



# DETERMINATION OF INTENSITY OF ENZYMES INHIBITION AND ACTIVATION

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It was established that at increasing concentration of inhibitors (or activators) in the enzyme-substrate system the vector lengths of enzyme inhibition (and activation) retain a permanent tendency to increase, while the constants of enzyme inhibition (and activation) either remain unchangeable or change in any direction by studying the dependence of a course of change of vector length ( $l_i$ ) and constants of enzyme inhibition ( $K_i$ ). Examples are given of using the  $K_i$  constants and the vector lengths for the characterization of the strength of enzyme binding to inhibitor and the intensity of enzyme inhibition, respectively.

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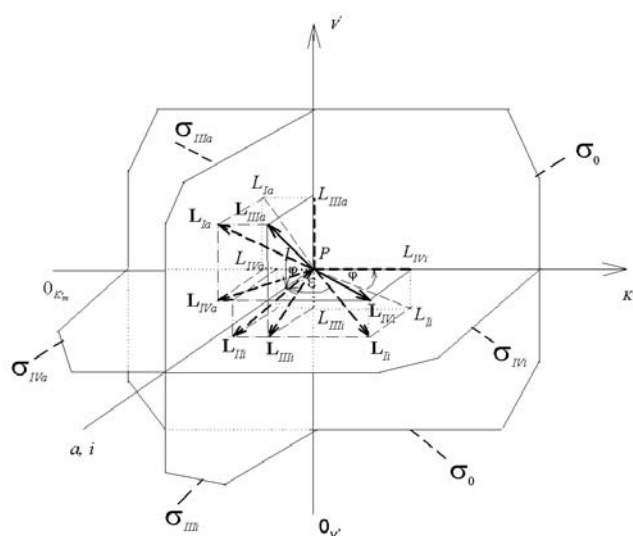
## Introduction

When studying the enzyme properties, a necessity often arises to characterize the intensity of effect of inhibitor ( $i$ ) or activator ( $a$ ) on enzyme. This question presents a great interest in experimental enzymology, because in the reacting enzyme-substrate system it is impossible to escape the presence of other components such as added activators and inhibitors and also the substances comprising a buffer part of the medium. Accumulated reaction products are also capable of decreasing enzyme activity. Before a vector method of representation of enzymatic reactions in the three-dimensional  $K'_mVI$  coordinate system (Figure 1),<sup>1-5</sup> where  $K'_m$  and  $V'$  – the effective determined in presence of inhibitor ( $i$ ) or activator ( $a$ ) Michaelis constant and maximum reaction rate,  $I$  – the common (printing) symbol of  $K'_mVI$  coordinate system, was unclear which parameters of catalyzed reaction can be used for these purposes: probably, the maximum reaction rate  $V'$  or – which is the same – the  $k_{+2}$  constant of reaction rate?

But simultaneously with change of this parameter of substrate cleavage the other important reaction parameter, i.e. the  $K_m^0$  Michaelis constant, also changes in most cases

$$v_0 = \frac{V^0}{1 + \frac{K_m^0}{S}} \quad (1)$$

where  $K_m^0$  and  $V^0$  – the Michaelis constant and maximum reaction rate determined in absence of inhibitor ( $i$ ) or activator ( $a$ ),  $S$  – substrate concentration,  $v_0$  – initial reaction rate.



**Figure 1.** A three-dimensional  $K'_mVI$  system of rectangular coordinates with the coincident  $P_a$  and  $P_i$  semi-axes of molar concentrations of  $i$  and  $a$  (the  $P_{a,i}$  semi-axis) convenient for analysis of the positions of three-dimensional vectors of enzyme inhibition: calf alkaline phosphatase – vector  $L_{Ii}$ , eel alkaline phosphatase – vector  $L_{Iii}$  and pyrimidine-specific RNase A-vector  $L_{IVa}$  analyzed as examples 1, 2 and 3 in the text. The symbols of the rest vectors  $L_{Iiii}$ ,  $L_{IVi}$ , ...,  $L_{Iia}$ ,  $L_{Vaa}$ , and their projections  $L_{Iiii}$ ,  $L_{IVi}$ , ...,  $L_{Iia}$ ,  $L_{Va}$  and the projections of directing planes  $\sigma_{Ivi}$ ,  $\sigma_{Iii}$ ,  $\sigma_{IVa}$ ,  $\sigma_{IIa}$  on the basic  $\sigma_0$  plane and slope angles of vectors are considered in the text.

Sometimes the authors make attempts to characterize the intensity of effect of inhibitor on enzyme using a numerical value of the respective constant of enzyme inhibition  $K_i$  by the principle: the smaller the value of a calculated constant of enzyme inhibition is the stronger is the effect of inhibition on enzyme.<sup>6-12</sup>

However, a course of change of constants of enzyme inhibition may completely contradict such their application, because:

- 1) the constants of enzyme inhibition may remain unchangeable at increasing concentration of inhibitor (Figures 3A, 3B and Table 2) and

- 2) their values may augment at increasing concentration of inhibitor in the enzyme-substrate system (Figures 2A, 2B and Table 1).

There are examples of using the  $K_i$  constants of enzyme inhibition for characterization of strength of enzyme binding to inhibitor.<sup>13-17</sup> In some articles<sup>18-21</sup> the Tables of a course of change in the parameters of enzyme inhibition (or activation) are analyzed for explanation of such processes without construction of plots of a course of simultaneous change of both parameters. Such plotting becomes extremely difficult, if these parameters change in different directions (Table 1).

**Table 1.** Inhibitory effect of increasing concentration of  $\text{WO}_4^{2-}$  anions on calf alkaline phosphatase

Inhibitor, $10^{-4}$ M	$K'_m$ , $10^{-5}$ M	$V'$ , $\mu\text{mol min}^{-1}$ $\mu\text{g protein}^{-1}$	$K_{ii}$ , $10^{-5}$ M	$l_{ii}$ , c.u.
0.0	4.45	2.56		
0.0625 (0.0625 c.u.)	5.28 (5.28 c.u.)	2.51 (2.51 c.u.)	3.33	0.834
0.125	5.39	2.30	5.28	0.983
0.250	5.97	2.15	6.39	1.59
0.50	6.56	1.74	7.48	2.32

At data analysis by taking into account of a course of change of only one  $K'_m$  (or  $V'$ ) reaction parameter (Fig. 4A), the situation is simplified. But the researchers usually construct plots of the data obtained either in the  $(\text{tg}\omega; i)$  coordinates of slopes, where  $\text{tg}\omega = K'_m/V'$  or in the  $(1/V'; i)$  coordinates of intercepts, which is only convenient for calculation of the so-called  $K_{is}$ -slope and  $K_{ir}$ -intercept constants of enzyme inhibition but not for the characterization of intensity of effect of enzyme inhibition.

A vector method representation of enzymatic reactions in the three-dimensional  $K'_m V' I$  coordinate system (Fig. 1) showed that the  $L$  vector length dependent on the concentration of inhibitor (Eq. 2) in the above coordinate system characterizes the intensity of effect of inhibitor (or activator) on enzyme, while the  $K_i$  constant of enzyme inhibition (or the  $K_a$  constant of enzyme activation) independent of the concentration of inhibitor – characterizes the strength of binding of enzyme to inhibitor.<sup>1-5</sup>

Let us consider the principle of construction and functioning of this system. If to designate the effective Michaelis constants determined in the presence of inhibitor ( $i$ ) or activator ( $a$ ) by the symbol  $K'_m$  and the Michaelis constants of initial (uninhibited  $i = 0$  and nonactivated  $a = 0$ ) enzymatic reaction by the symbol  $K_m^0$ , the  $OK'_m$  axis of numerical values of  $K'_m$  parameters of enzymatic reactions shall be obtained with the point  $K_m^0$  on it. The construction of a scale of numerical values of the  $V'$  and  $V^0$  maximum reaction rates of activated ( $V' > V^0$ ) and inhibited ( $V' < V^0$ ) enzymatic reactions is analogous.

If to intersect these axes at the right angle to each other at the points  $K_m^0$  and  $V^0$  and draw from the obtained  $P(K_m^0 V^0 0)$  point a  $Pa, i$  semiaxis of molar concentrations of inhibitor ( $i$ ) and activator ( $a$ ) perpendicular to the basic  $\sigma_0$  plane with the  $OK'_m$  and the  $OV'$  axes in it and also to draw through this

semiaxis and each of the directing  $PK'_m$ ,  $PV'$ ,  $PO_{K_m}$  and  $PO_V$  semiaxes a system of reciprocally-perpendicular  $\sigma_{iVi}$ ,  $\sigma_{iIi}$ ,  $\sigma_{iVa}$ ,  $\sigma_{iIa}$  planes, we shall obtain a three-dimensional  $K'_m V' I$  coordinate system with a coincident  $Pa, i$  semiaxis convenient for vector analysis of enzymatic reactions (Fig. 1).

According to the numerical values of  $K'_m$ ,  $V'$  and  $i$  (or  $a$ ) parameters, each inhibited or activated enzymatic reaction shall have in such a system its own vector of representation – a concrete three-dimensional  $L$  vector of this reaction.

Thus, the  $l$  length (module) of a vector:

$$l_i = \left( (K'_m - K_m^0)^2 + (V' - V^0)^2 + (i - 0)^2 \right)^{0.5} \quad (2)$$

shall characterize the intensity of proceeding of inhibited ( $i > 0$ ) or activated ( $a > 0$ ) enzymatic reaction, and a ratio of the lengths of  $L$  vector projections on the  $Pa, i$  semiaxis to the coordinates of this vector projection on the basic  $\sigma_0$  plane shall be an equation for calculation of the  $K_i$  constant of enzyme inhibition (or the  $K_a$  constant of enzyme activation).<sup>1-5</sup>

Let us consider the possibility of using vector lengths at data analysis of the intensity of effect of inhibitor and activator on enzyme.

## Materials and methods

Enzymes: calf and eel alkaline phosphatase (EC 3.1.3.1) – a preparation of Sigma (USA). Substrate: p-nitrophenyl phosphate-2CHA salt (pNPP) – a product of Serva (Germany). Inhibitors: sodium tungstate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ ) and isopropanol (i-PrOH) (high purity grade). The concentration of pNPP was changed within  $0.294 \cdot 10^{-4}$  –  $0.98 \cdot 10^{-4}$  M, the concentration of calf alkaline phosphatase and that of eel alkaline phosphatase were kept constant  $0.978 \mu\text{g mL}^{-1}$  and  $2.46 \mu\text{g mL}^{-1}$ , respectively.

The course of pNPP cleavage catalyzed by all tested alkaline phosphatases was registered by a CF-4 DR Optica Milano spectrophotometer (Italy). Reactions were carried out on 0.05 M Tris-HCl buffer (pH 9.0) at ionic strength 0.1 by NaCl of high purity grade under constant stirring<sup>1</sup> and thermostating ( $37^\circ\text{C}$ ) at the wave length  $+\Delta D_{400}$  of a solution containing the substrate, enzyme and inhibitor versus a solution of the same composition, but without the enzyme.

The 2<sup>nd</sup> enzyme: bovine pyrimidine-specific RNase A (EC 3.1.44.22) – a preparation of Sigma. Substrate of RNase A: cytidine-2',3'-monophosphate (C>p), a product of Sigma. Activator of RNase A: sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ), a domestic preparation of high purity grade. Reactions were carried out under constant stirring and thermostating ( $24^\circ\text{C}$ ) in 0.05 M Na-acetate buffer (pH 5.8) at ionic strength 0.1 using NaCl of high purity grade. The concentration of C>p was changed within  $6.58 \cdot 10^{-4}$  –  $1.645 \cdot 10^{-4}$  M and that of enzyme was kept constant  $8.46 \mu\text{g mL}^{-1}$ . A course of C>p cleavage was recorded by increase in the absorbance at the wave length  $\lambda = 260$  nm.

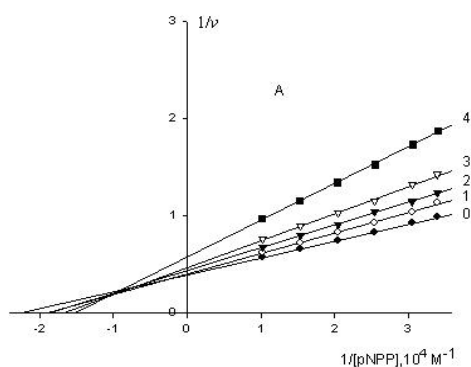
The initial reaction rates  $v_0$  ( $v_i$  and  $v_a$ ) of substrate cleavage were determined by a slope angle of tangents to the initial segments of curves representing a course of reaction change in not less than five parallel experiments.

The kinetic parameters  $K'_m$  and  $V'$  of enzyme inhibition (or activation) were calculated by constructing plots in the ( $v^{-1}$ ,  $S^{-1}$ ) coordinates of Lineweaver-Burk by using a computer program SigmaPlot, version 2000 (USA).

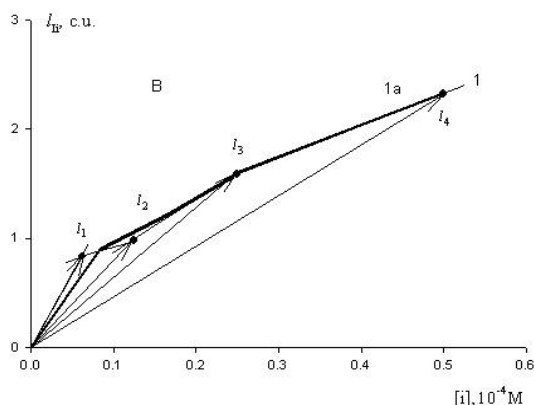
The mean-square deviation a fivefold determination was as follows:  $v = \pm 2.5\%$ ,  $K'_m$  and  $V' = \pm 7.5\%$ ,  $K_i = \pm 10\%$  and  $l = \pm 10\%$ .

## Results and Discussions

**Example 1.** As it can be seen from Figs. 2A, 2B and Table 1, the inhibitory effect of anions of tungstic acid ( $\text{WO}_4^{2-}$ ) on initial rates of pNPP cleavage by calf alkaline phosphatase is exhibited by increasing effective Michaelis constants and decreasing maximum reaction rates ( $K'_m > K^0_m$ ,  $V' < V^0$ ), in this case i.e. the  $K'_m$  and  $V'$  parameters change simultaneously in different directions.



**Figure 2A.** Plots of the inhibitory effect of anions  $\text{WO}_4^{2-}$  on initial rates of pNPP cleavage catalyzed by calf alkaline phosphatase in the coordinates of Lineweaver-Burk. Note: line 1 – the concentration of inhibitor ( $10^{-4}$  M) is 0.0625, line 2 – 0.125, line 3 – 0.250, line 4 – 0.500. Line (0) – the inhibitor is absent. Designation:  $v$ ,  $\mu\text{mol}\cdot\text{min}^{-1} \mu\text{g protein}^{-1}$ .



**Figure 2B.** Data representation of Figure 2A in the ( $l_i; i$ ) coordinates. Note:  $l_1, l_2, l_3$  and  $l_4$  are the lengths of  $L_i$  vectors. Line 1 – is a thin curve drawn by the positions of mobile vector ends, line 1a is a thick line drawn by taking into account the deviations at respective concentrations of  $\text{WO}_4^{2-}$ .

The attempt to use the  $K_i$  constants of enzyme inhibition (Table 1) for the characterization of intensity of inhibitory effect of  $\text{WO}_4^{2-}$  on enzyme indicated the weakening of binding of anions to enzyme, because the constants of enzyme inhibition augment at increasing concentration of  $\text{WO}_4^{2-}$ . But how to express the dependence of a course of change of the intensity of inhibitory effect of increasing concentrations of  $\text{WO}_4^{2-}$  on phosphatase as a function by using data of Fig. 2A and Table 1 seems unclear yet.

Substitution of the appropriate parameters of calf alkaline phosphatase inhibition in Equation (2) allows to obtain data on the dynamics of intensity of enzyme inhibition. For this purpose, it is necessary to express the dimensions of all parameters used in conventional units (c.u.).

It is noteworthy that because of using different definite ranges of concentration of the inhibitor ( $10^{-3} - 10^{-6}$  M) explained by both the convenience in the obtaining of a desired effect on enzyme, the ability of inhibitors to suppress enzyme activity and an essential difference in the catalytic activity of enzymes proper (a range of difference in the  $V^0$  and  $K^0_m$  parameters may be enumerated by orders in value), to choose a standard number of conventional units to be used in all possible cases of enzyme inhibition (and activation) is impossible now. The practice shows that a selection of conventional units (c.u.) when the determined vector lengths shall be expressed by small decimal digits ( $l_i = 1.000, 2.000 \dots$ ) would be advisable. So, the conventional units for experimental data analysis of (Fig. 2A – 4A) were chosen according to the above reasons (this is marked by brackets in the 3<sup>rd</sup> line of each of Tables 1, 2 and 3).

**Table 2.** Inhibitory effect of increasing concentration of isopropanol on eel alkaline phosphatase

Inhibitor, $10^{-3}$ M	$K'_m$ , $10^{-5}$ M	$V'$ , $\mu\text{mol}\cdot\text{min}^{-1} \mu\text{g protein}^{-1}$	$K_{III}$ , $10^{-3}$ M	$l_{III}$ , c.u.
0.0	4.82	3.16		
0.2	4.47	2.93	1.77	0.4641
0.2 c.u.	4.47 c.u.	2.93 c.u.		
0.5	4.07	2.66	1.89	1.0308
1.0	3.53	2.31	1.91	1.8403

**Table 3.** Activating effect of increasing concentration of anions  $\text{MoO}_4^{2-}$  on RNase A

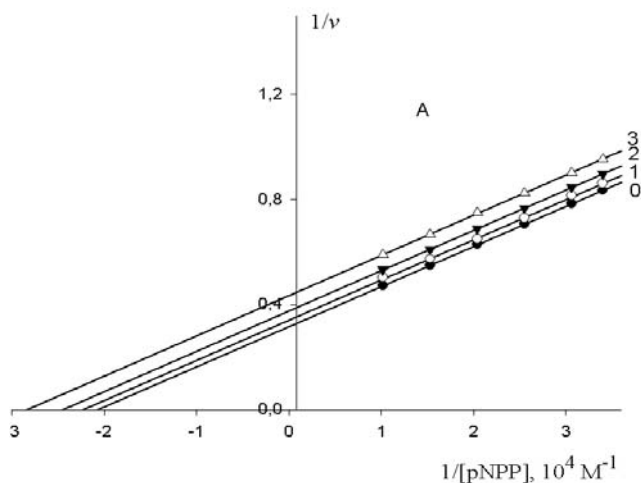
Activator, $10^{-3}$ M	$K'_m$ , $10^{-4}$ M	$V'$ , $\mu\text{mol}\cdot\text{min}^{-1} \mu\text{g protein}^{-1}$	$K_{IVa}$ , $10^{-3}$ M	$l_{IVa}$ , c.u.
0.0	5.446	8.17		
0.2	4.651	8.14	1.17	0.8198
0.2 c.u.	4.651 c.u.	8.14 c.u.		
0.5	3.732	8.13	1.09	1.7256
1.0	2.960	8.32	1.19	2.4940

Use of Equation (2) gives the possibility to calculate not only a course of change in the  $L$  vector lengths of calf alkaline phosphatase inhibition by  $\text{WO}_4^{2-}$ , but also to plot their vector length positions in the ( $l_i; i$ ) (Fig.2B–3B) or ( $l_a; a$ )

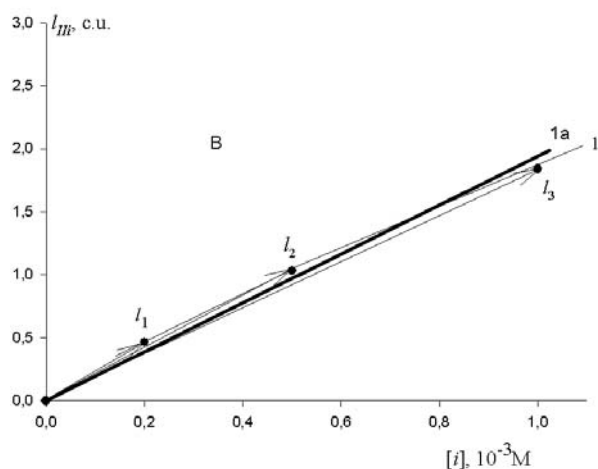
Fig. 4B coordinate systems, thus allowing to take into account a simultaneous course of change of the  $K'_m$  and  $V'$  reaction parameters, which was impossible without vector analysis of enzymatic reactions in the three-dimensional  $K'_m$   $V'$  coordinate system.

As seen from the obtained data (Table 1, Figs. 2A-B), the dynamics of intensity of enzyme inhibition ( $l_{ii}$ ) changed with retardation from linearity. This testifies to instability in the dynamics of effect of the above phosphatase by anions of tungstic acid and the disaccordance of this experiment with the criterion of stability of the mechanism of proceeding of this reaction, which should be characterized by constancy of the slope angles of  $L$  vectors of enzyme inhibition: azimuth ( $\text{tg } \varphi$ ) – to the horizontal  $\sigma_{iVi}$  plane of Fig. 1 and tangential ( $\text{tg } \varepsilon$ ) – to the vertical  $\sigma_{iii}$  plane of the same Figure 1:<sup>3</sup>

$$\text{tg } \varepsilon = \text{const}, \text{tg } \varphi = \text{const.} \quad (3)$$

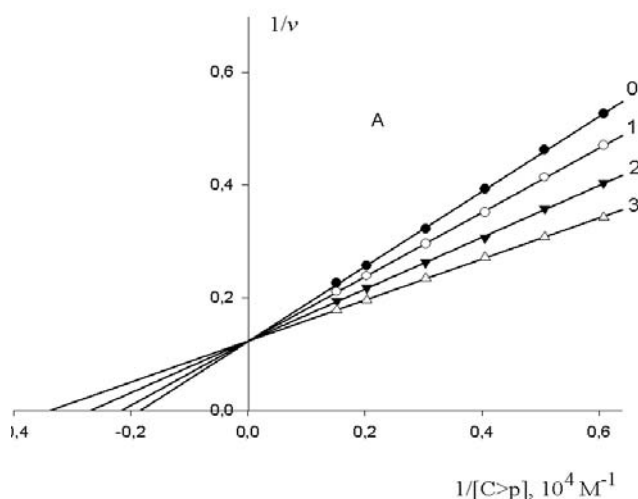


**Figure 3A.** Plots of the inhibitory effect of isopropanol on initial rates of pNPP cleavage catalyzed by eel alkaline phosphatase in the coordinates of Lineweaver-Burk. Note: line 1 – the concentration of inhibitor is 0.0002 M, line 2–0.0005 M, line 3–0.001 M. Line (0)–the inhibitor is absent. Designation:  $v$ ,  $\mu\text{mol} \cdot \text{min}^{-1} \mu\text{g protein}^{-1}$ .

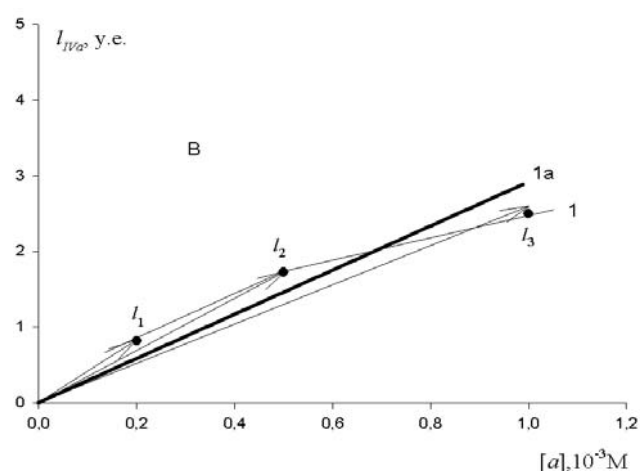


**Figure 3B.** Data representation of Figure 3A in the  $(l_{iii}; i)$  coordinates. Note:  $l_1$ ,  $l_2$  and  $l_3$  are the lengths of  $L_{iii}$  vectors. Line 1 – is a thin curve drawn by the positions of mobile vector ends, line 1a is a thick curve drawn by taking into account the deviations at respective concentrations of i-PrOH by analogy to Figure 2A.

**Example 2.** In a course of reaction the values of two ( $K'_m$  and  $V'$ ) parameters of inhibited reaction change in one direction. As an example of analysis of such situation, it is convenient to analyze data of the study on the inhibitory effect of increasing concentrations of i-PrOH on initial rates of pNPP cleavage catalyzed by eel alkaline phosphatase (Fig. 3A and Table 2). The results show that although the  $K'_m$  and  $V'$  parameters of enzyme inhibition changed in one direction and at simultaneous increasing length of each subsequent pair of segments cut off on the coordinate axes by  $n$  times, where  $n$  is a coefficient of similarity of respective rectangular triangles cut off by respective lines on the coordinate axes at increasing concentration of isopropanol. This puts additional questions: the 1<sup>st</sup> one – should a researcher in this case analyze a course of change by only one parameter of enzyme inhibition, if so, which of the parameters? And the 2<sup>nd</sup> question: what new information can be obtained from constancy of the values of  $K_{ii}$  constants of enzyme inhibition?



**Figure 4A.** Plots of the activating effect of anions  $\text{MoO}_4^{2-}$  on initial rates of C>p cleavage catalyzed by RNase A in the coordinates of Lineweaver-Burk. Note: line 1 – the concentration of inhibitor is 0.0002 M, line 2 – 0.0005 M, line 3 – 0.001 M. Line (0) – the inhibitor is absent. Designation:  $v$ ,  $\mu\text{mol} \cdot \text{min}^{-1} \mu\text{g protein}^{-1}$ .



**Figure 4B.** Data representation of Figure 4A in the  $(l_{IIa}; i)$  coordinates. Note:  $l_1$ ,  $l_2$  and  $l_3$  are the lengths of  $L_{IIa}$  vectors. Line 1 – is a thin curve drawn by the positions of mobile vector ends, line 1a is a thick curve drawn by taking into account the deviations at respective concentrations of  $\text{MoO}_4^{2-}$  by analogy to Figure 2B.

Construction of the positions of vector lengths  $l_{li}$  of calf alkaline phosphatase inhibition in the  $(l_{li};i)$  coordinate system (Fig. 3B) and the form of Eqn. (2) used for calculation of these lengths showed that since a change of the  $K'_m$  and  $V'$  parameters of inhibited reaction influences the position of each vector in this system and as these both parameters are found in Eqn. (2) used at calculation of vector modules, one shall have to obligatorily take into account a course of simultaneous change of the  $K'_m$  and  $V'$  parameters for characterization of the inhibitory effect of increasing concentrations of isopropanol on enzyme.

**Example 3.** Only the  $K'_m$  parameter of activated reaction is changed in the reaction course. Data analysis of the study on the activating effect of anions of molybdenic acid ( $\text{MoO}_4^{2-}$ ) on initial rates of C>p cleavage catalyzed by pyrimidine-specific RNase A (Figs, 4A – 4B, Table 3) showed that a gradually increasing concentration of activator was accompanied by decrease in only the  $K'_m$  reaction parameter ( $K'_m < K^0_m$ ,  $V' = V^0$ ). The attempt to use anions of molybdate for the characterization of the intensity of activating effect on enzyme by taking into account a course of change of the  $K_{IVa}$  constants of RNase A activation revealed that there was no such an effect on enzyme in that case because the values of the constants remained unchangeable at increasing concentration of activator on enzyme (Fig. 4A, Table 3).

At the same time, the calculation of vector  $l_{IVa}$  lengths of RNase A activation (Table 3) and analysis of vector positions in the  $(l_{IVa};a)$  coordinate system (Fig. 4B) showed that despite the constancy of values of the  $K_{IVa}$  constants of enzyme activation their vector lengths increased directly proportional to the concentration of  $\text{MoO}_4^{2-}$ , which indicates the presence of the activating effect of molybdate anions on enzyme as well as on the stability of dynamics of enzyme activation.

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