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Inhibition of jack bean urease by tetrachloro-*o*-benzoquinone and tetrachloro-*p*-benzoquinone

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Abstract

Tetrachloro-*o*-benzoquinone (TCoBQ) and tetrachloro-*p*-benzoquinone (TCpBQ) were studied as inhibitors of jack bean urease in 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, 25°C. The mechanisms of inhibition were evaluated by analysis of the progress curves obtained with two procedures: the reaction initiated by addition of the enzyme and the reaction initiated by addition of the substrate after preincubation of the enzyme with the inhibitor. The obtained results were characteristic of slow-binding inhibition. The effects of different inhibitor concentrations on the initial and steady-state velocities obeyed the relationships of two-step enzyme-inhibitor interaction, qualified as mechanism B. It was found that TCoBQ and TCpBQ are strong urease inhibitors. TCpBQ is more effective than TCoBQ with the overall inhibition constant of $K_i^\star = 4.5 \times 10^{-7}$ mM. The respective inhibition constant of TCoBQ was equal to: $K_i^\star = 2.4 \times 10^{-6}$ mM. The protective experiment proved that the urease active site is involved in the tetrachlorobenzoquinone inhibition process. High effectiveness of thiol protectors against inhibition by TCoBQ and TCpBQ indicates the strategic role of the active site sulfhydryl group in the blocking process. The stability of the complexes: urease-TCoBQ and urease-TCpBQ was tested in two ways: by dilution or addition of dithiothreitol. No recovery of urease activity bound in the urease-inhibitor complexes proves that the complexes are stable and strong.

Keywords: *Urease, inhibition, tetrachloro-*o*-benzoquinone, tetrachloro-*p*-benzoquinone, slow-binding*

Introduction

Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel metalloenzyme that catalyzes the hydrolysis of urea: $CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2$. Many organisms, plants, some bacteria, fungi and invertebrates, are able to synthesize urease. The best-characterised urease is that from jack bean. Jack bean urease exists as a homotrimer able to aggregate to a homohexamer. Each catalytic subunit contains the active site with two nickel ions. 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 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. On the other hand, the hydrolysis of urea causes production of ammonia and a pH increase, the major reason for the negative effects of the urease action on human and animal health, as well the environment [1–5]. The approach to reducing these problems is to find compounds that can inhibit urease. Many inhibitors have been searched for among various organic and inorganic compounds. A specific group of tested chemicals were the quinones, which are compounds of wide occurrence in nature where they serve as biological oxidation-reduction reagents. They are known for their bacteriostatic and fungicidal action as well as an inhibitory influence on certain enzymes, such as carboxylase and urease [6–9]. The effectiveness of substituted quinones as inhibitors, largely depends upon their substituent group. Halogen derivatives are usually effective urease inhibitors. Bundy and Bremner [8] found that chloro-, bromo- and fluoro-substituted p-benzoquinones have a marked inhibitory effect on soil urease activity. Lukens [6] reported that fungitoxicity increases with change in the quinone on halogenation and with the halogen

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according to the following order I < Br < Cl [6]. Chloranil (tetrachloro-*p*-benzoquinone) and dichlone (2,3-dichloro-*p*-naphthoquinone) are fungicides most frequently used.

Alternatively, quinones are applied for determination of the total proteins in different media or for selective determination of cysteine. The results of the reaction between quinones and amines, amino acids or proteins has been reported as a formation of a charge transfer complex or substituted quinone compound [10–11].

The present work was aimed at studying the inhibitory influence of tetrachloro-*o*-benzoquinone (TCoBQ) and tetrachloro-*p*-benzoquinone (TCpBQ) on jack bean urease. The inhibition was studied by monitoring reaction progress curves obtained with two techniques: the reaction initiated by addition of the enzyme and by addition of the substrate after preincubation of urease with the inhibitor (the preincubation technique). Moreover, thiols and inorganic inhibitors of urease were examined as protectors against the inhibition of urease. The stability of the urease-inhibitor complex was tested by dilution and by the use of dithiothreitol. The studies, based on the reaction with thiols have elucidated the role of the urease active-site sulfhydryl group in the inhibition by TCoBQ and TCpBQ.

Materials

The jack bean urease, Sigma type III of specific activity 16 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), L,D-dithiothreitol (DTT), glutathione (Glu), L-cysteine (L-cys), 2-mercaptoethanol (2-ME) were purchased from Sigma and the inhibitors, tetrachloro-*o*-benzoquinone (TCoBQ), and tetrachloro-*p*-benzoquinone (TCpBQ), from Aldrich. Other chemicals were obtained from POCh, Gliwice, Poland. All reagents used were of analytical grade. Since TCoBQ in aqueous solution is unstable, TCoBQ solution was freshly prepared for each experiment.

Methods

Ammonia determination

The hydrolysis of urea catalyzed by jack bean urease was monitored by measuring the ammonia concentration. The amount of ammonia was determined by the spectrophotometric, phenol-hypochlorite method and absorbance was measured at 630 nm [12].

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: $K_M = 2.74 \pm 0.15$ mM and $v_{max} = 0.13 \pm 0.01$ mM min^{-1}.

Residual activity measurement

A concentrated solution of urease was preincubated with a concentrated solution of inhibitor in the absence of substrate.

The preincubation solution contained 0.75 mg cm^{-3} of urease, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA and the inhibitor: TCoBQ – 7.5, 15 μM; TCpBQ – 0.29, 0.59 μM, respectively. The time when the enzyme and the inhibitor were mixed was marked as the zero time of preincubation. After appropriate periods of time, aliquots were withdrawn from the preincubation solution and diluted 50-fold into the reaction mixtures (50 mM urea, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA). After 5 min a sample of the reaction mixture was withdrawn and the amount of ammonia was determined.

The amount of ammonia released in the reaction mixture for 5 min after addition of uninhibited urease was taken as a control activity of 100%.

Reaction progress curves monitoring

The hydrolysis of urea catalyzed by jack bean urease was studied in 20 mM phosphate buffer, pH 7.0, 1 mM EDTA at 25°C. The initial concentration of urea in the reaction mixture was 50 mM and the concentration of urease was 0.015 mg cm^{-3} in all studied systems. The reaction was studied in the absence and presence of TCoBQ and TCpBQ as the inhibitors using two procedures.

(1) The progress curves were determined for the reactions initiated by the addition of the enzyme into the reaction mixtures containing different concentrations of TCpBQ (0.04, 0.24, 0.16, 0.12, 0.08 μM) and TCoBQ (0.5, 0.6, 0.75, 1.0 μM), respectively.
(2) In the steady-state analysis, the initiation of the enzymatic reaction followed a 15 min preincubation of the enzyme with the inhibitor. The reaction was initiated by the addition of a concentrated urea solution into the reaction preincubation mixtures containing different concentrations of TCpBQ (0.24, 0.12, 0.08 μM) and TCoBQ (0.5, 0.6, 0.75 μM), respectively.

In both procedures used for monitoring reaction progress curves, a sample of the reaction mixture was removed after an appropriate reaction time and the amount of ammonia was determined by the phenol-hypochlorite spectrophotometric method.

Protective experiment

In the protective experiment, all the preincubation mixtures contained 0.75 mg cm^{-3} urease, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, the inhibitor and the protector.

Conditions of the protective experiment (preincubation time, protector concentration) were chosen according to the inhibitory potency of the inhibitor.

(1) The TCoBQ preincubation mixture contained: 0.025 mM TCoBQ and the respective protector: 5 mM L-cysteine, 5 mM dithiothreitol, 5 mM glutathione, 5 mM 2-mercaptoethanol, 2.5 mM sodium fluoride, 5 mM boric acid. The mixture was incubated for 5 min.
(2) The TCpBQ preincubation mixture contained: 0.59 μM TCpBQ and the respective protector: 0.125 mM L-cysteine, 0.125 mM dithiothreitol, 0.125 mM glutathione, 0.125 mM 2-mercaptoethanol, 0.125 mM sodium fluoride, 0.125 mM boric acid. The mixture was preincubated for 30 min.

After preincubation a sample of the preincubation mixture was withdrawn and diluted 50-fold into the reaction mixture (50 mM urea, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA). The amount of ammonia released for 5 min was determined by the phenol-hypochlorite method.

Reactivation of inhibited urease

The reactivation of inhibited urease was studied in two ways, using DTT and by dilution of the reaction mixture containing urea.

(1) The preincubation mixture contained 0.75 mg cm^{-3} urease, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, 15 μM TCoBQ or 0.59 μM TCpBQ, respectively. After a 10 min preincubation DTT was added (DTT concentration in the preincubation mixture was equal to 5 mM). The activity of urease was determined before and after the addition of DTT. A sample of the preincubation mixture was withdrawn and diluted 50-fold into the reaction mixture: 50 mM urea, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA. After 5 min the amount of ammonia was measured.
(2) The sample of preincubation mixture (0.75 mg cm^{-3} urease, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA, 15 μM TCoBQ and 0.59 μM TCpBQ, respectively), after a 10 min preincubation, was diluted 50-fold into the reaction mixture: 50 mM urea, 20 mM phosphate buffer, pH 7.0, 1 mM EDTA.

After appropriate periods of time, aliquots were withdrawn and the amount of ammonia was determined.

Results and discussion

Residual activity measurement

The obtained results of residual activity measurement versus preincubation time are presented in Figure 1. Urease was preincubated with 0.29, 0.59 μM TCpBQ and 7.5, 15 μM TCoBQ, respectively. The ranges of the used concentrations differed about 25-fold which indicated the different potency of the inhibitors. TCpBQ was found to be a much more effective inhibitor of urease than TCoBQ as was clear from comparing the curves: 0.59 μM TCpBQ and 15 μM TCoBQ. For these concentrations, the residual activities of urease, after a 30 minute preincubation, were approximately equal. Moreover, the residual activity curves characterised the same type of inhibition. It was seen that increasing the of preincubation results in a decrease of urease activity. Initially, the loss of activity was quick until the achievement of the equilibrium between urease E, the inhibitor I and complexes urease-inhibitor EI and EI*: $E + I \Leftrightarrow EI \Leftrightarrow EI^{*}$. The equilibrium denoted is the

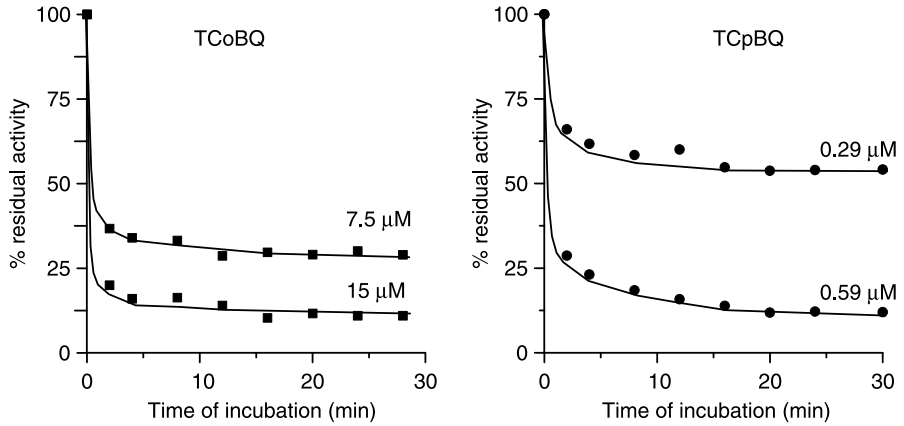

Figure 1. Dependence of residual activity of urease *vs* incubation time with 7.5, 15 μM TCoBQ and 0.29, 0.59 μM TCpBQ.

constant activity of urease. The final recorded state relates to the equilibrium. The obtained relationship of the residual activity versus time of preincubation are characteristic of slow binding inhibition.

Progress curves analysis, inhibition constants

The enzymatic hydrolysis of urea was studied by two different procedures: the reaction initiated by addition of urease, without preincubation of the enzyme with the inhibitor (unpreincubated system) and the reaction initiated by addition of urea, with preincubation of urease with the inhibitor (preincubated system, steady-state analysis). The unpreincubated system is represented by the reaction progress curves shown in Figure 2A. The curves were obtained for the enzymatic urea hydrolysis in presence TCpBQ. The curves indicates that the velocity of the reaction 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}. Such a behaviour is characteristic of slow-binding inhibition elaborated by the theory of Morrison and Walsh [13]. A curve fitting computer program was used to fit the experimental points to the integrated equation describing slow-binding inhibition progress curves:

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

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 for interconversion between v_o and v_s, and t is time. It was found that the initial velocity and steady-state velocity are inhibitor concentration-dependent.

The system with preincubation (steady-state analysis) is represented by the curves shown in Figure 2B. The obtained straight line progress curves proved that the reaction achieved the steady-state velocity (v_s), this being different for each studied inhibitor concentration.

The obtained relationship of the reaction velocities (v_o; v_s) versus the inhibitor concentration are characteristic of a two-step enzyme inhibitor interaction, mechanism B described by the Equation (2):

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

$$E + I \underset{k_4}{\overset{k_3}{\Leftrightarrow}} \underbrace{EI \underset{k_4}{\overset{k_3}{\Leftrightarrow}} EI^{\star}}_{\text{slow}} \qquad (2)$$

Where E is enzyme, S substrate, P product, I inhibitor, EI and EI* enzyme-inhibitor complexes, respectively. $k_1 - k_7$ are velocity constants.

Linear dependencies of $1/v_o$ and $1/v_s$ on the inhibitor concentration were used to asses the inhibition constants, K_i and $K_i^{\star}$:

$$\frac{1}{v_o} = \frac{K_M}{v_{max} S_o K_i} I + \frac{1}{v_{max}} \left(1 + \frac{K_M}{S_o}\right) \qquad (3)$$

$$\frac{1}{v_s} = \frac{K_M}{v_{max} S_o K_i^{\star}} I + \frac{1}{v_{max}} \left(1 + \frac{K_M}{S_o}\right) \qquad (4)$$

where K_M is the Michaelis constant and v_{max} is the maximum velocity given by the Michaelis-Menten equation for the uninhibited reaction, S_o denotes the initial concentration of urea, K_i and $K_i^{\star}$ are the inhibition constants defined as:

$$K_i = [E][I]/[EI]; K_i^{\star} = [E][I]/([EI] + [EI^{\star}]),$$

respectively [13].

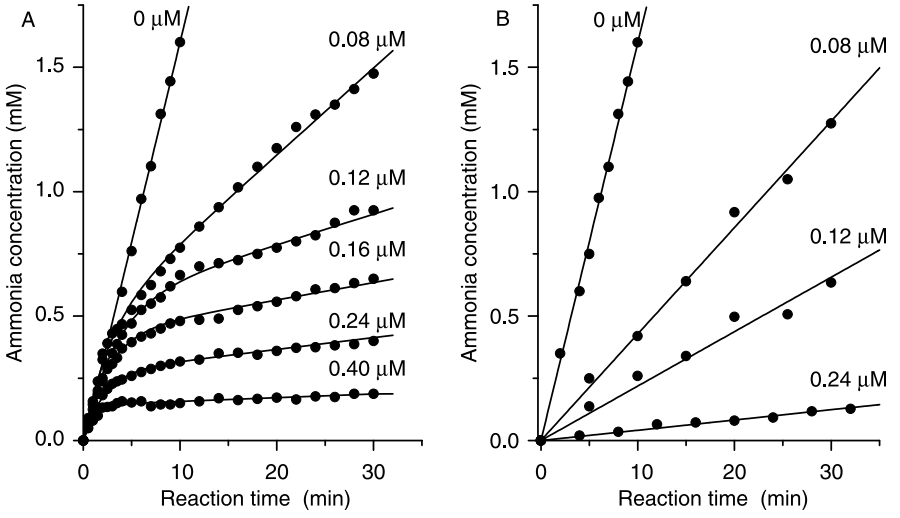

Figure 2. (A) Reaction progress curves of the urease-catalyzed hydrolysis of urea in the presence of TCpBQ. (B) Steady-state analysis: concentration of ammonia *vs* time. Concentration of TCpBQ [μM] is numerically given.

Table I. Inhibition constants for the slow-binding inhibitors of jack bean urease in phosphate buffer pH 7.0, 1 mM EDTA, 25°C.

Inhibitor	Initial inhibition constant K_i; mM	Overall inhibition constant K_i^*; mM	Ref.
1,4-benzoquinone	0.031 ± 0.003	$(4.5 \pm 0.4) \times 10^{-5}$	[7]
2,5-dimethyl-1,4-benzoquinone	0.42 ± 0.08	$(1.2 \pm 0.1) \times 10^{-3}$	[7]
Tetrachloro-o-benzoquinone	$(8.3 \pm 0.8) \times 10^{-5}$	$(2.4 \pm 0.2) \times 10^{-6}$	–
Tetrachloro-p-benzoquinone	$(2.2 \pm 0.2) \times 10^{-5}$	$(4.5 \pm 0.5) \times 10^{-7}$	–
Phenylphosphorodiamide	–	$(1.6 \pm 0.039) \times 10^{-7}$	[18]
Hg^{2+}	–	$(1.9 \pm 0.19) \times 10^{-6\dagger}$	[19]

†buffer HEPES, pH 7.0

That same procedure was followed for isomer TCoBQ (experimental data not shown). The calculated inhibition constants are listed in Table I. The obtained value for the TCpBQ inhibition constants classified that inhibitor among the strongest urease inhibitors. TCoBQ followed that same mechanism of inhibition as that for TCpBQ but it was found that TCoBQ is a weaker inhibitor than the para-isomer. The final inhibition constant is 10-fold higher than that for TCpBQ.

We showed in previous report that the inhibition of urease by 1,4-benzoquinone and 2,5-dimethyl-1,4-benzoquinone also followed a slow-binding mechanism [7]. That scheme seems to be a representative mechanism for the urease inhibition by quinones. The value of the overall inhibition constants of tetrachlorobenzoquinones classified them among the most potent urease inhibitors such as phenylphosphorodiamide and Hg(II) ions. A higher normal potential value for o-quinone than p-quinone suggests that TCoBQ should be a stronger inhibitor than TCpBQ; the experiments showed the opposite result. The lower inhibitory potency of TCoBQ might be due to a steric hindrance effect of the TCoBQ molecule.

Protective experiment

It was shown that the presence of monothiols: L-cysteine, 2-mercaptoethanol and glutathione as well dithiol: dithiothreitol, in the preincubation mixture protected urease against inhibition by TCoBQ and TCpBQ (Figure 3). The enzyme retained more than 80% of its control activity after preincubation with TCpBQ and the thiol-protector, while the preincubation without the thiol-protector resulted in a decrease in activity down to almost 10%. The protection against TCoBQ resulted in a 60–80% urease residual activity. Protection ability was also show for sodium fluoride (a competitive slow binding urease inhibitor [14]) and boric acid (a classical competitive urease inhibitor [15]). Sodium fluoride and boric acid inhibit the enzyme by interaction with active site nickel ions [16,17]. The protective experiments showed that the urease active site is involved in inhibition by TCoBQ and TCpBQ. Better prevention by thiols than inorganic compounds indicated that the active site cysteine is a residue responsible for urease inhibition. Quinones are highly reactive towards nucleophiles which explains the thiol-prevention effect. Moreover, halogenation increases the quinone oxygen reactivity.

Activity recovery of the inhibited urease

The stability of the urease-inhibitor complex was examined by using two methods. In the first approach, DTT was applied. Quinones are thiol-active reagent so the addition of DTT into the solution of complex inhibitor-urease (TCoBQ-urease or TCpBQ-urease, respectively), could release the enzyme and create a new complex inhibitor-thiol. The obtained results (data not shown) did not indicate that urease regained its activity. This result proved that the complexes TCoBQ-urease and TCpBQ-urease are strong and stable. That conclusion was confirmed by the next method where the inhibitor was preincubated with the enzyme to establish the equilibrium:

$$E + I \Leftrightarrow EI \Leftrightarrow EI^{\star} \qquad (5)$$

The choice of the preincubation time was made on the basis of the results of the residual activity measurement (Figure 1). The preincubated mixture was diluted in the mixture containing the substrate

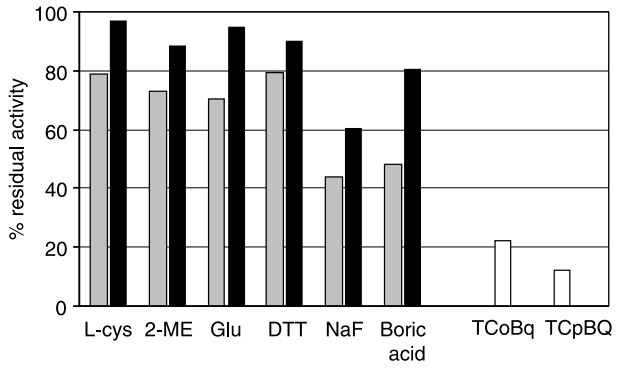

Figure 3. Protective effects of L-cysteine (L-cys), 2-mercaptoethanol (2-ME), glutathione (Glu), dithiothreitol (DTT), sodium fuoride and boric acid against urease inhibition by TCoBQ (grey) and TCpBQ (black), relative to the control activity. The percent of the residual activity of urease in the presence of TCoBQ and TCpBQ without the protector is given for comparison (white).

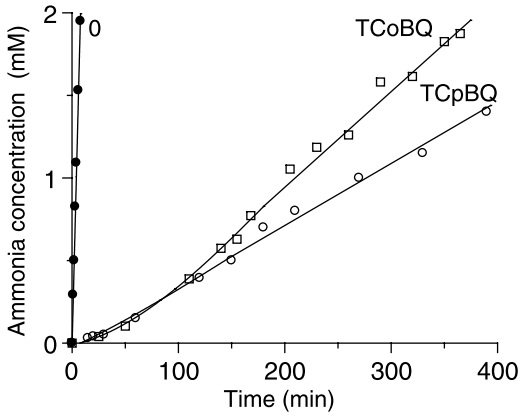

Figure 4. Reactivation progress curves of inhibited urease by TCoBQ (□) and TCpBQ (○), respectively, after 50-fold dilution in 50 mM urea. The progress curve of uninhibited urease is given by (●).

and the enzymatic reaction was initiated. The addition of the substrate perturbed the equilibrium and the system established a new equilibrium:

$$\begin{aligned} E + S &\Leftrightarrow ES \rightarrow P + E \\ E + I &\Leftrightarrow EI \Leftrightarrow EI^{\star} \end{aligned} \qquad (6)$$

The progress curves obtained after dilution of the preincubation mixture are presented in Figure 4. The curves displayed an initial shallow slope which turned over to the steady-state velocity. The early parts of the curves represent a slow process of achieving equilibrium. The later parts relate to the systems with the constant amount of the free enzyme. No increase of the steady-state velocity in the studied time range confirmed the stability of the complexes: TCoBQ-urease and TCpBQ-urease.

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