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THE INTERACTION OF *p*-NITROPHENYL CARBAMATE WITH UREASE

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Summary

1. *p*-Nitrophenyl carbamate and thiourea have been shown to be substrates for urease (urea amidohydrolase, EC 3.5.1.5)

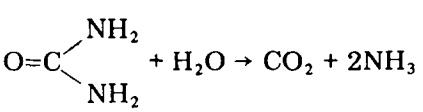
2. Urease has been shown to have a lower K_m, 0.67 mM, with *p*-nitrophenyl carbamate than with urea, 2.0 mM.

3. The V of urease for the hydrolysis of urea, *p*-nitrophenyl carbamate and thiourea has been shown to be the same, indicating a common rate-limiting step.

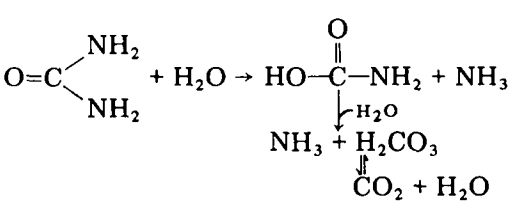
4. A mechanism has been proposed for urease where the initial rate-limiting step is the release of a molecule of ammonia from the substrates.

Introduction

Three mechanisms for the hydrolysis of urea by urease (urea amidohydrolase, EC 3.5.1.5) have been discussed [1]. These are the carbon dioxide mechanism:



the carbonic acid mechanism:



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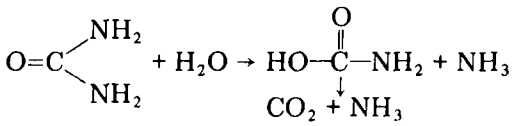
the carbamic acid mechanism:

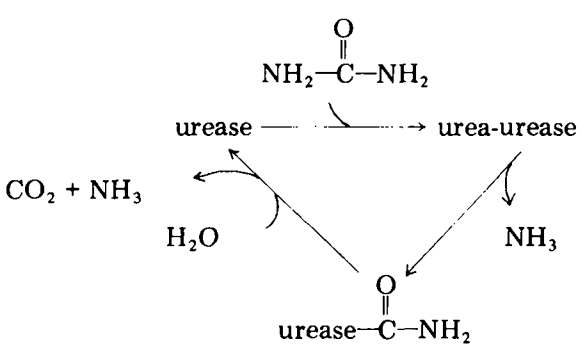


Sumner [2] first demonstrated the formation of ammonium carbamate in the products of the urease-catalysed hydrolysis of urea, but later he inclined to the view that the carbamate is synthesised from CO_2 and NH_3 [3]. It is, however, difficult to envisage how urea can be hydrolysed to carbon dioxide and ammonia without passing through a carbamic acid or carbamate stage, and extensive investigations by various workers [4—7] have established ammonium carbamate as a first free intermediate of the urease hydrolysis of urea. Ammonium carbamate logically arises from a carbamoyl transfer reaction, although attempts to demonstrate the formation of a carbamoyl enzyme intermediate, which was first postulated in 1937 by Brandt [8], have so far failed. Blakeley et al. [7] obtained supporting evidence for a carbamoyl transfer reaction in experiments measuring acidity changes during the enzymic hydrolysis of a limited amount of urea. Barth and Michel [9] investigated the dependence of the Km on pH and the action of inhibitors, in order to study the functional groups in the active site of the enzyme. On the basis of their results Barth and Michel proposed a mechanism which also includes the formation of a carbamoyl enzyme complex.

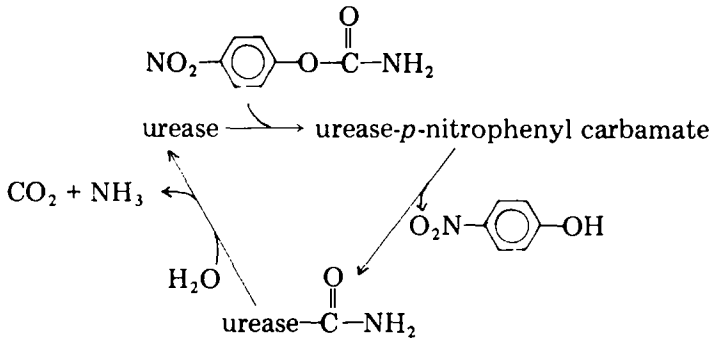
Currently, therefore, the majority of the evidence suggests a mechanism involving a carbamoyl-enzyme complex intermediate, without any direct evidence to support this.

Although for many years urease was thought to be absolutely specific for urea, other substrates for this enzyme have now been demonstrated. Fishbein [10] found hydroxyurea and dihydroxyurea were substrates for urease and Sundaram and Laidler [11] have demonstrated the urease-catalysed hydrolysis of some substituted ureas and esters of carbamic acid. In this work two possible substrates, thiourea and *p*-nitrophenyl carbamate, were investigated. It was thought that an investigation, in the stopped flow apparatus, of the hydrolysis of *p*-nitrophenyl carbamate, which can be followed spectrophotometrically by monitoring the release of *p*-nitrophenol, might demonstrate an enzymebound carbamate intermediate.

The postulated mechanism for the hydrolysis of urea is:



Therefore a similar procedure would be expected with *p*-nitrophenyl carbamate:



If the rate of production of a carbamoyl-enzyme intermediate is faster than the rate of its breakdown it should be possible to observe a biphasic rate using the stopped flow apparatus. An initial rapid release of *p*-nitrophenol would be followed by a slower rate of increase of *p*-nitrophenol concentration as the urease-carbamate complex breaks down.

Experimental

p-Nitrophenyl carbamate was prepared as described by Robillard et al. [12]. The product had a melting point of 163—165° C but was contaminated with *p*-nitrophenol (m.p. 114° C). The *p*-nitrophenyl carbamate was recrystallised from warm ethanol when white plate crystals were obtained.

p-Nitrophenyl carbamate is insoluble in water but soluble in acetonitrile, dimethylformamide, dimethylsulphoxide and dioxane. Acetonitrile and dioxane were found to have an inhibitory effect upon urease. Dimethylformamide and dimethylsulphoxide were not inhibitory at a concentration of 10% in the assay. In dimethylformamide, however, the *p*-nitrophenyl carbamate underwent rapid hydrolysis and this solvent was, therefore, unsuitable.

p-Nitrophenol concentration can be measured at 320 nm at pH values less then 7, or at 400 nm at pH values greater than 7 when it exists as the phenylate ion. The molar extinction coefficients for *p*-nitrophenol are 9000 $l \cdot mol^{-1} \cdot cm^{-1}$ at 320 nm and 15 000 $l \cdot mol^{-1} \cdot cm^{-1}$ at 400 nm. Because *p*-nitrophenyl carbamate also absorbs at 320 nm but shows little absorbance at 400 nm it was preferred to monitor the absorbance at 400 nm. However, *p*-nitrophenyl carbamate in 10% (v/v) dimethylsulphoxide showed some instability above pH 7.0 whereas there was no spontaneous hydrolysis at pH 6.0. Assays were therefore performed at pH 6.0, which is close to the enzyme optimum [13], but necessitated monotoring the *p*-nitrophenol released at 320 nm. The spectra of *p*-nitrophenyl carbamate and *p*-nitrophenol at pH 6.0 are shown in Fig. 1. The molar extinction coefficient for the change in the *p*-nitrophenol concentration was estimated to be 5700 $l \cdot mol^{-1} \cdot cm^{-1}$.

Urease was extracted from Jackbean meal (Sigma, London) by the method of Blakeley et al. [14] and purified by twice recrystallising from acetone by the method of Dounce [15] followed by filtration through a millipore membrane 0.22 μm [13].

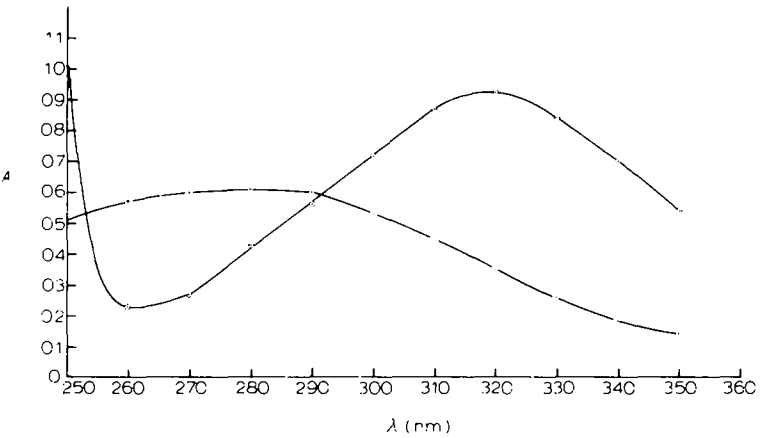


Fig. 1. The spectra of 100 μM solution in 10% dimethylsulphoxide at pH 6.0 of *p*-nitrophenol (○——○) and *p*-nitrophenyl carbamate (●——●).

At first, enzymic hydrolysis of *p*-nitrophenyl carbamate was followed in a Unicam SP1800 spectrophotometer. The assay system used involved the use of cells in tandem. In the reference beam one cell containing 0.1 mM *p*-nitrophenyl carbamate in dimethyl sulphoxide, 0.5 mM Tris/H_2SO_4 pH 6.0, 0.033 mM EDTA and 0.033 mM β-mercaptoethanol, the second containing 0.5 mM Tris/H_2SO_4, pH 6.0, 0.033 mM EDTA, 0.033 mM β-mercaptoethanol and 0.1 ml urease. In the sample beam one cell containing 0.1 mM *p*-nitrophenyl carbamate in dimethyl sulphoxide, 0.5 mM Tris/H_2SO_4, pH 6.0, 0.033 mM EDTA, 0.033 mM β-mercaptoethanol and 0.1 ml urease, the second containing distilled water. Total volume in each cuvette was 3.0 ml. The reaction was initiated by the addition of enzyme to the test cuvette. Assays were also carried out without enzyme but with the addition of buffer, and without substrate but with the addition of an equivalent volume of 10% dimethylsulphoxide. It was concluded that the observed increase in the absorbance obtained on addition of enzyme to a solution of *p*-nitrophenyl carbamate was due to the enzymic hydrolysis of *p*-nitrophenyl carbamate.

The assay was then adapted for use with the Durrum stopped flow apparatus. Syringe 1 contained the enzyme and buffer in 10% (v/v) dimethyl sulphoxide and syringe 2 contained 200 μM *p*-nitrophenyl carbamate in 10% (v/v) dimethylsulphoxide.

The hydrolysis of *p*-nitrophenyl carbamate was followed over 10 ms, when only one rate was observed, not the two rates expected if the breakdown of an enzyme-carbamate intermediate is rate-limiting. To determine whether urea hydrolysis and *p*-nitrophenyl carbamate hydrolysis were taking place at the same site, the rate was assayed in the presence of varying urea concentrations. It was found that the presence of urea did decrease *p*-nitrophenyl carbamate hydrolysis. Table I shows the rates of hydrolysis by urease of 100 μM *p*-nitrophenyl carbamate at pH 6.0 and the value for K_i (2.0 mM) for urea obtained from a Dixon plot is in agreement with the K_m determined for urease with urea under the same conditions (2.0 mM).

K_m and V were estimated for urease hydrolysis of *p*-nitrophenyl carbamate.

TABLE I

Urea (mM)	v ($A_{320nm} \cdot min^{-1}$)
0	0.26
0.2	0.22
1.0	0.17
2.0	0.09

The results obtained were reproducible and Lineweaver-Burk and s/v vs. s plots were linear. K_m was found to be 0.67 mM and V, for the enzyme sample used, was 87.0 μmol *p*-nitrophenyl carbamate hydrolysed/min per mg protein.

Enzyme activity with urea and thiourea was measured with a recording pH stat (Radiometer, Copenhagen). Assays were carried out at 25°C in the presence of 5 mM Tris/H_2SO_4 buffer and the pH was maintained at 6.0 by titration with 50 mM H_2SO_4. Using the same enzyme sample as that used for the *p*-nitrophenyl carbamate experiments, and with urea as substrate, the K_m was found to be 2.0 mM and the V was found to be 82.5 μmol urea hydrolysed/min per mg protein. When thiourea was used as substrate, s/v vs. s plots were linear the K_m was found to be 210 mM and V was 82.5 μmol thiourea hydrolysed/min per mg protein. The hydrolysis of urea in the presence of thiourea was investigated and thiourea was found to be a competitive inhibitor of urease.

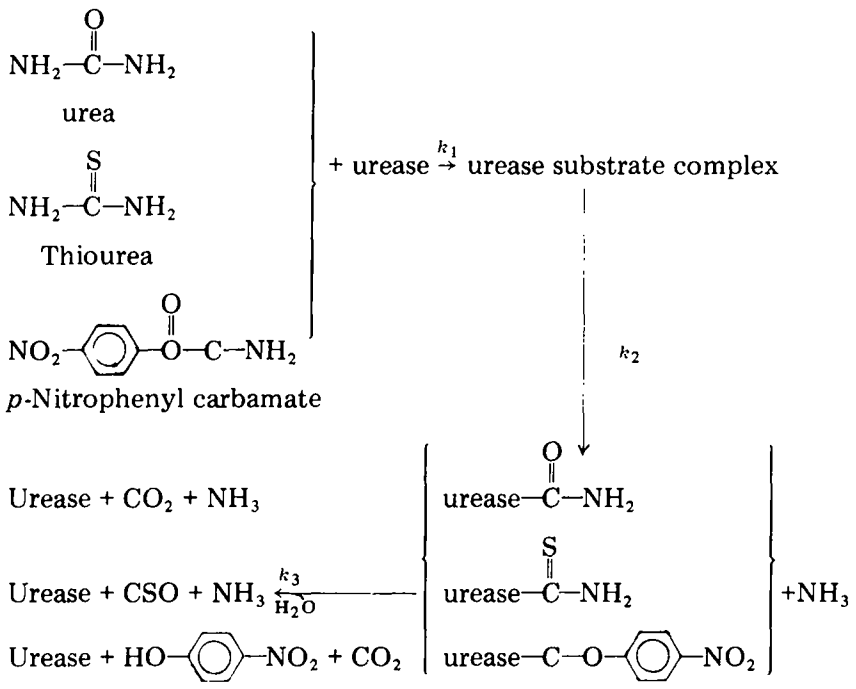
Discussion

Thiourea and *p*-nitrophenyl carbamate have been shown to be hydrolysed by urease. The K_m of the enzyme is of a similar order for *p*-nitrophenyl carbamate and urea but much higher for thiourea. In view of the fact that thiourea is a competitive inhibitor of urea hydrolysis and urea is a competitive inhibitor of *p*-nitrophenyl carbamate hydrolysis it may be assumed that both compounds are being hydrolysed at the same site as urea.

The K_m for urease with *p*-nitrophenyl carbamate has been determined as 0.67 mM compared to 2.0 mM for the same urease sample with urea. This suggests a greater affinity of the enzyme for *p*-nitrophenyl carbamate than for urea. In view of the low K_m for *p*-nitrophenyl carbamate as substrate, and the fact that a urea-splitting function for urease in the many plants, fungi and bacteria in which it is found has yet to be demonstrated, it is possible that urea is not the natural substrate for this enzyme.

It had been hoped that the hydrolysis of *p*-nitrophenyl carbamate would be shown to be biphasic with a rapid initial rate and a subsequent slower rate when examined using the Durrum stopped flow apparatus. This was not found to be so. As the substrates *p*-nitrophenyl carbamate, thiourea and urea exhibit the same V with urease it can be concluded that they share a common rate limiting step. The only step envisaged common to all three substrates is the release of an ammonia molecule and the following mechanism is therefore proposed.

The formation of the substituted enzyme intermediate (I) would be the rate-limiting step in this mechanism, to explain the observation of a single rate constant for the release of *p*-nitrophenol observed in the stopped flow experiments.



I

It might be expected that the thiol group would influence the rate of formation of enzyme-substrate complex, but the results suggest that the release of an ammonia molecule from this complex is the rate-limiting step. The results of previous workers [7,9], investigating the hydrolysis of urea by urease, would still be in agreement with these findings as with urea as substrate, an enzyme-carbamate intermediate would still be formed.

References

1 Barth, A. and Michel, H.-J. (1971) Pharmazie 26, 321—328
2 Sumner, J.B., Hand, D.B. and Holloway, R.G. (1931) J. Biol..Chem. 91, 333—341
3 Sumner, J.B. and Somers, G.F. (1953) Chemistry and Methods of Enzymes, 3rd edn., pp. 156—161, Academic Press, New York
4 Wang, J.H. and Tarr, D.A. (1955) J. Am. Chem. Soc. 77, 6205—6206
5 Gorin, G. (1959) Biochem. Biophys. Acta 34, 268—270
6 Jenks, W.P. (1963) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 6, pp. 914—928, Academic Press, New York and London
7 Blakeley, R.L., Hinds, J.A., Kunze, H.E., Webb, E.C. and Zerner, B. (1969) Biochemistry 8, 1991—2000
8 Brandt, W. (1937) Biochem. Z. 291, 99—104
9 Barth, A. and Michel, H.-J. (1972) Biochem. Physiol. Pflanz. 163, 103—109
10 Fishbein, W.N. (1969) J. Biol. Chem. 244, 1188—1193
11 Sundaram, P.V. and Laidler, K.J. (1970) Can. J. Biochem. 48, 1132—1140
12 Robillard, G.T.,Powers, J.C. and Wilcox, P.E. (1972) Biochemistry 11, 1773—1784
13 Bennett, J. (1973) Ph.D. Thesis, University of Birmingham, England
14 Blakeley, R.L., Webb, E.C. and Zerner, B. (1969) Biochemistry 8, 1984—1990
15 Dounce, A.L. (1941) J. Biol. Chem. 140, 307—308