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AN ANALYSIS AND INTERPRETATION OF THE MICHAELIS–MENTEN KINETICS OF IMMOBILIZED ENZYMES PERTURBED BY FILM DIFFUSION

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The determination of kinetic parameters such as the apparent Michaelis constant (K_m app.) of enzymic reactions occurring at a mixed interface, that is, at a solid/liquid interface where the catalyst is bound to the solid phase, can lead to values that are too high when an unstirred layer is present. The perturbation, for which an equation is derived in this paper, depends on the thickness of the unstirred layer, the maximal catalytic rate, and the diffusion constant. An additional error is introduced by the graphical determination of K_m using the Lineweaver–Burk plot since after transformation the relation is not linear if an unstirred layer is present. A numerical example shows that apparent Michaelis constants obtained from in vitro experiments involving immobilized enzymes can lead to misleading results originating from conditions used in experiments such as stirring rate (CSTR-continuous stirred tank reactors) or flow rate (packed-bed reactors) of substrate. This can partly be explained by a greater bias of the K_m determination caused by unstirred layers of the liquid medium (substrate) being present around the solid particles onto which the enzyme molecules are attached.

INTRODUCTION

Diffusion plays a significant part in the overall efficiency of catalytic reactions that occur at mixed interfaces. Since the early 1960s the work done with immobilized enzymes, that is, enzymes that have been attached to insoluble polymeric matrices of various chemical and physical properties (1–3), has often shown how the diffusion of the substrate onto the active site of the enzyme molecules present on the insoluble polymeric matrix affects not only the catalytic efficiency of the enzyme but has also led to some anomalous kinetics.

Diffusion may be classified largely into the following:

(a) External mass transport
(b) Internal mass transport
(c) Film diffusion—caused by the unstirred layer
(d) Pore diffusion

It has already been shown by Sundaram, Tweedale, and Laidler (3) that in a membrane or particle, in which enzyme molecules are spread uniformly over

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its entirety, partitioning of the substrate molecules and the diffusion constant of the substrate molecules can perturb the kinetic parameters of the system. In another treatment involving a microencapsulated enzyme Sundaram (4) showed that external and internal mass transport of the substrate/product do not perturb the Michaelis–Menten kinetics of the system. However the significance of the membrane resistance in such a system was pointed out.

A paper by Sundaram and Joy (5) deals with problems in the analysis and interpretation of kinetic data obtained with immobilized glutamate dehydrogenase and urease assayed in packed-bed reactors.

This paper deals with film diffusion or the diffusion of substrate molecules across the unstirred layer and the effects thereof on (1) the kinetic parameters which are measured by conventional means and (2) the significance of these kinetic parameters, in particular the Michaelis constant.

THEORY

The catalytic efficiency of an enzyme immobilized on the surface of an insoluble polymeric matrix will depend on how easily the substrate molecules become accessible to it from the bulk of the solution. The availability of the substrate molecules or the concentration of the substrate at the polymer surface will depend upon the rate of diffusion or external mass transport of the substrate through the unstirred layer next to the polymer surface. This mass transport rate may be denoted by:

$$\phi = D/\delta(s - s') \tag{1}$$

where ϕ is the transport rate per unit area (mol sec^{-1} cm^{-2}); D is the diffusion constant of the substrate in the unstirred layer (cm^2 sec^{-1}); δ is the thickness of the unstirred layer (cm); s' is the substrate concentration at the polymer surface (M); and s is the substrate concentration in the well-mixed bulk phase (M).

δ denotes an "equivalent" thickness because there is no sharp boundary but only a diffuse boundary between the bulk phase and the unstirred layer. Net flux of water is excluded and as such there is no solvent drag phenomenon.

Reaction rate of an immobilized enzyme may be represented by a modified Michaelis–Menten equation which incorporates into it the term ϕ representing the rate of mass transport or diffusion of the substrate through the unstirred layer as follows:

$$V = \phi(K_m + s')/s' \qquad \text{or} \qquad \phi = Vs'/(K_m + s') \tag{2}$$

Here the enzyme concentration term is omitted and is given a value of unity. V = apparent maximal enzymic rate per unit area (mol sec^{-1} cm^{-2}) of immobilized enzyme.

In the steady-state there are two cases: (1) the case of a membrane particle that contains a uniform distribution of enzyme, and (2) enzyme molecules which are attached to the surface groups of an insoluble polymer. In the former case, as has already been shown by Sundaram, Tweedale, and Laidler (3), the distribution of the substrate $[s']$ within the membrane dictates the efficiency of the bound enzyme as a catalyst whereas in the latter case the substrate molecules will be broken down as soon as they come into contact with the surface of the polymer containing the enzyme molecules.

In the former case it may be assumed that the transport across the unstirred layer and the partitioning of the substrate between the steady-state concentration in the unstirred layer and the polymeric matrix are the two key factors affecting the catalytic rate. In the latter case only the transport from the bulk phase on to the polymer surface through the unstirred layer need be considered since the substrate molecules are catalytically broken down as soon as they reach the active sites of enzyme molecules present on the polymer surface.

The concentration of the substrate s' at the surface of the polymer is unknown and cannot be directly measured. As such, s, the substrate concentration in the bulk phase which varies depending upon experimental conditions, is used.

Taking equations (1) and (2) we get a quadratic equation:

$$s'^2 + s'[K_m - s + (V\delta/D)] - sK_m = 0 \tag{3}$$

Equations (1) and (3) give

$$\phi = (D/\delta)\{0.5[K_m + s + (V\delta/D)] \pm [0.25[K_m - s + (V\delta/D)]^2 + sK_m]^{1/2}\} \tag{4}$$

For two special cases simpler equations can be derived:

Case 1. $s' \ll K_m$, that is, ϕ is related in a nearly linear fashion to the concentration at the polymer surface. With $K_m + s' \approx K_m$ we get from equation (1) and (2)

$$s' = s/[1 + (V\delta/K_m D)] \tag{5}$$

and

$$\phi = s/(\delta/D) + (K_m/V) \tag{6}$$

The apparent mass transport or diffusion rate is proportional to the bulk phase concentration but the rate is less when the unstirred layer is present

because of the additional term δ/D. The denominator of equation (6) is the transport resistance.

Case 2. $s' \gg K_m$, that is, the region of saturation for the catalytic system. With $K_m + s' \approx s'$ we get from equation (1) and (2)

$$s' = s - (V\delta/D) \tag{7}$$

and

$$\phi = V \tag{8}$$

The apparent mass transport coincides with the catalytic rate and the K_m app. is equal to half the maximal catalytic rate. Setting $\phi = 0.5\ V$ in equation (4) we get

$$s\ (\text{for } \phi = 0.5\ V) = K_m + (0.5\ V\delta/D) \tag{9}$$

Using the bulk phase concentration instead of the concentration at the surface a too-high value for K_m app. is obtained as indicated by the "bias term" in equation (9). The error increases with δ, the thickness of the unstirred layer, and the V_{max}.

In vigorously stirred cell suspensions the thickness of the unstirred layer can be as small as 5 to 10 μm (6, 7). Larger values of 20 to 170 μm have been meaasured in well-stirred in vitro preparations consisting of millipore filters (8) and lipid bilayer (9). Thus the values of 0.1 to 0.5 mm assumed for δ in the calculations involved in Figs. 1 and 2 are quite reasonable.

Figure 1 graphically demonstrates the influence of the unstirred layer on the kinetics of an immobilized enzyme. From curve 1 (unstirred layer absent) K_m is determined correctly. From curves 2 and 3 (unstirred layer present) a double and six-fold higher K_m is obtained. Assuming $V_{max} = 0.06$ mol sec^{-1} cm^{-2}, $K_m = 3$ mM, and $D = 0.5 \cdot 10^{-5}$ cm^2 sec^{-1}, curve 2 corresponds to an unstirred layer of thickness $\delta = 0.1$ mm and curve 3 to $\delta = 0.5$ mm. The curves demonstrate that an unstirred layer reduces the mass transport or diffusion at low substrate concentrations, thus reducing the rate of enzymic catalysis. Approaching the region of saturation the reduction of the rate of mass transport decreases. The conditions used in this figure represent a reaction catalyzed by urease immobilized on an uncharged polymer.

Figure 2 shows additional error being introduced in obtaining K_m from a Lineweaver–Burk plot. The unstirred layer introduces a nonlinearity in double reciprocal plots and different values of K_m app. may be obtained depending upon where the extrapolation is made from (curves 2 and 3 in Fig. 2). Curve 2 yields a four-fold to-high K_m app. value if extrapolated from $\phi = 0.5\,V$ and data from below $0.5\,V$ yields eight-fold too-high a K_m app. value.

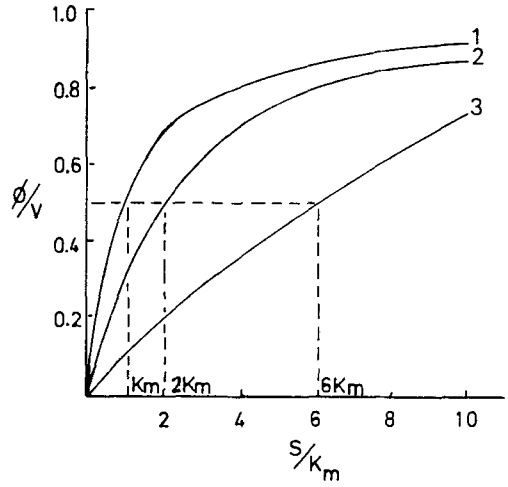

FIG. 1. The influence of the unstirred layer on the kinetics of an immobilized enzyme. Abscissa—the concentration of the substrate in the bulk phase relative to the Michaelis constant s/K_m. Ordinate—the transport rate of substrate relative to catalytic rate ϕ/V. Curve 1, no unstirred layer ($\delta = 0$); curve 2, 0.5 $V\delta/D = K_m$; curve 3, 0.5 $V\delta/D = 5\ K_m$. Assuming $V = 0.06\ \mu\text{mol} \cdot \text{sec}^{-1}\ \text{cm}^{-2}$, $K_m = 3$ mM, and $D = 0.5 \cdot 10^{-5}\ \text{cm}^2\ \text{sec}^{-1}$. Curve 2 corresponds to an unstirred layer of thickness $\delta = 0.1$ mm and curve 3, $\delta = 0.5$ mm.

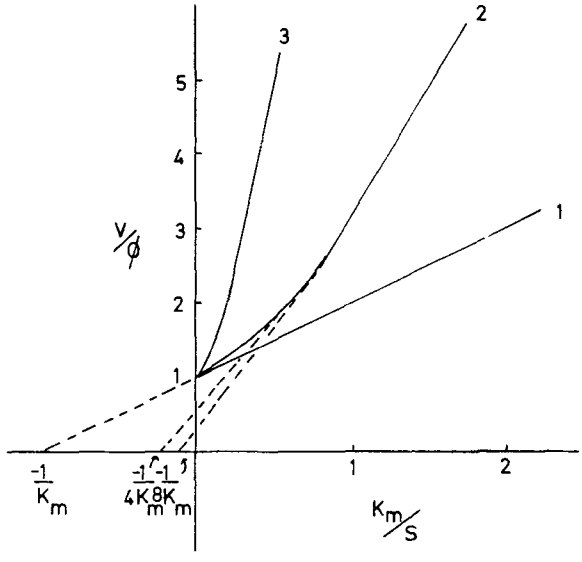

FIG. 2. Influence of the unstirred layer on the Lineweaver–Burk plot of the kinetic data obtained from an immobilized enzyme. Abscissa—reciprocal of relative concentration; ordinate—reciprocal of relative transport rate. Other details in Fig. 1.

In conclusion it must be said that K_m app. values are not adequate for the definitive analysis of heterogenous catalysis. However the challenge at hand is (a) to separate the effects of diffusion on the kinetic parameters such as K_m app. from true catalytic effects and (b) to develop the means of operation to obtain and use classical enzyme parameters correctly. This point is discussed by Sundaram and Joy (5).

It is also implicit that reciprocal effects between the diffusion of product and reaction can exist in the active layer. However, an analysis of either a substrate or a product gradient cannot be undertaken to the total exclusion of electrostatic interactions, for example, in the case of AE-cellulose–urease mentioned in the previous paper (5) the inhibitory effects of NH_4^+ ions on urease in its positively charged microenvironment will be very different from those in homogeneous reactions or in the case of a negatively charged polymer. Admittedly an analysis of this nature is very complicated and perhaps cannot be driven globally.

Thus the assumption in this paper is that the reaction at the mixed interface is not perturbed by inhibition or activation by either the substrate and/or the product. It is considered essential to understand a simpler system first before going on to more complicated ones.

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