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DETERMINATION OF THE UREASE ACTIVITY AND THE RELATIVE INHIBITION IN THE PRESENCE OF SOME METAL IONS: A MICROCALORIMETRIC STUDY

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SUMMARY

Batch microcalorimetry has been employed to obtain a calibration curve for the enzymatic activity of urease in solution.

This method is simplier, more reliable and easier to handle than the more common techniques (spectrophotometry and potentiometry), because it is based on direct investigation of the enzymatic reaction.

By comparison with calorimetric studies employing the thermistor combined with the immobilized enzyme, this method also allows the catalytic activity to be measured.

Variations in the urease activity in the presence of nine metal ions [Hg(II), Ag(I), Cu(II),Zn(II), Cd(II), Ni(II), Co(II), Mn(II) and Mg(II)] are also described.

A graphic method has been devised for immediate identification of the minimum inhibitor concentration,determining the start, 50% and complete inhibition of ureasic activity.

INTRODUCTION

Enzymatic activity can be experimentally determined in several ways. Spectrophotometry (ref.1) is generally employed, since it is very simple and fast. It is often necessary to use more than one enzymatic reaction, however, with the result that inaccurate responses may be generated through the summation of errors. Moreover, the use of several reactions in the study of inhibition of catalytic activity, may prove incorrect because the inhibiting agents could affect the activity of more than one enzyme.

Calorimetry is a very suitable way of avoiding these drawbacks, because it allows direct investigation of the enzymatic reaction itself and no complementary reactions are needed (ref.2).

Batch microcalorimetry with the enzyme in solution was employed in the study described in this paper.

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Calorimetric studies using thermistors with immobilized enzymes (refs.3,4) and also a comparative study of solubilized and immobilized enzyme (ref.5) have been reported. This is a complicated procedure, however, because the calorimetric vessels must be changed each time a new reaction is to be examined. In addition, immobilized enzyme calorimetry is generally concerned with determination of the substrate concentrations and not with the measurement of the catalytic activity.

Nine metal ions are considered: mercury(II), silver(I), copper(II), zinc(II), cadmium(II), nickel(II), cobalt(II), manganese(II) and magnesium(II). The effects of Zn(II), Cd(II), Ni(II), Co(II), Mn(II) and Mg(II) on urease activity have never been studied by microcalorimetry, though inhibition of the first three have been investigated by immobilized enzyme (ref.6).

The microcalorimeter employed in this work is of the "heat conduction" type (ref.7). Its output is thus directly related to the heat change rate during the reaction and hence to the enzymatic activity (EU):

$$V = \mathfrak{S} \Delta (q/\Delta t) \qquad (1)$$

therefore:

$$V = \mathfrak{S}\,(EU)\,\Delta H_R \qquad (2)$$

where V is the thermopile voltage, $\mathfrak{S}$ is the calibration constant of the calorimeter and EU are the enzymatic units expressed in international units per ml (IU/ml). A calibration curve for the determination of urease activity is worked out from eqn.(2) and the effects of each ion are simply quantified and expressed graphically.

EXPERIMENTAL

Materials and apparatus

All chemicals for buffer solutions were commercial products of analytical grade.

Metal ion solutions were prepared with the corresponding nitrate salts (Merck pro anal.) and the stock solutions were titrated by EDTA disodium salt (Merck puriss.) using the appropriate indicators.

Urea crystalline used was from Sigma and its purity was checked by thermal-analysis.

Urease liophilized product (Sigma type IV from jeack beans) was stored at - 20°C until used. Fresh solutions were prepared daily and were stored at 0°C.

The calorimetric apparatus was an LKB mod.2107 batch microcalorimeter equipped with two gold vessels (total volume about 7ml), a multitemp cooling circulator (LKB 2209) and a potentiometric recorder (LKB 2210). The system was completely housed in a thermostated room and all measurements were carried out at 25± 0.01°C.

Calorimetric accuracy was checked by determining the sucrose dilution. The heat values were in good agreement with the reported values (ref.7), within the 0.5 %.

A Perkin Elmer TGS-2 thermanalytical apparatus was used to check the purity of both substrate and enzyme products.

The buffer solution pH was measured with a Metrohm 605 pHmeter.

Microcalorimetric measurements

The effect of each ion on urease activity was studied with the aid of a calibration curve, previously plotted in the absence of ions.

The results of eqns. (1) and (2) are reported in the figure 1, where Δ_{max} corresponds to the maximum deviation of the curves (V = thermopile voltage vs time) and it is directly related to EU.

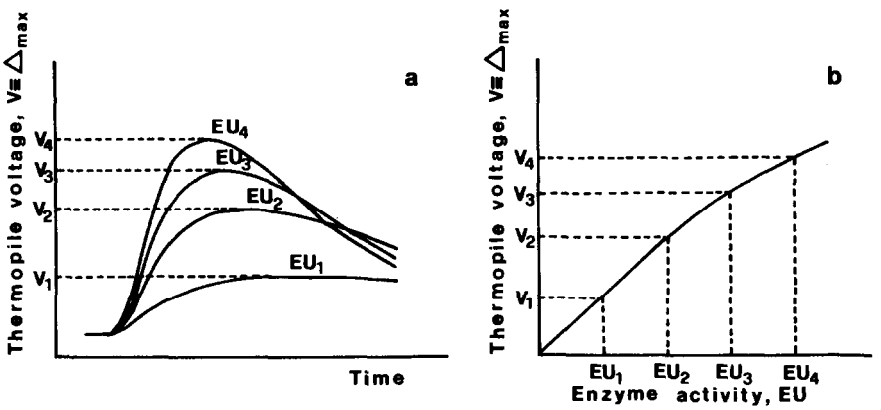

Fig.1. (a) Calorimetric curves relative to a series of enzyme activity levels (EU): $EU_1 < EU_2 < EU_3 < EU_4$; V = thermopile voltage; Δ_{max} = peak height. (b) Corresponding analytical calibration plot of peak height versus enzyme activity.

The calibration curve for urease activity is therefore obtained by plotting the calorimetric datum (Δ_{max}) versus EU in IU/ml (Fig. 2). Each point is the mean of three measurements performed in pseudo-zero order conditions of reaction (excess of substrate). The equation (3):

$$v = v_{max} \cdot [S] / (K_M + [S]) \qquad (3)$$

where v is the reaction rate, [S] is the substrate concentration in mole per liter (M),and K_M is the Michaelis constant, shows that the substrate concentration of $10 \cdot K_M$ is itself sufficient to give a rate of $0.91 \cdot v_{max}$, while concentrations of 100 or 1000 times K_M do not raise it much further. The value $10 \cdot K_M$ was therefore chosen for $[S_o]$.

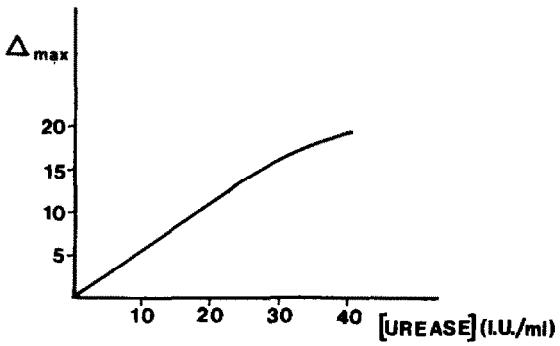

Fig. 2. Calibration curve for determination of the enzyme activity at 25°C, pH = 6, [urea] = $6 \cdot 10^{-2}$ M, assuming pseudo-zero order kinetics.

The following experimental conditions were established: [urea] = $6 \cdot 10^{-2}$ M, [urease] from 2.5 to 40 IU/ml in tris-maleic acid-NaOH 0.2 M buffer at pH 6, T = 25°C.

The pH and the buffer solutions were the same used in a previous work (ref. 8), where the optimum experimental conditions were found to give the maximum calorimetric response and the maximum catalytic activity of urease.

The calorimetric measurements for the calibration curve were obtained by filling the two compartments of the measuring vessel (detector 1) with 1 ml of the substrate solution and 1 ml of the enzyme solution, respectively. The reference vessel (detector 2) was filled with 2 ml of buffer solution only, because preliminary experiments had shown that no heat effects arose from dilution of the solutions.

The calibration curve (Fig. 2) was used to measure the inhibition effects with [urea] = $6 \cdot 10^{-2}$ M and [urease] = 20 IU/ml. Detector 1 was filled as before. The metal ion was mixed with the enzyme in the same solution at varying concentrations. Detector 2 was again filled as before, though if any metal dilution heat was observed, one compartment was filled with 1 ml of buffer solution containing the appropriate metal ion concentration.

The ion concentrations were extensively experimented over a wide range: from

10^{-7} to 10^{-1} M .

The minimum concentration value causing i) a calorimetric response and ii) a total enzyme inhibition were found for each metal ion.

The appropriate microvolume of the stock titrated solution was withdrawn and added to the enzyme solution because this gave best calorimetric responses. Furthermore, preliminary experiments showed an absence of undesiderable heat due to a possible interaction between the ions and individual reagents.

All experiments were run twice and all concentrations refer to the reagents in the calorimetric vessels before mixing.

RESULTS AND DISCUSSION

Our results are summarized in table 1 and figure 3. As expected (refs. 6, 9,10), mercury(II), silver(I) and copper(II) are the strongest inhibitors. Zinc(II) and cadmium(II) also produce total inhibition of urease activity.

TABLE 1

Inhibition effects of metal ions on urease activity, T = 25°C, pH = 6, [urea] = $6 \cdot 10^{-2}$ M, [urease] = 20 IU/ml.

Inhibition	Hg^{2+}	Ag^{+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Ni^{2+}	Co^{2+}	Mn^{2+}	Mg^{2+}
	(10^{6} M)			(10^{6} M)					
start	0.1	0.1	0.1	0.9	0.9	0.4	0.7	0.5	10
50 %	0.3	1.7	13.2	5.2	7.2	6.8	53.0	---	---
100 %	0.5	5.0	26.0	40.0	53.0	---	-----	---	---

Our data agree with those of the literature. The method here described, however, is much more suitable because it is based on measurement of an experimental quantity (the heat involved in the reaction) directly related to the trend of the enzymatic reaction, without any mathematical elaboration and without complicated manipulations of the samples and/or instrumental apparatus.

In some cases, we found that total inhibition required a higher metal concentration. This is probably due to experimental differences between calorime-

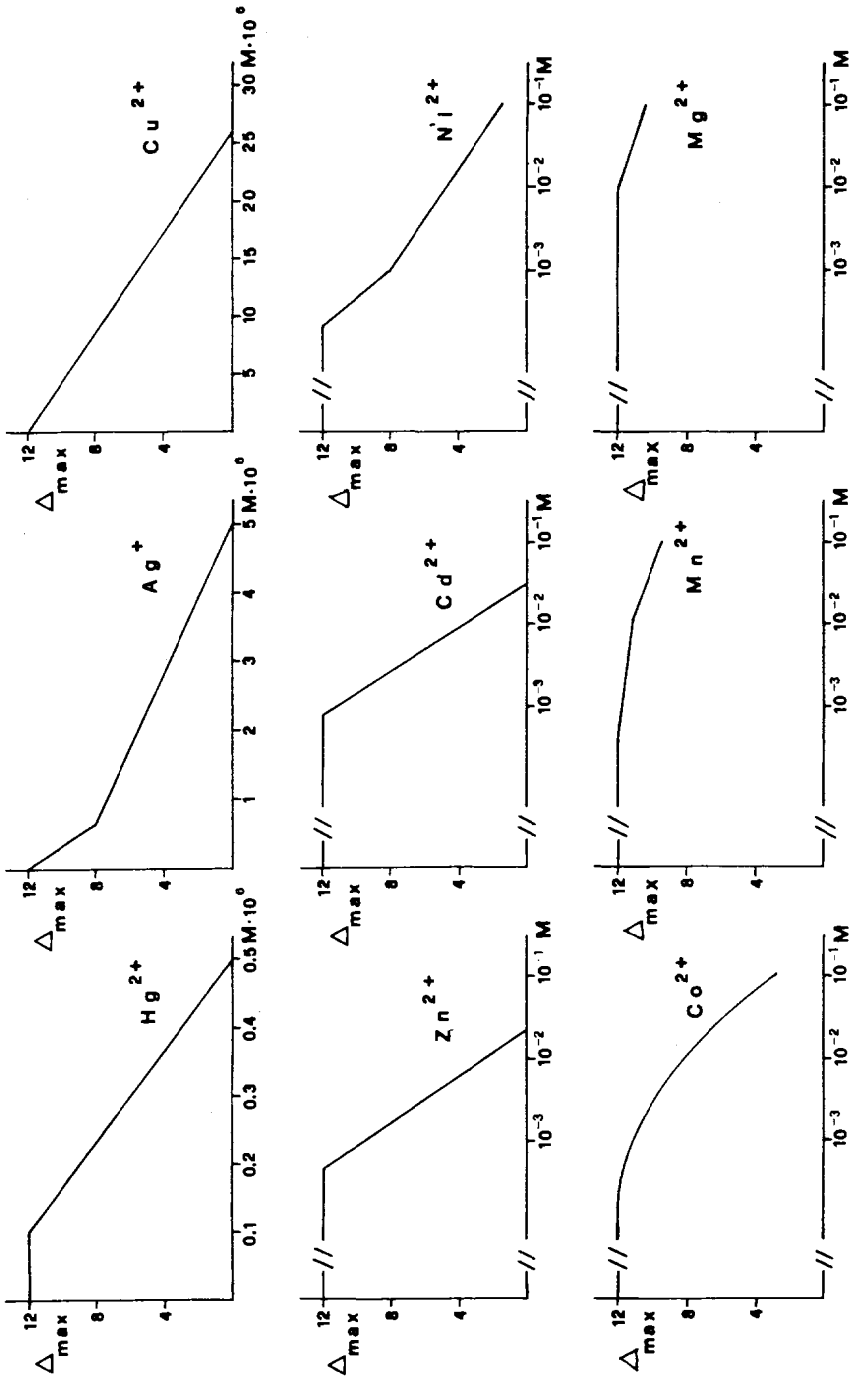

Fig. 3. Inhibition effects of metal ions on urease activity, T = 25°C, pH = 6, [urea] = 6.10^{-2} M, [urease] = 20 IU/ml.

try and other techniques. Our study, infact, is based upon direct investigation of the enzyme reaction without the complementary reactions required for indirect methods, such as spectrophotometry and/or potentiometry. On using the more common techniques, therefore, the inhibitor quantities necessary to reach the 100 % inhibition could prove less than the real ones, because the metal ion might influence the revealing reaction and not only the reaction under examination.

The effect of nickel(II), cobalt(II), manganese(II) and magnesium(II) was very weak (see Tab.1 and Fig.3). Particular attention must be directed to that of magnesium(II) and nickel(II). The former is generally considered as a biologically active ion and there have been no studies of its possible inhibition of urease activity. Our experimental evidence shows that a 10 % inhibition is observed in the presence of about 10^{-1} M of Mg^{2+} and the picture for Mn^{2+} was quite the same.

Some papers (refs. 11-13) report the presence of nickel(II) inside the urease molecule. This metal ion, therefore, cannot be regarded as a real urease inhibitor. Its influence (positive or negative) strongly depends on the relative concentrations with respect to the enzyme activity. Our data show a 50 % inhibition in the presence of about $7 \cdot 10^{-2}$ M of Ni^{2+} ion . The literature data confirm this effect on the activity of soil urease (ref.14).

The source of the enzyme is another important factor and great differences may depend on the type of urease examined (ref.15). Soil urease is activated by the same metal ions (ref.16) usually regarded as strong inhibitors of jeackbean urease, e.g. the copper(II) ion.

Calorimetry also helps to show which molecule (enzyme and/or substrate) will be attacked by the inhibitor molecule. It is known (ref.17) that an E-I complex is formed between urease and metal ions. The calorimetric results are in line with this observation, because measurements performed with high metal concentrations show no variations in the heat quantities involved (Q_{tot}). Since the areas subtended by the calorimetric curves (Fig.4) obtained in the absence (curve a) or in the presence (curve b) of inhibitor are the same: it must be concluded that the free substrate is still the same (i.e. not bonded to the inhibitor) while the initial rate of reaction is varied (see the initial slope of the two curves in Fig.4), meaning that a part of the enzyme is really bonded to the inhibitor. Moreover blank measurements with metal ions and urea without enzyme

gave no heat at all.

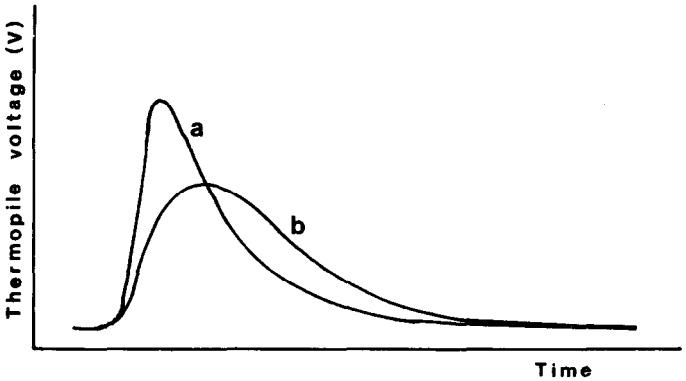

Fig. 4. Calorimetric responses V = thermopile voltage versus time: a) in the absence of inhibitor, b) in the presence of inhibitor; $A_a = A_b$ (A = curve area).

The inhibtion effect on urease can be assumed to be reversible, as known (ref.10). The calorimetric data confirm this statement, because activity is re-established by adding the appropriate amount of EDTA: in such conditions, the heat effect returns normal, i.e. equal to that obtained in the absence of meta ions. With regard to the inhibition mechanism, our data do not accord with the view that the metal inhibition of urease is non-competitive below pH 7 and hence that the -SH groups of the protein do not lie in the active site (refs.9, 10). By contrast, the final aminoacid sequence shows that there are five sulphydryl groups non essential and one essential for each enzyme subunit (ref.11). Our evidences, at fixed enzyme and inhibitor concentrations and with variable urea concentrations, do not display the same trend: the inhibition mechanism may thus supposed to be of a mixed type (ref.2).

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