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KINETIC ANALYSIS FOR SOLID-SUPPORTED ENZYMES

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SUMMARY

On the basis of theoretical treatments of the kinetics of solid-supported enzymes in which diffusion effects are significant, methods are suggested for analyzing experimental results. The procedures can be applied to membranes, spherical particles, and rods. The Michaelis–Menten law applies to such systems but the kinetic parameter K_m(app.) is only an apparent one, being influenced by partitioning and diffusional effects. The methods suggested allow the true parameters, k'_c and K'_m, relating to the behavior of the enzyme within the support, to be derived from the experimental results. Method 1 is applicable when the experimental results relate to various substrate concentrations at a constant membrane thickness or particle diameter. Methods 2 and 3 are useful when there are data for various membrane thicknesses or particle diameters, at a constant substrate concentration.

INTRODUCTION

Enzymes immobilized on solid supports are of great scientific and technological importance. The many methods of immobilizing enzymes have been reviewed by Silman and Katchalski[1], the theories by Sundaram and Laidler[2] and by Goldman *et al.*[3]. The effects of immobilization on the kinetic behaviour can be classified as follows:

(1) Conformational effects: the enzyme may be conformationally different in the support.

(2) Environmental effects: the enzyme–substrate interactions occur in a different environment when the enzyme is supported.

(3) Partitioning: the substrate concentration within the support will be different from that in the solution.

(4) Diffusional, or mass-transfer effects: the kinetics may not be dominated by the enzyme–substrate interactions, but may be influenced to a greater or lesser extent by the speed of diffusion of the substrate through the support.

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As a result of effects (1) and (2) the kinetic parameters for the supported enzyme will not be the same as in free solution and will be denoted as k'_c and K'_m. As a result of effects (3) and (4) the Michaelis constant actually observed will be different from K'_m, and will be denoted as K_m(app.). Theories which deal in particular with effects (3) and (4) have been developed by Goldstein *et al.*[4]; Wharton *et al.*[5]; Hornby *et al.*[6]; Sundaram *et al.*[7]; Goldman *et al.*[8]; Kasche *et al.*[9]; Kobayashi and Moo-Young[10,11], and Shuler *et al.*[12]. In homogeneous solution even the fastest of enzyme-catalyzed reactions appear to be little influenced by diffusion effects, but with supported enzymes, especially when the enzyme is fairly active and the membrane thickness or particle diameter is not too small, diffusion effects are quite significant.

Moo-Young and Kobayashi[13] have in particular analyzed the kinetic characteristics of a supported enzyme in terms of an effectiveness factor, E_f, defined as the ratio of the total rate when there are diffusional effects within the support to the rate if diffusion within the support were infinitely rapid. When $E_f \approx 1$, diffusional effects are negligible; when E_f is very small, diffusional effects are significant. This concept has been found to be very useful for the description of the importance of diffusional effects in supported enzymes and for an analysis of the experimental results in terms of kinetic parameters, the thickness of the membrane or the particle diameter, and the diffusivity of the substrate within the support. The effectiveness factor can be determined most readily by measuring reaction rates at several support thicknesses, under otherwise identical conditions. The effectiveness factor approaches unity when no increase in rate for unit quantity of support occurs on subdivision. If rate data are available on very thin membranes, or finely divided particles having $E_f \approx 1$, then the ratio of the rate per unit quantity of support for a larger size to that for the smaller is equal to E_f for the larger size; this is the most reliable method of determining E_f.

However, there are many cases in which it is difficult or impossible to make the support sufficiently thin. When the microencapsulation technique is employed, it is difficult to go to sufficiently small sizes. Enzymes acting in living systems may be embedded in membranes or attached to subcellular particles which are sufficiently large for diffusional effects to be important, and which cannot be subdivided without damage.

The present paper is concerned with examining alternative methods for evaluating the true kinetic parameters k'_c and K'_m from data in which there are substantial diffusional effects. Bunting and Laidler[14] have recently made an experimental attack on this problem.

THEORETICAL

Estimates of times required for a supported enzyme system to attain a steady state have been given by Sundaram *et al.*[7]. In what follows it will be assumed that the steady state has been established. When this is so the local concentration of substrate does not vary with time, since the disappearance of substrate by enzymic reaction is compensated by the net flow of substrate due to diffusion. Use of Fick's law to describe the diffusion of the substrate leads to the following mass-balance equation:

$$D_s \left(\frac{d^2 s}{dx^2} + \frac{n}{x} \frac{ds}{dx} \right) - \frac{k_c' [E]_m s}{K_m' + s} = 0 \qquad (1)$$

Here D_s is the diffusion constant for the substrate in the support, s the local concentration of substrate in the support and $[E]_m$ is the enzyme concentration within the support; $n = 0, 1, 2$ for membrane, rod and spherical particle, respectively. The boundary condition is $s = [S]'$ at the surface of the support. Moo-Young and Kobayashi[13] have solved the equation and find that the effectiveness factor is

$$E_f = \frac{E_0 + \varrho E_1}{1 + \varrho} \tag{2}$$

where

$$\varrho = \frac{K_m'}{[S]'} \tag{3}$$

and E_0 and E_1 are the effectiveness factors for reaction in the zero- and first-order limiting regions, being as follows:

(a) For a membrane

$$E_0 = \begin{cases} 1 & (m \leqslant 1) \\ 1/m & (m > 1) \end{cases} \tag{4}$$

$$E_1 = \frac{\tanh m}{m} \tag{5}$$

(b) For a sphere:

$$E_0 = \begin{cases} 1 & (m \leqslant \sqrt{3}) \\ 1 - \left(\frac{1}{2} + \cos\frac{\psi + 4\pi}{3}\right)^3 & (m > \sqrt{3}) \end{cases} \tag{6}$$

$$\psi = \cos^{-1}\left(\frac{6}{m^2} - 1\right) \tag{7}$$

$$E_1 = \frac{3}{m}\left(\frac{1}{\tanh m} - \frac{1}{m}\right) \tag{8}$$

(c) For a rod:

$$E_0 = \begin{cases} 1 & (m \leqslant \sqrt{2}) \\ 1 - \zeta & (m > \sqrt{2}) \end{cases} \tag{9}$$

$$\zeta - \zeta \ln \zeta = 1 - \frac{2}{m^2} \tag{10}$$

$$E_1 = \frac{2I_1(m)}{mI_0(m)} \tag{11}$$

where

$$m = \frac{h}{(1+\varrho)\sqrt{1 - \varrho \ln(1 + 1/\varrho)}} \tag{12}$$

and

$$h = R\sqrt{\frac{k_c'[E]_m}{2 D_s [S]'}} \tag{13}$$

R is the half thickness of the membrane, or radius of the rod or spherical particle. Eqn 11 has been given by Aris[15]; the derivation of E_0 for a rod (Eqns 9 and 10) is

given in the Appendix; I_0 and I_1 are the modified Bessel functions of the zero and first order.

On the basis of the definition of the effectiveness factor, the overall reaction rate per unit gross volume of support is given by

$$v = E_f \frac{k_c' [E]_m [S]'}{k_m' + [S]'} \tag{14}$$

Here $[S]'$, the concentration of substrate just inside the surface, is related to $[S]$, the concentration in the solution, by

$$[S]' = P[S] \tag{15}$$

where P is the partition coefficient; values of P have been measured in the study of Bunting and Laidler[14].

Suppose that we have a set of experimental data, involving rates at various $[S]'$ values, *i.e.* $(v_{obs})_j$, $[S]'_j$, where $j = 1,2,3 \ldots\ldots J$. By assuming appropriate values of $k'_c [E]_m$ and K'_m the theoretically estimated overall rate, v_{theo}, is evaluated from Eqn 14 and 2–13. If we define the variance as the sum of $[(v_{obs})_j - (v_{theo})_j]^2$ for $j = 1$ to $j = J$, we may adjust the estimated $k'_c [E]_m$ and K'_m values so as to minimize the variance (*cf.* Deming[16]). However, this procedure is tedious, since E_f is a complex function of $[S]'$, K'_m, *etc.* and Eqn 14 is not linear. More convenient methods are therefore needed.

Elimination of $k'_c [E]_m$ between Eqns 12, 13 and 14 leads to

$$\frac{v R^2}{D_s [S]'} = 2E_f(1+\varrho)\,[1 - \varrho \ln (1+1/\varrho)]m^2 \tag{16}$$

The dimensionless modulus on the left-hand side may be written as Φ:

$$\Phi \equiv \frac{vR^2}{D_s [S]'} \tag{17}$$

All of the factors in Φ can be determined experimentally or estimated.

The modulus m is expressed as a function of E_f through Eqns 2–11; the new modulus Φ is expressed as a function of ϱ and E_f. For a particular value of E_f there is, for each shape, a unique dependence of Φ on ϱ. If we take $E_f = 0.95$ as a criterion for insignificant diffusional effects we can relate Φ and ϱ; plots for this case are shown in the lower curves of Figs 1, 2 and 3 for membranes, rods and spheres, respectively. Similarly, $E_f = 0.6$ can be taken to represent an arbitrary dividing line between moderate and large diffusion control; the upper curves in Figs 1, 2 and 3 show the relationships in this case. In these three figures, Region 1 corresponds to insignificant diffusional effects, while Region 3 corresponds to strong diffusional control; Region 2 represents the intermediate behaviour. These diagrams are therefore helpful in arriving at a preliminary evaluation of the importance of diffusional effects.

The theories of Sundaram *et al.*[7] and of Moo-Young and Kobayashi[13], indicate that the overall reaction rate will still be given to a good approximation by an equation of the Michaelis–Menten form*,

$$v = \frac{k_c' [E]_m [S]'}{K_m \text{ (app.)} + [S]'} \tag{18}$$

* Note that K_m (app.) is here defined with respect to $[S]'$, not $[S]$; the apparent K_m defined with respect to $[S]$ is equal to K_m (app.)/P.

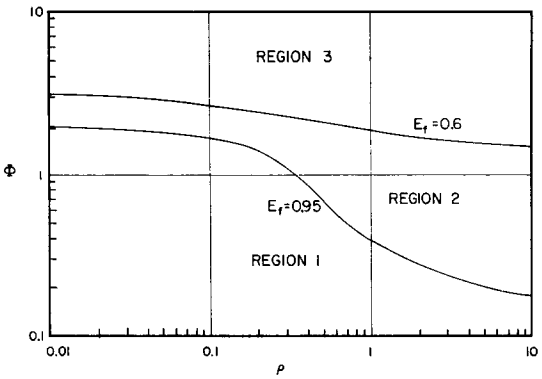

Fig. 1. Double-logarithmic plots of Φ against ϱ for a membrane. The two lines correspond to the effectiveness factors shown, and divide the diagram into three regions corresponding to: 1, small; 2, moderate and; 3, large diffusional effects.

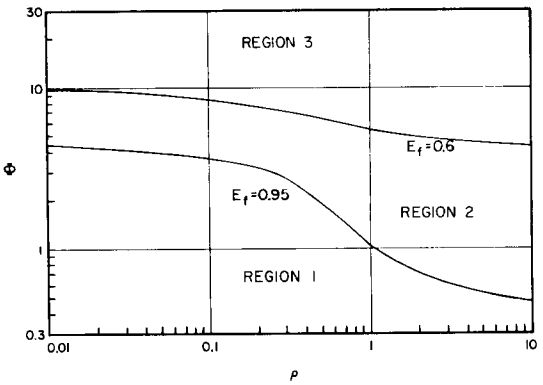

Fig. 2. Similar diagram to Fig. 1, for rods.

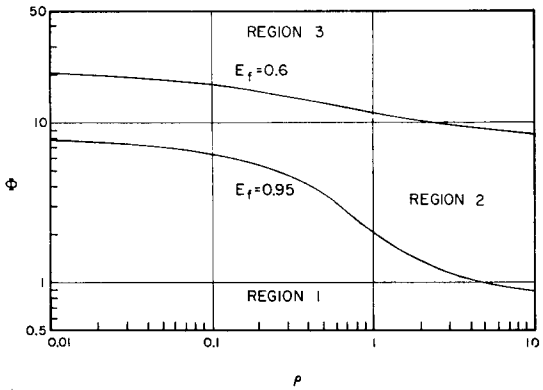

Fig. 3. Similar diagram to Fig. 1, for spheres.

even if the system is in Region 2 or 3. The value of K_m(app.) is now different from the true value K'_m, owing to diffusion effects. For example, K_m(app.) is in general larger than K'_m. If we define an apparent parameter ϱ(app.) by

$$\varrho\,(\text{app.}) = \frac{K_m\,(\text{app.})}{[S]'} \tag{19}$$

it follows that

$$\varrho\,(\text{app.}) \geqslant \varrho \tag{20}$$

Since the lines for $E_f = 0.95$ in Figs 1, 2 and 3 decrease monotonically as ϱ increases, it is safe to obtain $k'_c[E]_m$ and K'_m values from the Lineweaver–Burk or Eadie–Hofstee plots if the plots for Φ *vs* ϱ(app.) lie in Region 1. In Regions 2 and 3, however, the situation is more complex. Three methods, now to be discussed, are applicable to the analysis of systems in Regions 2 and 3.

Method 1

The first method to be considered can be used when there are data at various substrate concentrations on a given support geometry, (*e.g.* membranes of the same thickness, or rod or particles of the same diameter.) The k'_c and K_m(app.) values are first determined from the Lineweaver–Burk plot of $1/v$ against $1/[S]'$. The K_m(app.) value corresponds to a substrate concentration at which the rate is equal to $k'_c\,[E]_m/2$, so that from Eqn 14

$$\frac{k_c'\,[E]_m}{2} = E_f\,\frac{k_c'\,[E]_m\,K_m\,(\text{app.})}{k_m' + K_m\,(\text{app.})} \tag{21}$$

This rearranges to

$$\omega = \frac{K_m'}{K_m\,(\text{app.})} = 2E_f - 1 \tag{22}$$

where

$$E_f = \frac{E_0 + \omega E_1}{1 + \omega} \tag{23}$$

If, by analogy with Eqn 13 for h, we define a parameter h^* as

$$h^* = R\sqrt{\frac{k_c'\,[E]_m}{2D_s K_m\,(\text{app.})}} \tag{24}$$

it is then found that

$$m = \frac{h^*}{(1+\omega)\sqrt{1-\omega\ln(1+1/\omega)}} \tag{25}$$

E_0 and E_1 are evaluated from Eqns 4–11 and 25. Since Eqn 22 explicitly expresses the relationship between ω and h^*, through Eqns 23–25, it is possible to obtain the relation between ω and h^* by trial and error; the results are plotted, for the three shapes of support, in Figs 4, 5 and 6.

It is possible to obtain an approximate explicit equation relating ω and h^*. The expression $(1+\omega)\sqrt{1-\ln(1+(1/\omega))}$ is approximately unity; if it is set equal to unity, $m = h^*$, which means that E_0 and E_1 are functions of h^* only, not of ω. Introduction of Eqn 23 into 22 gives, after rearrangement,

$$\omega = E_1 - 1 + \sqrt{(E_1-1)^2 + 2(E_0-1)} \tag{26}$$

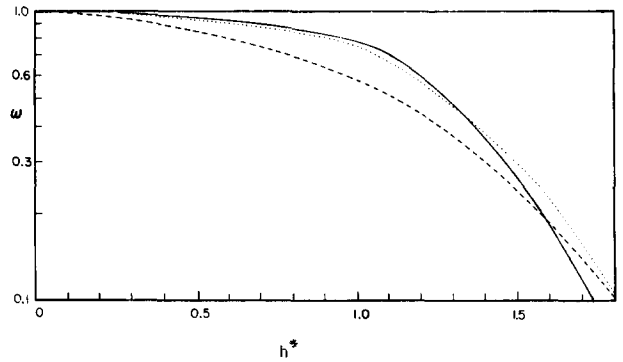

Fig. 4. Relationship between the parameters ω and h^*, for a membrane (Method 1). The firm line is the exact relationship. The dotted line results from one approximate solution (Eqn 26), the dashed line from another, based on the theory of Sundaram *et al.*[7].

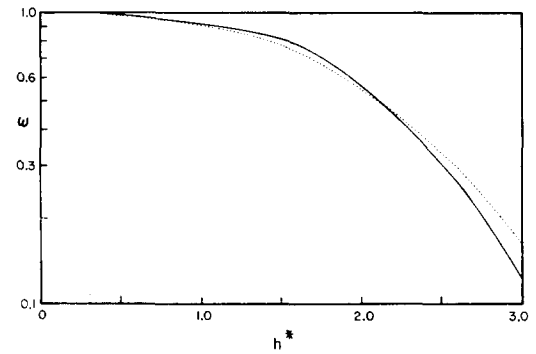

Fig. 5. Similar diagram to Fig. 4, for rods.

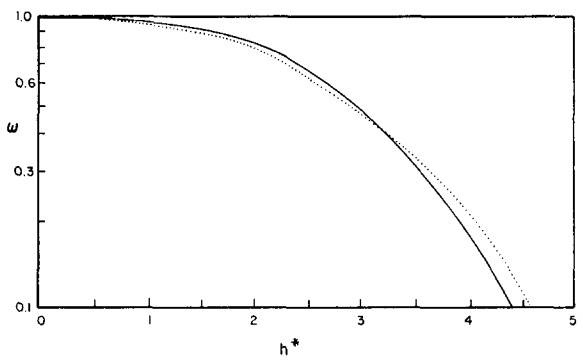

Fig. 6. Similar diagram to Fig. 4, for spheres.

where E_0 and E_1 are evaluated from Eqns 4–11 and $m = h^*$. This relationship is shown by the dotted lines in Figs 4, 5 and 6; the dotted lines are seen to fall quite close to the solid lines.

Lineweaver–Burk or other plots of the data yield values of $k'_c[E]_m$ and K_m (app.), and hence h^* can be calculated. Use of Figs 4–6 then yields ω, from which K'_m can be calculated.

Sundaram *et al.*[7] proceeded slightly differently; for a membrane of thickness $l(=2R)$ they obtained the following relationship between K'_m and K_m(app.):

$$F = \frac{K_m'}{K_m \text{ (app.)}} \tag{27}$$

where

$$F = \frac{2}{\alpha l} \frac{\cosh \alpha l - 1}{\sinh \alpha l} \tag{28}$$

$$\alpha l = 2R \sqrt{\frac{k_0' [E]_m}{k_m' D_s}} \tag{29}$$

If γ is written for $\alpha/2$, Eqn 28 takes the simpler form

$$F = \frac{\tanh \gamma l}{\gamma l} \tag{30}$$

Introduction of $h^* \sqrt{8}/\omega$ for γl, and application of the trial and error method for Eqn 27, yields the relationship between ω and h^* that is shown in Fig. 4 as a dashed line. This curve does not greatly differ from those given by the other procedures.

Method 2

This method, based on the "triangle method" of Weisz and Prater[17], can be applied to experimental rate data for three or more support geometries, at constant substrate concentration.

Suppose, for example, that we have spherical particles having radii R_1, R_2 and R_3. Consider first one pair of particles of radii R_1 and R_2; the ratio of the rates (per unit volume of support) on these particles is the ratio of the effectiveness factors, $(E_f)_1/(E_f)_2$, and the ratio R_1/R_2 is h_1/h_2. The simultaneous equations expressing $(E_f)_1$, as a function of h_1 and $(E_f)_2$ as a function of h_2 can then be solved to obtain $(E_f)_1$ and $(E_f)_2$ for an assumed constant value of ϱ.

The triangle method is based on the fact that on a logarithmic plot of E_f *versus* h, as shown in Fig. 7, the ratio $(E_f)_1/(E_f)_2$ forms a line of fixed length on the ordinate and the ratio h_2/h_1 another fixed length on the abscissa. These two lengths form a triangle which can be fitted to the E_f *versus* h plot for an assumed value of ϱ, resulting

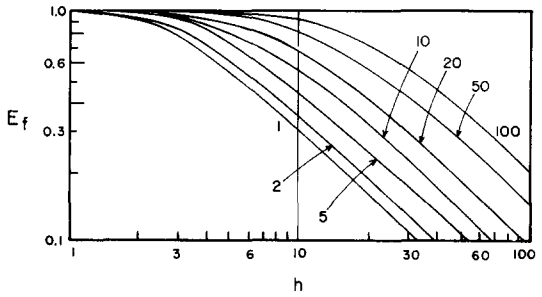

Fig. 7. Double-logarithmic plot of effectiveness factor, E_f, against h, for various values of the parameter ϱ, which are noted on the curves (Method 2). These curves are recommended for $\varrho \geqslant 1$ (compare Fig. 9).

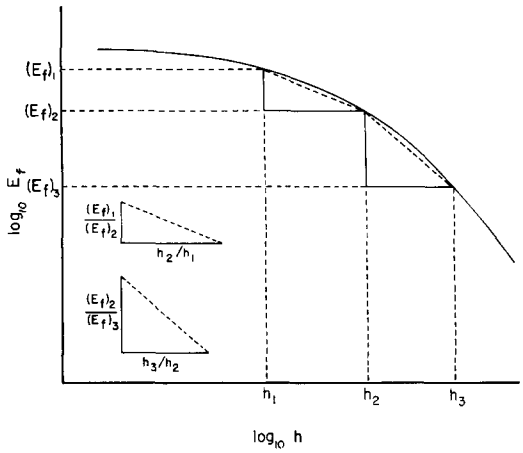

Fig. 8. Schematic double-logarithmic plot of E_f against h, for a fixed value of the parameter ϱ; this plot illustrates the use of the triangle method.

in the determination of $(E_f)_1$, $(E_f)_2$, h_1 and h_2. This is demonstrated in Fig. 8. The same procedure is then carried out for another pair of particles, of radii R_2 and R_3, and individual values of $(E_f)_2$, $(E_f)_3$, h_2 and h_3 obtained in the same way. If the assumption of ϱ is correct, the values of h_2 or $(E_f)_2$ should coincide with each other. From the values of ϱ and h_2 the values of K'_m and $k'_c[E]_m$ can be calculated.

When ϱ is less than 1, use of Fig. 9 is recommended, instead of Fig. 7. For accuracy, however, the equivalent analytical expression, such as Eqns 2–12, should be used instead of a graphical solution.

This method is inapplicable if a pair of the experimental data falls in Region 3, because in this region the rate per unit support volume is inversely proportional to support thickness or diameter, and the log E_f *vs* log h (or log γl) curve becomes a straight line.

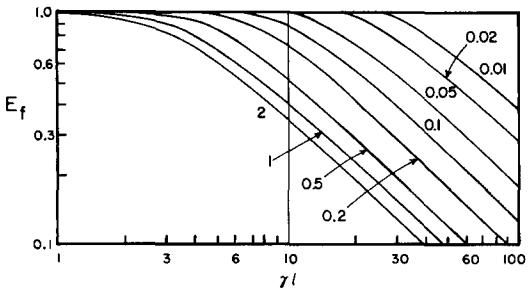

Fig. 9. Double-logarithmic plot of effectiveness factor, E_f, against γl, for various values of the parameter ϱ (Method 2). These curves are recommended for $\varrho \leqslant 1$ (compare Fig. 7).

Method 3

This method is applicable to a situation where rates are known at two different geometries of support and at constant substrate concentration at the surface of the support; the diffusion coefficient within the support must be known.

Rearrangement of Eqn 16 leads to

$$\Phi = \frac{vR^2}{D_s[S]'} = \frac{2E_f h^2}{1+\varrho} = \frac{\varrho E_f \gamma^2 l^2}{1+\varrho} \tag{31}$$

By the use of Eqns 2–12 the relationships between Φ and h (or, when ϱ is less than 1, between Φ and γl) are obtained, and are shown in Figs 10 and 11 for spherical particles.

For example, suppose we have two spherical particles of radii R_1 and R_2. First, Φ_1 and Φ_2 are evaluated from the experimental data. With the assumption of an appropriate value of ϱ, h_1 and h_2 are then evaluated from Figs 10 and 11. If the assumption of ϱ is correct, the ratio R_1/R_2 will coincide with h_1/h_2. This method is also inapplicable if the two rates fall in Region 3.

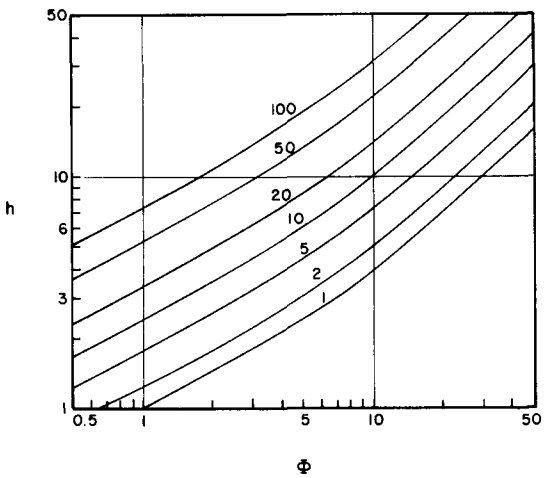

Fig. 10. Double-logarithmic plots of h agaisnt Φ, for spherical particles and various values of ϱ (Method 3). These curves are recommended for $\varrho \leqslant 1$.

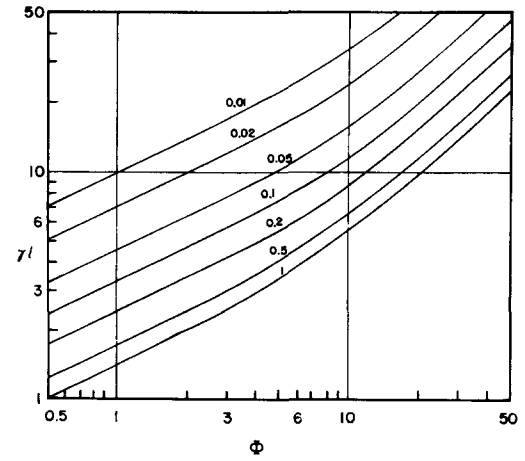

Fig. 11. Double-logarithmic plots of Φ against γl, for spherical particles and various values of ϱ (Method 3). These curves are recommended for $\varrho \leqslant 1$.

CONCLUSIONS

The statistical method is the most accurate method of obtaining values of $k_c[E]_m$ and K'_m; however, as previously pointed out, the method is tedious, particularly if the data are in Region 3 in Figs 1, 2 and 3. The present paper is therefore concerned with presenting alternative methods.

Of the three methods described, the use of Method 1 is to be recommended. Since K_m(app.) is first evaluated, it is possible to know in which region, with respect to Figs 1–3, the experimental data fall. Furthermore, Method 1 provides the most convenient method for obtaining values of $k'_c[E]_m$ and K'_m. When Methods 2 or 3 are used it is difficult to know in which region the experimental data fall.

A test of Method 1 is to be found in the paper of Bunting and Laidler[14].

APPENDIX

When $K_m \ll [S]'$ (*i.e.* $\varrho \approx 0$), the enzyme reaction is of zero order and Eqn 1 becomes, for a rod,

$$D_s\left(\frac{d^2s}{dx^2} + \frac{1}{x}\frac{ds}{dx}\right) - k_c'[E]_m = 0 \tag{A-1}$$

This equation is to be solved for the following conditions:

at $x = R$ (surface of the support)

$$s = [S]' \tag{A-2}$$

and

at $x = 0$ (center of the support)

$$\frac{ds}{dx} = 0,\ s > 0 \tag{A-3}$$

or

at $x = x_c$ $(0 \leqslant x_c < R)$

$$\frac{ds}{dx} = s = 0 \tag{A-4}$$

On introducing dimensionless variables ($y = s/[S]'$, $\varrho = x/R$ and $p = dy/d\varrho$), these equations become

$$\frac{d(\varrho p)}{d\varrho} = 2h^2\varrho \tag{A-5}$$

with the boundary conditions

$$\text{at } \varrho = 1;\ y = 1 \tag{A-6}$$

and

$$\text{at } \varrho = 0;\ p = 0,\ y > 0 \tag{A-7}$$

or

$$\text{at } \varrho = \varrho_c\ (0 \leqslant \varrho_c < 1);\ p = y = 0 \tag{A-8}$$

After integrating Eqn A-5 twice, the dimensionless concentration profile of the substrate is expressed as

$$y = \frac{h^2\varrho^2}{2} + C_1 \ln \varrho + C_2 \tag{A-9}$$

where C_1 and C_2 are the constants of integration. From its definition, the effectiveness factor for this case is given by

$$E_f = \frac{2\pi R D_s \left.\frac{ds}{dx}\right|_{x=R}}{\pi R^2 k_c'[E]_m} = \frac{1}{h^2}\left.\frac{dy}{d\varrho}\right|_{\varrho=1} = 1 + \frac{C_1}{h^2} \tag{A-10}$$

When Eqns A-6 and A-7 are used, $C_1 = 0$, $C_2 = 1 - h^2/2$ and $E_0 = 1$. When Eqns A-6 and A-8 are used, $C_1 = -h^2\zeta$, $C_2 = 1 - h^2/2$ and $E_0 = 1 - \zeta$, where $\zeta = \varrho_c^2$ and the value of ζ is the root of the equation

$$\zeta - \zeta \ln \zeta = 1 - \frac{2}{h^2} \tag{A-11}$$

When $h = \sqrt{2}$, $\zeta = 0$; *i.e.* under this condition the substrate is depleted at the center of the rod. When h is larger than $\sqrt{2}$, the depletion of substrate occurs at $\varrho = \sqrt{\zeta}$.

The value of m approaches h when ϱ becomes zero in Eqn 12.

ACKNOWLEDGEMENT

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REFERENCES

1 Silman, I. H. and Katchalski, E. (1966) *Annu. Rev. Biochem.* 35, 873–908
2 Sundaram, P. V. and Laidler, K. J. (1971) in *Chemistry of the Cell Interface* (Brown, H. D., ed.), pp. 255–296, Academic Press, New York
3 Goldman, R., Goldstein, L. and Katchalski, E. (1971) in *Biochemical Aspects of Reactions on Solid Supports* (Stark, G. R., ed.), pp. 1–78, Academic Press, New York
4 Goldstein, L., Levin, Y. and Katchalski, E. (1964) *Biochemistry* 3, 1913–1919
5 Wharton, C. W., Crook, E. M. and Brocklehurst, K. (1968) *Eur. J. Biochem.* 6, 572–578
6 Hornby, W. E., Lilly, M. D. and Crook, E. M. (1968) *Biochem. J.* 107, 669–674
7 Sundaram, P. V., Tweedale, A. and Laidler, K. J. (1970) *Can. J. Chem.* 48, 1495–1504
8 Goldman, R., Kedem, O. and Katchalski, E. (1971) *Biochemistry* 10, 165–172
9 Kasche, V., Lundqvist, H. and Bergman, R. (1971) *Biochem. Biophys. Res. Commun.* 45, 615–621
10 Kobayashi, T. and Moo-Young, M. (1971) *Biotechnol. Bioeng.* 13, 893–910
11 Kobayashi, T. and Moo-Young, M. *Biotechnol. Bioeng.*, in the press
12 Shuler, M. L., Aris, R. and Tsuchiya, H. M. (1972) *J. Theor. Biol.* 35, 67–76
13 Moo-Young, M. and Kobayashi, T. (1972) *Can. J. Chem. Eng.* 50, 162–167
14 Bunting, P. S. and Laidler, K. J. (1972) *Biochemistry* 11, 4477–4487
15 Aris, A. (1957) *Chem. Eng. Sci.* 6, 262–268
16 Deming, W. E. (1964) *Statistical Adjustment of Data*, Dover Publications, New York
17 Weisz, P. B. and Prater, C. D. (1954) *Adv. Catal.* 6, 143–170