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ARTICLES

A New Approach to the Enzymatic Determination of Magnesium, Calcium, and Barium

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Abstract—The effects of magnesium, calcium, and barium ions on the catalytic activity of alkaline phosphatase from the small intestine of the Greenland seal in the reaction of *p*-nitrophenyl phosphate hydrolysis in the presence of a number of complexones (ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, 1,2-cyclohexanediaminetetraacetic acid, and nitrilotrimethylenephosphonic acid) were studied. As a result, a new approach to the enzymatic determination of the above metals was developed. It consists in the use of the liberative effects of these metals on alkaline phosphatase preinhibited by nitrilotrimethylenephosphonic acid. Sensitive procedures for the enzymatic determination of magnesium, calcium, and barium ions were developed (c_{min} = 14 ng/mL, 24 ng/mL, and 0.8 μg/mL, respectively).

Enzymatic methods of analysis have long been successfully used for determining transition metal ions [Cu(II), Zn(II), Ni(II), and Fe(III)] and heavy metal ions [Hg(II), Cd(II), Pb(II), and Bi(III)] in various water, soil, food, and biological fluid (blood and urine) samples [1–3]. As a rule, the inhibiting or activating effects of the above metals on the catalytic activity of native or immobilized enzymes from various classes (usually, oxidoreductases and hydrolases) have been used for this purpose. Enzymatic procedures for determining cofactor metal ions, which enter into the active centers of enzymes and are responsible for their high catalytic activity, exhibited the highest sensitivity and selectivity. These procedures are based on the reactivation of apoenzymes. Procedures for determining Fe(III) with peroxidase [4]; for determining Cu(II) with polyphenol oxidase [5]; and for determining Zn(II) with aminopeptidase [6], alcohol dehydrogenase [7], carboanhydrase [8], and alkaline phosphatase [9–11] are well known. A target-oriented change in the sensitivity and selectivity of enzymatic procedures for determining transition metals and heavy metals became possible because of the use of the combined action of two effectors (a metal ion and an organic compound) [12], various analytical signals (reaction rate and the duration of an induction period) [1], various buffer solutions [13], and enzymes from the same class but isolated from different sources [7, 12, 14].

Enzymes have been used very rarely for determining magnesium and alkaline earth metals, although magnesium is a constituent of alkaline phosphatases and ATPases (it is responsible for the high stability of enzymes in storage and regulates (modulates) the catalytic activity of biocatalysts), whereas calcium is a constituent of peroxidases, α-amylases, and phytases (it stabilizes their structures).

An analysis of data published in the last decade demonstrated that only a few procedures for the enzymatic determination of the above metals are known. Thus, for example, a highly sensitive procedure was developed for determining magnesium (2.4–2400 μg/mL); it is based on the activation of firefly luciferase immobilized on BrCN-activated Sepharose [15]. Firefly luciferase catalyses the bioluminescence reaction of luciferin conversion into oxyluciferin in the presence of adenosinetriphosphoric acid and magnesium. This procedure is characterized by high accuracy; however, its wide use in the actual practice of chemical analysis is restricted by time-consuming and labor-intensive enzyme preparation and immobilization, as well as by the use of a luminescence technique for monitoring the rate of the indicator process. In the determination of Mg(II) at a level of the determination limit, Ca(II), Sr(II), and Ba(II) in 200- and 400-fold amounts, respectively, caused no interference.

A procedure for determining magnesium by its activating effect on the catalytic activity of alkaline phosphatase from chicken intestine in the hydrolysis reaction of *p*-nitrophenyl phosphate (*p*-NPP) [16] is highly sensitive (c_L = 0.6 ng/mL), selective, and rapid; the experimental procedure and instrumentation are simple. This procedure was applied to the determination of magnesium in urine.

The reactivating effect of calcium ions on alkaline phosphatase from chicken intestine preinhibited with EDTA in the above indicator reaction formed the basis for a procedure for the enzymatic determination of these ions in the concentration range 0.1–0.6 μg/mL [9]. The selectivity of the procedure is low because commensurable amounts of Ba(II), Sr(II), Mn(II), Be(II), Co(II), Hg(II), Cu(II), and Pb(II), as well as a

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tenfold amount of Mg(II), interfered with the determination of 0.1 μg/mL of Ca(II).

A search for new ways to target-oriented changes in the analytical characteristics of procedures for determining magnesium and alkaline earth metals promises to expand the possibilities of enzymatic methods of analysis.

The aim of this work was to develop new approaches to the determination of magnesium, calcium, and barium ions with alkaline phosphatase isolated from the small intestine of a seal as an example based on a study of individual and combined effects of the metal ions and complexones on the enzyme.

EXPERIMENTAL

Reagents. Alkaline phosphatase (EC 3.1.3.1) isolated from the small intestine of the Greenland seal *Phoca groenlandica* (henceforth, alkaline phosphatase from seal intestine) with an activity of 13 sp. units/mg (M_r = 67 kDa) was used in this study. The enzyme preparation was a solution in a 2.5 M ammonium sulfate solution (NPO Biolar, Latvia). Working solutions of the enzyme were prepared immediately before use by successively diluting the stock solution with a 0.05 M Tris–HCl buffer solution (pH 9.8).

p-Nitrophenyl phosphate disodium salt hexahydrate (Sigma, United States) was used without additional purification. The working solutions of *p*-NPP were prepared daily by dissolving weighed portions of the solid preparation in water. The solutions of alkaline phosphatases and the *p*-NPP preparation were kept in a refrigerator at 4°C.

Tris–HCl buffer solutions (0.05 M) with different pH values were prepared by dissolving weighed portions of tris(hydroxymethyl)aminomethane (Tris) (Serva, Germany) in water and adjusting the required pH values with 0.1 M solutions of high-purity grade HCl or KOH (Skeron, Russia).

The following solid aminopolycarboxylic acids were used without additional purification: ethylenediaminetetraacetic acid (Fluka, Switzerland), diethylenetriaminepentaacetic acid (DTPA), 1,2-cyclohexanediaminetetraacetic acid (CDTA), and nitrilotrimethylenephosphonic acid (NTP) (Sigma, United States) of analytical grade. The solutions of DTPA, CDTA, and NTP were prepared by dissolving weighed portions of the acids in water. The stock solution of sodium ethylenediaminetetraacetate (EDTA) was prepared by dissolving a weighed portion of ethylenediaminetetraacetic acid in a small volume of a 0.1 M NaOH solution followed by dilution with water (pH 5.0) to a required volume.

The working solution of Mg(II) ($n \times 10^{-2}$ mM) was prepared by diluting a stock standard solution of $MgCl_2$ (Sigma, United States) with water. The 7.2 mM stock solutions of Ca(II) and Ba(II) were prepared by dissolving weighed portions of the salts $CaCl_2 \cdot 2H_2O$ and $BaCl_2 \cdot 2H_2O$ (both of high-purity grade), respectively, in water. The solutions with lower concentrations of the metals were prepared daily by successively diluting the stock solutions with water.

Deionized water with a specific resistance of 18.2 MΩ purified on a Simplicity system (Millipore, France) was used for preparing all of the aqueous solutions.

Instrumentation. The absorbance of reaction solutions was measured on a Shimadzu UV-2201 spectrophotometer (Japan) or on a KFK-2 photoelectric colorimeter (Russia). The pH values of solutions were measured to within ±0.005 using an Ekoniks-Ekspert-001 pH meter–ion meter (Russia). Biohit pipettors (Finland) were used for taking small solution volumes (<0.25 mL).

Experimental procedures. *Procedure 1: Hydrolysis of p-NPP catalyzed by alkaline phosphatase from seal intestine.* A 0.05 M Tris–HCl buffer solution (with a corresponding pH value) (4.9 mL) and 0.1 mL of a 0.6 μM enzyme solution were successively introduced into a graduated ground-glass stopper tube. The resulting mixture was stirred, and 1 mL of a 3.3 mM *p*-NPP solution was added. At the instant the *p*-NPP solution was added, the reaction solution was stirred and transferred to a cell, and a stopwatch was started simultaneously. The absorbance of the reaction solution was measured at regular 15-s intervals for 2 min (400 nm; l = 1 cm). Based on these data, kinetic curves were plotted in the absorbance (A)–time (t, s) coordinates and the value of tanα and the initial rate v_0 of the indicator reaction were calculated using Eq. (1), which is given below.

Procedure 2: Enzymatic hydrolysis of p-NPP in the presence of complexones. A 0.05 M Tris–HCl buffer solution (with a corresponding pH value) (4.8 mL), 0.1 mL of a 0.6 μM enzyme solution, and 0.1 mL of a complexone solution with a corresponding concentration were successively introduced into a graduated ground-glass stopper tube. The resulting mixture was stirred and incubated for 15 min. Next, 1 mL of a 3.3 mM *p*-NPP solution was added to the mixture. The subsequent operations were performed as described in procedure 1. The rate v_I of the indicator reaction in the presence of the complexone was calculated by Eq. (1).

Procedure 3: Reactivation of alkaline phosphatase apoenzyme by magnesium (calcium) ions. A 0.05 M Tris–HCl buffer solution (4.7 mL; pH 9.8), 0.1 mL of a 0.6 μM enzyme solution, and 0.1 mL of a 3.6 mM complexone solution were successively introduced into a graduated ground-glass stopper tube. The resulting mixture was stirred and incubated for 15 min. Next, 0.1 mL of a metal ion solution of a certain concentration was introduced into the reaction solution, and 1 mL of a 3.3 mM *p*-NPP solution was added. The subsequent operations were performed as described in procedure 1. In the cases when two metal ions should be added to the indicator system, the buffer solution vol-

ume was 4.6 mL. A blank experiment in the presence of a complexone but in the absence of metal ions was performed analogously; in this case, the buffer solution volume added was 4.8 mL.

Procedure 4: Enzymatic hydrolysis of p-NPP in the presence of NTP and a metal (magnesium, calcium, or barium). A 0.05 M Tris–HCl buffer solution (4.7 mL; pH 8.5, 9.0, or 9.8), 0.1 mL of a 0.6 μM enzyme solution, 0.1 mL of a metal (magnesium, calcium, or barium) solution with a certain concentration, and 0.1 mL of a 3.6 mM NTP solution were successively introduced into a graduated ground-glass stopper tube. The resulting mixture was incubated for 15 min; 1 mL of a 3.3 mM *p*-NPP solution was added, and a stopwatch was started simultaneously. The subsequent operations were performed as described in procedure 1. The initial rate v of the indicator reaction in the presence of a metal ion and NTP was calculated using Eq. (1).

The absolute values of the initial rates of the enzymatic reaction were calculated from the equation

$$v_0 = \Delta c/\Delta t = \Delta A/\Delta t(1/\varepsilon \times 1) = \tan\alpha/(\varepsilon l), \quad (1)$$

where ε is the molar absorption coefficient of sodium *p*-nitrophenolate at 400 nm, which corresponds to the absorption band maximum of sodium *p*-nitrophenolate formed as a result of the reaction ($\varepsilon_{400} = 1.7 \times 10^4$); l is the cell thickness (1 cm).

The degree of the inhibiting effect (I, %) of complexones on the catalytic activity of alkaline phosphatase was calculated from the equation

$$I, \% = 1 - (v_I/v_0) \times 100. \quad (2)$$

The degree of the reactivating effect (ReA, %) of a metal ion on apophosphatase was calculated from the equation

$$\text{PeA}, \% = (v - v_I)/(v_0 - v_I) \times 100, \quad (3)$$

where v_I is the initial rate of the indicator reaction in the presence of a complexone; v_0 and v are the initial rates of the indicator reaction in the absence and in the presence of a metal ion and an organic compound, respectively. The degree of the liberative effect (ΔI, %) of a metal ion on the catalytic activity of alkaline phosphatase preinhibited with NTP was calculated from the equation

$$\Delta I = I_{\text{M + NTP}} - I_{\text{NTP}}, \quad (4)$$

where I_{NTP} and $I_{\text{M + NTP}}$ are the degrees of the inhibition of alkaline phosphatase in the presence of NTP and in the absence or in the presence of a metal ion, respectively.

RESULTS AND DISCUSSION

As noted above, procedures for determining metal ions based on the reactivation of the apoforms of various enzymes exhibited the highest sensitivity and selectivity. Biocatalysts that simultaneously contain the ions of two chemically different metals are of particular interest. Alkaline phosphatase belongs to these enzymes (zinc ions are present at the catalytic center of the enzyme, and magnesium ions are present at the allosteric center). The removal of one or two metal ions from the active center of alkaline phosphatase by binding into a stable complex with an organic reagent resulted in the production of an apoenzyme and, as a consequence, in the partial or complete loss of the catalytic activity of the biocatalyst. On the external addition of zinc and/or magnesium ions to the apophosphatase, the catalytic activity of the enzyme was restored; that is, the reactivation of the enzyme took place. The above reactivation effect formed the basis for the previously developed procedure for determining zinc ions with alkaline phosphatase from seal intestine and EDTA as a chelating agent in the hydrolysis of *p*-NPP [11]. It seemed reasonable to apply this approach to the determination of magnesium ions because procedures based on its reactivating effect on apophosphatases were not described in the literature, although many researchers observed this effect.

The hydrolysis of *p*-NPP catalyzed by alkaline phosphatase from seal intestine was chosen as an indicator reaction. EDTA, DTPA, CDTA, and NTP were used for the preparation of the apophosphatase; they form stable chelates not only with zinc ions but also (and most important) with magnesium ions: logβ is 8.69, 9.34, 11.07, or 7.52 using EDTA, DTPA, CDTA, or NTP as a ligand, respectively [17, 18]. Note that there is no published data on organic reagents that are capable of binding only magnesium ions into stable complexes.

Effects of oxygen- and phosphorus-containing complexones on the catalytic activity of alkaline phosphatase. Previously [11], we found the following optimum conditions for the preparation of the apoform of alkaline phosphatase from seal intestine using EDTA: 0.05 M Tris–HCl buffer solution, pH 9.8; incubation time of the enzyme (E) with the complexone, 15 min; EDTA concentration, 60 μM; and concentration ratio E : EDTA = 1 : 6000.

It was found that the dependence of the degree of the inhibition of alkaline phosphatase on the concentration of DTPA was rectilinear at the incubation time of DTPA with the enzyme equal to 15 min (Fig. 1). However, experimental results were poorly reproducible. Note that the DTPA concentrations that caused 90% inhibition (1–3 μM) were two times lower than those in the case of EDTA. This may be explained by the DTPA (which is prone to form polynuclear, in particular, binuclear, complexes with high stability constants for 3*d* cations), unlike EDTA, not forming mononuclear complexes by the interaction with alkaline phosphatase metal ions [18].

We studied the effect of 0.23 mM CDTA solutions on the catalytic activity of alkaline phosphatase from seal intestine. These data demonstrated that the degree of the inhibition of the enzyme was low (15%) even

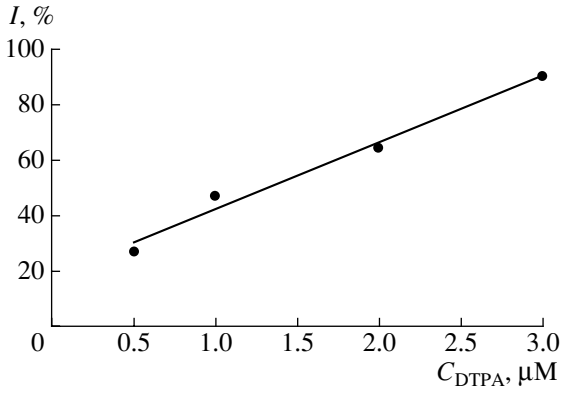


Fig. 1. The degree of the inhibition (I, %) of alkaline phosphatase from seal intestine as a function of the concentration of DTPA (optimum conditions of the indicator reaction; incubation time of the enzyme with DTPA, 30 min).

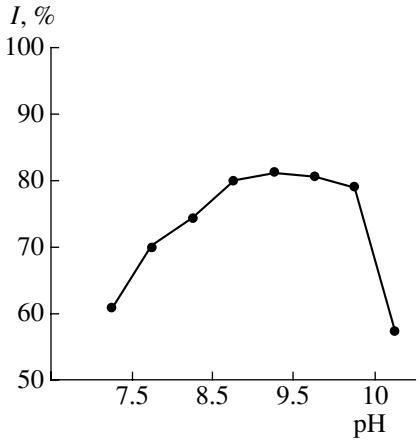


Fig. 2. The degree of the inhibition (I, %) of alkaline phosphatase from seal intestine by NTP in the reaction of *p*-NPP hydrolysis as a function of the pH of the reaction mixture (optimum conditions of the indicator reaction; NTP concentration, 60 μM; τ_{inc} = 15 min).

upon incubation for 30 min. This can be likely explained by the role of the kinetic factor increasing compared to that of the thermodynamic factor in the course of formation of CDTA complexes with the metal ions of the active centers of the enzyme: complexation occurred slower than that with EDTA by a factor of 10–100. The cyclohexane ring in the CDTA molecule inhibited not only ligand exchange because of a decrease in the flexibility of the ethylenediamine moiety but also the processes of glycinate-ring opening and carboxyl-group exchange within the coordination spheres of cations [18]. Consequently, although the stability of CDTA complexes with metals is higher than that in the case of EDTA primarily because of the less strained structures of the complexes [18], for the effective interaction of CDTA with alkaline phosphatase from seal intestine, a longer incubation (>30 min) of a mixture of the reactants is required. However, this may subsequently result in a regular increase in the time required for the reactivation of the apoenzyme thus prepared or even in the impossibility of restoring its catalytic activity in the presence of metals. Because the aim of this study was to use a reactivating effect for determining magnesium(II), we considered the use of the above chelating agents to be unreasonable for the production of apophosphatase.

We studied the dependence of the rate of the indicator reaction on the incubation time of alkaline phosphatase with NTP. We found that the higher the NTP concentration in the reaction mixture and the longer the incubation time, the stronger the inhibition of this enzyme by NTP (Table 1). The following conditions were chosen for the preparation of apophosphatase from seal intestine with NTP and the subsequent reactivation by magnesium ions: ratio of E : I = 1 : 6000 and τ_{inc} = 15 min. Under these conditions, the degree of the inhibition of the enzyme was high (I = 75%) (Table 1).

At the above concentration of NTP and incubation time, we studied the effect of NTP on the catalytic activity of alkaline phosphatase under the optimum conditions of *p*-NPP hydrolysis at pH 7–10. In this case, the operations were performed in accordance with procedure 2. Note that studies of this kind were not performed previously; at the same time, it is well known [18] that NTP forms the stable protonated chelates MHL with magnesium and calcium ions. This is a fundamental difference between complexes with phosphonic groups and complex compounds with amino and carboxyl groups, for example, with EDTA.

To evaluate the character and degree of the effect of NTP on the catalytic activity of alkaline phosphatase, we also studied the effect of pH in the absence of NTP. Based on the experimental data, we plotted the degree of the inhibiting effect of NTP on the catalytic activity

Table 1. Dependence of the degree of the inhibition of alkaline phosphatase from seal intestine by NTP on concentration and incubation time (τ) with the enzyme (0.05 M Tris–HCl buffer solution, pH 9.8; enzyme and *p*-NPP concentrations were 10 nM and 0.55 mM, respectively)

c_I (in a mixture), μM	E : I ratio	I, % at τ, min				
		5	10	15	30	60
1.5	1 : 150	0	0	0	8	15
3	1 : 300	0	0	0	5	20
6	1 : 600	0	9	19	23	35
15	1 : 1500	11	22	25	41	43
30	1 : 3000	16	18	25	34	44
60	1 : 6000	29	51	75	81	81
120	1 : 12000	43	70	78	82	82

of the enzyme as a function of the pH of the reaction mixture (Fig. 2).

It can be seen that NTP effectively inhibits the catalytic activity of alkaline phosphatase from seal intestine over the entire test pH range. In this case, the degree of inhibition gradually increased as pH was increased from 7.0 to 8.5; it reached a maximum and remained practically independent of pH over the range 8.5–9.8, whereas it somewhat decreased at pH > 10. Note that, according to published data [18], magnesium, calcium, and barium ions form stable normal and monoprotonated complexes with EDTA and NTP in the range of pH 8–10.

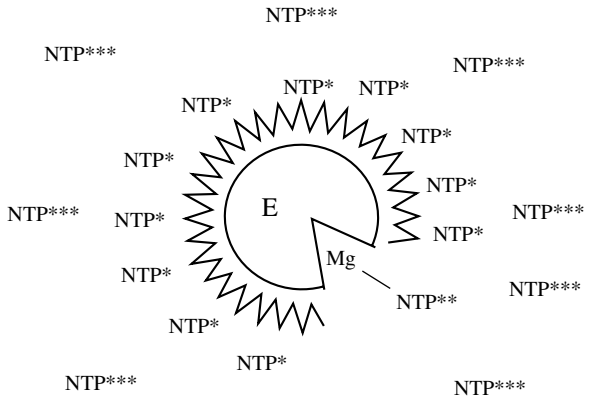
Reactivation of the alkaline phosphatase apoenzyme from seal intestine prepared with EDTA by magnesium, calcium, and barium ions. First, we studied the individual effects of magnesium(II) and calcium(II) on apophosphatase under the optimum conditions of the indicator reaction (procedure 3). Previously [14], we found that the above metals had no effect on native alkaline phosphatase from seal intestine. Therefore, we chose the concentrations of these metals such that, according to published data [16], they exerted a noticeable reactivating effect on other intestinal alkaline phosphatases. The addition of magnesium(II) [or calcium(II)] in a concentration of 0.2 (0.4) mg/mL immediately to the alkaline phosphatase apoenzyme from seal intestine resulted in its reactivation. The reactivating effect of calcium(II) was more pronounced than that of magnesium(II), all other factors being the same. This may be explained by the calcium ions externally added to the alkaline phosphatase apoenzyme occupying its catalytic and allosteric centers, whereas magnesium ions occupied only the latter centers.

The degree of the reactivating effect of calcium(II) increased by 30% upon incubation with the apoenzyme for 5 min, whereas the reactivating effect of magnesium(II) remained unchanged under these conditions. On the simultaneous addition to the apoenzyme, these ions activated the apo form, as well as calcium(II) alone; that is, their combined reactivating effect was nonadditive. The reason could be the competition of calcium(II) and magnesium(II) for the interaction with the same metal-binding centers of the enzyme. As the concentration of magnesium(II) or calcium(II) in the reaction mixture was decreased to 2 or 4 μg/mL, respectively, the degree of the reactivation of the apoenzyme by either of these metals remained unchanged. The reactivating effect on the apoenzyme was absent at all when the concentrations of magnesium(II) and calcium(II) in a mixture were 0.2 and 0.4 μg/mL, respectively. The independence of the degree of the reactivation of the apoenzyme from seal intestine from magnesium(II) concentration over the range 2–20 μg/mL demonstrates that the same amount of magnesium ions, which is required for the restoration of the catalytic activity of apophosphatase, was likely bound to the apoenzyme every time. An analogous behavior of calcium(II) may be explained by the similarity of the ionic radii of magnesium and calcium. It is likely that, consequently, the partial replacement of magnesium ions by calcium ions at the regulatory centers of the alkaline phosphatase molecule is possible.

As a consequence of the above consideration, attention was subsequently focused on studying the effect of magnesium ions on the alkaline phosphatase apoenzyme from seal intestine prepared using NTP. The operations were performed in accordance with procedure 3 at an NTP concentration in the reaction mixture equal to 60 μM, which provided enzyme inhibition by 75–80%; the incubation time of the biocatalyst with the complexone was 15 min; a 0.05 M Tris–HCl buffer solution (pH 9.8) was used.

Reactivation of the alkaline phosphatase apoenzyme from seal intestine prepared with NTP by magnesium ions. On the addition of magnesium ions (2 μg/mL) to the alkaline phosphatase apoenzyme from seal intestine prepared in accordance with procedure 2, the degree of the individual reactivating effect was 10%. An increase in the concentration of magnesium ions by a factor of 100 resulted in only an insignificant increase (by no more than 15%) rather than the complete restoration of the catalytic activity of the enzyme.

In our opinion, the interaction of magnesium(II) with alkaline phosphatase from seal intestine modified with NTP (exemplified by an enzyme subunit) in the test system can be represented by the following scheme:



NTP*… E-Mg-NTP** + NTP***

A. MgNTP*** + Mg NTP** + NTP* … E-Mg

B. MgNTP*** + Mg NTP** + Mg NTP* + NTP*… E-Mg

where E is the enzyme subunit; NTP* and NTP** are NTP molecules bound to the protein globule of the enzyme that form a hydrophobic sphere and interact with the magnesium ions of the allosteric center of the

enzyme, respectively; and NTP*** is an excess amount of NTP in the system.

Thus, the externally added magnesium ions in low concentrations form a complex primarily with NTP molecules that are not bound to the enzyme. On the addition of magnesium ions in concentrations (200 μg/mL or 8.3 μM) comparable with the concentration of NTP in the indicator system, their amount is sufficient for not only the binding of free NTP molecules but also the partial degradation of the hydrophobic sphere around the enzyme. This hydrophobic sphere consists of NTP molecules coordinated by specific and nonspecific binding near the active centers of phosphatases.

Thus, the concentrations of magnesium ions at which the reactivating effect of magnesium on the alkaline phosphatase apoenzyme is observed are high. The determination of these concentrations is of no analytical interest. Therefore, to improve the sensitivity of determining magnesium with NTP, we used the technique of competitive complexation and replaced the direct order of adding the components of the indicator system by the reverse order (that is, initially, the test metal ion was added to the enzyme, and NTP was then added). The other conditions of the indicator reaction were the same as in the case of the direct order of adding the components (procedure 3).

Varying the order of adding the components of the indicator reaction allowed us to find that magnesium ions added to the system before NTP decreased the inhibiting effect of the chelating agent by binding it into a complex. Thereby, the catalytic activity of alkaline phosphatase was restored (Fig. 3). This effect of magnesium on the enzyme in the presence of NTP is designated a liberative effect. The degree of the liberative effect of metal ions was characterized by the value of ΔI, which was calculated from Eq. (4).

Data given in Table 2 indicate that, with the reverse order of adding the components of the indicator reaction, the inhibiting effect of NTP on the enzyme was partially removed in the presence of low magnesium concentrations (≈14 ng/mL) and a 100-fold amount of the complexone in the reaction mixture. However, the degree of the liberative effect was as low as 7%. In this case, it is likely that the inhibiting effect of the complexone on the enzyme dominated over the formation of a complex with magnesium. At complexone concentrations higher than metal concentrations by a factor of 10–50, the magnesium added effectively bound the complexone present in the indicator system ($\Delta I \approx 40\%$). At the ratio Mg : NTP = 1 : 1, the inhibiting effect of NTP was almost completely suppressed. A small difference (7%) between the degrees of the liberative effect of magnesium at magnesium-to-NTP ratios equal to 1 : 1 and 2 : 1 can be explained by the formation of binuclear complexes with NTP in the latter case [18].

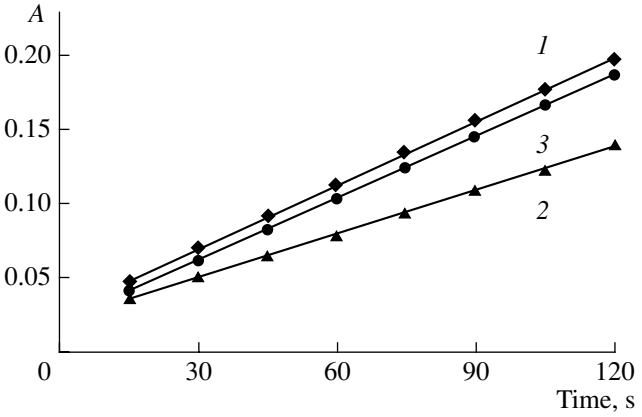


Fig. 3. Schematic diagram of kinetic curves of the reaction of *p*-NPP hydrolysis catalyzed by alkaline phosphatase from seal intestine (*1*) in the absence and (*2*) in the presence of NTP or (*3*) in the presence of NTP and magnesium (the concentrations of magnesium ions and NTP were 60 μM).

Thus, the higher the concentration of magnesium in the indicator system, the weaker the inhibiting effect of NTP on the catalytic activity of alkaline phosphatase from seal intestine. The direct proportionality between

Table 2. Dependence of the degrees of the liberative effects of magnesium, calcium, and barium ions on the catalytic activity of alkaline phosphatase from seal intestine in the presence of NTP on the concentration of metal ions and metal-to-complexone concentration ratio (0.05 M Tris–HCl buffer solution, pH 9.8; NTP concentration, 60 μM; incubation time of the enzyme with the metal and NTP, 15 min)

Concentration of M(II), μM	M(II) : NTP ratio (M)	I_{M+NTP}, %			ΔI^*_{Me}, %		
		Mg(II)	Ca(II)	Ba(II)	Mg(II)	Ca(II)	Ba(II)
120	2 : 1	6	6	64	69	69	11
60	1 : 1	13	13	70	62	62	5
30	1 : 2	24	27	72	51	48	2
6	1 : 10	45	44	74	30	31	1
1.2	1 : 50	61	60	75	14	15	0
0.6	1 : 100	68	69	75	7	6	0

* ΔI, % = $I_{NTP} - I_{M+NTP}$, where I_{NTP} and I_{M+NTP} are the degrees of the inhibition of alkaline phosphatase from seal intestine by NTP in the absence and in the presence of magnesium or calcium, respectively; $I_{NTP} = 75\%$.

Table 3. Correlation of the degrees of the liberative effects (ΔI, %) of metal (M) ions on the catalytic activity of alkaline phosphatase from seal intestine inhibited by NTP (L) with the ionic radii (r) and real stability constants of complexes with NTP (0.05 M Tris–HCl buffer solution)

Metal ion	r, Å [17]	log β [19]		ΔI, % at c_M (μM)					
		ML	MHL	pH 8.5			pH 9.0		
				60	90	120	60	90	120
Mg(II)	0.78	7.52	9.42	14	23	35	18	28	37
Ca(II)	1.06	7.86	8.80	6	6	9	5	9	21
Ba(II)	1.43	6.34	9.72	0	0	2	0	2	4

the degree of the liberative effect of magnesium and magnesium concentration was observed in the following two magnesium concentration ranges with different slopes of linear portions: 0.6–6 and 6–60 μM (Table 2).

A similar study in the presence of calcium, which also forms a stable complex with NTP as magnesium, pointed to the identity of the effects of both metal ions (see Table 2).

Upon the simultaneous addition of magnesium and calcium ions at a concentration ratio of 1 : 1 to the indicator reaction in the presence of tenfold amounts of NTP, the catalytic activity of alkaline phosphatase from seal intestine increased by only 15% as compared with that in the case of the separate addition of metal ions. That is, there was no synergetic liberative effect on the enzyme. The liberative effect of barium ions was much lesser than that of magnesium and calcium ions (Table 2); it was observed in the concentration range 6–120 μM.

The found liberative effect of magnesium or calcium ions, as well as barium, on alkaline phosphatase from seal intestine in the presence of NTP suggested that these metals can be determined in principle in the concentration ranges 0.6–6 (or 6–60) and 6–120 μM, respectively.

Dependence of the degrees of the liberative effects of magnesium and calcium on the pH of the reaction mixture and on metal concentrations. To improve the selectivity of the determination of magnesium, calcium, and barium ions, we studied the effects of these ions (in the concentration range 0.6–60 μM) on the catalytic activity of alkaline phosphatase from seal intestine in the presence of NTP at pH 8.5 and 9.0. These pH values were chosen based on published data [18], according to which magnesium ions primarily form stable monoprotonated complexes with NTP at pH 9–10, whereas calcium and barium complexes with this complexone are formed at pH 8–9. The operations were performed in accordance with procedure 4.

As can be seen in Table 3, at the specified pH values of the reaction solution, magnesium and calcium ions restored the catalytic activity of NTP-preinhibited alkaline phosphatase from seal intestine. The degree of the liberative effect of either of the ions increased as the pH of the reaction mixture or the concentration of these ions was increased. The greatest difference (26%) between the degrees of the liberative effects of magnesium and calcium was observed at pH 8.5. As the pH of the solution was increased to 9.8 (Table 2), the degrees of the liberative effects of magnesium and calcium became virtually equal. Note that the liberative effect of magnesium at the pH values under consideration was more pronounced than that of calcium, whereas barium ions restored, however, incompletely, the catalytic activity of the enzyme only at pH 9.8 (Tables 2, 3).

This is inconsistent with published data on the real stability constants of the ML complexes of magnesium and calcium ions with NTP, which are summarized in Table 3. At the same time, a correlation of experimental data with the real stability constants of the protonated complexes of these ions with NTP was observed. In this case, the following regularity was observed: the higher the stability constant of an MHL complex, the greater the liberative effect. It can also be seen in Table 3 that the greater the ionic radius of a metal, the smaller the liberative effect. In the case of barium ions, a correlation of the degree of the liberative effect with the stability constant of the MHL complex was not observed because, although the stability constants of the corresponding barium and magnesium complexes are comparable (Table 3), the latter restored the catalytic activity of the enzyme more effectively by a factor of almost 12 at pH 9.8 and a concentration of 60 μM (Table 2).

The experimental data indicate that the liberative effect of barium ions can be completely suppressed in the determination of magnesium (in a concentration of 60 μM or lower) if the indicator reaction is performed at pH 9.0. In this case, the degree of the liberative effect of calcium (in commensurable concentrations with magnesium) can be decreased by a factor of 3, as compared with that at pH 9.8.

Thus, the study performed allowed us to conclude that the degrees of the liberative effects of magnesium, calcium, and barium ions primarily depend on the nature of the metal ion. It is likely that two processes simultaneously occurred in the restoration of the catalytic activity of NTP-preinhibited alkaline phosphatase from seal intestine by magnesium, calcium, and barium ions. In the case of magnesium, its complex with NTP

Table 4. Performance characteristics of procedures for determining magnesium, calcium, and barium based on their liberative effects on alkaline phosphatase from seal intestine inhibited by NTP in the reaction of *p*-NPP hydrolysis (0.05 M Tris–HCl buffer solution, pH 9.8)

Test metal ion	Analytical range		Calibration equation	RSD (%) at c_L (n = 3)
	μM	μg/mL		
Mg(II)	0.6–6	0.014–0.14	$y^* = -0.20 + 12.10x$	2
	10–120	0.23–2.8	$y = -0.51 + 0.33x$	5
Ca(II)	0.6–6	0.024–0.24	$y = -0.20 + 11.85x$	2
	10–90	0.40–3.6	$y = 0.02 + 0.09x$	7
Ba(II)	6–120	0.8–16	$y = -0.09 + 0.09x$	3

* y is the degree of the liberative effect (ΔI, %), and x is the metal ion concentration (μM).

was formed. In the case of calcium and barium, along with the above process, the replacement of the magnesium ions of the allosteric centers of the enzyme freed from the effect of NTP by calcium and barium ions was also possible. As a result of this replacement, the liberative effect decreased.

Procedure for the enzymatic determination of magnesium, calcium, and barium ions. Based on the found liberative effect of magnesium, calcium, and barium ions on the catalytic activity of NTP-preinhibited alkaline phosphatase from seal intestine at the pH values of maximum liberative effects of the metals, we developed procedures for the enzymatic determination of each of these metals. Table 4 summarizes the performance characteristics of these procedures.

The accuracy of the enzymatic determination of magnesium and calcium was checked using the standard addition technique. Thus, on the addition of 0.60 μM magnesium (or calcium) to the indicator system, 0.58 ± 0.03 or (0.57 ± 0.04) μM magnesium (or calcium) was found.

The procedure proposed for the enzymatic determination of magnesium(II) based on its liberative effect on alkaline phosphatase from seal intestine is superior to the determination procedures based on atomic emission or atomic absorption spectrometry [20, 21] and the formation of a fluorescent complex of magnesium(II) with 8-hydroxyquinoline-5-sulfonic acid (c_{min} = 12 ng/mL) [22] in sensitivity. The procedure proposed is comparable to the enzymatic procedure based on the activation of a bioluminescence reaction catalyzed by firefly luciferase by Mg(II) (c_L = 0.1 ng/mL) [15].

The enzymatic procedures proposed allowed us to determine magnesium, calcium, and barium ions over almost the same analytical range (Table 4). Therefore, unfortunately, the selectivity of the determination of these ions is low. Nevertheless, these highly sensitive enzymatic procedures can be useful in the determination of the above ions in the solutions of pure metal salts or in a mixture obtained after sample dilution (for the removal of the interfering effect of barium ions) and masking calcium ions with glycol ether diaminetetraacetic acid [23].

Thus, we experimentally found that the liberative effects of magnesium, calcium, and barium ions on alkaline phosphatase from seal intestine in the presence of NTP in the hydrolysis reaction of *p*-NPP at pH 9.8 in a 0.05 M Tris–HCl buffer solution are promising for the use in the sensitive determination of the above metals. The selectivity of the determination of these metal ions can be improved by varying the pH of the reaction mixture.

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