

## Chemical aspect of the influence of cobalt ions on ATPase activity

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The influence of  $\text{Co}^{2+}$  ions on the activities of  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Mg}^{2+}$ -ATPase, enzymes from rat brain synaptic plasma membrane, was studied. The aim of this study was to investigate the inhibition of both ATPases activities by exposure to cobalt ions as a function of experimentally added  $\text{CoSO}_4$ . The "free"  $\text{Co}^{2+}$  concentrations in the reaction mixture were also calculated and discussed.  $\text{CoSO}_4$  induced a dose-dependent inhibition of both enzymes. The  $IC_{50}$  values of  $\text{Co}^{2+}$ , as calculated from the experimental curves, were 168  $\mu\text{M}$  for  $\text{Na}^+/\text{K}^+$ -ATPase and 262  $\mu\text{M}$  for  $\text{Mg}^{2+}$ -ATPase, and for the recalculated free  $\text{Co}^{2+}$  concentration 75.4  $\mu\text{M}$  for  $\text{Na}^+/\text{K}^+$ -ATPase and 136  $\mu\text{M}$  for  $\text{Mg}^{2+}$ -ATPase. The obtained linear Dixon's plot for  $\text{Na}^+/\text{K}^+$ -ATPase implies equilibrium binding of cobalt with inhibitory sites on the enzyme. The kinetic parameters for both enzymes in presence and absence of  $\text{CoSO}_4$  were calculated from the experimental data. The results of the kinetic analysis show that inhibition of  $\text{Na}^+/\text{K}^+$ -ATPase induced by  $\text{CoSO}_4$  is non-competitive, and for  $\text{Mg}^{2+}$ -ATPase that there are two sites of different sensitivities or two different enzymes.

**Keywords:** cobalt,  $\text{Na}^+/\text{K}^+$ -ATPase,  $\text{Mg}^{2+}$ -ATPase, kinetics.

### INTRODUCTION

Phosphate esters are crucial components of all living matter and they play a vital role in many cell processes, such as protein synthesis, genetic coding, photosynthesis, nitrogen fixation and innumerable other pathways. The biophosphorous compound on which all cell functions depend is adenosine triphosphate (ATP). ATP is involved as a substrate in the functioning of two types of membrane-bound enzymes: sodium, potassium-adenosine triphosphatase ( $\text{Na}^+/\text{K}^+$ -ATPase) and magnesium-adenosine triphosphatase ( $\text{Mg}^{2+}$ -ATPase) that mediate the active transport of ions across the plasma membrane of most animal cells. The ouabain sensitive  $\text{Na}^+/\text{K}^+$ -ATPase is pretty well characterized, while the ouabain insensitive  $\text{Mg}^{2+}$ -ATPase is much less well characterized. Recent results<sup>1</sup> show that  $\text{Mg}^{2+}$ -ATPase apparently consists of at least two forms with different sensitivity to metal ions.

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A hydrolysis of this ionic triphosphate ester to adenosine diphosphate (ADP) or adenosine monophosphate (AMP), in which  $\{P_3O_{10}^{5-}\}$  is replaced by  $\{P_2O_7^{4-}\}$  or  $\{PO_4^{3-}\}$  supplies the energy required for many biochemical processes. The energy changes associated with these hydrolyses are very dependent on pH, temperature, and the presence of metal ions. It has been shown that these membrane enzymes are very sensitive and alter their activity under the influence of some metal ions, organic pollutants and several drugs.<sup>2-12</sup>

It is remarkable that the metals of the first transition series (Fe, Co, Cu, Zn) are very important biological metals and are apparently necessary for all physiological processes. They take place in biochemical processes with enzymes and enable their normal functioning. Thus, understanding the chemical processes between these metals and ATPase, which cause changes in the enzyme activity, is of great interest. Cobalt is an essential micro element required for metabolic functions<sup>13</sup> but it is also potentially toxic if its internal concentration exceeds a limit and can cause the inhibition of  $Na^+/K^+$ -ATPase activity.<sup>14</sup>

The aim of this work was to examine the effects of  $CoSO_4$  on the activity of  $Na^+/K^+$ -ATPase and  $Mg^{2+}$ -ATPase in synaptic plasma membranes (SPM), prepared from the whole brain of adult male rats.

## EXPERIMENTAL

### Chemicals

All chemicals were of analytical grade and were purchased from Sigma Chemicals Co.

### Synaptic plasma membranes preparation and ATPase assay

The synaptic plasma membranes (SPMs) were isolated according to a standard method.<sup>15,16</sup> The ATPase activities were determined by a modified spectrophotometric method for inorganic phosphate<sup>9</sup> liberated by the hydrolysis of ATP. The standard assay medium for the investigation of the ATPase activity contained (in mM): 50 Tris-HCl, pH 7.4; 100 NaCl; 20 KCl; 5  $MgCl_2$ ; 2 ATP; 25  $\mu g$  SPM proteins. The activity obtained in the presence of  $Mg^{2+}$  alone was attributed to  $Mg^{2+}$ -ATPase activity. The  $Na^+/K^+$ -ATPase activity was calculated by subtracting the  $Mg^{2+}$ -ATPase activity from the total ATPase activity in the presence of  $Na^+$ ,  $K^+$  and  $Mg^{2+}$  ions. All the experiments were performed at 37 °C in the presence of various concentrations of  $CoSO_4$ .

TABLE I. The complexes formed by the components in the assay mixture for ATPase in the presence of  $CoSO_4$  and the equilibrium reactions. The values of the association constants were taken from Refs. 17 and 18

Reaction	$K/M^{-1}$
$ATP^{4-} + H^+ \rightleftharpoons HATP^{3-} + H^+$	$1.09 \times 10^7$
$HATP^{3-} + Mg^{2+} \rightleftharpoons MgHATP^-$	$5.42 \times 10^2$
$Mg^{2+} + ATP^{4-} \rightleftharpoons MgATP^{2-}$	$3.48 \times 10^4$
$Mg^{2+} + MgATP^{2-} \rightleftharpoons Mg_2ATP$	40
$Co^{2+} + ATP^{4-} \rightleftharpoons CoATP^{2-}$	$5.13 \times 10^4$

### Calculation of the free ionic concentrations

The concentrations of the ionic species were calculated according to a well-known method<sup>17</sup> taking into account all the equilibrium reactions involving  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ , Tris and ATP (Table I). The stability constants were taken from the literature.<sup>17,18</sup> In all the measurements the free  $\text{Co}^{2+}$  levels and the levels of  $\text{MgATP}^{2-}$  and  $\text{CoATP}^{2-}$  were controlled.

### Kinetic analysis

Kinetic investigations were undertaken to determine the nature of both ATPase enzyme inhibitions induced by  $\text{CoSO}_4$ . The experiments were performed in the presence of increasing concentrations of ATP (0.25–5 mM), and the absence or presence of  $\text{CoSO}_4$ . The  $\text{CoSO}_4$  concentration was 168  $\mu\text{M}$  and 262  $\mu\text{M}$  in the  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Mg}^{2+}$ -ATPase experiments, respectively, while the concentrations of the other ions ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ) were kept constant. The kinetic parameters ( $K_m$ ,  $V_{\text{max}}$ ) were determined using a computer program developed in our laboratory.

## RESULTS AND DISCUSSION

### $\text{CoSO}_4$ - induced inhibition of synaptosomal ATPases

The neuroactive potency of a cobalt ion was estimated by determining its ability to affect the  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Mg}^{2+}$ -ATPase activity as a function of experimental and recalculated free cobalt concentration. In the reaction mixture,  $\text{CoSO}_4$  was present in the concentration range from  $1 \times 10^{-7}$  to 0.01 M. Increasing the concentration of cobalt results in an inhibition of the SPM  $\text{Na}^+/\text{K}^+$ -ATPase and

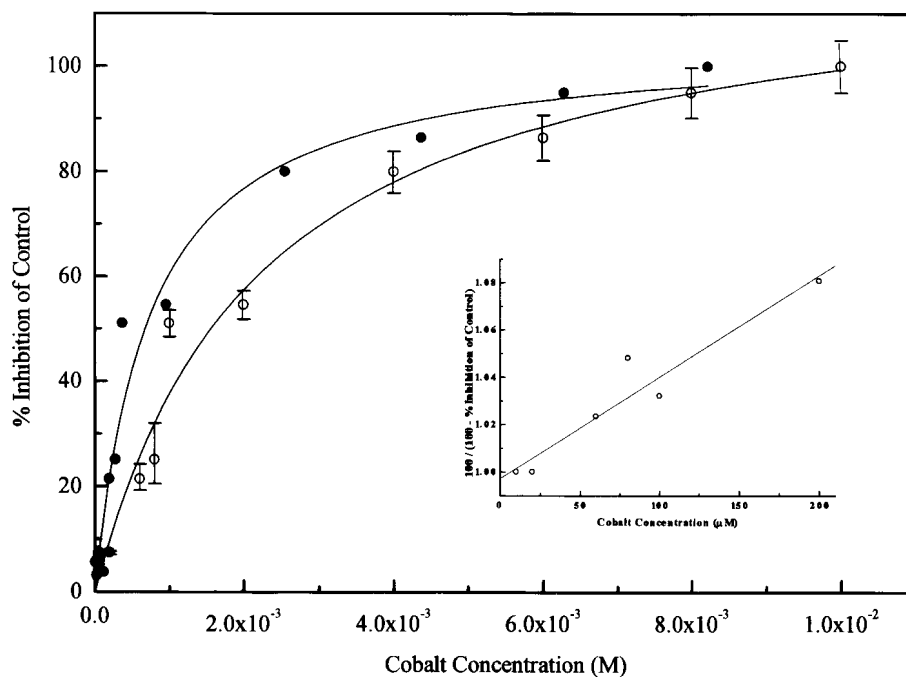


Fig. 1. Effects of  $\text{Co}^{2+}$  on the activity of  $\text{Na}^+/\text{K}^+$ -ATPase as a function of the experimentally added  $\text{CoSO}_4$  (open circles) and of the free  $\text{Co}^{2+}$  (solid circles) concentration. The experimental values are given as the means of at least three experiments  $\pm$  S.E.M. The Dixon's plot is shown in the inset.

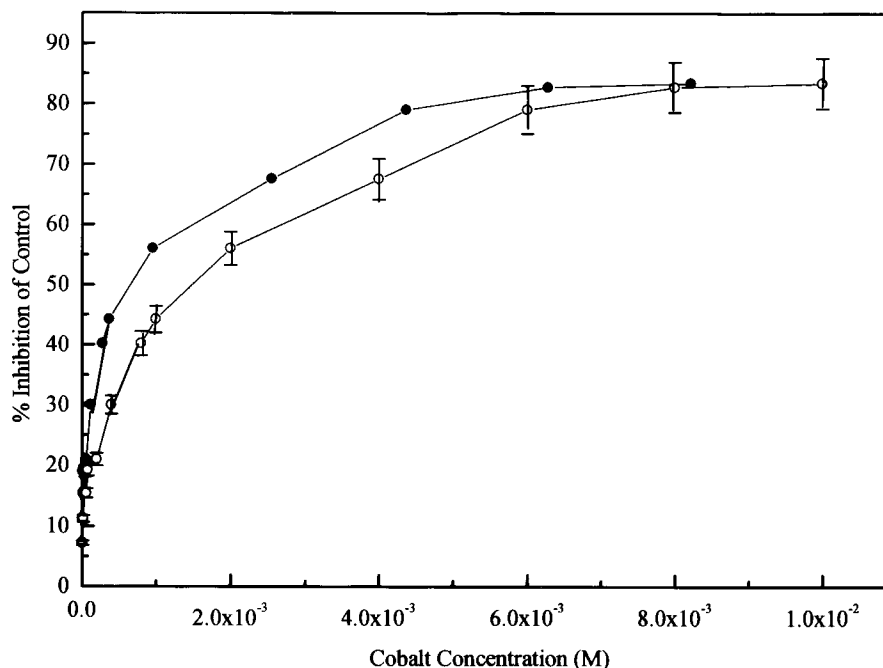


Fig. 2. Effects of  $\text{Co}^{2+}$  on the activity of  $\text{Mg}^{2+}$ -ATPase as a function of the experimentally added  $\text{CoSO}_4$  (open circles) and of the free  $\text{Co}^{2+}$  (solid circles) concentration. The experimental values are given as the means of at least three experiments  $\pm$  S.E.M.

$\text{Mg}^{2+}$ -ATPase activities relative to the control samples which were incubated with the same volume of bidistilled water. The inhibition of the activity is concentration dependent, in a hyperbolic fashion (Figs. 1 and 2). The half-maximum inhibitory activities ( $IC_{50}$ ) of the enzymes were determined as parameters of rectangular hyperbolas giving the value of  $168 \mu\text{M}$  for  $\text{Na}^+/\text{K}^+$ -ATPase and  $262 \mu\text{M}$  for  $\text{Mg}^{2+}$ -ATPase. The  $IC_{50}$  for the recalculated free  $\text{Co}^{2+}$  concentrations for both ATPases is half the value compared with the experimental concentration and is  $75.4 \mu\text{M}$  and  $136 \mu\text{M}$  for  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Mg}^{2+}$ -ATPase, respectively. In addition, comparison of the  $IC_{50}$ -values of  $\text{Co}^{2+}$  for  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Mg}^{2+}$ -ATPase indicates that  $\text{Na}^+/\text{K}^+$ -ATPase is more sensitive to  $\text{Co}^{2+}$  than  $\text{Mg}^{2+}$ -ATPase, since  $IC_{50}$ -value for  $\text{Na}^+/\text{K}^+$ -ATPase is about 2 times lower than the value obtained for  $\text{Mg}^{2+}$ -ATPase.

To establish whether the cobalt binding was in equilibrium with inhibitory sites on the enzyme,  $\text{Na}^+/\text{K}^+$ -ATPase, a Dixon's plot,<sup>19</sup>  $100/(100 - \% \text{ inhibition})$  versus metal ion concentration, was constructed. The obtained linear plot implies equilibrium binding (inset in Fig. 1.). The Dixon's plot cannot be employed yet for  $\text{Mg}^{2+}$ -ATPase as investigations leading to the definition of the active sites are still in progress.

#### *Influence of $\text{Co}^{2+}$ ion on $\text{MgATP}^{2-}$ concentration*

Cobalt ions, as well as magnesium ions form complexes with ATP. These two complexes have similar stabilities (Table I), and so  $\text{Co}^{2+}$  may compete with  $\text{Mg}^{2+}$

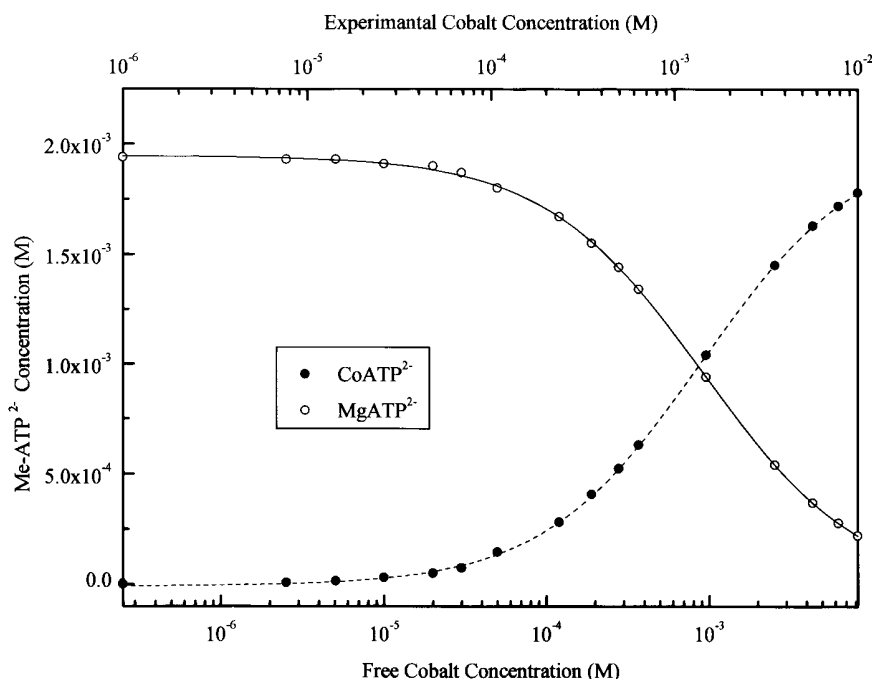


Fig. 3. Concentration of  $\text{MgATP}^{2-}$  and  $\text{CoATP}^{2-}$  complexes as a function of the experimentally added  $\text{CoSO}_4$  and of the free  $\text{Co}^{2+}$  concentration.

in the formation of ATP complexes. To function properly,  $\text{Na}^+/\text{K}^+$ -ATPase requires  $\text{MgATP}^{2-}$ . Therefore, it is necessary to know the cobalt ion concentration which affects  $\text{MgATP}^{2-}$  formation. The results of the investigation of the influence of both the experimental and recalculated free concentration of  $\text{Co}^{2+}$  ions on the concentration of  $\text{MgATP}^{2-}$  in reaction mixture are shown on Fig. 3. The results show that cobalt concentrations lower than 0.1 mM have little effect on the  $\text{MgATP}^{2-}$  concentration. That implies that in the kinetic analyses the  $\text{Co}^{2+}$  concentration should be 0.1 mM or lower.

#### Kinetic analysis

The results presented in Figs. 1 and 2 show that  $\text{Co}^{2+}$ -induced inhibition of  $\text{Mg}^{2+}$ -ATPase activity asymptotically approaches 85 % in contrast to 100 % for  $\text{Na}^+/\text{K}^+$ -ATPase. The incomplete inhibition may imply two kinds of  $\text{Mg}^{2+}$ -ATPase activities, or two enzymes.<sup>1,20</sup>

The results of the kinetic analysis of  $\text{Na}^+/\text{K}^+$ -ATPase, in the absence and presence of 0.1 mM of  $\text{CoSO}_4$ , are shown in the Fig. 4. The  $\text{MgATP}^{2-}$  concentrations were varied from 0.1 to 5 mM. The initial velocities of the  $\text{Na}^+/\text{K}^+$ -ATPase activity vs. the concentrations of  $\text{MgATP}^{2-}$  follow Michealis-Menten kinetics and are rectangular hyperbolas. The kinetic parameters ( $K_m$  and  $V_{\max}$ ) were calculated as hyperbolas parameters. The parameters were also determined from Eadie-Hofstee

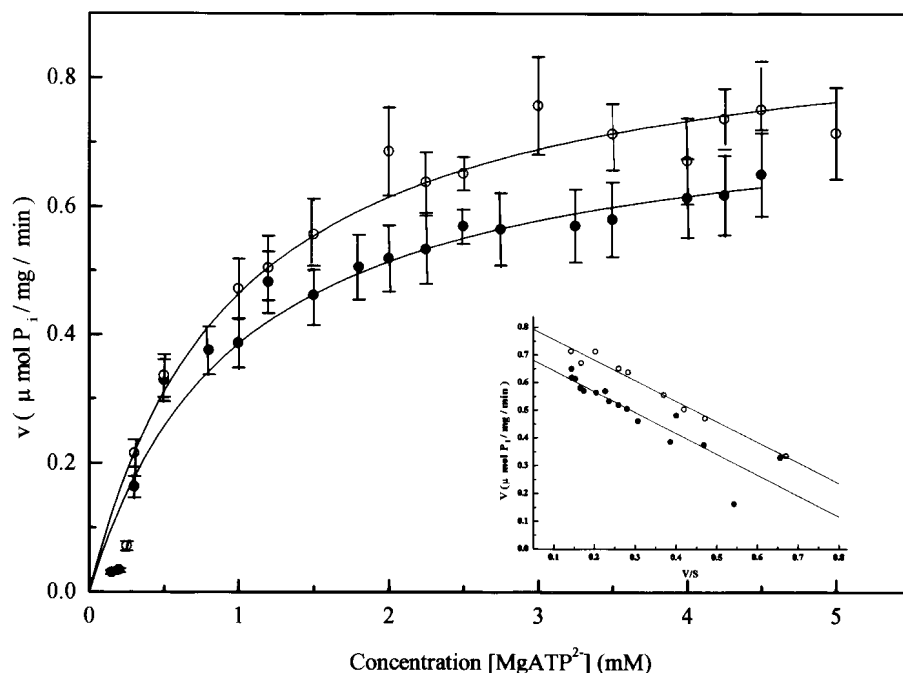


Fig. 4.  $\text{Na}^+/\text{K}^+$ -ATPase activity dependence on the  $\text{MgATP}^{2-}$  concentration in the presence (solid circles) and absence (open circles) of  $1 \times 10^{-3}$  M  $\text{CoSO}_4$ . The values given are the means of at least three experiments  $\pm$  S.E.M. The Eadie-Hofstee transformation of the data is shown in the inset.

transformation of the data (inset in Fig. 4) and the agreement between the results is within experimental error (Table II). Comparison the values of the kinetic parameters in the absence and presence of 0.1 mM  $\text{CoSO}_4$  show that inhibition induced by  $\text{Co}^{2+}$  ions decreases the value of  $V_{\max}$  but, at the same time, the apparent affinity for ATP,  $K_m$ , is not changed. These results indicate the non-competitive nature of the inhibition of the enzyme by cobalt. This means that  $\text{Co}^{2+}$  does not interfere with the specific binding of ATP to the enzyme.

TABLE II. Kinetic analyses of the  $\text{Na}^+/\text{K}^+$ -ATPase and  $\text{Mg}^{2+}$ -ATPase activities in the absence (control) and presence of 1 mM  $\text{CoSO}_4$

Enzyme	Control		$\text{Co}^{2+}$	
	$V_{\max}$ $\mu\text{mol Pi/mg/min}$	$K_m$ mM	$V_{\max}$ $\mu\text{mol Pi/mg/min}$	$K_m$ mM
$\text{Na}^+/\text{K}^+$ -ATPase	$0.90 \pm 0.04^1$	$0.69 \pm 0.05^1$	$0.76 \pm 0.04^1$	$0.77 \pm 0.04^1$
	$0.83 \pm 0.02^2$	$0.74 \pm 0.04^2$	$0.72 \pm 0.03^2$	$0.75 \pm 0.09^2$
$\text{Mg}^{2+}$ -ATPase high affinity	$1.28 \pm 0.03$	$0.29 \pm 0.03$	$1.42 \pm 0.07$	$0.41 \pm 0.09$
$\text{Mg}^{2+}$ -ATPase low affinity	$1.62 \pm 0.03$	$0.68 \pm 0.06$	$1.69 \pm 0.10$	$0.86 \pm 0.19$

<sup>1</sup>Kinetic parameters obtained from hyperbolas; <sup>2</sup>Kinetic parameters obtained by Eadie-Hofstee plot

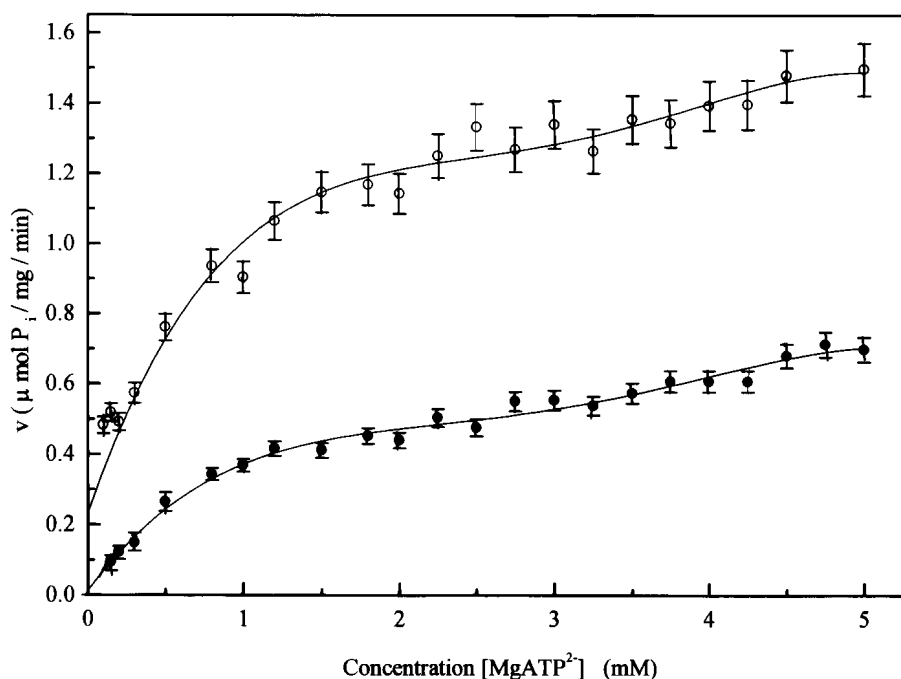


Fig. 5.  $\text{Mg}^{2+}$ -ATPase activity dependence on the  $\text{MgATP}^{2-}$  concentration in the presence (solid circles) and absence (open circles) of  $1 \times 10^{-3}$  M  $\text{CoSO}_4$ . The values given are the means of at least three experiments  $\pm$  S.E.M.

Investigation of the kinetic behavior of  $\text{Mg}^{2+}$ -ATPase was performed by varying the  $\text{MgATP}^{2-}$  concentration from 0.1 to 5 mM in the absence and presence of 0.1 mM  $\text{Co}^{2+}$  ion. The results are presented in Fig. 5. The obtained functions are not purely of the Michaelis-Menten type and have two saturation plateaus indicating that there are either two kinds of  $\text{Mg}^{2+}$ -ATPase activities or two different  $\text{Mg}^{2+}$ -ATPases.<sup>1,20</sup> Eadie-Hofstee transformation of the kinetic data of the  $\text{Mg}^{2+}$ -ATPase in the absence and presence of 1 mM  $\text{CoSO}_4$  are concave curved lines. Two straight lines can be drawn to describe the two portions of the curve and illustrate two sites of different sensitivity (high and low).<sup>21</sup> The parameters of the straight lines represent initial approximations of the kinetic parameters of each site. By using a computer program developed in our laboratory, the final values of the kinetic parameters  $V_{\max}$  and  $K_m$  were calculated after five iterations (Table II). The cobalt ion is a nonselective inhibitor for  $\text{Mg}^{2+}$ -ATPase and it can only be concluded that there are two sites of different sensitivity.

In conclusion, our results show that the  $\text{Co}^{2+}$   $IC_{50}$  values for both ATPases are much higher compared with the other metals of the first transition series, *i.e.*, for  $\text{Na}^+/\text{K}^+$ -ATPase ( $1.68 \times 10^{-4}$  M) it is about three orders of magnitude higher than the value for  $\text{Cu}^{2+}$  ( $5.9 \times 10^{-7}$  M).<sup>21</sup> The apparent lack of effect of  $\text{Co}^{2+}$  ions on the ATPase activity suggests that this metal may be prevented from affecting the active sites of the enzyme, perhaps by binding to proteins not native to the enzyme. Since

$\text{Co}^{2+}$  has a coordination number of six, it could bind simultaneously to proteins not native to ATPases as well as to CH. Further work is underway to elucidate the mechanism of the interaction of the metal with SPM.

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#### ИЗВОД

#### ХЕМИЈСКИ АСПЕКТ УТИЦАЈА ЈОНА КОБАЛТА НА АКТИВНОСТ АТР-АЗА

ЉУБИЦА ВУЈИСИЋ, ДАНИЈЕЛА КРСТИЋ И ЈОВАН ВУЧЕТИЋ\*

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Испитан је утицај  $\text{Co}^{2+}$  јона на активност  $\text{Na}^+/\text{K}^+$ -АТРазе и  $\text{Mg}^{2+}$ -АТР-азе, ензима синаптозомалне мембране мозга пацова. Циљ рада је био да се испита инхибиција активности оба ензима изазвана излагањем јонима кобалта као функција експериментално додатог  $\text{CoSO}_4$ . Такође је израчуната и дискутована "слободна" концентрација  $\text{Co}^{2+}$  у реакционој смеси. Утврђено је да кобалт инхибира ензиме у концентрационо зависном смислу. Вредности  $IC_{50}$  израчунате из експерименталних кривих су:  $168 \mu\text{M}$  за  $\text{Na}^+/\text{K}^+$ -АТР-азу и  $136 \mu\text{M}$  за  $\text{Mg}^{2+}$ -АТР-азу. Линеаран Dixon-ов плот за  $\text{Na}^+/\text{K}^+$ -АТР-азу указује на равнотежно везивање кобалта. Израчунати су кинетички параметри оба ензима у присуству и одсуству  $\text{CoSO}_4$ . Инхибиција  $\text{Na}^+/\text{K}^+$ -АТР-азе изазвана  $\text{CoSO}_4$  је некомпетитивна, док  $\text{Mg}^{2+}$ -АТР-аза има два места везивања различитог афинитета или пак два различита ензима.

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