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Inhibition of Jack Bean Urease by N-(n-butyl) thiophosphorictriamide and N-(n-butyl) phosphorictriamide: Determination of the Inhibition Mechanism

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N-(n-butyl)thiophosphorictriamide (NBPT) and its oxygen analogue N-(n-butyl)phosphorictriamide (NBPTO) were studied as inhibitors of jack bean urease. NBPTO was obtained by spontaneous conversion of NBPT into NBPTO. The conversion under laboratory conditions was slow and did not affect NBPT studies. The mechanisms of NBPT and NBPTO inhibition were determined by analysis of the reaction progress curves in the presence of different inhibitor concentrations. The obtained plots were time-dependent and characteristic of slow-binding inhibition. The effects of different concentration of NBPT and NBPTO on the initial and steady-state velocities as well as the apparent first-order velocity constants obeyed the relationships for a one-step enzyme-inhibitor interaction, qualified as mechanism A. The inhibition constants of urease by NBPT and NBPTO were found to be 0.15 μM and 2.1 nM, respectively. The inhibition constant for NBPT was also calculated by steady-state analysis and was found to be 0.13 μM. NBPTO was found to be a very strong inhibitor of urease in contrast to NBPT.

Keywords: Urease; Inhibition; Kinetic constants; N-(n-butyl) thiophosphorictriamide; N-(n-butyl)phosphorictriamide

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.5) catalyzes the hydrolysis of urea: $CO(NH_2)_2+H_2O \rightarrow 2NH_3+CO_2$. Urease is widely distributed in a variety of bacteria, algae, fungi and plants. Structure, number and type of subunits, molecular weight and amino acid sequence of urease depend on its origin.[1–3] Apart from these differences the amino acid sequences of active sites are the same and each active site contains two nickel ions which proves that different kinds of urease have the same mechanism of enzymatic activity.[4] The distance between nickel ions in the urease active site is approximately 3.5 Å.[5] The study of urease inhibition has medical, environmental and agronomic significance, as well as providing insight into the urease catalytic mechanism. Without an efficient degradation process urea would rapidly accumulate causing

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severe environmental problems. In another context, urea is the most used nitrogen fertilizer in world agriculture and too high activity of soil urease decreases the efficiency of fertilization with urea since the rapid hydrolysis of urea can result in significant N loss through NH_3 volatilisation. Moreover, the increase of the ammonia concentration in soil due to urea hydrolysis can be toxic to germinating seeds or growing plants.[6] The application of urease inhibitors has been considered as one of the solutions to these problems. Despite the considerable effort to identify urease inhibitors for application with fertilizer urea, most of the tested compounds were ineffective for use in soil. The only compounds that have proved to be viable for soil application are the structural analogues of urea phosphoroamides[7–9,12] and thiophosphoroamides.[10–12] Hydroquinone, benzoquinones and substituted benzoquinones[13,14] were also considered as additives to urea fertiliser but they have to be used at much higher concentrations than phosphoroamides.

Many phosphoroamide compounds has been extensively studied.[9,15–17] In contrast, there is no kinetic data on NBPT in spite of the fact that NBPT is frequently mentioned in agricultural literature.

In this paper the inhibitory influence of NBPT and its oxygen analogue NBPTO on jack bean urease was studied. The kinetic parameters, inhibition constants and mechanisms of the inhibition were determined.

MATERIALS AND METHODS

Materials

Jack bean urease, Sigma type III of specific activity 22 units/mg protein was used. One unit is the amount of enzyme that liberates 1.0 μmol of NH_3 from urea per minute at pH 7.0 and 25°C. NBPT and urea (Molecular Biology Reagent) were purchased from Sigma. Other chemicals were obtained from POCh, Gliwice, Poland. All reagents used were of analytical grade.

Enzymatic Reaction

The hydrolysis of urea catalyzed by jack bean urease was studied in phosphate buffer pH 7.0 (50 mM, 2 mM EDTA) at 25°C. The initial concentration of urea was 50 mM and the concentration of urease was 0.05 mg cm^{-3}. The reaction was studied in the absence and presence of NBPT and NBPTO as inhibitors. A sample (0.5 cm^3) of the reaction mixture was removed after an appropriate reaction time and the amount of ammonia was determined by the phenol-hypochlorite colorimetric method.[18] The absorbance was measured at 625 nm. The effect of NBPT on the phenol-hypochlorite method was tested and no interference was shown.

Progress-curve Studies

Progress curves were obtained for the reactions initiated by addition of enzyme into the reaction mixtures containing different concentrations of NBPT (0.3, 0.4, 0.5, 0.8, 1.0 mM) and NBPTO (0.75, 1.25, 2.5, 5.0 μM).

Steady-state Kinetic Studies

The initiation of the enzymatic reaction was preceded by a 20 min incubation of the enzyme with the inhibitor during which the equilibrium between enzyme E, inhibitor I and enzyme-inhibitor complex EI* was attained ($E + I \Leftrightarrow EI^*$). The reaction was initiated by addition of 1 cm^3 of a concentrated solution of urea (5 M) into the reaction mixture containing 50 mM phosphate buffer, 2 mM EDTA, 0.05 mg cm^{-3} urease and different concentrations of NBPT (0.005, 0.006, 0.007, 0.01 mM).

Determination of K_M And v_{max}

The Michaelis constant K_M and the maximum velocity v_{max} in the absence of the inhibitor were determined by measuring the initial reaction velocities at urea concentrations in the range 2–50 mM. The values obtained by applying non-linear regression to the Michaelis-Menten equation were: K_M=7.4 ± 0.2 mM, v_{max}=0.023 ± 0.001 mM s^{-1}.

Conversion of NBPT to NBPTO

Under aerobic conditions NBPT undergoes conversion to the oxygen analogue NBPTO[19,20] $C_2H_9NHP(S)(NH_2)_2 \rightarrow C_2H_9NHP(O)(NH_2)_2$.

Since NBPTO is a stronger inhibitor of urease than NBPT[20–22] the conversion increases the inhibitory effect. The evolution of inhibitory activity in a 5 mM solution of NBPT (stored at room temperature) was tested over 3 months. Each day alignment of the NBPT solution was added to the freshly prepared reaction mixture. The final concentration of NBPT in the reaction mixture was 0.5 mM. The reaction was initiated by addition of the enzyme. The concentration of ammonia was measured after 15 min of the reaction course. The results are presented in Fig. 1. It was observed that within the first few days the change in inhibitory activity was negligible. In this work all experiments concerning NBPT were realized within 3 days from preparing the NBPT solution.

After 8 weeks the inhibitory activity reached its maximum and became constant. This indicated that the process of conversion of NBPT into NBPTO was completed. It was assumed that the conversion was quantitative and the concentration of produced NBPTO was equal to the initial concentration of NBPT. All the experiments concerning NBPTO were realized within the twelfth week after preparation of the NBPT solution.

Data Analysis

The theory of reactions involving enzymes and slow-binding inhibitors has been presented by

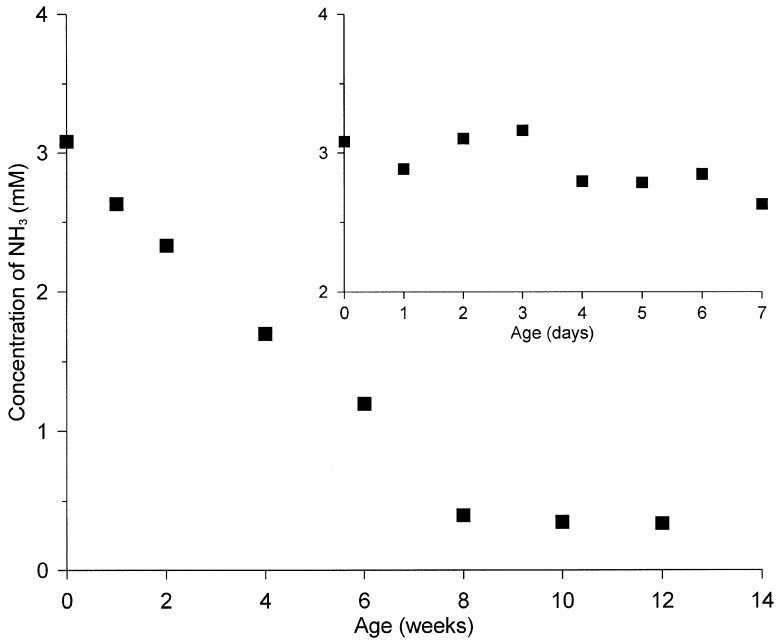

FIG. 1 The dependence of released ammonia in the fifteenth minute of the reaction in the urea–urease–NBPT/NBPTO system vs ageing time of NBPT solution.

Morrison and Walsh.[23] Two mechanisms have been assumed for the analysis of slow-binding inhibition. Mechanism A is illustrated by Eq. (1):

$$E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_7} E + P$$

$$\underbrace{E + I \underset{k_4}{\overset{k_3}{\Leftrightarrow}} EI^*}_{\text{slow}} \tag{1}$$

Inhibitor I and substrate S compete for the active-site of enzyme E. The velocity of EI^* complex formation is slow compared with the velocity of product P formation. The inhibition constant is equal to the dissociation constant of EI^* complex.

Mechanism B is described by Eq. (2):

$$E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_7} E + P$$

$$E + I \underbrace{\underset{k_4}{\overset{k_3}{\Leftrightarrow}} EI \underset{k_6}{\overset{k_5}{\Leftrightarrow}} EI^*}_{\text{slow}} \tag{2}$$

The step that determines the velocity of formation of the final complex EI^* is the isomerization of the EI complex into more stable form EI^*. Two inhibition constants, K_i and K_i^* characterises the inhibition. K_i is the dissociation constant of the initial complex EI. K_i^* is the dissociation constant of the final complex EI^*.

For the condition when the inhibitor concentration is much higher than that of the enzyme, the integrated Eq. (3) describes product concentration in the presence of slow-binding inhibitor which interacts with enzyme according to mechanism A or B:

$$P = v_s t + (v_o - v_s)(1 - e^{-k_{app} t})/k_{app} \tag{3}$$

where P is the amount of product accumulated at time t after initiation of the reaction, v_o and v_s are the initial and steady-state velocities, respectively. k_{app} is the apparent first-order velocity constant for the establishment of equilibrium between EI and EI^*. The initial and steady-state velocities are given by the following equations:

$$v_o = \frac{v_{max} S_o}{K_M(1 + I/K_i) + S_o} \tag{4}$$

$$v_s = \frac{v_{max} S_o}{K_M(1 + I/K_i^*) + S_o} \tag{5}$$

A curve-fitting programme (Cleland, W.W., BURSTO computer programme) was used to fit the experimental data to Eq. (3). The best fitting curves were found by calculation of the set of parameters (v_o, v_s, k_{app}) for each concentration of the inhibitor. It has been possible to distinguish between mechanism A and B by analysis of the reaction progress curves for different concentrations of inhibitor. The following relationships characterise the mechanism A: v_o is independent of I while $1/v_s$ is a linear function of I and $1/k_{app}$ is a linear function of $1/I$ with zero Y-intercept. The inhibition followed by mechanism B results in linear functions: $1/v_s$ vs I and $1/v_o$ vs I while k_{app} vs I is a hyperbolic function. The linear double reciprocal plot $1/k_{app}$ vs $1/I$ has a nonzero Y-intercept.

For the mechanism A the following equations were used to calculate the association (k_3) and dissociation (k_4) velocity constants:

$$k_4 = k_{app} v_s / v_o \tag{6}$$

$$k_{app} = k_4 + \frac{k_3 I}{1 + S_o/K_M} \tag{7}$$

The ratio k_4 and k_3 gives the inhibition constant:

$$K_i^* = k_4/k_3 \tag{8}$$

RESULTS

Analysis of Reaction Progress Curves, Calculation of the Kinetic Constants and Inhibition Constants

The results of the analytical studies and the curves generated by the curve-fitting programme

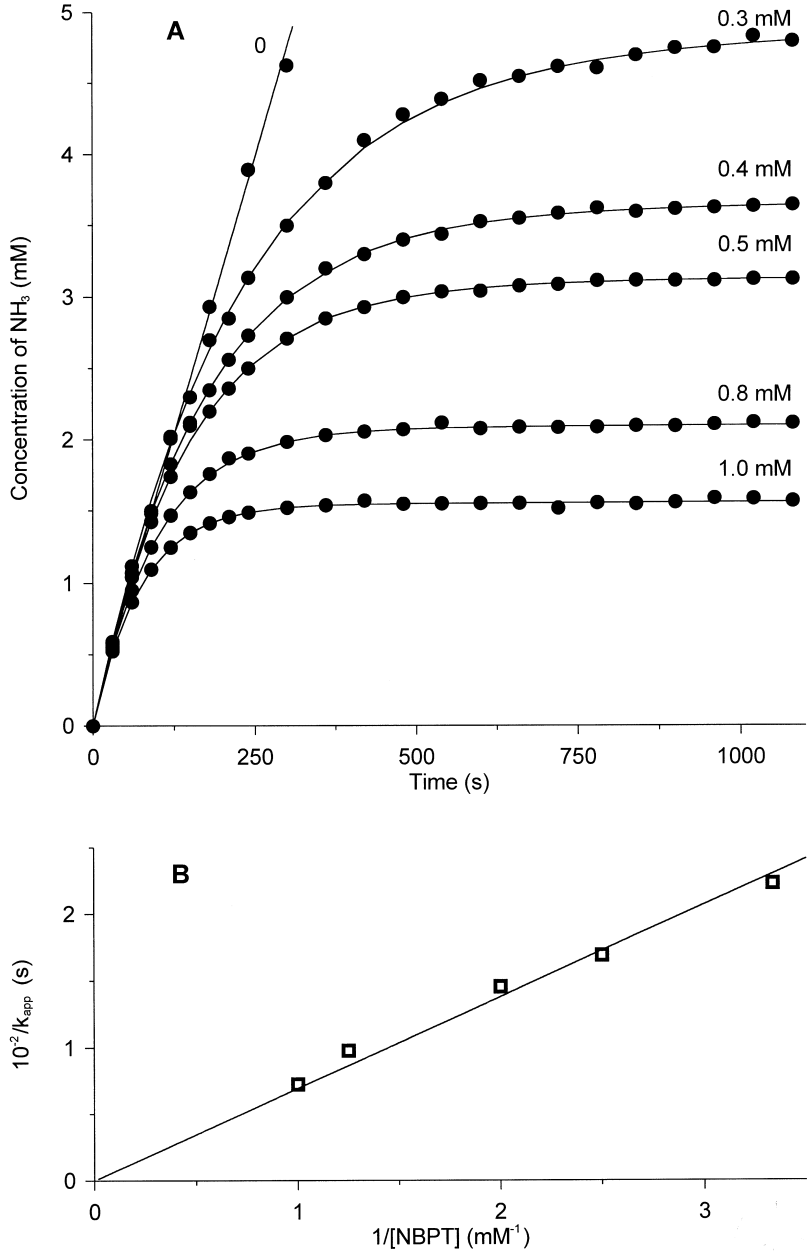

FIG. 2 (A) Reaction progress curves of urease-catalyzed hydrolysis of urea in the presence of NBPT. NBPT concentration [mM] is numerically given. (B) The double reciprocal plot of dependence of apparent velocity constant in the presence of different NBPT concentrations.

are shown in Fig. 2A for NBPT and in Fig. 3A for NBPTO. The curves for both inhibitors show the time-dependent character characteristic of the slow-binding inhibition. The velocity of urea hydrolysis decreased from an initial velocity (v_o) to a much slower steady-state velocity (v_s) according to the apparent first-order velocity constant k_{app} (Eq. 3). It was observed that v_o did not vary with the inhibitors concentration for examined ranges. The initial velocities for NBPT

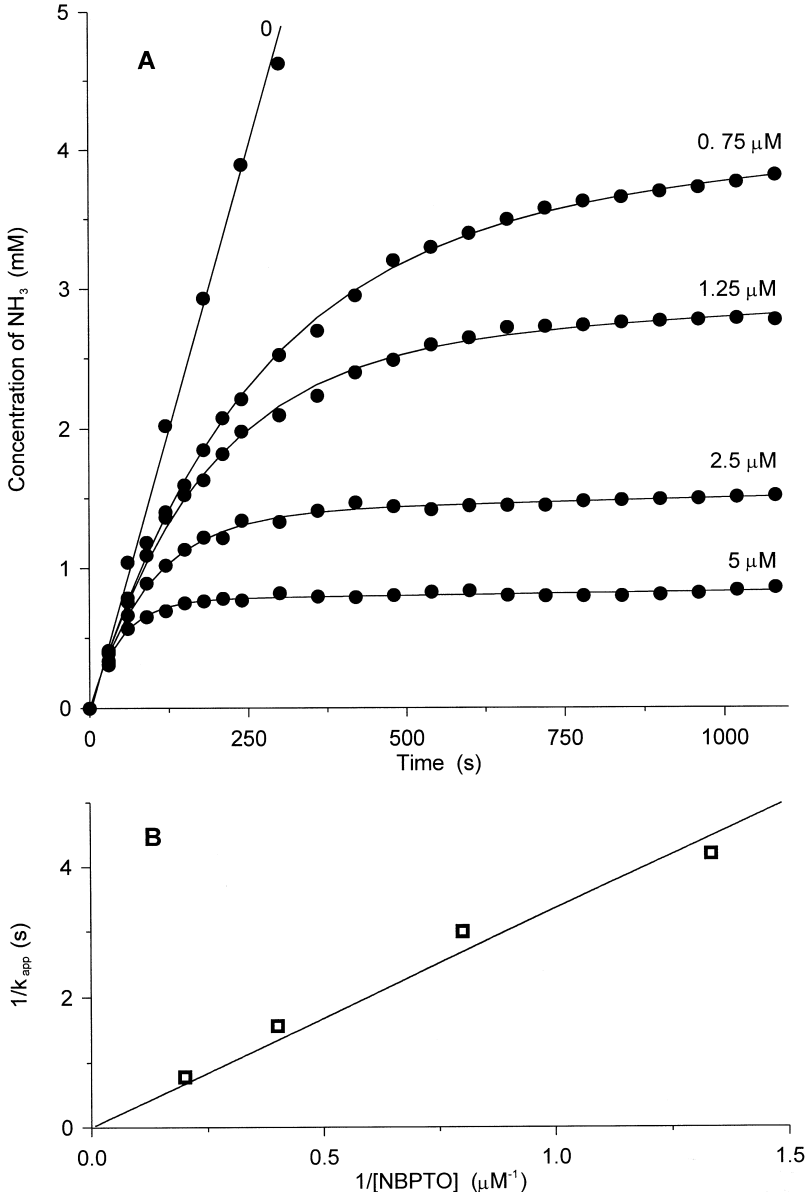

FIG. 3 (A) Reaction progress curves of urease-catalyzed hydrolysis of urea in the presence of NBPTO. NBPTO concentration [μM] is numerically given. (B) The double reciprocal plot of dependence of apparent velocity constant in the presence of different NBPTO concentration.

and NBPTO were equal to 0.021 ± 0.002 and $0.015 \pm 0.001\,\mathrm{mM\,s^{-1}}$, respectively. This observation proved that there was no significant accumulation of the *EI* complex. In this case it is considered that slow-binding arises not because of the isomerization of the *EI* complex but because of the slow interaction between enzyme and inhibitor. The plots of k_{app} vs I (not shown) and $1/k_{app}$ vs $1/I$ shown in Fig. 2B and Fig. 3B are linear. Moreover, the double reciprocal plots pass through the origins. According to the Morrison-Walsh theory this case occurs when the velocity of dissociation constant (k_4) of the enzyme-inhibitor complex is very low and

becomes insignificant. All relationships found were consistent with mechanism A for the processes of binding NBPT and NBPTO to urease. The sets of parameters (v_o, v_s, k_{app}) generated for each concentration of NBPT and NBPTO were used for calculating the individual reaction velocity constants k_4 (Eq. 6). Obtained average values of k_4 were equal to $(0.17 \pm 0.03) \times 10^{-4}\,s^{-1}$ for NBPT and $(0.82 \pm 0.05) \times 10^{-4}\,s^{-1}$ for NBPTO. Fig. 2B and Fig. 3B illustrate the relationships $1/k_{app}$ vs $1/I$ approximated with the linear functions. These functions refer to the transformed form of the equation for apparent first-order velocity constant for mechanism A:

$$1/k_{app} = \frac{1 + S_o/K_M}{k_3}\frac{1}{I} \qquad (9)$$

Linear regression analysis provided an accurate estimation of $(1+S_o/K_M)/k_3$. The values of k_3 were calculated using S_o=50 mM, K_M=7.40 mM and were equal to 0.11 ± 0.01 $(mM\,s)^{-1}$ and 38.6 ± 2.4 $(mM\,s)^{-1}$ for NBPT and NBPTO, respectively. The ratio k_4 and k_3 gives the inhibition constant. The inhibition constant of urease by NBPT was equal to K_i^*=0.15 ± 0.03 μM. The inhibition constant of NBPTO was two orders lower and equal to K_i^*=2.1 ± 0.2 nM.

Calculation of the Inhibition Constant of NBPT by Steady-state Analysis

The steady-state curves for the reaction initiated by the addition of urea after a 20 min incubation of NBPT with urease are presented in Fig. 4A. The incubation resulted in the equilibrium between enzyme, inhibitor and enzyme-inhibitor complex. The reaction achieved the steady-state velocity (v_s) and curves became linear. Equation (5) can be rearranged in the following form, where the reciprocal of v_s is a linear function of the inhibitor concentration:

$$\frac{1}{v_s} = \frac{1}{v_{max}}\left(1 + \frac{K_M}{S_o}\right) + I\frac{K_M}{S_o v_{max} K_i^*} \qquad (10)$$

Figure 4B presents the dependence of $1/v_s$ vs I. The value of the slope, given by linear regression analysis, was used for calculating the inhibition constant. The substitution of the kinetic constants and the initial concentration of urea gave the inhibition constant K_i^* equal to 0.13 ± 0.02 μM. This value is in good agreement with that determinated by the analysis of reaction progress curves.

DISCUSSION

NBPT has been extensively studied as an inhibitor of soil urease. In soil NBPT is quickly converted into NBPTO which is a much stronger inhibitor than NBPT. NBPTO was isolated and identified in soil after application of NBPT.[19,24] Factors that affect the rate of NBPT conversion into its oxygen analogue have not been elucidated so far. Keerthisinghe *et al.*[19] tested the inhibition influence of NBPT on jack bean urease. NBPT has been classified as an inhibitor with a minimum inhibitory activity. Our research provides the evidence that this activity is noticeable and cannot be neglected. Interestingly, under the laboratory controlled conditions the change of inhibitory effectiveness of NBPT remains insignificant within the first few days (Fig. 1). This means that conversion of NBPT under laboratory conditions occurs much slower than in soil. The plots of the reaction progress curves of urease-catalyzed hydrolysis of urea in the presence of NBPT and its oxygen analogue NBPTO are time-dependent and characteristic of the slow-binding inhibition. The effects of different concentrations of NBPT and NBPTO on the initial and steady-state velocities and the apparent first-order velocity constants obey the equations for a one-step enzyme-inhibitor

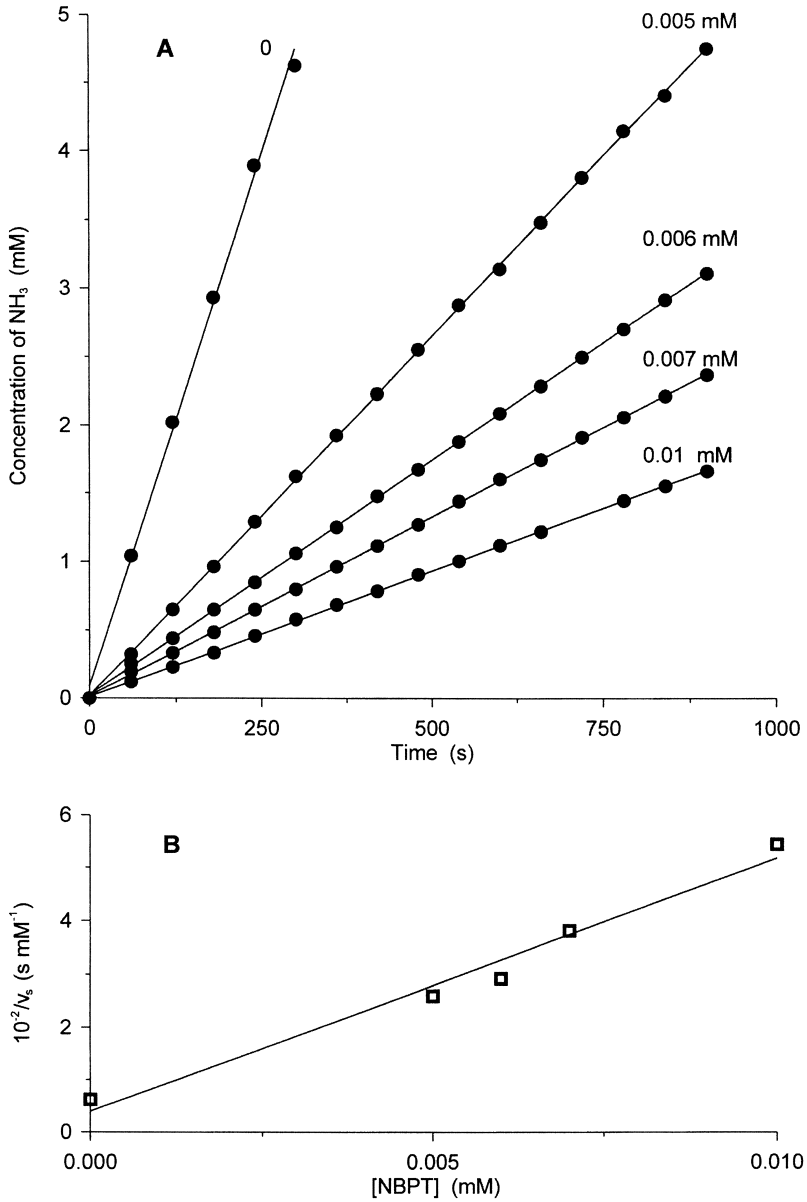

FIG. 4 Incubated urea–urease–NBPT system: (A) Concentration of ammonia vs time. NBPT concentration [mM] is numerically given. (B) The reciprocal plot of dependence of steady-state velocity in the presence of different NBPT concentration.

interaction, qualified as mechanism A (Eq. 1). Calculated association (k_3) and dissociation (k_4) velocity constants for the formation of urease-NBPT and urease-NBPTO complexes are presented in Table I. For both inhibitors, the values of k_4 are several orders lower than k_3. This indicates that the urease-inhibitor intermediate complex forms very fast and slowly dissociates into intact enzyme and inhibitor. The effect is a long equilibration time between enzyme, inhibitor and urease-inhibitor complex. As a result of the establishment of the equilibrium there is steady-state velocity of the reaction. This fact explains the time-dependent character of the

TABLE 1 Kinetic and inhibition constants of jack bean urease by NBPT and NBPTO (pH 7.0, 25°C)

Inhibitor	Method	K_i* (μM)	k_3 ($mM^{-1} s^{-1}$)	k_4 (s^{-1})
NBPT	Progress curves studies	0.15 ± 0.03	0.11 ± 0.01	$(0.17 \pm 0.03) \times 10^{-4}$
NBPT	Steady-state kinetic studies	0.13 ± 0.02		
NBPTO	Progress curves studies	$(2.1 \pm 0.2) \times 10^{-3}$	38.6 ± 2.4	$(0.82 \pm 0.05) \times 10^{-4}$

progress curves. It is well known that the bond angles and lengths of the amide groups of the phosphoric di- and triamides are similar to those found in urea. Apart from their structural similarity to urea, they are not substrates of urease. According to Andrews *et al*.[16] all phosphorictriamides react with urease to produce a diamidophosphate–urease complex following Eq. (11):

$$\begin{aligned} &E + R{-}NH{-}PO{-}(NH_2)_2 \\ &\rightarrow E{\cdot}R{-}NH{-}PO{-}(NH_2)_2 + OH^- \\ &\rightarrow E{\cdot}(NH_2)_2PO_2^- + R{-}NH_2 \end{aligned} \quad (11)$$

The reaction is a two stage process. In the first stage the molecule of phosphorictriamide binds with urease and the hydroxyl group is removed from the urease active site. In the second stage the urease–phosphorictriamide intermediate converts into urease–diamidophosphate complex and a molecule of amine is released. The size and the type of the R-group has a significant influence on the inhibition.[20] One of the strongest inhibitors of urease is phenylphosphorodiamidate (PPD). Kinetics of PPD were thoroughly studied by McCarty *et al*.[9] and Todd *et al*.[15] It was found that PPD is a slow, tight-binding inhibitor of jack bean and microbial urease. Dixon *et al*.[17] proved that diamidophosphate binds to the active-site nickel ions. An analogous model has been suggested for PPD by Todd *et al*.[15]

Our studies prove that NBPT is a much weaker inhibitor of urease than PPD but stronger than acetohydroxamic acid and sodium fluoride (Table II). In contrast, the inhibitory activity of NBPTO is very high, compared to PPD. The geometry of the thiophosphorictriamide group of NBPT and the phosphorictriamide group of NBPTO reflect the geometry of urea. It is quite probable that both of them create the tetrahedral intermediate complex, roughly similar to that which has been suggested as formed upon urea hydrolysis. The structure of that intermediate might correspond to the enzyme–phosphorictriamide complex (Eq. 11). By analogy to the postulated mechanism for all phosphorictriamides, complex urease–NBPTO may convert into a urease-$(NH_2)_2PO_2^-$ complex. Observed mechanism A indicates that conversion is a very fast process. NBPT is not a simple analogue of phosphorictriamides and additionally is a sulphur analogue so that Eq. (11) need not be directly followed. It seems likely that NBPT creates a urease–NBPT complex which does not undergo further change. The big difference in inhibitory activity between NBPT and NBPTO may be a result of a different type of final urease–inhibitor complex. The inhibition of urease by NBPTO may lead to a stable urease-$(NH_2)_2PO_2^-$ complex (the direct analogue of urease–urea intermediate) whereas in contrast the urease–NBPT complex may be the final complex of the inhibition.

TABLE 2 Inhibition constants of jack bean urease by slow-binding inhibitors

Inhibitor	K_i (mM)	K_i^* (mM)	Ref.
PPD	–	1.6×10^{-7}	9
NBPT	–	1.5×10^{-4}	This paper
NBPTO	–	2.1×10^{-6}	This paper
Acetohydroxamic acid	1.3	0.034	25
Sodium fluoride	1.04	0.03	26

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