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*ATP*ases as Multi-Response Sensing System for Various Organic and Inorganic Analytes

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Summary. The possibility of using synaptic plasma membrane (SPM) enzymes Na^+/K^+-*ATP*ase and Mg^{2+}-*ATP*ase, isolated from rat brain, as a biological component of multi-response sensing system for detection of different compounds (alkaline and heavy metal salts, organic compounds) was studied. The method is based on the spectrophotometric determination of inorganic *ortho*-phosphate (P_i) that serves as a measure of the enzymatic activity in the presence of various analytes. The concentration of P_i, liberated by enzyme catalysed hydrolysis of adenosinetriphosphate (*ATP*), was followed spectrophotometrically, by single exposure to analytes or in the mixture. P_i was dose dependent on the analyte concentration. Alkaline elements (Na, K, Mg), heavy metals (Pb, Cd, Hg, Cu, Fe, Co, Zn), toxic organic compounds (pyridine, urea, chlorpyrifos), and some drugs (digoxin, gitoxin) showed diverse effects, inducing the inhibition or stimulation of the enzymes activity. Development of simple test method for simultaneous detection of the investigated analytes based on the variation of medium assay composition was discussed.

Keywords. ATPase; Heavy metals; Alkaline elements; Organic compounds; Detection.

Introduction

Different organic compounds and metal ion salts widely used in agricultural, pharmaceutical, and other industrial processes, are considered to be one of main sources of pollution in the environment. A great number of biologically important metal ions which are, at trace levels, necessary to support life, at elevated levels become toxic, built up in biological systems, and induce significant health hazards [1–3].

Highly sensitive and specific techniques are widely used for their control [4, 5], but less specific and less tedious methods for determination of toxicity of heavy

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metals and organic compounds are required [6, 7]. Because these pollutants are known to influence the activity of many enzymes, the development of respective detection systems becomes increasingly interesting [8–13]. They usually use enzymes immobilized on solid support or simply enzyme assays in the water solution [14].

Synaptic plasma membrane enzymes (SPM) Na^+/K^+-*ATP*ase and Mg^{2+}-*ATP*ase seem to be promising for quick tests of various compounds, since a great number of metal ions and some organic compounds (pesticides, drugs) inhibit or stimulate their activity in a concentration dependent manner [15–17]. Moreover, their proper functioning depends strongly on the concentration of Na^+, K^+, and Mg^{2+} ions [18]. These proteins exist in plasma membranes of all higher organisms, especially in synaptic plasma membranes [19], and transfer chemical energy of hydrolysis of *ATP* to potential energy of electrochemical ion gradients. The temporal change of the concentration of inorganic *ortho*-phosphate (P_i), the main product of the enzyme catalyzed adenosinetriphosphate (*ATP*) hydrolysis, can be easily determined spectrophotometrically and serves as a measure of the enzymatic activity [16, 17, 20].

Because of the diverse effects of many organic and inorganic compounds on the enzymatic activity, development of respective biosensing system for their selective detection using the *ATP*ase assay becomes interesting. Recently, an integral membrane protein Na^+/K^+-*ATP*ase was functionally immobilized in its native environment on waveguide sensor surfaces for the application in pharmaceutical research [21]. SPM were also immobilized on polystyrene microtiter plates and nitrocellulose support by adsorption in order to improve the temperature and time stability of *ATP*ases. The immobilization increased the stability, and preserved the sensitivity to divalent heavy metals, such as Cd and Hg [22, 23].

The present paper deals with the study of the effect of various metal ions and organic compounds on the change of Na^+/K^+-*ATP*ase and Mg^{2+}-*ATP*ase activity by single and simultaneous exposure to the enzymes. The aim was to investigate the possibility of using an *ATP*ases assay as the semi-quantitative and qualitative test for multianalyte selective detection of enzyme activity modulators based on the color reaction for P_i determination. The detection scheme was adapted to a cuvette system along with optical determination of the activity change.

Results and Discussion

Influence of Na^+, K^+, and Mg^{2+} on ATPases Activity

The influence of Na^+, K^+, and Mg^{2+} ions on the activity of Na^+/K^+-*ATP*ase and Mg^{2+}-*ATP*ase was investigated in the concentration range from $1\times10^{-5}\,M$ to $0.1\,M$. The enzymatic activity was followed by changing the concentration of the corresponding ion in the incubation mixture containing all reaction medium components as described in Experimental, except the tested element. The relative absorbance of the colorimetric assay (*i.e.* the ratio between the absorbance of the sample and the control solution) increased with increasing the concentration of the tested ion (Fig. 1). The effect of heavy metals below $1\times10^{-3}\,M$ in the presence of $1\times10^{-3}\,M$ *EDTA* was negligible.

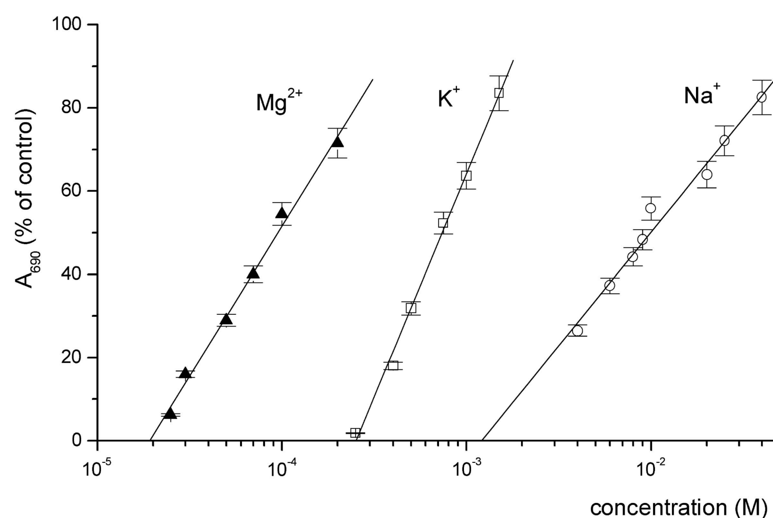

Fig. 1. Relative absorbance of Na^+/K^+-*ATP*ase (open symbols) and Mg^{2+}-*ATP*ase (solid symbols) assay depending on the concentration of Na^+, K^+, and Mg^{2+} ions

Table 1. Medium assay composition and concentration range for determination of Na^+, K^+, and Mg^{2+} ions by *ATP*ase assay

	target enzyme	assay composition[a]	concentration range (*M*)
Na^+	Na^+/K^+-*ATP*ase	K^+, Mg^{2+}, *ATP*, *EDTA*	8×10^{-3}–3×10^{-2}
K^+	Na^+/K^+-*ATP*ase	Na^+, Mg^{2+}, *ATP*, *EDTA*	2×10^{-4}–1×10^{-3}
Mg^{2+}	Mg^{2+}-*ATP*ase	*ATP*, *EDTA*	2×10^{-5}–2×10^{-4}
Mg^{2+}	Na^+/K^+-*ATP*ase	Na^+, K^+, *ATP*, *EDTA*	2×10^{-5}–2×10^{-4}

[a] standard concentrations of constituents and buffer as described in Experimental

The concentration range and the composition of the reaction mixture for selective detection of Na^+, K^+, and Mg^{2+} ions by *ATP*ase assay are given in Table 1. The lowest concentrations of the ions that produced a measurable effect (20% of the absorbance of the control solution) were close to that obtained by the ion selective membrane electrodes [24].

Inhibition of ATPases by Heavy Metals

The influence of Pb^{2+}, Cd^{2+}, Hg^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, and Zn^{2+} on Na^+/K^+-*ATP*ase and Mg^{2+}-*ATP*ase activity was investigated by single exposure to inhibitors in the concentration range from $1\times10^{-9}\,M$ to $1\times10^{-3}\,M$. The medium assay had the standard composition as described in *ATPase assay.* All metal ions induced the inhibition of the enzymatic activity in a concentration dependent manner, with the IC_{50} values (inhibitor concentration that produced the inhibition of 50% of the enzymatic activity) ranging from 10^{-7}–$10^{-4}\,M$. The linear part of the inhibition curves spanned about one order of magnitude near IC_{50}. Table 2 collects IC_{50} values and limits of detection (concentration that induced at least 20% inhibition of enzymatic activity). The results indicate that Na^+/K^+-*ATP*ase is more sensitive to low heavy metals than Mg^{2+}-*ATP*ase [25].

Table 2. The inhibitory efficiency (IC_{50} values) and limits of detection of heavy metals by Na^+/K^+-*ATP*ase and/or Mg^{2+}-*ATP*ase assay

	IC_{50} (*M*)		limit of detection (*M*)	
	Na^+/K^+-*ATP*ase	Mg^{2+}-*ATP*ase	Na^+/K^+-*ATP*ase	Mg^{2+}-*ATP*ase
Pb	$(7.1 \pm 0.2)\times10^{-6}$	$(8.1 \pm 0.2)\times10^{-6}$	1×10^{-7}	1×10^{-6}
Cd	$(2.3 \pm 0.5)\times10^{-6}$	$(2.5 \pm 0.2)\times10^{-4}$	1×10^{-7}	1×10^{-5}
Hg	$(3.0 \pm 0.1)\times10^{-6}$	$(2.3 \pm 0.4)\times10^{-4}$	5×10^{-7}	1×10^{-5}
Cu	$(6.5 \pm 1.0)\times10^{-7}$	$(3.6 \pm 0.7)\times10^{-6}$	2×10^{-7}	5×10^{-5}
Fe	$(3.4 \pm 0.9)\times10^{-5}$	$(5.4 \pm 0.3)\times10^{-4}$	5×10^{-6}	5×10^{-5}
Co	$(1.8 \pm 0.1)\times10^{-4}$	$(2.1 \pm 0.1)\times10^{-4}$	5×10^{-5}	5×10^{-5}
Zn	$(2.2 \pm 0.9)\times10^{-5}$	$(1.0 \pm 0.5)\times10^{-4}$	1×10^{-6}	1×10^{-5}

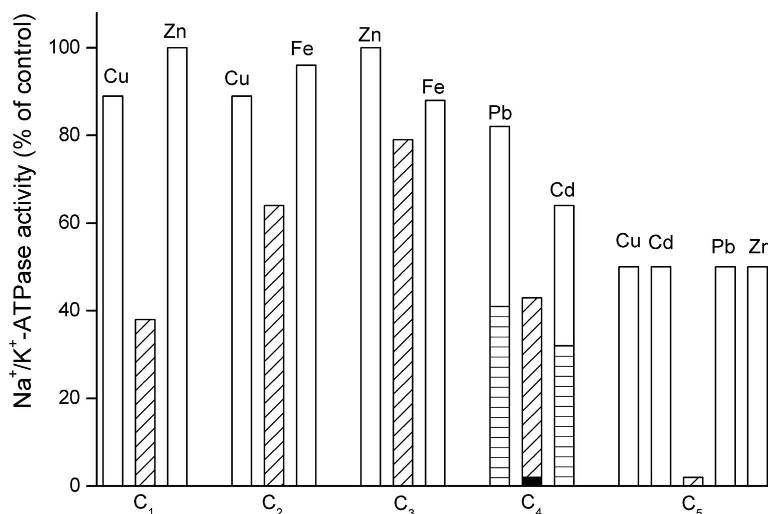

Fig. 2. Effects of combination of Cu(II), Fe(II), Zn(II), Pb(II), and Cd(II) on the relative absorbance of Na^+/K^+-*ATP*ase; C_1 – 0.5 μM Cu, 2 μM Zn; C_2 – 0.5 μM Cu, 20 μM Fe; C_3 – 2 μM Zn, 2 μM Fe; C_4 – 1 μM Pb, 2 μM Cd; C_5 – IC_{50} concentrations of Cu, Pb, Cd, and Zn; open bars – single ions, dashed bars – mixture; incubation time – 5 min, C_4 – 20 min (solid bar)

The effect of binary combinations of heavy metals on the activity of Na^+/K^+-*ATP*ase was studied using the mixtures of Cu/Zn, Cu/Fe, Zn/Fe, Pb/Cd, and Cu/Pb/Zn/Cd ions. The results obtained are presented in Fig. 2. A synergistic effect was obtained in all cases. The inhibition induced by combination of Pb with Cd was time dependent (Fig. 2, C_4 combination). The relative absorbance decreased when incubation time was prolonged, and after 20 min the mixture of 1×10^{-6} *M* Pb and 2×10^{-6} *M* Cd inhibited the activity completely. This finding offers the possibility of increasing the sensitivity and selectivity of the assay. Moreover, all metal ions in the mixture at concentration levels near IC_{50} values inhibited the activity of both enzymes completely (Fig. 2, C_5 combination). Addition of 1 m*M* *EDTA* in the medium assay recovered 100% of the inhibited activity of both enzymes [6, 27].

Effect of Organic Compounds on ATPases Activity

The effect of two groups of organic compounds on Na^+/K^+-*ATP*ase and Mg^{2+}-*ATP*ase was investigated by single exposure to the enzymes: some pesticides and their breakdown products (chlorpyrifos, urea, pyridine) and ouabain like cardiac glycosides (digoxin, gitoxin). Typical graphs of the relative absorbance *vs*. concentration in the linear concentration – dependent range are presented in Fig. 3. 1 m*M EDTA* was added to the medium assay in order to prevent the metal ions influence the enzymatic activity. The results are presented in Table 3, together with the IC_{50} values obtained from inhibition curves.

As can be seen from Table 3, chlorpyrifos induced the inhibition of both enzymes, whereas urea and pyridine stimulated Na^+/K^+-*ATP*ase activity. Moreover, urea had no effect on Mg^{2+}-*ATP*ase activity.

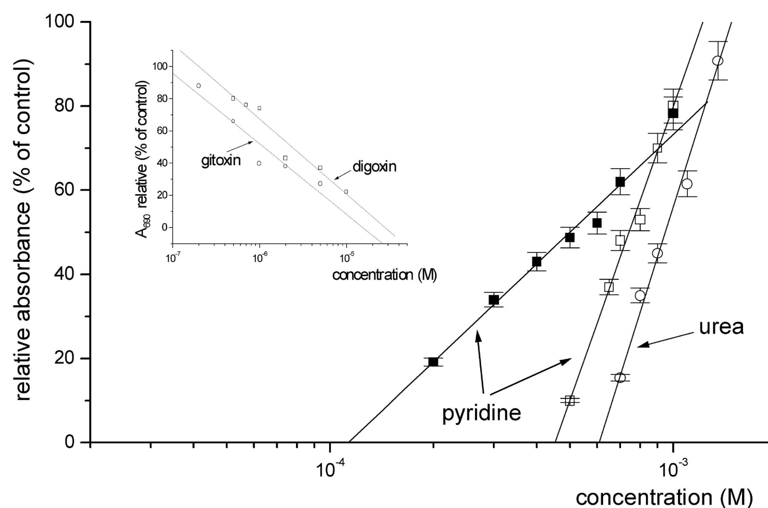

Fig. 3. Influence of organic compounds on relative absorbance of Na^+/K^+-*ATP*ase (open symbols) and Mg^{2+}-*ATP*ase (solid symbols) assay

Table 3. Concentration range for detection of organic compounds by *ATP*ases assay and their IC_{50} values

	Na^+/K^+-*ATP*ase		Mg^{2+}-*ATP*ase	
	concentration range (*M*)	IC_{50} (*M*)	concentration range (*M*)	IC_{50} (*M*)
chlorpyrifos	1×10^{-5}–5×10^{-4}	$(1.0\pm0.2)\times10^{-4}$	1×10^{-5}–1×10^{-4}	$(3.0\pm0.1)\times10^{-5}$
urea[a]	6×10^{-4}–1×10^{-3}	$(9.1\pm0.6)\times10^{-4}$	no effect	no effect
pyridine[a]	4×10^{-4}–1×10^{-3}	$(5.4\pm0.3)\times10^{-4}$	1×10^{-4}–1×10^{-3}	$(7.2\pm0.1)\times10^{-4}$
digoxin	5×10^{-7}–5×10^{-5}	$(2.2\pm0.2)\times10^{-6}$	no effect	no effect
gitoxin	1×10^{-7}–5×10^{-6}	$(3.4\pm0.4)\times10^{-7}$	no effect	no effect

[a] stimulation of enzymatic activity

Table 4. Spectrophotometric response of the colorimetric assay as function of the reaction mixture volume containing 25 μg of protein in the presence of Pb^{2+} ions

V/cm^3	protein/$\mu g/cm^3$	incubation time/min	A_{690} (control)	Pb^{2+}	
				c (M)	A (% of control)
0.2	125	10	0.720	7×10^{-6}	52 ± 2
1	25	20	0.695	7×10^{-7}	60 ± 3

The investigated glycosides inhibited Na^+/K^+-*ATP*ase enzymatic activity, whereas they had no effect on Mg^{2+}-*ATP*ase activity.

Optimisation of the Medium Assay

The spectrophotometric response of the colorimetric assay was tested as the function of the reaction mixture volume and the incubation time, in the presence and absence of Pb^{2+} ions as an example. The reaction mixture contained 25 μg of protein. The aim was to find out the optimum conditions for the absorbance reading. The optimum absorbance value about 0.7 at 690 nm was obtained for the standard medium assay composition as described in *ATPase assay*.

The results presented in Table 4 show that by dilution of the medium assay from 0.2 cm^3 to 1 cm^3 volume, the signal did not change considerably by increasing the incubation time to 20 min. At the same time, the actual protein concentration was proportionally reduced, since its amount retained constant. At lower protein concentration the detection limit for the analytes decreased. As can be seen from Table 4, the concentration of Pb^{2+} ions, which produced the same inhibition, was lowered for one order of magnitude.

Detection of Various Analytes by ATPases Assay by Single and Simultaneous Exposure

The series of ATPase assays containing the components as given in Table 5 were prepared in order to reconsider the response of the enzymes in the presence of the investigated compounds by single exposure. After addition of the tested analyte to the reaction mixture in 0.2 cm^3 volume the enzymatic reaction was allowed to proceed and the reagents for color development were added. The absorbance was measured and compared to the control value. The results are presented in Table 5, together with the expected relative absorbance corresponding to the concentration from Figs. 1 and 3. It is obvious from Table 5 that by single exposure all analytes can be recognised in the range of the experimental error.

The efficiency of SPM Na^+/K^+-*ATP*ase and Mg^{2+}-*ATP*ase to detect different groups of organic compounds or inorganic cations by simultaneous exposure on the enzymatic activity was tested on two synthetic mixtures containing the combinations of various analytes: a) 1×10^{-2} *M* Na^+, 1×10^{-3} *M* K^+, and heavy metals (Cu, Hg, Zn, Co, total concentration 1×10^{-5} *M*) and b) 5×10^{-6} *M* digoxin and the same mixture of heavy metals. The samples were parallely exposed to the enzymatic assay of various compositions for Na^+/K^+-*ATP*ase and Mg^{2+}-*ATP*ase, with the

Table 5. Spectrophotometric response of the colorimetric assay in the presence of single analytes as the function of composition of ATPases assay

analyte	conc./*M*	composition of *ATP*ase assay[a]	target enzyme	relative absorbance (% of control)	
				Found	Expect.[b]
Na^+	1×10^{-2}	K, Mg, *EDTA*, *ATP*	Na^+/K^+-*ATP*ase	50 ± 3	47
K^+	1×10^{-3}	Na, Mg, *EDTA*, *ATP*	Na^+/K^+-*ATP*ase	68 ± 2	65
Mg^{2+}	1×10^{-4}	*ATP*, *EDTA*	Mg^{2+}-*ATP*ase	49 ± 3	52
Heavy metals[c]	1×10^{-5}	Na, K, Mg, *ATP*	Na^+/K^+-*ATP*ase	0 ± 1	0
		Na, K, Mg, *ATP*, *EDTA*	Na^+/K^+-*ATP*ase	100 ± 1	100
pyridine	1×10^{-3}	Na, K, Mg, *ATP*, *EDTA*	Na^+/K^+-*ATP*ase	130 ± 5	130
		Mg, *ATP*, *EDTA*	Mg^{2+}-*ATP*ase	120 ± 4	120
urea	1×10^{-3}	Na, K, Mg, *ATP*, *EDTA*	Na^+/K^+-*ATP*ase	130 ± 5	130
		Mg, *ATP*, *EDTA*	Mg^{2+}-*ATP*ase	100	100
chlorpyrifos	3×10^{-5}	Mg, *ATP*, *EDTA*	Mg^{2+}-*ATP*ase	52 ± 3	50
digoxin	1×10^{-6}	Na, K, Mg, *ATP*, *EDTA*	Na^+/K^+-*ATP*ase	90 ± 2	90
		Mg, *ATP*, *EDTA*	Mg^{2+}-*ATP*ase	100	100
gitoxin	1×10^{-6}	Na, K, Mg, *ATP*	Na^+/K^+-*ATP*ase	35 ± 3	35
		Mg, *ATP*, *EDTA*	Mg^{2+}-*ATP*ase	100	100

[a] concentrations of the components are given in ATPase assay; [b] from Figs. 1 and 3; [c] summary concentration of ions

Table 6. Relative absorbance of the mixture containing a) 1×10^{-2} *M* Na^+, 1×10^{-3} *M* K^+, and heavy metals (Cu^{2+}, Hg^{2+}, Zn^{2+}, and Co^{2+}, total concentration 1×10^{-5} *M*); b) 5×10^{-6} *M* digoxin, 2.5×10^{-6} *M* Hg^{2+}, 2.5×10^{-6} *M* Cu^{2+}, 2.5×10^{-6} *M* Ni^{2+}, and 2.5×10^{-6} *M* Zn^{2+}

		target enzyme (medium assay composition[a])		relative absorbance (% of control)	
synthetic mixture	tested analyte	Na^+/K^+-*ATP*ase	Mg^{2+}-*ATP*ase	Na^+/K^+-*ATP*ase	Mg^{2+}-*ATP*ase
Na, K, heavy metals	control	Na, K, Mg, *ATP*, *EDTA*	Mg, *ATP*, *EDTA*	100	100
	Na^+	K, Mg, *ATP*, *EDTA*	Mg, *ATP*, *EDTA*	56.5 ± 0.5	100
	K^+	Na, Mg, *ATP*, *EDTA*	Mg, *ATP*, *EDTA*	65.2 ± 1.2	100
	heavy metals	Na, K, Mg, *ATP*	Mg, *ATP*	21.7 ± 0.9	38.6
digoxin, heavy metals	control	Na, K, Mg, *ATP*, *EDTA*	Mg, *ATP*, *EDTA*	9.5 ± 0.2	100
	digoxin	Na, K, Mg, *ATP*, *EDTA*	Mg, *ATP*	9.5	38.6 ± 1.5
	heavy metals	Na, K, Mg, *ATP*	Mg, *ATP*, *EDTA*	0	100 ± 0.5

[a] concentrations of medium assay components are given in *ATPase assay*

Table 7. Application of *ATP*ases assay to real samples

target analyte	sample	labelled content/M[a]	found content/M[b]
Na^+	mineral water	1.96×10^{-2}	$(1.5\pm0.5)\times10^{-2}$
K^+	mineral water	1.64×10^{-3}	$(2.2\pm0.4)\times10^{-3}$
Mg^{2+}	mineral water	2.58×10^{-3}	$(2.4\pm0.4)\times10^{-3}$
heavy metals	mineral water	–	not found
digoxin	Lanoxin[R] injection	0.32×10^{-3}	$(0.31\pm0.02)\times10^{-3}$

[a] contents were checked by standard methods (metal ions) [34] and HPLC (digoxin); [b] mean of 3 replicates

aim to recognize the analytes on the basis of the response of the target enzyme. The results are shown in Table 6. As can be seen, all the substances were identified according to the response of the corresponding reaction mixture.

Application of ATPases Assay to Real Samples

The method was applied to the determination of Na^+, K^+, Mg^{2+}, and heavy metals (as the group of elements) in mineral water. Four reaction mixtures were prepared containing the sample. Each mixture contained the standard concentrations of medium assay components, excluding the target ion (Na^+, K^+, and Mg^{2+}, respectively for alkaline elements detection). After 10 min preincubation the enzymatic reaction was allowed to proceed for 10 min, the colorimetric assay was added, and the resulting mixed solution was transferred to the reaction cell of a spectrophotometer. The absorbance at 690 nm was measured and compared to the absorbance of the control solution. The results are given in Table 7.

The proposed method was also applied to the quality control of digoxin. The tested sample of Lanoxin injection was added to the standard medium assay ($0.2\,cm^3$ final value), containing 1 m*M EDTA*. The activity was measured as described, and compared to the control value. The results presented in Table 7 showed the same result as obtained by standard method (HPLC) with the mean standard deviation of 6.45%.

As shown in our study *ATP*ase system is sensitive to a great number of different groups of analytes tested up to now, including cardiovascular drugs, biologically important elements, heavy metals, organic solvents, and some toxic organic compounds [15–17, 19, 28]. The possibility of using *ATP*ase system as a biological component for a multi-response sensing system for detection of different compounds is based on the level of the change of enzyme activity by simply changing the composition of the reaction mixture. Moreover, the neurotoxicity of the sample can be evaluated.

In conclusion, multi-analyte sensing requires highly parallel analysis [29, 30]. The concept of a matrix assay for a large number of samples at various concentrations, besides controls, blanks, and references for the quality control by cardiotonic drugs production, as well as for detection of heavy metals, K^+, Mg^{2+}, and Na^+ ions in biological fluids is the subject of further investigations.

Experimental

Chemicals and Material

All chemicals for medium assay were commercially available from Sigma (St. Louis, MO, USA) and of reagent grade. The metal ion salts, mercury(II) nitrate, lead(II) nitrate, cobalt(II) nitrate, zinc(II) nitrate, cadmium(II) nitrate, copper(II) sulphate, iron(II) chloride, NaCl, KCl, stannous chloride, and ammonium molybdate, were from Merck (Darmstadt, Germany). All solutions were prepared using de-ionized water.

SPM were isolated from the whole rat brain. Experiments were performed on 3-month-old male Wistar albino rats from the local colony. Animals were kept under controlled illumination (lights on: 5:00 am–5:00 pm) and temperature (23 ± 2°C), and had free access to food and water. After decapitation, brains were rapidly excised and pooled (6/pool) for immediate preparation of synaptic plasma membranes (SPM). The SPM were isolated according to the method of *Cohen et al.* [31], as modified by *Towle* and *Sze* [32]. The preparation procedure and the purity of SPM preparations were described previously [33]. The mitochondrial contamination and protein content were determined according to the standard procedure [16]. SPM were stored at −70°C until use. SPM lost about 30% of the activity when keeping at +4°C for one week and complete loss of activity was detected after 24 hours at +20°C [21].

ATPase Assay

Total ATPase activity was determined in a standard incubation medium (0.2–1 cm^3), containing 0.05 *M* *Tris*-HCl (*pH* 7.4), 0.1 *M* NaCl, 0.02 *M* KCl, 5×10^{-3} *M* $MgCl_2$, 2×10^{-3} *M* *ATP*, and 25 μg protein in the presence or absence (control) of the desired concentration of inhibitors or their mixtures. The reaction was initiated by the addition of *ATP*, preincubated for 5 min, allowed to proceed for 5–20 min at 37°C, and interrupt by the addition of 22 mm^3 ice cold 3 *M* $HClO_4$, and by immediate cooling on ice. The activity obtained without NaCl and KCl in the medium assay was attributed to Mg^{2+}-*ATP*ase. Na^+/K^+-*ATP*ase activity was calculated as a difference between the total *ATP*ase and Mg^{2+}-*ATP*ase activity. In all experiments the enzymatic activity in the presence of the tested elements or compounds was calculated as the percentage of the control value of the standard incubation mixture.

Monitoring Enzyme Activity

The *ATP*ase assay was placed in the 3 cm^3 thermostated glass cuvette. After the enzymatic reaction was stopped, the reagents for color development using the modified spectrophotometric procedure [16] based on the modified stannous chloride method were added. The solution was diluted to 3 cm^3 volume with bidistilled water and mixed. P_i liberated from the hydrolysis of *ATP* during the course of the reaction was measured by reading the absorbance at 690 nm after 20 min. The control test cuvette contained no analytes. The results are expressed as the mean percentage of the relative absorbance, *i.e.* the absorbance of the sample compared to the absorbance of the corresponding control value (100% ± SE) from at least three independent experiments.

Apparatus

The spectrophotometric measurements were performed on a Beckman 5260 UV VIS spectrophotometer.

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