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# Deactivation of Jack Bean Urease in Urea Hydrolysis

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## ABSTRACT

Deactivation studies of jack bean urease immobilized on porous alumina beads in the hydrolysis of urea were conducted in a continuously stirred tank reactor (CSTR) at a temperature of 25°C and pH 7.0. Though the mechanism of poisoning of urease by product ammonia is fairly well understood from the literature, the nature of the poisoning of urease by urea is presented in this article. These studies were conducted by adsorbing the ammonia formed in the hydrolysis reaction. The results indicate that, in the presence of the adsorbent Zeolite W, the deactivation rate is reduced by a factor of almost two, and thus provide a technique for prolonging the life of the enzyme. The deactivation model suggests that the free form of the enzyme is most susceptible to attack by the substrate urea. The experimental data suggest that deactivation by combined ammonia and urea is fairly complex.

**Index Entries:** Urease deactivation; Zeolite W; self-poisoning.

**Nomenclature:** $a$, activity of the enzyme urease ($[E_T]_0$ / $[E_T]_t$); $E$, free form of the enzyme urease; $[E_T]$, total enzyme concentration, IU/g; $[E_T]_t$, total enzyme concentration at time t, IU/g; $[E_T]_0$, total enzyme concentration at time t = 0, IU/g; $ES$, enzyme-substrate complex; $I$, inactive enzyme complex; $k_d$, urea deactivation rate constant, (m$M$-min)$^{-1}$; $k_d'$, ammonia deactivation rate constant, (m$M$-min)$^{-1}$; $K_I$, urea inhibition constant, m$M^{-1}$; $K_I'$, ammonia inhibition constant, m$M^{-1}$; $K_m$, Michaelis constant, $M$; $n$, order of the deactivation rate equation; $P$, product ammonia; $[P]$, product (ammonia concentration), m$M$; $Q$, flow rate of feed solution used in each experiment, mL/min; $S$, substrate urea; $[S]$, substrate (urea) concentration, m$M$; $[S_0]$, feed

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urea concentration, m$M$; $V$, hydrolysis reaction rate, mmol/min·g; $v$, volume of reaction mixture in CSTR balance, mL; $V_{max}$, maximum reaction rate velocity, IU/g; $V_d$, deactivation reaction rate due to urea poisoning, $min^{-1}$; $V_d'$, deactivation reaction rate due to ammonia poisoning, $min^{-1}$; $W$, weight of the immobilized enzyme used in each experiment, g.

## INTRODUCTION

An important aspect associated with the use of soluble and immobilized enzymes is their susceptibility to poisoning or deactivation. A good deal of work has been done in the past on the poisoning of enzymes, but, in the main, it has tended to be empirical rather than fundamental (*1,2,3,4,5,6*). This has led to deactivation rate expressions having little physical basis, thus limiting their use to the range of conditions within which measurements have been made.

In postulating any mechanism for enzyme action, the enzyme can exist in only a very limited number of forms (enzyme substrate complex, free enzyme, oxidized or reduced form of the enzyme, and so on) and at least one of these forms must be the one in which the enzyme exists when it undergoes poisoning. This simple conjecture led Do and Weiland (*7*) to consider the possible rate forms for the poisoning reactions that could arise in a number of elementary mechanistic situations. It turns out that regardless of the basic mechanism that one postulates for the main reaction, all possible mechanisms of poisoning lead unerringly to deactivation kinetics of the same general form as the main reaction. Experimental work on the enzyme systems glucose-oxidase (*8*), urease (*9*), and catalase (*10*) has corroborated this hypothesis, thereby demonstrating the importance of mechanism in determining deactivation kinetics.

Studies with jack bean urease immobilized on nonporous glass, in order to exclude both external and pore-diffusional resistances, have clearly shown that the enzyme is deactivated by both substrate urea and product ammonia (*9*). Deactivation studies conducted with the product ammonia alone (no urea present) have indicated a model in which the free form of the enzyme appears to be most susceptible to attack by ammonia. In the above studies, the poisoning of urease by the reactant urea could not be ascertained. This was primarily due to the complication introduced by poisoning of urease by ammonia, and the inability to design experiments in which poisoning and inhibition by ammonia could be excluded. In this article, the results of experiments in which poisoning of urease by ammonia is minimized are discussed. A model for the poisoning of urease by urea is also presented.

## MATERIALS AND METHODS

### Materials

Urease from jack beans (Type IV from Sigma, St. Louis, MO) was used in all experiments. Urea, THAM-sulfate, EDTA, TRIZMA base, and the urea assay BUN (Blood Urea Nitrogen) reagents were all obtained from Sigma. Zeolite W was purchased from Universal Oil Products (UOP, Des Plaines, IL). All chemicals employed were reagent grade or better and solutions were prepared in distilled deionized water.

Urease was immobilized by glutaraldehyde coupling to a silanized support as described by Vasudevan and Thakur (*11*). For the immobilization step, 10 g of the silanized-glutaraldehyde-treated support were immersed in the enzyme solution (0.25 g of jack bean urease powder, having an activity of 74,000 IU/g, was dissolved in 15 mL of 0.1*M* THAM-sulfate buffer solution at pH 7.5) for 5 h. The beads were intermittently stirred during this period. After 5 h, the beads were thoroughly washed with distilled deionized water and stored at 4°C in 0.1*M* THAM-sulfate buffer at pH 7.0 until further use.

Urea concentrations were measured using the Sigma Blood Urea Nitrogen (BUN) diagnostic kit. Urea reacts with diacetyl monoxime to yield a pink-colored complex. At sufficiently low concentrations of urea, the concentration is directly proportional to the intensity of the pink color produced. The absorbance of the pink-colored solution was measured using a Milton Roy 1001 Spectronic spectrophotometer (Rochester, NY) at 535 nm.

### Experimental Set-up

A small CSTR (50 mL) was employed in all the deactivation experiments. The feed to the reactor was metered by a Cole-Parmer Masterflex peristaltic pump from a 5 L feed storage tank. The reactor outlet was connected to an automated Gilson fraction collector. For the experiments with Zeolite W, the concentration of the zeolite in the feed was maintained constant by agitating the solution in the feed tank continuously for the entire period of the experiment.

### Experimental Procedure

Deactivation studies on immobilized jack bean urease were conducted in a CSTR, operated at steady state conditions. In the first set of experiments, the enzyme was exposed to the ammonia produced during the reaction. A second set of experiments was conducted in which Zeolite W was added to the feed solution to adsorb any ammonia produced during the reaction. Cattaneo and Chang have developed a microencapsulated urease-zeolite oral sorbent for the removal of urea in uremia (*12*). They

found that the use of zeolites alleviated the problems encountered with zirconium phosphate and that Zeolite W, in particular, had a high ammonium capacity. Zeolite W shows a rapid rate of ammonium uptake compared to zirconium phosphate. We reasoned that deactivation experiments with urease and urea in which Zeolite W is present in the reactor will lead to an environment in which the poisoning and inhibition of urease by ammonia can be minimized to a large extent. The zeolite has a theoretical ammonium capacity of 5.3 mEq/g. However, the actual capacity determined from the following equation is about 2.5 mEq/g.

$A = (C_i - C_{eq})\ V/W$, where $A$ is the zeolite capacity, $C_i$ is the initial ammonium concentration, $C_{eq}$ is the equilibrium ammonium concentration, $V$ is the volume of the solution, and $W$ is the weight of dry zeolite (g). The above value was used to determine the quantity of zeolite to be added with the feed to the reactor, assuming 100% conversion of urea to ammonia.

Experiments were conducted with 10, 20, 50, and 100 m*M* urea solutions. All solutions were prepared in 0.1*M* THAM-sulfate and 1 m*M* EDTA in distilled deionized water adjusted to pH 7.0 and stored in the feed storage tank. The reactor was filled with the feed urea solution, along with 4 grams of the immobilized urease. The reactor was immediately sealed off, and the feed was then metered to the reactor at a rate of 0.8 mL/min. The reactor effluent was sent to the fraction collector and analyzed for urea conversion every 2 h. The timer for the fraction collector was set to collect sample fractions of the effluent over a 7.5 min period. The deactivation experiment was carried out over a 48 h period.

## RESULTS AND DISCUSSION

The experiments were performed in a reaction medium containing an inert buffer, to exclude inhibition of urease by various metal ions and organic substances (urease inhibition by metal ions is well-documented [*13*)]. Recent studies have also shown that the stability of urease against metal ion inactivation is considerably improved upon immobilization (*14*).

The reasons for our current focus on self-poisoning studies of urease by urea and ammonia are as follows. Past studies by Vasudevan et al. (*9*) have clearly indicated that product ammonia is mainly responsible for the long-term activity loss of the enzyme. This is not to say that deactivation may not be caused by other independent poisons. It is well known that jack bean urease has two nickel ions present per catalytic unit (*15*). The urease nickel ion is tightly bound to the protein, as evidenced by retention of the metal ion during enzyme isolation in buffers containing 1 m*M* EDTA. However, nickel can be released under acidic conditions, leading to an irreversible loss of activity. In our experiments, the pH was maintained at 7.0. Since urease is inactivated by heavy metals and by oxidation, most purification procedures include low levels of EDTA or a thiol (such as

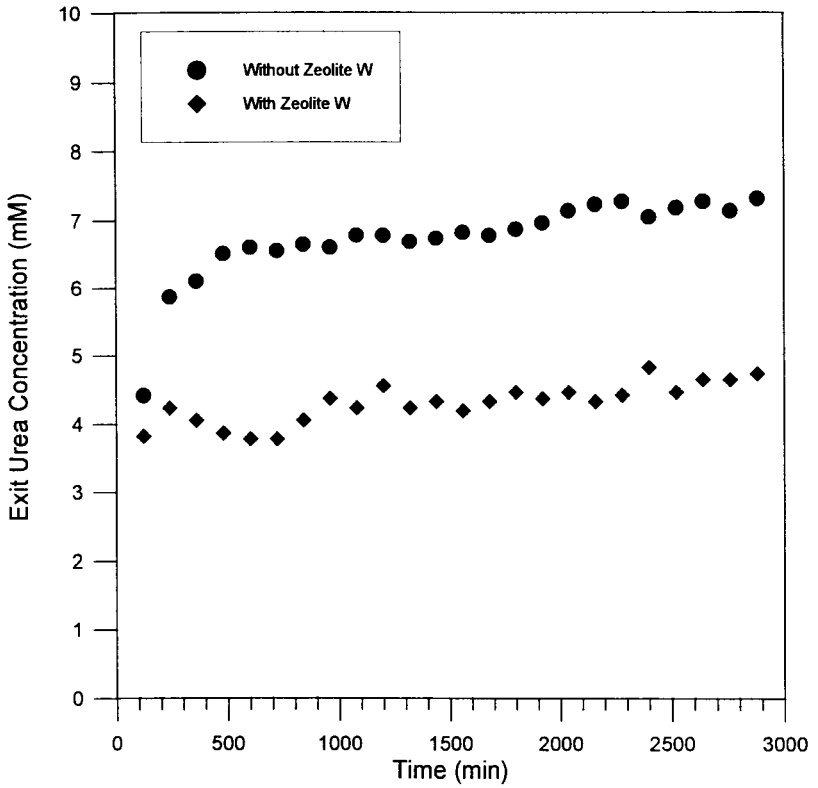


Fig. 1. Exit urea concentration vs time. Feed urea concentration = 20 m*M*, zeolite loading = 16 g/L, wt. of enzyme = 4 g, temperature = 25°C.

2-mercaptoethanol) to preserve activity. Urease inhibitors can be used as probes to unravel the enzyme mechanism. Thiols have been shown to competitively inhibit urease from jack beans (*16,17*). Since nickel–sulfur interaction is observed with a competitive inhibitor, this provides strong evidence that urea binds to the nickel ion. Similarly, hydroxamic acids and phosphoroamides are potent inhibitors of urease. However, experiments conducted in our laboratory suggest that in long-term kinetic studies (not dealing with inhibition of the enzyme by various inhibitors, but in the hydrolysis of urea by urease), deactivation of urease by ammonia and urea is a major problem, and the focus in the following paragraphs is to examine this.

Representative plots of the data are shown in Figs. 1 and 2, in which with exit urea concentration is plotted against time. Results from experiments conducted with and without Zeolite W are plotted on the same graph for comparison. In separate control experiments, it was found that the background level of deactivation (determined with buffer alone) was less than 0.5% over the 2 d period. In another control experiment, the effluent sample was analyzed for urea concentration immediately upon collection, and after a 24 h period. No leaching of the enzyme from the

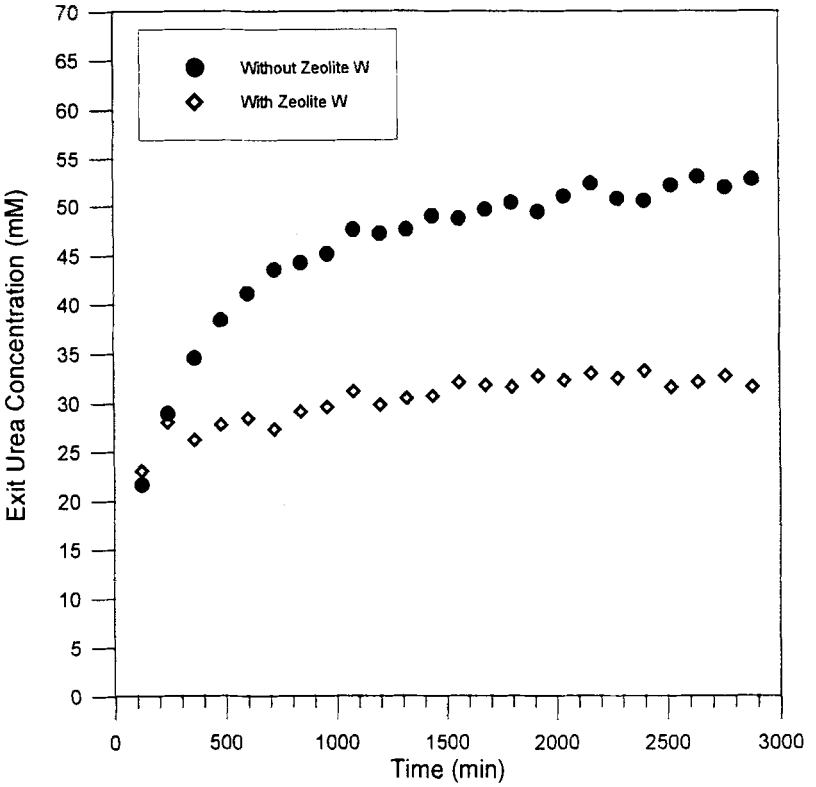


Fig. 2. Exit urea concentration vs time. Feed urea concentration = 100 m*M* (without Zeolite W), and 94.34 m*M* (with Zeolite W), zeolite loading = 80 g/L, wt. of enzyme = 4 g, temperature = 25°C.

beads was detected, as evidenced by the lack of change in urea concentration in the samples collected.

It is clear from the plots that deactivation of urease is reduced in the presence of zeolite. As a quick check on the extent of deactivation, a linear regression analysis was performed to quantify the deactivation in both sets of experiments (with and without Zeolite W). Though this is a simplistic approach, it sheds light on the rate of deactivation both in the presence and in the absence of ammonia. A representative plot is shown in Fig. 3 for 50 m*M* urea concentration. The data are tabulated in Table 1. Results are compared in terms of the % loss in conversion per h.

The results from Table 1 indicate that there is at least a twofold reduction in deactivation rate in experiments conducted with Zeolite W. It therefore appears that deactivation of urease by ammonia is reduced in the presence of Zeolite W. Previous studies by Vasudevan et al. (9) on the deactivation of urease have indicated that both ammonia and urea are responsible for the deactivation of the enzyme. The authors further proposed a deactivation model to explain the deactivation mechanism for the ammonia attack on urease wherein the free form of urease (*E*) was found to be most susceptible to attack by ammonia.

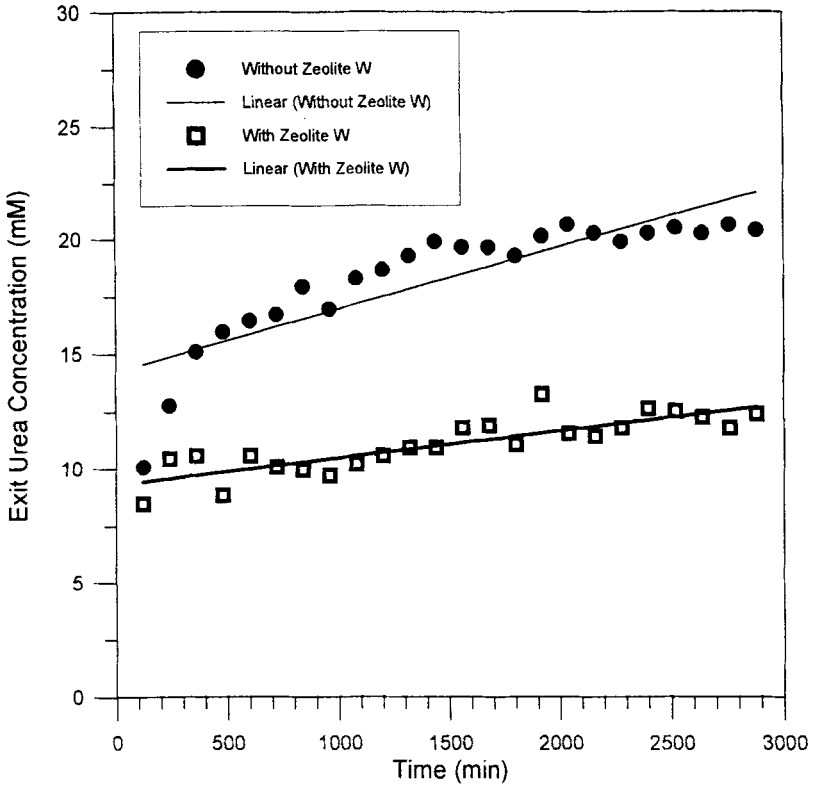


Fig. 3. Regression analysis: exit urea concentration vs time. Feed urea concentration = 50 m*M* (without Zeolite W), and 48.54 m*M* (with Zeolite W), zeolite loading = 40 g/L, wt. of enzyme = 4 g, temperature = 25°C.

Table 1
Comparison of Deactivation Data

| Feed urea concentration | Without Zeolite W, % conv/hr | With Zeolite W, % conv/hr |
|---|---|---|
| 10 | 0.33 | 0.1 |
| 20 | 0.18 | 0.09 |
| 50 | 0.33 | 0.15 |
| 100 | 0.48 | 0.15 |

The deactivation model for the deactivation of urease by substrate urea as proposed by Vasudevan et al. (*9*) is now considered. For substrate poisoning of urease in the absence of ammonia, the following deactivation equations can be written:

$$E + S \xrightarrow{k_d(1)} I \qquad n = 1 \tag{1}$$

$$ES + S \xrightarrow{k_d(2)} I \qquad n = 2 \tag{2}$$

Here, $n = 1, 2$ corresponds to the poisoning of the enzyme in either the free form or in a form complexed with the substrate, and $I$ is an inactive complex. Using a quasi-steady-state approximation (*9*), the following deactivation rate expression for urease deactivation by substrate urea is obtained:

$$V_{d(n)} = k_d K_m [E_T][S]^n / \{(K_m + [S] + K_I[S]^2)(1 + K_I'[P])\} \quad (3)$$

The above deactivation rate expression is of the same general form as the main rate reaction expression (see [*9*] for details)

$$V = V_{max}[S] / \{(K_m + [S] + K_I[S]^2)(1 + K_I'[P])\} \quad (4)$$

Since the experiments were conducted with urea concentrations ranging from 10 m*M* to only 100 m*M*, the contribution of the substrate inhibition term is small and, hence, can be neglected. The substrate inhibition becomes significant only when the urea concentration is above 0.3*M* (*18*). Likewise, the ammonia inhibition term can be neglected as the ammonia produced in the reaction is adsorbed by Zeolite W. Furthermore, ammonia inhibition becomes significant only above an ammonium ion concentration of 100 m*M* (*19*). The deactivation rate expression can therefore be written as

$$V_{d(n)} = k_d K_m [E_T]_t [S]^n / (K_m + [S]) \quad (5)$$

Assuming that the free form of the enzyme is most susceptible to poisoning by urea ($n = 1$), and expressing the deactivation rate expression in terms of the activity, $a$, we get the following expression:

$$-da/dt = k_d K_m [S] a / (K_m + [S]) \quad (6)$$

Taking deactivation into account, the main reaction rate expression can be expressed as

$$V = V_{max}[S] a / (K_m + [S]) \quad (7)$$

An unsteady state CSTR balance for the hydrolysis reaction in terms of urea can be written as

$$Q([S_o] - [S]) - V_{max}[S] W a / (K_m + [S]) = v\, d[S]/dt \quad (8)$$

where $Q$ is the flow rate, $[S_0]$ is the feed urea concentration, $[S]$ is the exit urea concentration, $v$ is the volume of the reactor, and $W$ is the weight of the enzyme. Eqs. 6 and 8 were solved numerically using a Runge Kutta scheme to obtain the urea concentration and activity-time profile. The deactivation rate constant is a fitted constant in the equation. The computer-generated profile of the exit urea concentration vs time was then compared with the experimental data at the same feed urea concentration. This trial and error procedure was followed until a reasonable fit between the predicted profile and the experimental data was obtained. The other parameters used in solving the equations were: $Q = 0.8$ mL/min, $v = 35$ mL/min, $W = 4$ g, and $S_0 = 10, 20, 48.54, 94.34$ m*M*, $K_m = 182$ m*M* and $V_{max} = 154$ IU/g. The values of the substrate concentration are slightly different for

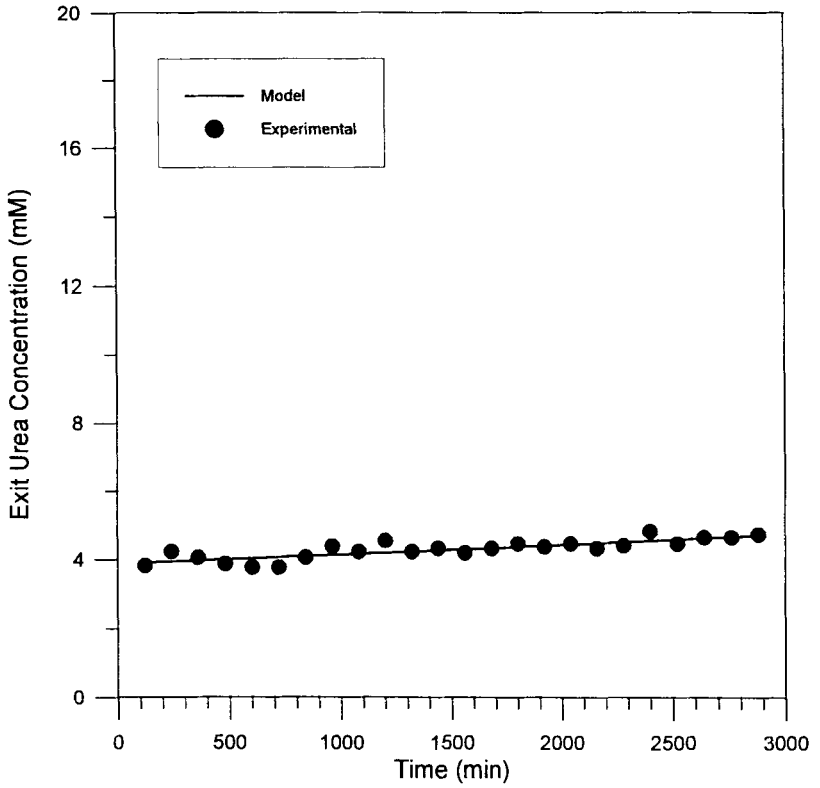


Fig. 4. Experimental vs model: experimental – feed urea concentration = 20 m*M*, zeolite loading = 16 g/L, wt. of enzyme = 4 g, temperature = 25°C.

the 50 and 100 m*M* concentrations, since these have been adjusted for the volume change encountered when zeolite is added. The volume change for the 10 and 20 m*M* concentrations is negligible. Pore diffusion was not considered to be a problem, since fluorescent spectroscopic studies with the same alumina support and the enzyme catalase have indicated that the enzyme is confined to a thin outer shell (*11*).

A similar procedure was followed with the assumption that the enzyme–substrate complex, and not the free enzyme, is more susceptible to attack by the substrate urea ($n = 2$). A representative plot of the model vs experimental data is shown in Fig. 4 for $n = 1$. The values of the deactivation constant for $n = 1$ and $n = 2$, which gave the best fit to the experimental data, are compiled in Table 2 as a function of the urea concentration.

It is clear from Table 2 that for $n = 1$, the deactivation rate constant is of the same order of magnitude over the range 10 m*M* to 100 m*M* of urea. However, for $n = 2$, there is a two-order-of-magnitude difference in the deactivation constant. This suggests that the deactivation of urease by urea follows a mechanism in which the free form of urease appears to be most susceptible to attack by the substrate urea. The slight difference in the values of the deactivation constant at different urea concentrations,

Table 2
Dependence of Urea Deactivation Constant ($k_d$) on $n$

| Urea concentration, m$M$ | $k_d$, m$M$-min$^{-1}$ ($n$ = 1) | $k_d$, m$M^{-2}$ min$^{-1}$ ($n$ = 2) |
|---|---|---|
| 10 | $3.4 \times 10^{-5}$ | $2.2 \times 10^{-5}$ |
| 20 | $1 \times 10^{-5}$ | $0.5 \times 10^{-5}$ |
| 48.54 | $0.5 \times 10^{-5}$ | $9 \times 10^{-7}$ |
| 94.34 | $0.5 \times 10^{-5}$ | $4 \times 10^{-7}$ |

observed in Table 2, may be attributed to incomplete or slow trapping of the ammonia by the zeolite.

The results presented in the previous paragraphs reiterate the conclusions of Vasudevan et al. (*9*) that both ammonia and urea are responsible in deactivating urease. In both cases, it appears that the free form of the enzyme is most susceptible to attack by ammonia (in the absence of urea) and by urea (in the absence or at low ammonia concentrations). The combined effect of simultaneous poisoning of the enzyme urease by both substrate and product is not known. As a first step, the combined effect can be viewed as the sum of the individual deactivation rates (Occam's razor). Although this approach is simplistic, it is certainly worth examining. As proposed by Vasudevan et al. (*9*), the deactivation rate expression for ammonia attack is

$$V_d' = -da/dt = k_d' K_m [P] a / \{ (K_m + [S] + K_I [S]^2)(1 + K_I' [P]) \} \quad (9)$$

Neglecting substrate inhibition for the reasons cited earlier, the additive model can be written as

$$-da/dt = k_d K_m [S] a / (K_m + [S]) + k_d' K_m [P] a / \{ (K_m + [S])(1 + K_I' [P]) \} \quad (10)$$

An unsteady state substrate balance for a CSTR is given by

$$Q([S_0] - [S]) - V_{max} [S] W a / \{ (K_m + [S])(1 + K_I' [P]) \} = v\, d[S]/dt \quad (11)$$

Eqs. 10 and 11 were solved numerically using a Runge Kutta scheme. The additive model was evaluated using the experiments conducted without Zeolite W. The only unknown in Eqs. 10 and 11 is the ammonia deactivation rate constant $k_d'$. The values for $k_d$ (urea deactivation constant) used in the simulation are the ones shown in Table 2. Two cases were considered while solving the above differential equations. In case I, the ammonium inhibition constant, $K_I'$ was set to zero, and in case II, the ammonia inhibition was taken into account. The value of $K_I'$ used in this case was 8.7-$M^{-1}$. Representative plots for the two cases are shown in Figs. 5 and 6.

It is clear from the figures that the additive model is somewhat inadequate in explaining the deactivation of urease in the presence of both urea

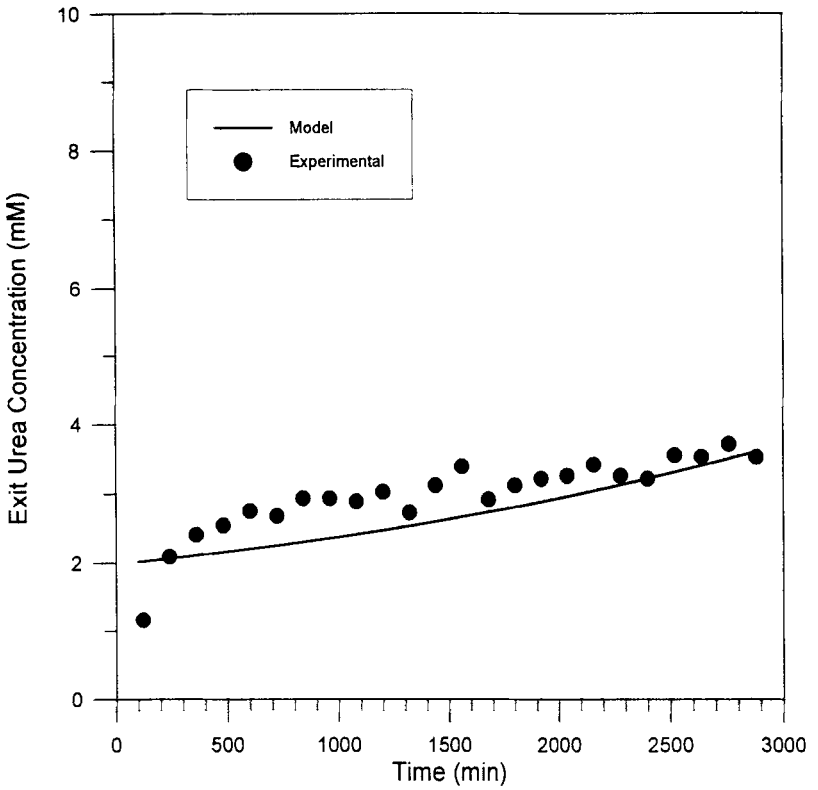


Fig. 5. Experimental vs additive model: experimental – feed urea concentration = 10 m*M*, wt. of enzyme = 4 g, temperature = 25°C.

and ammonia, especially at higher concentrations of urea. It is important to note that before starting the feed to the reactor, the reactor was first filled with substrate of the desired concentration and a known amount of the immobilized urease. In Figs. 5 and 6, for example, the starting concentrations are 10 m*M* and 50 m*M*, respectively. At the higher concentration of 50 m*M*, it is possible to adjust the value of $k_d'$ to get a good fit during the initial time period or towards the end of the run, but not over the whole time period. The ammonia inhibition term appears to have little effect on the deactivation rate in the 10–50 m*M* urea concentration range. For the 10, 20, and 50 m*M* urea concentrations, the ammonia deactivation rate constants (Table 3) are almost the same for cases I and II. For the 100 m*M* urea concentration, however, the ammonia deactivation rate constants in the two cases are markedly different. This is not surprising, since, at this concentration, inhibition by ammonia can be expected to be significant (*19*). It is clear that combined deactivation by ammonia and urea is much more complex than deactivation by ammonia or urea alone. Previous studies by Vasudevan et al. (*9*) in a packed bed reactor have clearly demonstrated that the greatest loss in activity occurred with a feed

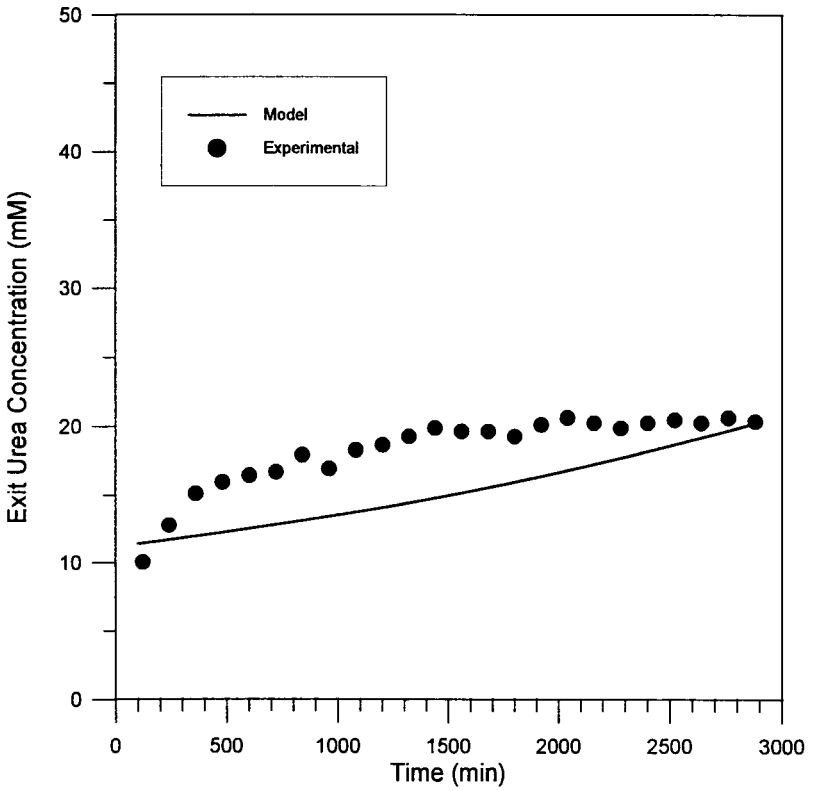


Fig. 6. Experimental vs additive model: experimental – feed urea concentration = 50 m*M*, wt. of enzyme = 4 g, temperature = 25°C.

Table 3
Deactivation Constants

| Feed urea concentration, m*M* | Urea deactivation constant, $k_d \times 10^5$ (1/m*M*-min) | CASE I, ($K_I = 0$) Ammonia deactivation rate constant, $k'_d \times 10^5$ (1/m*M*-min) | CASE II, ($K_I = 8.7M^{-1}$) Ammonia deactivation rate constant, $k'_d \times 10^5$ (1/m*M*-min) |
|---|---|---|---|
| 10 | 3.4 | 4 | 3.8 |
| 20 | 1 | 2.4 | 2.2 |
| 50 | 0.5 | 1.1 | 0.8 |
| 100 | 0.5 | 0.65 | 0.3 |

containing only ammonia at 0.1*M*; the loss in activity experienced was 20.3% over an 8 d period. The loss in activity with 0.25*M* urea alone (without the adsorption of product ammonia) was 4%. This suggests that the presence of urea shields the enzyme partially from attack by ammonia, and gives some indication of the complexity of the deactivation process which is not represented by the additive model. The additive model is, however, reasonable at concentrations of urea around 10 m*M* or less. The above deactivation studies clearly shed light on the nature of the deactivation process by urea in the absence of ammonia.

## CONCLUSIONS

Deactivation studies on jack bean urease immobilized on porous alumina pellets have shown that the enzyme is deactivated both by substrate urea and the product of the hydrolysis reaction, ammonia. Deactivation experiments conducted with Zeolite W show at least a two-fold decrease in the deactivation rate of urease by ammonia when compared with experiments conducted without Zeolite W. The experimental runs conducted with Zeolite W facilitated the design of experiments for the study of urease deactivation by the substrate urea alone; the poisoning and inhibiting effect of ammonia were reduced by its effective adsorption on Zeolite W. The results also indicate that, in the absence of ammonia, the free form of urease appears to be most susceptible to attack by urea. The results from the study suggest that the life of the enzyme can be prolonged by adsorbing the ammonia as soon as it is formed.

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