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THE KINETIC BEHAVIOUR OF ACTIVE SITES PRESENT IN A STRUCTURED ENVIRONMENT

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SUMMARY

We show that an enzyme, when embedded in a matrix which allows access to the active site only via an unstirred layer of fluid will, as a direct approximation, still show rectilinear Lineweaver-Burk plots. However, the apparent K_m will depend upon Δ , the reduced diffusional transport coefficient. This is defined as D/δ , where D is the diffusion coefficient of the substrate and δ is the thickness of the layer through which diffusion has to take place. Different Lineweaver-Burk plots obtained at different numbers of active sites per unit area of matrix will intercept at $(-1/K_m, -1/\Delta K_m)$. K_m and Δ can then be determined directly.

INTRODUCTION

In all instances where an active site is located in a molecule that is large compared to the solvent and substrate molecules, the site will be surrounded by a layer of unstirred solvent. Diffusion of the substrate through this layer will cause a bias in the estimation of the kinetic properties of the active site [1]. This is a very common problem in biology. It will occur in all active or transport sites located in membranes for instance.

It will be shown here that under these conditions the data obtained from rate measurements still can be used to obtain the true Michaelis constant of the active site as well as the coefficient governing the diffusion.

Unstirred layers disturb the kinetics of several types of experiments viz. carrier mediated transport systems [1-3], enzymes immobilized in solid matrices [4], enzymes adsorbed at an interphase [5], enzymes located in (particles derived from) natural membranes, enzymes arising from the interaction of 2 or more proteins at an interphase [6]. The results shown here pertain to these different fields.

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The diffusional transport through the unstirred layer will be proportional to the difference in concentration of the substrate in the bulk phase and at the active site. We call the proportionality constant involved the reduced diffusional transport coefficient (Δ). The real situation can be depicted by a model in which the active site is surrounded by a completely unstirred layer of thickness δ in contact with a completely stirred bulk phase. Because of the stirring, diffusion in the bulk phase can be neglected. If the diffusion coefficient in the unstirred layer is D , $\Delta = D/\delta$. As there will be no sharp border to the unstirred layer, and as the diffusion coefficient is not necessarily uniform throughout the layer — e.g., because of changes in water structure near the interphase — we prefer to use the reduced diffusional transport coefficient as such. That means that we model the boundary layer as a layer of defined thickness δ and uniform diffusion coefficient D .

We will use the following formalism:

- v = transport rate per unit area ($\text{mol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$)
- S = concentration of the substrate in the bulk phase ($\text{mol} \cdot \text{cm}^{-3}$)
- S_s = concentration of the substrate at the active site ($\text{mol} \cdot \text{cm}^{-3}$)
- V = maximal chemical turnover rate per unit area ($\text{mol} \cdot \text{sec}^{-1} \cdot \text{cm}^{-2}$)
- Δ = reduced diffusional transport coefficient ($\text{cm} \cdot \text{sec}^{-1}$)
- K_m = Michaelis constant ($\text{mol} \cdot \text{cm}^{-3}$).

Under steady state conditions the velocity of transport to the active site (v_{diff}) and the conversion at the active site (v_{chem}) will be equal. Movement from the active site can be either via transport through a membrane (in case of carrier mediated transport systems) or diffusion of the product into free solution (in case of enzymes on a solid support). We will assume simple steady state kinetics to apply to the chemical conversion. The basic equations are:

$$v_{\text{chem}} = \frac{VS_s}{K_m + S_s} \quad (1)$$

$$v_{\text{diff}} = \Delta (S - S_s) \quad (2)$$

$$v_{\text{chem}} = v_{\text{diff}}$$

from which it follows that

$$v^2/\Delta - (S + K_m + V/\Delta)v + VS = 0 \quad (3)$$

so that,

$$v = \frac{1}{2}(\Delta S + \Delta K_m + V) - \frac{1}{2}\sqrt{(\Delta S + \Delta K_m + V)^2 - 4\Delta VS} \quad (4)$$

a positive sign after the first term would not be realistic as $v = 0$ when $S = 0$.

$$\text{We define } \alpha = 4\Delta VS/(\Delta S + \Delta K + V)^2, \quad (5)$$

hence

$$v = \frac{1}{2}(\Delta S + \Delta K_m + V) (1 - \sqrt{1 - \alpha}). \quad (6)$$

It can be easily proven that $\alpha < 1$, hence the square root can be developed into a series

$$\sqrt{1 - \alpha} = 1 - \frac{1}{2}\alpha - \frac{1}{8}\alpha^2 \text{ etc.}$$

When this series is approximated by its first 2 terms, Eqn. 6 reduces to the convenient equation

$$v = \Delta VS/(\Delta S + \Delta K_m + V). \quad (7)$$

Before applying this formula it will be investigated under what conditions the approximation $\sqrt{1 - \alpha} = 1 - \frac{1}{2}\alpha$ is acceptable. The error brought about by this approach will in first approximation be equal to the first neglected term. The relative error (e) therefore will be

$$e = \alpha^2/(8 - 4\alpha). \quad (8)$$

Eqn. 5 can be written as

$$4/\alpha = \Delta S/V + V/\Delta S + (\Delta K_m/V + 2)(K_m/S + 2) - 2 \quad (9)$$

introducing the new variable $f = \Delta S/V$ this becomes

$$4/\alpha = 1/f + f(K_m/S + 1)^2 + 2(K_m/S + 1) \quad (10)$$

this will be maximal for $f = S/(K_m + S)$

$$\text{in that case also } \alpha = S/(K_m + S). \quad (11)$$

As long as $S \leq K_m$, α will be ≤ 0.5 , hence e will be less than $\approx 4\%$. This in most cases will be smaller than the experimental error. Even under unfavourable circumstances Eqn. 7 therefore will give an acceptable approximation as long as $S \leq K_m$. It must also be borne in mind that the most unfavourable condition only occurs at one fixed concentration (viz., at $\Delta S = V$), smaller errors will be involved at all other concentrations used.

When the numerical values given by Winne [1] are used to calculate the error brought about by the series development, we calculate $\Delta (=D/\delta)$ to be about $5 \times 10^{-5} \text{ cm} \cdot \text{sec}^{-1}$. ΔS will be $5 \times 10^{-7} \text{ M} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$ maximally. V is given as about $2 \times 10^{-8} \text{ M} \cdot \text{cm}^{-2} \cdot \text{sec}^{-1}$. For this case, the first term of the right hand part of Eqn. 9 is calculated to be about 25. Via Eqn. 8 one

then finds that the error will remain below 0.5% as long as S is larger than $0.025 K_m$. The use of Eqn. 7 therefore seems justified to evaluate the kinetic data.

Eqn. 7 predicts a straight line relationship between $1/v$ en $1/S$. That part of a Lineweaver-Burk plot that cannot be distinguished from a straight line can be used to estimate K_m , V and Δ when it is possible to carry out experiments at different V , i.e., at different numbers of active sites per unit area. With enzymes on a solid support this can be realised by incorporating more or less enzyme per unit area of the interphase and, in carrier mediated transport, by irreversible inhibition of transporting sites.

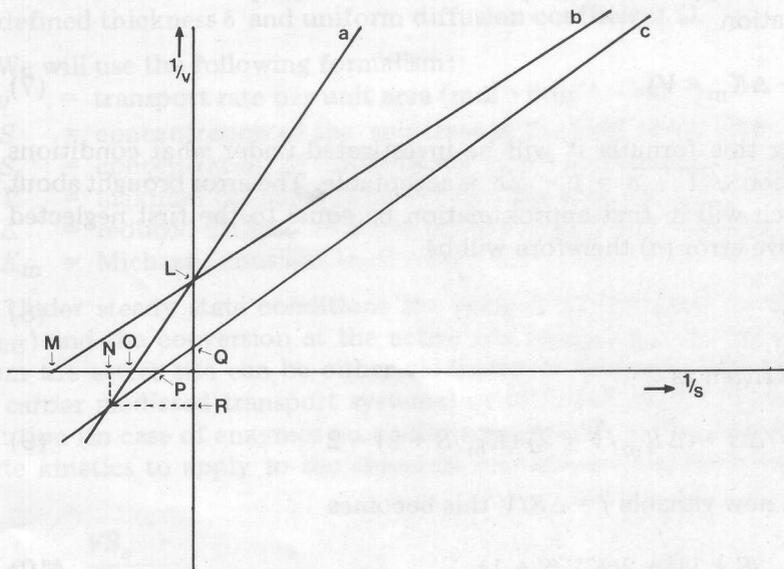


Fig. 1. Theoretical Lineweaver-Burk plots obtained in a system where diffusion is rate-limiting.

(a) Relationship obtained in a system with given V , Δ and K_m

$$1/v = \left(1/V + \frac{K_m}{V} + \frac{1}{\Delta} \right) \frac{1}{S}$$

(b) The same at a higher value of Δ ($= \Delta'$) and same V and K_m

$$1/v = 1/V + \left(\frac{K_m}{V} + \frac{1}{\Delta'} \right) \frac{1}{S}$$

(c) The same at a higher value of V ($= V'$) and same Δ and K_m

$$1/v = 1/V' + \left(\frac{K_m}{V'} + \frac{1}{\Delta} \right) \frac{1}{S}$$

TABLE I

Summary of the data obtained from Fig. 1.

$$L = 1/v;$$

$$M = -1/(K_m + V/\Delta');$$

$$N = -1/K_m;$$

$$O = -1/(K_m + V/\Delta);$$

$$P = -1/(K_m + V'\Delta);$$

$$Q = -1/V';$$

$$R = -1/\Delta K_m.$$

With different values for V in the Lineweaver-Burk plot lines are obtained that intersect at the point $(-1/K_m; -1/\Delta K_m)$, yielding both K_m and Δ . Changing v by changing the rate of stirring (and thereby Δ) does not allow to estimate K_m . All lines obtained by varying S at different constant values of Δ will intersect at $(0; 1/V)$ and the intercept with the abscissa will be at $(-1(K_m + \Delta V); 0)$.

The observation of an apparent K_m diminishing with the stirring rate will be indicative of unstirred layers disturbing the kinetics of a given system. In any case this is an experimental check, that should be carried out whenever possible because Lineweaver-Burk plots at varying enzyme concentrations intercepting at a point in the third $(-, -)$ quadrant can be obtained under circumstances where there is no unstirred layer present, e.g., when the substrate is not present in a concentration much larger than the enzyme [7].

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