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Inhibition of Jack Bean Urease by 1,4-benzoquinone and 2,5-dimethyl-1,4-benzoquinone. Evaluation of the Inhibition Mechanism

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1,4-benzoquinone (BQ) and 2,5-dimethyl-1,4-benzoquinone (DMBQ) were studied as inhibitors of jack bean urease in 50 mM phosphate buffer, pH 7.0. The mechanisms of inhibition were evaluated by progress curves studies and steady-state approach to data achieved by preincubation of the enzyme with the inhibitor. The obtained reaction progress curves were time-dependent and characteristic of slow-binding inhibition. The effects of different concentrations of BQ and DMBQ on the initial and steady-state velocities as well as the apparent first-order velocity constants obeyed the relationships of two-step enzyme-inhibitor interaction, qualified as mechanism B. The rapid formation of an initial BQ–urease complex with an inhibition constant of $K_i = 0.031$ mM was followed by a slow isomerization into the final BQ–urease complex with the overall inhibition constant of $K_i^* = 4.5 \times 10^{-5}$ mM. The respective inhibition constants for DMBQ were $K_i = 0.42$ mM, $K_i^* = 1.2 \times 10^{-3}$ mM. The rate constants of the inhibitor-urease isomerization indicated that forward processes were rapid in contrast to slow reverse reactions. The overall inhibition constants obtained by the steady-state analysis were found to be 5.1×10^{-5} mM for BQ and 0.98×10^{-3} mM for DMBQ. BQ was found to be a much stronger inhibitor of urease than DMBQ. A test, based on reaction with L-cysteine, confirmed the essential role of the sulfhydryl group in the inhibition of urease by BQ and DMBQ.

Keywords: Urease; Inhibition; Kinetic constants; 1,4-Benzoquinone; 2,5-Dimethyl-1,4-benzoquinone

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.5) is a well-known nickel metalloenzyme that catalyzes the hydrolysis of urea: $CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2$. Urease is widely distributed in plants and microorganisms. Ureases isolated from different sources have highly similar amino acid sequences although they may be comprised of one, two or three distinct polypeptide chains. On the basis of the similarities in the sequence and reaction kinetics it has been assumed that known ureases have a common structure for the active site and the same catalytic mechanism. The enzyme metallocenter contains two nickel ions, which are ~3.5 Å apart and liganded by three and four protein atoms, respectively. This metallocenter is directly involved in binding of substrates and inhibitors. The supporting role in the catalytic mechanism is played by the cysteine residue in the side chain. The modification of this essential cysteine by chemical reagents blocks the enzymatic activity of urease. The knowledge of the properties of urease is largely based on the extensive studies of jack bean urease.[1–5]

The primary environmental role of urease is to allow the organism to use urea as the nitrogen source and moreover, urease participates in systemic nitrogen transport pathways in plants. The common use of urea as nitrogen fertilizer involves problems associated with the activity of urease in soil.[6–8] The simplest approach to reduce these negative effects is the application of inhibitors. Bremner and Douglas have evaluated more than 100 compounds as inhibitors of soil urease, including substituted *p*-benzoquinones. They provided evidence that the effectiveness of *p*-benzoquinones depends upon their substituted groups.[9,10] Bundy and Bremner reported 1,4-benzoquinone as the most promising

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inhibitor of soil urease. The methyl-, chloro-, bromo- and fluoro-substituted *p*-benzoquinones have a marked inhibitory effect, whereas phenyl-, t-butyl- and hydroxy-substituted *p*-benzoquinones have only little, if any effect. Methyl-substituted *p*-benzoquinones differ from the others as their inhibitory effect usually increases markedly with the increase in time of their contact with soil.

Studies of the effectiveness of agriculturally applicable urease inhibitors were mainly carried out with the native urease present in soil. This specific kind of urease is more stable and protected by organic soil constituents against inactivation or decomposition processes. Several workers have suggested that the resistance of the soil urease arises through immobilization of the enzyme with organic colloids during humus formation. Unfortunately, it is difficult to account for the remarkable stability of urease in heterogeneous environments such as soils that is a necessary condition for obtaining reliable kinetic data.[8,11] Most probably this is the reason for the lack of kinetic data regarding the inhibition of urease by benzoquinones and their derivatives.

The purpose of the present work was to study the inhibitory influence of 1,4-benzoquinone (BQ) and 2,5-dimethyl-1,4-benzoquinone (DMBQ) on jack bean urease. The kinetic parameters, inhibition constants and mechanisms of the inhibition were determined. The studies, based on reaction with L-cysteine, have elucidated the role of the urease active-site sulfhydryl group in the inhibition by BQ and DMBQ.

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. Urea (Molecular Biology Reagent), Hepes, L-cysteine and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma, and the inhibitors, BQ and DMBQ from Fluka Chemika. 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 50 mM phosphate buffer, pH 7.0, 2 mM EDTA at 25°C. The initial concentration of urea was 50 mM and the final concentration of urease was 0.05 mg cm^{-3} in all studied systems. The reaction was studied in the absence and presence of BQ and DMBQ as inhibitors in two ways. In the first, the progress curves were obtained for the reactions initiated by addition of enzyme into the reaction mixtures containing different concentrations of BQ (0.01, 0.015, 0.02, 0.03, 0.05, 0.07, 0.12 mM) and DMBQ (0.17, 0.34, 0.68, 1.35, 2.0 mM). In the steady-state analysis the initiation of the enzymatic reaction followed a 20 min preincubation of the enzyme with the inhibitor (the equilibrium between enzyme E, inhibitor I and enzyme-inhibitor complexes EI and EI* : $E + I \Leftrightarrow EI \Leftrightarrow EI^*$). The reaction was initiated by addition of a concentrated solution of urea (5 M) into the reaction mixtures containing different concentrations of BQ (0.001, 0.002, 0.003, 0.004 mM) and DMBQ (0.017, 0.034, 0.085 mM). 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.[12] The absorbance was measured at 625 nm. The effect of BQ and DMBQ on the phenol-hypochlorite method was tested and no interference was seen.

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 different urea concentrations in the range of 2–50 mM. The values obtained by applying nonlinear regression to the Michaelis-Menten equation were: $K_M = 5.8 \pm 0.3$ mM and $v_{max} = 1.6 \pm 0.1$ mM min^{-1}.

Quantitative Determination of Inhibitor-Cysteine Interaction

Reactions of BQ (10, 7.5, 5, 2.5 mM) with 20 mM cysteine, and DMBQ (1, 0.75, 0.5, 0.25 mM) with 4 mM cysteine were carried out at 25°C in 50 mM Hepes buffer (pH 7.5). After 10 min, the residual concentration of cysteine in the mixture was determined by reaction with DTNB. Cysteine reacts with DTNB to produce 2-nitro-5-thiobenzoic acid (NTB), according to Equation (1):

$$HOOC{-}CH(NH_2)CH_2SH + X{-}S{-}S{-}X \rightarrow HOOC{-}CH(NH_2)CH_2S{-}S{-}X + HS{-}X \quad (1)$$

where $X = C_6H_3(NO_2)COOH$.

A sample (0.05 cm^3) of the reaction mixture was removed and incubated with 10 cm^3 of 50 mM Hepes buffer containing 0.15 mM DTNB. The concentration

of cysteine was determined indirectly by spectrophotometric measurement ($\lambda = 412$ nm) of liberated NTB.[13]

Data Analysis

To obtain kinetic parameters and inhibition constants for inhibitor–urease interaction, the method described for slow-binding inhibitors was used.[14,15] There are two basic mechanisms of slow-binding inhibition (A and B).

Mechanism A:

$$E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_7} E + P \qquad (2)$$

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

Mechanism B:

$$E + S \underset{k_2}{\overset{k_1}{\Leftrightarrow}} ES \xrightarrow{k_7} E + P \qquad (3)$$

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

The product concentration in the presence of slow-binding inhibitor, for mechanism A and B, if $[I_o] \gg [E_o]$ and $[P] \ll [S_o]$, is given by the general integrated Equation (4):

$$P = v_s t + (v_o - v_s)(1 - e^{-k_{app} t})/k_{app} \qquad (4)$$

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, and k_{app} is the apparent first-order velocity constant.

In mechanism A (Equation 2) the inhibitor and enzyme interaction results in the direct formation of the enzyme-inhibitor complex EI*. The inhibition constant is equal to the dissociation constant of the EI* complex.

Mechanism B (Equation 3) involves a two-step interaction. In the first step, a loosely bound enzyme–inhibitor complex EI is formed. In the next step, complex EI converts into the tightly bound final complex EI*. The evidence for accordance with the reaction mechanism (A or B) is given by the analysis of respective functions. In 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 a zero Y-intercept. The inhibition followed by mechanism B results in linear functions: $1/v_s$ vs I and $1/v_o$ vs I. The linear double reciprocal plot $1/k_{app}$ vs $1/I$ has a non-zero Y-intercept.

Linear dependencies of $1/v_o$ and $1/v_s$ on the inhibitor concentration are used to assess the inhibition constants, K_i and K_i^*:

$$\frac{1}{v_o} = \frac{K_M}{v_{max} S_o K_i} I + \frac{1}{v_{max}} \left(1 + \frac{K_M}{S_o}\right) \qquad (5)$$

$$\frac{1}{v_s} = \frac{K_M}{v_{max} S_o K_i^*} I + \frac{1}{v_{max}} \left(1 + \frac{K_M}{S_o}\right) \qquad (6)$$

The dependence of the apparent velocity constant on inhibitor concentration follows Equation (7):

$$k_{app} = k_6 + \frac{k_5 I}{K_i(1 + S_o/K_M) + I} \qquad (7)$$

If k_6 is neglectable as compared to the other term, then:

$$k_{app} \approx \frac{k_5 I}{K_i(1 + S_o/K_M) + I} \qquad (8)$$

A double reciprocal plot of $1/k_{app}$ vs $1/I$ yields a straight line that gives the value of the forward velocity constant k_5.

The ratio of k_5 and k_6 gives the relationship between K_i and K_i^*:

$$k_5/k_6 = K_i/K_i^* - 1 \qquad (9)$$

RESULTS AND DISCUSSION

Analysis of Reaction Progress Curves: Kinetic and Inhibition Constants

The results of the analytical studies and the curves generated by the curve-fitting programme (Cleland, W.W., BURSTO computer programme), according to Equation (4), are shown in Fig. 1A for BQ and in Fig. 2A for DMBQ. 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}. It was observed that for both studied inhibitors, v_o decreased with the inhibitor concentration over the examined ranges. This observation indicated that there was accumulation of the EI complex which undergoes transformation into the final EI* complex. The linear replots of $1/v_o$ and $1/v_s$ vs I, $1/k_{app}$ vs $1/I$ (Figs. 1 and 2, inserts B, C, D) proved that mechanism B is a good description of the urease inhibition by BQ and DMBQ. The values of the inhibition constants K_i and K_i^* were calculated from reciprocal dependence of v_o and v_s on the inhibitor concentration using Equations (5) and (6), respectively. The inhibition constants are listed in Table I.

The forward velocity constants (k_5) were calculated from equations provided by linear regression of a double reciprocal replots: $1/k_{app}$ vs $1/I$ (Figure 1D, Figure 2D) according to transformed Equation (8). The reverse velocity constants k_6 were obtained with

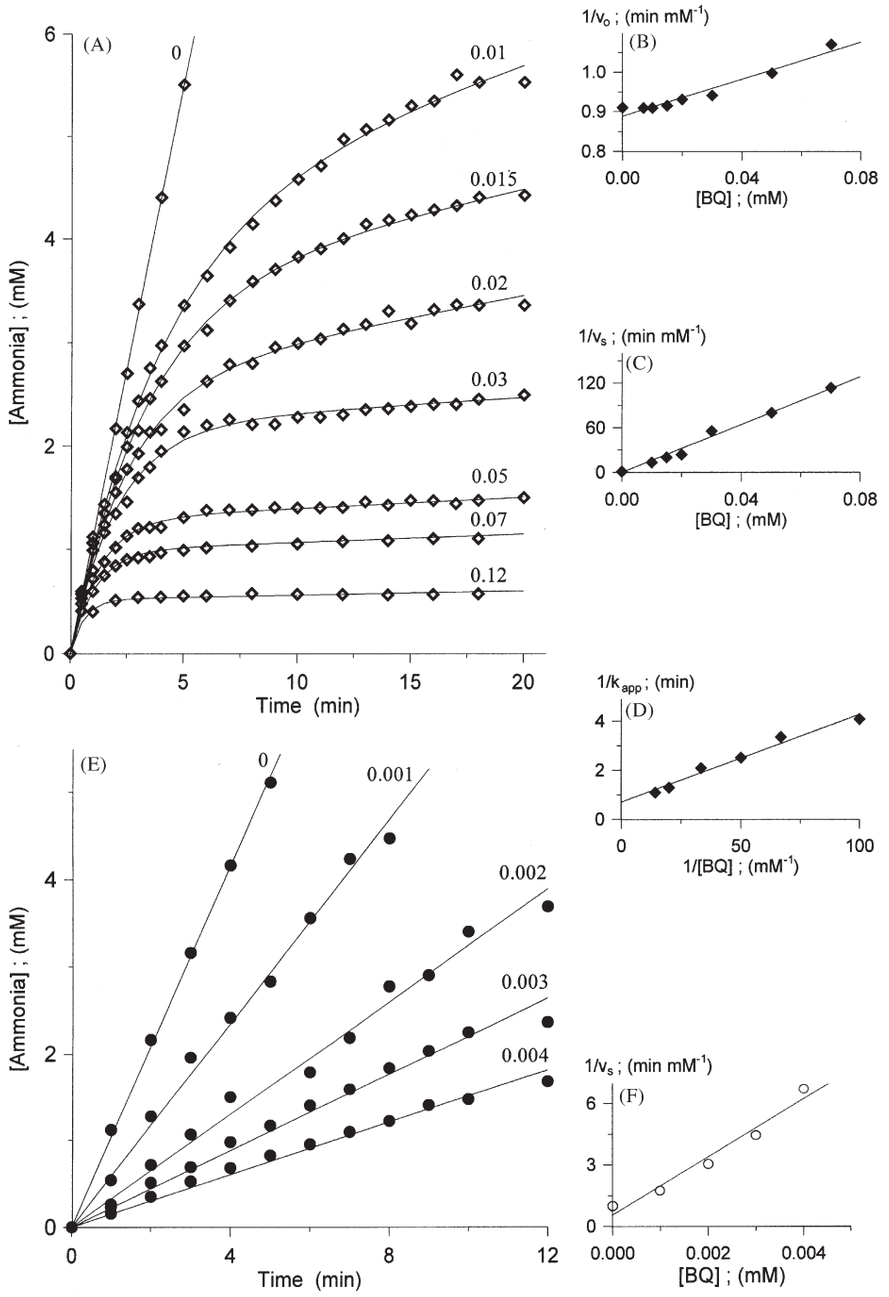


FIGURE 1 (A) Reaction progress curves of urease-catalyzed hydrolysis of urea in the presence of BQ. (B) The reciprocal plot of dependence of initial velocity vs BQ concentration. (C) The reciprocal plot of dependence of steady-state velocity vs BQ concentration. (D) The double reciprocal plot of dependence of apparent velocity constant vs BQ concentration. Steady-state analysis: (E) concentration of ammonia vs time. (F) The reciprocal plot of dependence of steady-state velocity vs BQ concentration. BQ concentration [mM] is numerically given.

the use of the inhibition constants, forward velocity constants (k_5) and Equation (9).

The ratio of EI and EI* complexes in the final steady-state conforms to the ratio of the forward and reverse velocity constants of the isomerization process (Equation 9). It was found that the amount of the final complex EI* was predominant in relation to the amount of the initial EI complex. The contribution of the EI* complex for BQ and DMBQ was over 99% while the remaining inhibitor was loosely bound in the EI complex. All kinetic constants and the other parameters are compiled in Table I.

Calculation of the Inhibition Constant by Steady-state Analysis

The steady-state curves for the reaction initiated by the addition of urea after a 20-minute preincubation

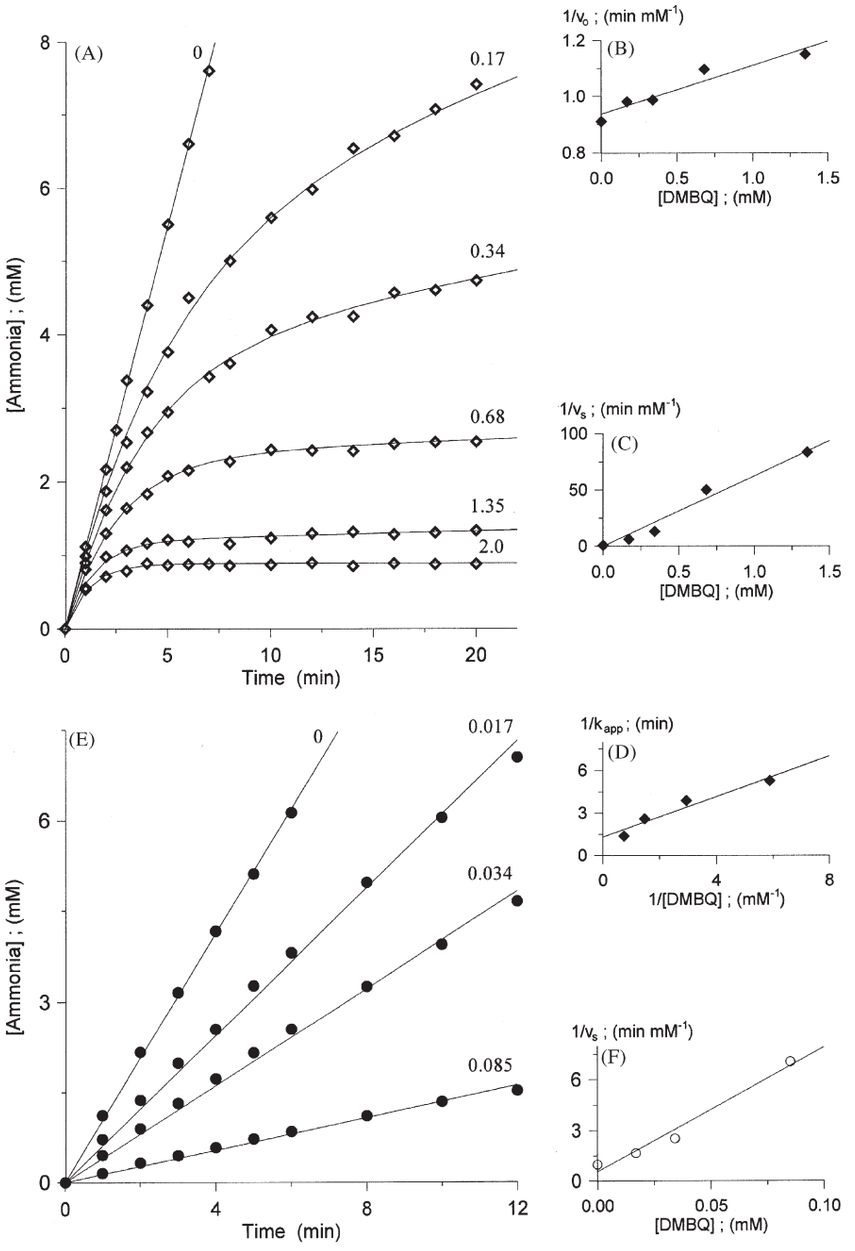


FIGURE 2 (A) Reaction progress curves of urease-catalyzed hydrolysis of urea in the presence of DMBQ. (B) The reciprocal plot of dependence of initial velocity vs DMBQ concentration. (C) The reciprocal plot of dependence of steady-state velocity vs DMBQ concentration. (D) The double reciprocal plot of dependence of apparent velocity constant vs DMBQ concentration. Steady-state analysis: (E) concentration of ammonia vs time. (F) The reciprocal plot of dependence of steady-state velocity vs DMBQ concentration. DMBQ concentration [mM] is numerically given.

of BQ and DMBQ with urease are presented in Figures 1E and 2E, respectively. The attainment of the equilibrium conditions for the mixture of urease and the inhibitor by preincubation produced the linear steady-state curves after initiation of the reaction. Figures 1F and 2F show the dependence of $1/v_s$ vs I, according to Equation (6). The values of the slope, provided by linear regression analysis, were used for calculation of the inhibition constants. The substitution of the kinetic constants and the initial concentration of urea gave the inhibition constants K_i^* listed in Table I. The inhibition constants provided by complementary methods, reaction progress curves and steady-state approach, are in good agreement.

Inhibitor–Cysteine Interaction

The experiments were conducted to monitor changes in the concentration of cysteine following incubation with varying concentration of BQ and DMBQ. In this method, the inhibitor was reacted first with an

TABLE I Kinetic and inhibition constants for jack bean urease with BQ and DMBQ, pH 7.0, 25°C

Inhibitor	Method of studies	K_i (mM)	K_i^* (mM)	k_5 (min^{-1})	k_6 (min^{-1})	$t_{1/2}$ (h)
BQ	Progress curves	0.031 ± 0.003	$(4.5 \pm 0.4) \times 10^{-5}$	1.4 ± 0.3	$(2.0 \pm 0.5) \times 10^{-3}$	5.8
	Steady-state	–	$(5.1 \pm 0.6) \times 10^{-5}$			
DMBQ	Progress curves	0.42 ± 0.08	$(1.2 \pm 0.1) \times 10^{-3}$	0.73 ± 0.02	$(2.1 \pm 0.5) \times 10^{-3}$	5.5
	Steady-state	–	$(0.98 \pm 0.10) \times 10^{-3}$			

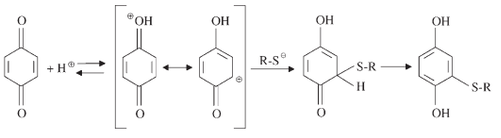
excess amount of cysteine. The residual cysteine created NTB as the result of the reaction with DTNB. The concentration of NTB was determined spectrophotometrically. The results are shown in Table II.

The concentration of cysteine which reacted with the inhibitor decreases with the decrease in the inhibitor concentration. The mean ratios of reacted cysteine to the concentration of BQ and DMBQ were found to be 0.98 and 1.0, respectively. These results indicate that BQ and DMBQ react with cysteine with a 1:1 stoichiometry.

DISCUSSION

BQ and DMBQ have been frequently mentioned as inhibitors of soil urease. Among tested quinones and their derivatives BQ has been found to be the most effective inhibitor. The inhibitory strength of DMBQ has been assessed as significant but less effective. Our research on BQ and DMBQ activities as jack bean urease inhibitors proved that BQ is two orders in magnitude stronger as an inhibitor than DMBQ. Both compounds showed the same mechanism of inhibition. The plots of the reaction progress curves in the presence of the inhibitor showed slow, time-dependent onset of enzyme inhibition. The analysis of the dependencies of the kinetic parameters on the concentration of the inhibitor indicated that the inhibition obeyed the two-step enzyme-inhibitor interaction, qualified as mechanism B. In the final steady-state complexes BQ-urease and DMBQ-urease were predominant (99%) as compared to the amount of the initial inhibitor-urease complexes (less than 1%). Apart from that, the replots $1/v_o$ vs concentration of the inhibitor proved that the presence of the initial urease-inhibitor complex was evident and detectable. The final forms of the BQ-urease and DMBQ-urease complexes were stable with half-lives of 5.8 and 5.5 h, respectively (Table I). A long lifetime enables experiments with such complexes. The calculated forward (k_5) and reverse (k_6) velocity constants were consistent with the values predicted for slow-binding inhibition. The high values of the forward velocity constant k_5 and low value of the reverse velocity constant indicated that the final urease-inhibitor complex was formed very fast and dissociated slowly into the initial enzyme-inhibitor complex. A long equilibration time resulted in the time-dependent character of the progress curves.

It is generally assumed that quinones are highly sulfhydryl-reactive compounds. The reaction follows the general mechanism of multistep nucleophilic substitution. The last step is an isomerization process into an aromatic product.



(10)

Ureases are thiol rich enzymes. Urease jack bean contains in total 15 cysteine residues per subunit. One of them, cysteine-592, is recognized to be essential for enzymatic activity and is placed in the urease active site.[16] The interaction of the inhibitor with that unique sulfhydryl group elucidates the inhibitory influence of BQ and DMBQ on urease.

TABLE II Reaction of L-cysteine with BQ and DMBQ in 50 mM Hepes, pH 7.5, 25°C

Initial concentration (mM)		Residual cysteine (mM)	Reacted cysteine (mM)	Ratio of reacted cysteine (mM) to inhibitor (mM)
Cysteine	BQ			
20	10	10.4	9.6	0.96
20	7.5	12.9	7.1	0.95
20	5	14.8	5.2	1
20	2.5	17.5	2.5	1
Cysteine	DMBQ			
4	1	3.04	0.96	0.96
4	0.75	3.25	0.75	1
4	0.5	3.45	0.55	1.1
4	0.25	3.72	0.28	1.1

The mechanism of the reaction of quinone and thiol bound to protein might be much more complicated than with free thiol. A quantitative test proved that the stoichiometry of the reaction of BQ and DMBQ with free cysteine was 1:1. The same stoichiometry is suggested for the reaction with the urease sulfhydryl group(s).

Quinones and hydroquinones in solutions create reversible redox system. The oxidizing potential of quinone depends on the substituted group. The groups that enrich an aromatic ring with electrons, e.g. methyl group, decrease the oxidizing ability of the quinone. On the other hand, groups that withdraw electrons from an aromatic ring increase the oxidizing potential of quinones. This effect elucidates the difference in the inhibition strength between BQ and DMBQ.

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