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THE KINETIC PROPERTIES OF MICROENCAPSULATED UREASE

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SUMMARY

1. Spherical microcapsules of nylon (diameters ranging from 10 to 30 μm) enclosing aqueous solutions of urease (urea amidohydrolase, EC 3.5.1.5) were prepared by interfacial polymerization.

2. Urease was encapsulated both in the presence and absence of a foreign protein, haemoglobin.

3. The encapsulated enzyme follows Michaelis–Menten kinetics with optimum activity occuring at lower pH values than for the native enzyme in free solution.

4. The K_m values increase upon encapsulation, larger capsules showing a greater increase.

5. A theoretical treatment included deals with the effects of diffusion and mass transfer on the Michaelis–Menten kinetics of the encapsulated enzyme.

INTRODUCTION

Most enzymes occur in nature either attached to discrete particles such as the mitochondria within the cells or are contained in single-membrane vesicles such as the lysosomes and peroxisomes. The degree of freedom that these enzymes possess *in situ*, and the effect of the microenvironment upon them, has a bearing on their efficiency as catalysts and their mode of action. This is borne out by the fact that many of these enzymes show a change in their properties upon isolation and purification.

Recently, model systems of particle-bound enzymes have been prepared[1–3] with a view to investigating the influence of the microenvironment on the behaviour of these enzymes. However, simulating a lysosome with a model system is more complicated. Therefore, a relatively simple system was chosen for this study, in which microcapsules of nylon membrane enclosing aqueous droplets of enzyme were prepared by the method of Chang *et al.*[4].

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Microcapsules enclosing aqueous solutions of enzymes catalyze reactions that are restricted to the small volume of the capsules. The semipermeable membrane of the capsule is permeable only to small molecules of substrates and products whereas the large molecules of the enzyme protein are trapped inside.

Enzymic reactions that take place within such structures will encounter and be affected by principal factors such as diffusion, Donnan effects at the interface and the surface to volume ratio of the capsules.

Urease was chosen for this study because urea and NH_4^+ diffuse rapidly through the nylon membrane. Also, by virtue of its high turnover number the enzymic activity of urease may be easily estimated.

MATERIALS AND METHODS

Urease

Three different samples of urease of varying specific activities were used: Urease V (3500 Sigma Units/g or 306 I.U./mg) was a Sigma Chemical Co. product. Urease S and Urease S_2 were prepared from the first and second extracts, respectively, of Jack Bean meal with 0.014 M mercaptoethanol in 31.6% (v/v) acetone by the method of Mamiya and Gorin[5]. The specific activities of the two samples were 255 and 2970 I.U./mg, respectively. Bovine haemoglobin was a Worthington Chemical Corp. product. All other chemicals were of reagent grade.

Urease-loaded nylon capsules

Two types of urease-loaded capsules were made: one contained the enzyme dissolved in 10% haemoglobin solution made in 0.9% NaCl and the other type was loaded with an aqueous solution of urease. These will be referred to as Type-A and Type-B capsules, respectively. The total concentration inside the capsules has to be high to maintain a critical turgor pressure. This condition may be satisfied either by including a foreign protein or a high concentration of enzyme protein.

The microcapsules were made by the method of Chang *et al.*[4] with minor modifications as follows:

Type-A capsules. 25 mg of urease V were dissolved in 1.5 ml of 10% haemoglobin solution made in 0.9% NaCl containing 10^{-3} M EDTA and an equal volume of alkaline 1,6-hexamethylenediamine (0.4 M in 0.45 M $NaHCO_3$–Na_2CO_3 buffer of pH 9.8). This mixture kept at 0 °C was emulsified for 1 min with 15 ml of a mixed solvent of chloroform–cyclohexane (1:4, v/v) containing 12.5% (v/v) Span 85 as emulsifying agent. While this solution was still being stirred 15 ml of 0.018 M solution of sebacyl chloride made in chloroform–cyclohexane mixture mentioned above was injected from a syringe into it. To this mixture, 30 ml of the mixed solvent was added quickly and the mixture centrifuged at 350 × *g* for 15 s; following this the microcapsules in the suspension were dispersed in 25 ml of 50% (v/v) Tween 20 solution. This suspension was stirred for 1 min before 50 ml of 0.9% NaCl was added and centrifuged at 350 × *g* and the supernatant discarded. These microcapsules (average diameter 30 μm) were finally suspended in 0.9% NaCl containing 10^{-3} M EDTA and stored at 2 °C.

Type-B capsules. Essentially the same procedure as above was used in the preparation of Type-B capsules excepting that (1) the initial enzyme solution was

made at a concentration of 250 mg in 1.5 ml, (2) 15% (v/v) Span 85 was used as emulsifiying agent and (3) the capsules were finally suspended in 10^{-3} M EDTA solution. These smaller capsules (average diameter 10 μm) were made of urease S and S_2.

Microcapsule diameter

An aqueous suspension of microcapsules was placed in a hemocytometer chamber and photographed including the hemocytometer scale. The diameters of at least 200 microcapsules were measured[6], and in each case more than 85% of the capsules were of the diameter specified.

Enzyme activity measurements

Initial rates of activity of the native as well as the encapsulated enzyme were measured by the titrimetric method[7,8]. A uniform suspension of the capsules was first introduced into the reaction vessel of the titrator and the reaction was started by addition of the substrate. The rates were measured at 30 °C, Type-A and Type-B capsules being assayed at $I = 0.15$ and 0, respectively.

Protein measurements

Protein estimations were made by the method of Lowry *et al.*[9]. Enzyme concentrations in Type-A capsules were calculated by subtracting the residual amounts of protein left behind in the solution after the capsules formed were removed. However, a more satisfactory method was devised for the Urease S and S_2 capsules, which involved a hydrolysis of the nylon capsules with 4 M HCl before protein estimation. The original solutions used in making the capsules were used as controls. Enzyme concentrations were calculated as in Sundaram and Laidler[8].

RESULTS

Figs 1a and 1b show the pH-activity profiles of Type-A and Type-B microcapsules compared with the activity profiles of the respective samples of urease in free solution. The optimum activity in Type-A and Type-B capsules shift down to pH 5.8 and 5.0, respectively. Above pH 6.0 the enzyme Urease S_2 shows nearly identical pH-dependance in free solution as it does in capsules (Type-B) even though below pH 6.0 a divergence is seen. The curves of Type-A capsules and the free enzyme (Urease V) display similarity on the alkaline side, the capsules showing less noticeable drop in activity above pH 6.5 than the enzyme in free solution. The haemoglobin in Type-A capsules may be offering a slight buffering action even though there is no appreciable effect seen in free solution (unpublished results).

Rates varied linearly with enzyme concentration in the case of both Type-A and Type-B capsules. The Michaelis parameters of the reactions are given in Tables Ia and Ib. The specific activities of Type-A and Type-B capsules average around 35 and 90%, respectively.

The Michaelis constants of the enzymes increase upon encapsulation, the effect being more pronounced with the larger capsules. However, K_m values did not vary appreciably with pH thus resembling the native enzyme in free solution. These K_m values as well as the V values shown in Tables Ia and Ib were determined through

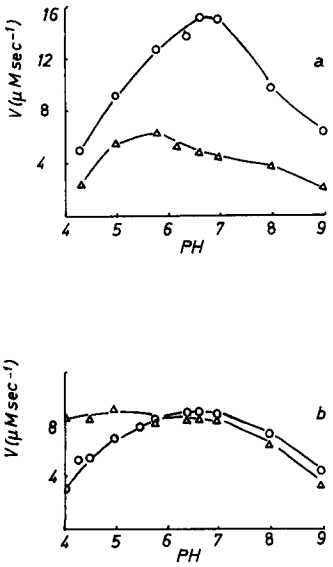

Fig. 1. (a) ○ and △ represent free Urease V and Type A capsules, respectively, ($E_0 = 8.55 \cdot 10^{-10}$ M). (b) ○ and △ represent free Urease S_2 and Type-B capsules, respectively, ($E_0 = 4.8 \cdot 10^{-10}$ M).

TABLE Ia

MICHAELIS PARAMETERS OF UREASE V AND TYPE-A CAPSULES

pH	*Free enzyme**		*Encapsulated enzyme***	
	V (μmoles/l per s)	*K_m (mM)*	*V (μmoles/l per s)*	*K_m (mM)*
4.3	4.0	2.4	2.22	6.89
5.0	7.3	2.7	5.46	8.06
5.8	10.06	2.5	6.17	11.22
6.4	10.9	2.1	5.12	9.4
6.65	12.08	2.1	4.69	7.66
7.0	12.0	2.76	4.32	9.09
8.0	7.65	2.35	3.63	7.68
9.0	5.04	2.2	2.04	5.89

TABLE Ib

MICHAELIS PARAMETERS OF UREASE S_2 AND TYPE-B CAPSULES

pH	*Free enzyme†*		*Encapsulated enzyme†*	
	V (μmoles/l per s)	*K_m (mM)*	*V (μmoles/l per s)*	*K_m (mM)*
4.0	3.08	2.0	8.48	2.5
4.3	5.3	2.1		
4.5	5.35	2.5	8.4	3.2
5.0	6.9	3.0	9.18	3.8
5.5	7.8	2.7		
5.8	8.5	2.0	8.25	3.5
6.4	8.6	2.4	8.3	3.4
6.65	9.0	2.2	8.41	2.77
7.0	8.8	2.4	8.33	3.4
8.0	7.25	2.5	6.55	3.4
9.0	4.35	3.2	3.22	3.2

* $E_0 = 6 \cdot 10^{-10}$ M
** $E_0 = 7.55 \cdot 10^{-10}$ M
† $E_0 = 4.8 \cdot 10^{-10}$ M

Lineweaver–Burk plots (Fig. 2) with rates obtained at substrate concentrations ranging between 10^{-1} and 10^{-3} M.

Microcapsules (average diameter 10 μm) of Urease S, the purest enzyme among the samples, had a specific activity averaging around 90% of that of the enzyme in free solution at pH 6.65. The k_{cat} and K_m values were $1.44 \cdot 10^4$ s^{-1} and 2.72 mM for the native enzyme and $1.3 \cdot 10^4$ s^{-1} and 3.2 mM for the encapsulated enzyme, respectively.

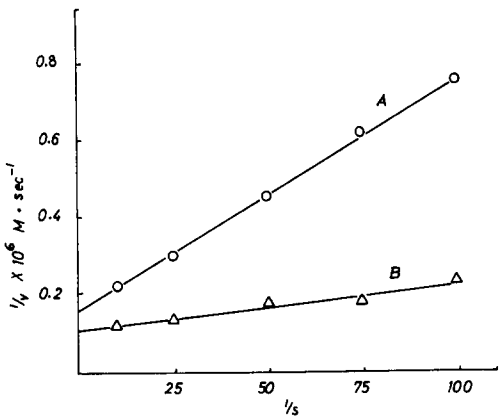

Fig. 2. Lineweaver–Burk plots of Type-A and Type-B capsules at pH 5.8 and 5.0, respectively.

DISCUSSION

The kinetic parameters of an encapsulated enzyme may be perturbed by diffusion, ion-enchange and permselective properties of the capsule membrane, change if any of the conformation of the enzyme suffered during the process of encapsulation and effects of the microenvironment.

Shifts in pH optima may be caused by the presence of a pH gradient in enzyme reactions at solid–liquid interfaces[10] as seen in the case of particle-bound and membrane-bound enzymes[3].

A pH gradient usually develops as a result of the presence of charges on the solid phase. Except at extremes of pH the net effective charge on the nylon is zero so that no pH gradient should develop for this reason. Besides it is seen by calculation that proton diffusion across the nylon membrane is several orders of magnitude faster than the rate at which the product is formed in a capsule. A downward shift in pH optimum would occur if the pH inside the capsule is higher than that outside, and this may be caused by NH_3 liberated from urea hydrolysis being spontaneously protonated to form NH_4^+. Even though proton diffusion across the membrane is rapid enough to offset the build up of any pH gradient at most pH values, permeability studies (Chang, T. M. S., personal communication) show that the membrane becomes cation exclusive at acid pH values. This is due to the alignment of the terminal NH_3^+ and COO^- groups of the amphoteric nylon in such a way that the product NH_4^+, resulting from urea hydrolysis are trapped inside the capsules; at the same time the protons from outsise are prevented from entering the capsules. If indeed the shift

in pH optima is due to an increase in NH_4^+ inside the capsule, then the less pronounced shift in Type-A capsules may be due to (1) a certain degree of buffering provided by the haemoglobin even though it does not otherwise influence urease activity in free solution, and (2) a reduction in cation exclusion caused by the screening effect of high ionic strength.

Whereas the diffusion of the product H^+ affects the apparent pH characteristics of the encapsulated enzyme, substrate diffusion would perturb the initial rates and K_m values. The diffusion of urea through the nylon membrane is sufficiently rapid and the rate varies with the diameter of the capsules[11]. Type-A capsules, which are larger in size, have higher K_m values and lower specific activity, whereas the smaller, Type-B capsules are more active and show less enhancement in their K_m values.

Conformational changes may be induced in the enzyme due to the cross-linking of the protein to the capsule membrane to some extent. However, since the enzyme recovered from the capsule still retains the original kinetic properties (Chang, T. M. S., personal communication) it appears that if there is any change in conformation it is reversible.

The presence of a foreign protein such as haemoglobin reduces the extent of cross-linking of the enzyme to the capsule membrane. This could be an additional cause for the less pronounced shift in the pH optimum of Type-A capsules. pK values of the ionizing groups on the active site of the enzyme obtained from plotting log V against pH (ref. 12) gave values of 3.75 and 9.0 for pK_b' and pK_a' for the Type-A capsules, thus showing a change in pK_b' from 4.5 obtained for the native enzyme[8]. For Type-B capsules the change must be even more appreciable since below pH 7.0 the rates remain constant.

Organic solvents and the surfactants used in the preparation of the capsules may have a dual effect on the enzyme, one directly on the physicochemical structure of the protein and the other which may act indirectly through a change in the dielectric constant of the immediate environs of the enzyme.

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APPENDIX

Theoretical

In the experimental section we discussed the results obtained with microencapsulated urease and here we examine mainly the implications and the effects of mass transfer and diffusion on the Michaelis–Menten kinetics of the enclosed enzyme.

Fig. 3 represents a microcapsule with the substrate concentrations inside and

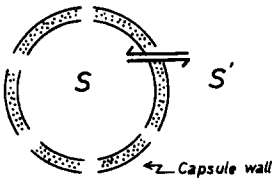

Fig. 3. A graphic representation of a semipermeable nylon microcapsule with the substrate concentrations inside and outside being denoted by S and S'.

outside being denoted by S and S'. The external solution is assumed to be well stirred and the capsules dilute enough so that there is little interaction between capsules. A material balance in a capsule yields

$$4\pi r^2 K(S' - S) = \frac{4}{3}\pi r^3 \left(\frac{VS}{K_m + S} + \frac{dS}{d\theta}\right) \tag{1}$$

and in the steady state,

$$4\pi r^2 K(S' - S) = \frac{4}{3}\pi r^3 \frac{VS}{K_m + S} \tag{2}$$

The reaction rate is assumed to be given by Michaelis–Menten kinetics and K is an overall mass transfer coefficient which accounts for the mass transfer resistance in the external fluid, the capsule wall and the internal fluid. Explicitly,

$$\frac{1}{K} = \frac{1}{k'} + \frac{1}{P} + \frac{1}{k} \tag{3}$$

where k' is the external mass transfer coefficient. It will be seen later that $K \simeq P$ for the conditions of interest here so the possible dependence of k on the chemical reaction is not troublesome.

The solution to Eqn 2 which yields positive values of S is

$$\frac{S}{S'} = \frac{1}{2}\left(M - 1 + \frac{K_m}{S'}\right)\left(-1 + \sqrt{1 + \frac{4K_m/S'}{\left(M - 1 + \frac{K_m}{S'}\right)^2}}\right) \tag{4}$$

The quantity M is given by

$$M = \frac{Vr}{3DS'} \tag{5}$$

and this dimensionless number is a measure of the rate of the reaction relative to the rate of the mass transfer.

If V_c is the reaction rate per unit volume in the capsules,

$$\frac{V_c}{V} = \frac{S}{K_m + S} = \frac{S/K_m}{1 + S/K_m} \tag{6}$$

The reaction rate per unit volume of total solution (capsules *plus* substrate) is then given by

$$V = V_c \cdot \frac{\text{volume of capsules}}{\text{volume of total solution}} \tag{7}$$

combining Eqns 4 and 6

$$\frac{V_c}{V} = \frac{\left(M - 1 + \frac{K_m}{S'}\right)\left(-1 + \sqrt{1 + \frac{4K_m/S'}{\left(M - 1\frac{K_m}{S'}\right)^2}}\right)}{\frac{2K_m}{S'} + \left(M - 1 + \frac{K_m}{S'}\right)\left(-1 + \sqrt{1 + \frac{4K_m/S'}{\left(M - 1 + \frac{K_m}{S'}\right)^2}}\right)} \tag{8}$$

For small values of M

$$S \to S', \text{ and } \frac{V_c}{V} \to \frac{S'}{K_m + S'} \tag{9}$$

At this limit the diffusion is rapid enough to keep the substrate concentration inside the capsule at the same concentration as in the free solution. Since the reaction rate is proportional to the amount of enzyme present it follows that the reaction rate per unit volume of total solution is the same as it would be if the capsules were not present and the enzyme in the capsules was dispersed throughout the total volume.

For large values of M

$$\frac{V_c}{V} \to \frac{1}{M} \tag{10}$$

Figs 4a and 4b which are plots of Eqn 8, show how V_c/V varies as M varies between the two limits. Fig. 4b is drawn to a larger scale which corresponds to the initial, linear region of the curves in Fig. 4a.

On the other hand if maximal rate in free solution is represented by

$$V^* = \frac{VS'}{K_m + S'} \tag{10a}$$

and $V_c = k^{\ddagger} V$ (where $k^{\ddagger}$ is a constant)

$$\text{then } V_c = k^{\ddagger}\frac{VS'}{K_m + S'} \text{ and} \tag{10b}$$

$$k^{\ddagger} = \frac{V\left(\frac{S}{K_m + S}\right)}{V\left(\frac{S'}{K_m + S'}\right)} \tag{10c}$$

then it may be shown as in Fig. 5 as $M \to 0$ the rate asymptotes to the values of $k^{\ddagger} = 1$.

Measurement of K

If K is assumed for the moment to be independent of the chemical reaction, it may be measured by carrying out transient experiments on the uptake of substrate by the capsules in the absence of reaction. For this case Eqn 1 yields

$$\frac{S}{S'} = 1 - e^{\frac{-3K\theta}{r}} \tag{11}$$

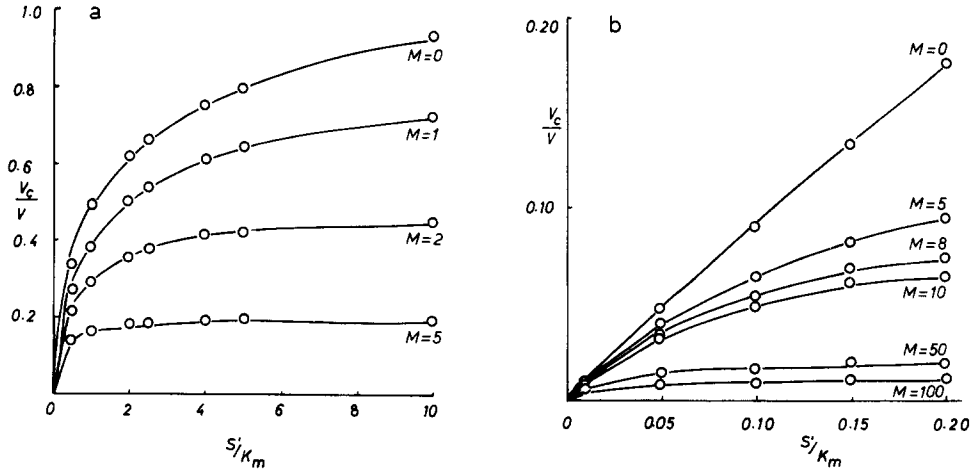

Fig. 4. (a) Plot of Eqn 8 to show how V_c/V varies as M varies for various values of S'/K_m. (b) A plot of Eqn 8 expanding the initial linear region of the curves in (a).

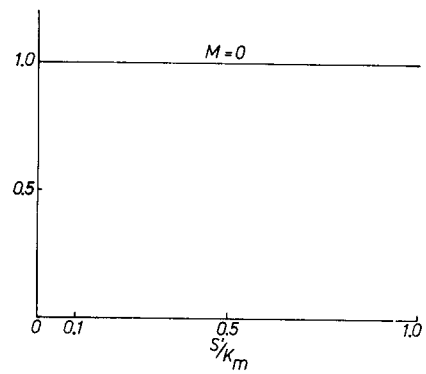

Fig. 5. Plot of Eqn 10c to show the effect of $k^{\pm}$ (ordinate) and M on V_c the reaction rate of the microencapsulated enzyme.

so

$$\frac{3K}{r} = \frac{\ln 2}{\theta_{1/2}} \tag{12}$$

where $\theta^1/_2$ is the time for S/S' to rise to 1/2.

Experiments with 10 μm radius nylon microcapsules using 0.05 M urea as the substrate gave a half life of 4.5 s (ref. 4). Hence for this system Eqn 12 yields a value of K of 10^{-4} cm/s.

The maximum value of k' is given by the value obtained for the steady diffusion of the substrate from an infinite medium to the surface of the microcapsule. This value is

$$k' = \frac{D}{r} \tag{13}$$

where D is the diffusivity of the substrate in the solution.

The maximum value of k' is given by the value obtained for the steady diffusion of substrate through a liquid film of thickness $r/2$ which in this case is twice the value given by Eqn 13. Hence Eqn 3 becomes

$$\frac{1}{K} \simeq \frac{3}{2}\frac{r}{D} + \frac{1}{P} \tag{14}$$

Taking $D \simeq 10^{-5}$ cm^2/s and using the experimental value of K

$$10^4 \simeq 10^2 + \frac{1}{P}$$

Hence $K \simeq P$

so that membrane transport is controlling relative to internal and external mass transfer. This suggests that the assumption that K is independent of the chemical reaction is justified. Because P is so much smaller than k and k', it appears that with the present system, at least, membrane resistance will control for capsules whose radii are in the microcapsule range, $< 100\ \mu$m.

If we consider a urea-urease reaction in the microcapsules considered earlier with a value of $V = 8 \cdot 10^{-6}$ M$\cdot$s^{-1} and a value of S' equal to 0.05 M we obtain

$$M = 2.5 \cdot 10^{-4}$$

which is so much less than 1 and as suggested by Fig. 4a and 4b the reaction approaches that in free solution and so the reaction is controlling.

Transient period

When the substrate is brought into contact with the enzyme containing microcapsules there is a transient period during which the substrate concentration in the capsule is rising to its steady value. This transient is given by the solution to Eqn 1.

For small values of M (slow reaction) the transient is described by Eqn 11 since negligible reaction takes place during the transient period. Hence, in this case, the time to reach 99% of the steady state substrate concentration is given by

$$\theta = 1.54\frac{r}{K}$$

which yields a transient time about 1.5 s for the earlier example.

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