nature and the contents of macro and trace components)

Table 8. Results of determination of metal ions and organic compounds in various samples by enzymatic (I) and alternative (II) methods ($n = 5$, $P = 0.95$)[2,19,26,29,30,32,39,41,53]

Sample	Analyte (enzyme)	Analyte content		
		I	II	Comparison method
Waters		c/pg mL^{-1}		
Sea of Azov	Hg^{II} (nat. HRP)	450±60	480	Cold vapor AAS
Caspian Sea	The same	810±100	850	
Mediterranean Sea	«	650±50	680	
river Moskva	«	746±4	749±5	AFS
river Moskva	Hg^{II} (immob. HRP)	210±30	190	MS
river Ob	The same	410±30	390	Cold vapor AAS
river Esit´, Ekaterinburg	«	288±72	336±84	IVA
undersoil, Moscow Region	«	820±20	660±40	AFS
tap water, Moscow	«	3.4±0.3	3.5±0.4	Method of additives
Cara sea (estuary, river Yenisei)	CH_3Hg^+ (nat. HRP)	1.3±0.2	1.1±0.1	Enzymatic test method
	CH_3Hg^+ (immob. HRP)	3.6±0.2	3.4±0.3	Method of additives

(*to be continued*)

Table 8 (*continued*)

Sample	Analyte (enzyme)	Analyte content		
		I	II	Comparison method
Soils		c/mg kg^{-1}		
Podzolic	Hg^{II} (immob. HRP)	3.4±0.3	3.5	Cold vapor AAS
Clay	The same	2.2±0.6	2.0	
Clay	Cd^{II} (immob. HRP)	3.2±0.2	3.5	
Black soil	Pb^{II} (immob. AP-I)	14.5±0.4	16±5	Sorptional AAS
Red soil		15.5±0.3	15.0±0.9	Sorptional AAS
Sward-podzolic		68.4±0.4	67±3	
Volga bottom-land		6.5±0.3	7.2±0.6	AAS
Plant samples				
(grass extracts)		c/ng mL^{-1} (c/μg mL^{-1})		
Reed grass (*Calamagrostis arundinacea*)				
a park, western district, Moscow	Cd^{II} (immob. HRP)	4±2	1.6	AAS
	Zn^{II} (nat. HRP)	(0.33±0.02 μg)	(0.45)	
near a highway, western district, Moscow	Cd^{II} (immob. HRP)	4±1	3.0	AAS
	Zn^{II} (nat. HRP)	(0.40±0.03)	(0.48)	
		c (%)*		
common St. John's wort (*Hupericum perforatum*)	Succinic acid	5.3±0.2	5.2	IEC
Garden sage (*Salvia officinalis*)	(nat. ADH-II)	4.9±0.4	5.8	IEC
Biological fluids		c/ng mL^{-1}		
Urine	Hg^{II} (nat. HRP)	4.8±0.4	4.9±0.5	Method of additives
	Mg^{II} (nat. AP-I)	37.4±0.4	33.76	SP, AAS
Blood serum	Hg^{II} (nat. HRP)	0.052±0.002	0.055±0.004	Method of additives
	Zn^{II} (nat. ADH-I)	3.9±0.3	3.8±0.3	AAS
Pharmaceuticals		c/μg mL^{-1}		
Zinc insulin suspension	Zn^{II} (nat. AP-II)	24.3±0.7	24	AAS
Foodstuffs		c/mg kg^{-1} (method of additives)		
Pea	Cd^{II} (immob. HRP)	0.0062±0.0006		
	Pb^{II} (immob. AP-I)	0.13±0.03		
Grapes	Hg^{II} (immob. HRP)	2.4±0.4		
	Pb^{II} (immob. AP-I)	0.23±0.02		
Beef	Hg^{II} (immob. HRP)	1.5±0.3		
	Cd^{II} (immob. HRP)	0.19±0.02		
	Zn^{II} ((immob. AP-II)	9.4±0.4		
Pork	Pb^{II} (immob. AP-I)	0.09±0.02		
	Zn^{II} (immob. AP-II)	4.9±0.4		
		c/μmol L^{-1}		
Rainbow trout skin extract	3-Aminophenol (nat. SP)	1.59	1.5	Method of additives
	4-Aminophenol (nat. SP)	3.86	4.0	
Juices		c/mg·100 g^{-1}		
Apple (fresh)	Ascorbic acid (nat. HRP)	2.20±0.01	2.23	Titration (GOST)
Apple (J-7)		0.28±0.01	0.29	
Pomegranate		2.30±0.08	2.36	
Infant food				
Milk		0.52±0.06	0.56	HPLC
Sour-milk formula		2.2±0.8	3.0	HPLC

Note. Abbreviations: HRP is horseradish peroxidase, SP is soybean peroxidase, AP-I is alkaline phosphatase from chicken intestine, AP-II is alkaline phosphatase from seal small intestine, AAS is atomic absorption spectrometry, AFS is atomic fluorescence spectrometry, MS is mass spectrometry, IVA is inversion voltammetry, IEC is ion exclusion chromatography, SP is spectrophotometry; nat. is native, immob. is immobilized.

* Of the dry raw material weight.

on the results were studied in detail and methods for minimization of the interfering factors were developed.

The results of determination of biologically active compounds in the above-listed materials obtained using native and immobilized enzymes were confirmed by alternative methods: atomic absorption spectrometry including cold vapor atomic absorption, atomic fluorescence spectrometry, mass spectrometry, ion exclusion chromatography, HPLC, inversion voltammetry, spectrophotometry, and titration. Examples of determination of inorganic and organic compounds in various samples are given in Table 8.

9. Conclusion

The results of our investigations and the discussion considering enzymes of two classes (oxidoreductases and hydrolases) attest to the necessity and importance of detailed investigation of the dependence of the indicator reaction kinetics, catalytic properties of native and immobilized enzymes, and enzyme sensitivity to biologically active compounds (inhibitors and activators) on the following factors

— the nature of the substrate,

— the nature and the concentration of the buffer solution,

— source of the enzyme preparation,

— simultaneous presence of two inhibitors (inorganic and organic) or an inhibitor and a second substrate in the reaction system,

— the nature of the support and agents used to immobilize the biocatalyst.

The substantiated choice of each of the components of the indicator reaction and thorough optimization of the reaction conditions both in solution and on the surface of a solid support result in a considerable increase in the sensitivity, selectivity, and rapidity of the enzymatic procedures.

The broad range of procedures with deliberately controlled metrological characteristics and selectivity allows one to choose the most appropriate procedure to solve a particular analytical task, to specify a broad range of inorganic and organic biologically active compounds in different concentration ranges for diverse samples taking into account the composition of the matrix.

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