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A FAST EVALUATION OF DIFFUSION EFFECTS ON BOUND ENZYME ACTIVITY

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Summary

As the kinetic behavior of bound enzymes is frequently affected by substrate diffusion between the bulk solution and the catalytic sites, a fast and simple method is proposed to detect and, subsequently, to remove diffusion effects on measured enzymic activities.

The procedure makes use of the effectiveness factor concept and essentially involves the direct determination on two diagrams of the magnitude of both external and internal diffusion limitations. It requires a prior estimation of the volume and external surface area of the matrix, of the substrate external transport coefficient and internal diffusivity, and of the intrinsic Michaelis constant of the bound enzyme. However, it does not necessitate the knowledge of the quantity of bound enzyme.

The two basic graphs have been calculated for Michaelis-Menten kinetics. They can also be used to evaluate diffusional effects on two-substrate reactions, as illustrated with previously published data.

Introduction

The kinetic behavior of enzymes bound to artificial or natural membranes is frequently affected by the diffusion of substrates or products in the enzyme microenvironment. Diffusional limitations were found to change observed affinities and maximal activities, modify inhibition and pH profiles and increase apparent stabilities [1—3]. Thus, when a kinetic study is aimed at determining the influence of binding on the intrinsic properties of the enzyme, it is imperative to first remove possible diffusion effects on the measured enzymic activity.

For enzymes embedded inside a porous matrix, both the external diffusion between the solution and the outside membrane or particle surface and the internal diffusion inside the pores can have a kinetic influence. Elaborate

mathematical treatments have been presented to quantitatively describe the contributions of diffusional interferences on bound enzyme kinetics [4—7]. But no simple method has yet been proposed to remove combined external and internal diffusional effects from experimental data in order to obtain the intrinsic activity that would be measured in the absence of diffusion restrictions.

This paper presents two diagrams which, in the case of Michaelis-Menten kinetics, can be easily used to detect the possible influence of substrate diffusion and, furthermore, to successively remove the kinetic contributions of external and internal diffusion on the measured enzymic activity. The simplicity and rapidity of the method is illustrated with previously published kinetic data on single and two-substrate enzymic reactions.

Method

External and internal diffusion limitations

When a relatively high amount of enzyme is immobilized inside a porous matrix, diffusion of the substrate becomes limiting and, as a result, the substrate concentration gradually decreases from the bulk solution to the inside of the matrix. Fig. 1 schematically illustrates the evolution of the substrate concentration profile inside and outside the matrix with an increasing amount of bound enzyme. Of course, when enzymes are attached at the surface of a nonporous support, only external concentration gradients are present.

It is possible to evaluate the kinetic contribution of substrate concentration gradients provided quantitative information on the ability of the substrate to diffuse from the bulk solution to the enzyme are available. Substrate transport, by molecular diffusion and turbulent convection, to the external surface is generally expressed by an external transport coefficient, h. The rate of external substrate transport is then equal to the product of the transport coefficient, h, and the substrate concentration difference between the bulk phase and the out-

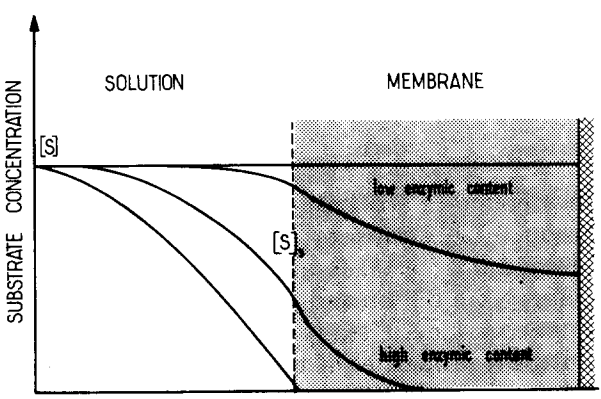

Fig. 1. Schematic illustration of the variation of the substrate concentration in a porous membrane containing an immobilized enzyme and in the surrounding solution. The different profiles correspond, at a given bulk concentration, [S], to increasing enzymic activity in the membrane, thus to increasing substrate diffusion limitations.

side particle or membrane surface. The value of h can be estimated with good precision for most configurations currently used for bound enzyme kinetic analysis, as detailed in Appendix I.

The rate of internal diffusion is commonly expressed by Fick's diffusion law using an effective substrate diffusivity, D_{eff}, inside the porous matrix. It can often be evaluated from the bulk phase diffusivity, D, by the following relationship:

$$D_{eff} = \frac{D\epsilon}{\tau} \tag{1}$$

The porosity, ϵ, represents the volume fraction of the porous medium occupied by the liquid and depends on the structure and the swelling of the matrix, its value is often between 0.8 and 0.4. The tortuosity, τ, on the otherhand, takes into consideration the non linear geometry of the pores and its value generally varies between 1 and 2. Consequently, the value of D_{eff} is frequently between a half and a fifth of that of D.

Effectiveness factors

The quantification and, consequently, the removal of diffusion effects is greatly facilitated by introducing the concept of effectiveness factors. Such parameters have been currently used in chemical engineering to analyse diffusion effects in heterogeneous reactors [8].

As a result of substrate depletion in the vicinity of the bound enzyme, the actual enzyme activity, v, is usually lower than the activity, v_{chem}, that would be measured in the absence of any diffusion limitations, i.e for a uniform substrate concentration equal to the bulk concentration. The relative decrease of enzyme activity due to diffusion limitations is expressed by the effectiveness factor, η, defined as

$$\eta = \frac{v}{v_{chem}} \tag{2}$$

Accordingly, the value of the effectiveness factor is smaller than one and approaches unity in the absence of diffusion effects.

The respective contributions of external and internal concentration gradients on the lowered enzyme activity can be separated by further defining an external and internal effectiveness factor, η_e and η_i, so that:

$$\eta = \eta_e \eta_i \tag{3}$$

The values of the external and internal effectiveness factors have been previously calculated and tabulated for known transport parameters and intrinsic kinetic constants of the enzymic reaction [4–6]. From a practical point of view, however, such graphs are of limited applicability since the maximal enzyme activity for the whole matrix, which is proportional to the amount of immobilized active enzymes, is difficult to evaluate a priori, especially if the measured activity is far from saturation.

The present method to remove diffusional effects is based on the evaluation of both external and internal effectiveness factors directly from the measured enzymic activity. More precisely, two series of curves have been calculated to

TABLE I

VARIABLES AND PARAMETERS WHICH MUST BE KNOWN OR ESTIMATED TO DETERMINE THE MAGNITUDE OF EXTERNAL AND INTERNAL DIFFUSION EFFECTS

External diffusion effects	External and internal diffusion effects
observed enzymic activity, v	observed enzymic activity, v
bulk substrate concentration, [S]	bulk substrate concentration, [S]
external surface area, $\mathcal{A}$	particle radius, r_p, membrane thickness, l
substrate external transport coefficient, h	external surface area, $\mathcal{A}$
intrinsic Michaelis constant of bound enzyme, K_m	volume of porous medium, ν
	substrate external tranport coefficient, h
	substrate internal diffusivity, D_{eff}
	intrinsic Michaelis constant of bound enzyme, K_m

permit the graphical determination of η_e and η_i for given measured activity, v, substrate bulk concentration, [S], transport parameters, h and D_{eff}. Knowing η_e and η_i, the overall effectiveness factor η is obtained from Eqn. 3, and, subsequently, the enzymic activity in the absence of diffusion limitations, v_{chem}, is simply calculated from Eqn. 2.

Evaluation of external diffusion effects

Table I lists the various variables and parameters that must be known or estimated before the magnitude of substrate diffusion effects can be determined. To evaluate the importance of external diffusion, in addition to the observed activity, v, the bulk substrate concentration, [S], and the previously discussed external transport coefficient, h, it is necessary to have an estimate of the external surface area of the particle and the intrinsic K_m of the bound enzyme. As illustrated in Fig. 2, the external surface area, $\mathcal{A}$, of a particle or membrane is not the actual interfacial area between the solid matrix and the liquid medium taking into account the pores of the matrix, but the macroscopic geometrical area of the support. For a spherical particle, for example, $\mathcal{A}$ is the external surface area of the sphere. The intrinsic K_m of the bound enzyme, on the other hand, as has been verified with several bound enzymes, can in a first approximation be taken equal to the K_m of the soluble enzyme.

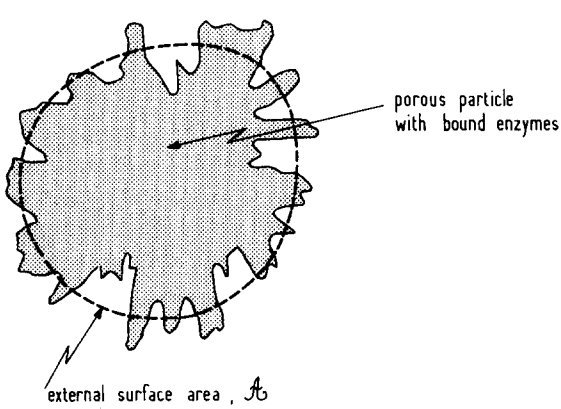

Fig. 2. Schematic representation of a porous particle and of its external surface area, $\mathcal{A}$.

With the values of the above parameters it is possible to calculate the ratio of the observed activity, v, and the maximum rate of external substrate transport, $\mathcal{A}h[S]$, and, consequently, to determine from Fig. 3 the external effectiveness factor, η_e, at a given normalized bulk concentration, $[S]/K_m$. Of course, when the value of η_e is found close to unity, external diffusion effects are negligible.

Evaluation of internal diffusion effects

According to Table I, to further determine the kinetic contribution of internal diffusion, it is also necessary to know the radius, r_p, in the case of a spherical particle, or the thickness, l, for a membrane, the total volume, v, of the porous medium, and the previously introduced substrate internal diffusivity, D_{eff}. In a first step the substrate concentration at the external surface, $[S]_S$, is calculated as

$$[S]_S = [S] - \frac{v}{\mathcal{A}h} \tag{4}$$

Fig. 4, after calculation of the abscissa parameter, then allows the determination of the internal effectiveness factor, η_i, for either a spherical particle or a flat membrane. As seen, the importance of diffusion effects depends on the value of the normalized substrate concentration at the surface, $[S]_S/K_m$.

Having thus evaluated the respective effects of external and internal diffusion, it is then possible to calculate the overall effectiveness factor, η, as the product of η_e and η_i and, consequently, to estimate the activity that would be measured in the absence of any limitations of diffusion as the ratio of the measured activity to the overall effectiveness factor.

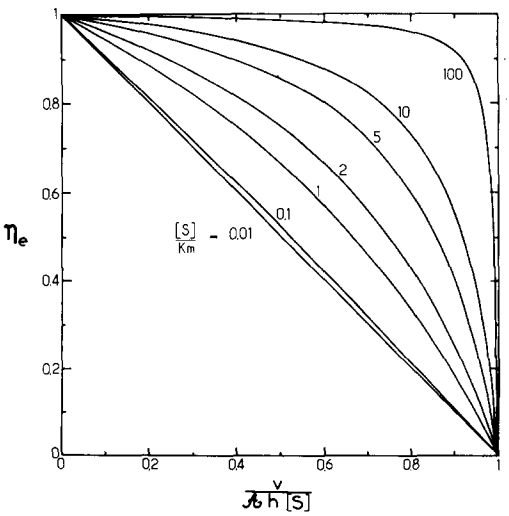

Fig. 3. Diagram for the evaluation of the external effectiveness factor with Michaelis-Menten kinetics. The abscissa ratio is first calculated from the measured enzymic activity, v, and bulk substrate concentration, [S], and from the estimated external surface area, $\mathcal{A}$, and substrate external transport coefficient, h. The external effectiveness factor, η_e, is then graphically evaluated at the given normalized bulk concentration, $[S]/K_m$.

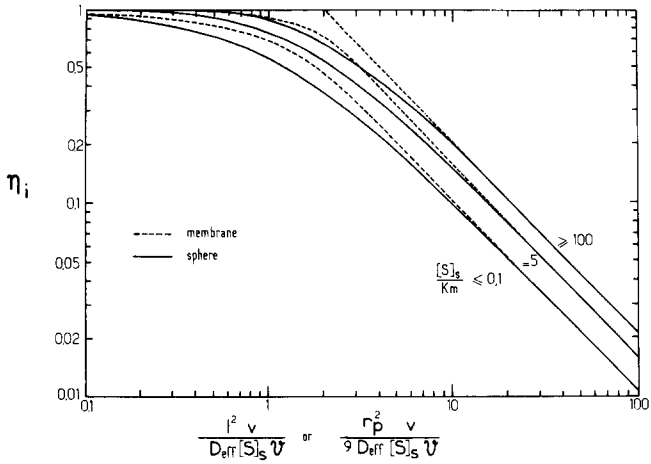

Fig. 4. Diagram for the evaluation of the internal effectiveness factor with Michaelis-Menten kinetics. The two sets of curves correspond to a spherical particle and to a flat membrane exposed on one side to the substrate solution. The abscissa ratio is first calculated from the measured enzymic activity, v, the calculated substrate surface concentration, $[S]_S$, and the estimated membrane thickness, l, or particle radius, r_p, substrate effective internal diffusivity, D_{eff}, and membrane or particle volume, ν. When the membrane is on both sides exposed to the substrate solution, l must be replaced by $l/2$. The internal effectiveness factor, η_i is then graphically evaluated at the given normalized surface concentration, $[S]_S/K_m$.

Examples

1. Immobilized invertase on ion-exchange resin beads

Kobayashi and Moo-Young [9] measured the rate of sucrose hydrolysis by invertase immobilized in an ion-exchange resin. Beads, 747 μm diameter, were packed in a glass column, 2.5 cm internal diameter. At a flow rate of 19.7 ml $\cdot$ min^{-1} and an average sucrose concentration of 0.085 M in the column, an activity of 0.8 mol sucrose $\cdot$ $s^{-1} \cdot l^{-1}$ of beads was obtained. In this concentration domain, sucrose hydrolysis follows Michaelis-Menten kinetics with a K_m of 0.115 M.

The voidage of the reactor being 0.41, the linear fluid velocity is 0.17 cm $\cdot$ s^{-1}. The external transport coefficient in the packed column is then estimated from Eqn. I-3 as $2.3 \cdot 10^{-3}$ cm $\cdot$ s^{-1} for a bulk sucrose diffusivity of $5 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$. The effective diffusivity in the resin beads was determined as $2 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$. Table II shows the values that are graphically obtained for the internal and external effectiveness factors.

Accordingly, bound invertase kinetics is mainly affected by internal

TABLE II

EXTERNAL AND INTERNAL EFFECTIVENESS FACTORS FOR IMMOBILIZED INVERTASE AS EVALUATED FROM FIGS. 3 AND 4 USING EXPERIMENTAL DATA OF KOBAYASHI AND MOO-YOUNG [9]

$\frac{[S]}{K_m}$	$\frac{v}{\mathcal{A}h\,[S]}$	η_e	$\frac{[S]_S}{K_m}$	$\frac{r_p^2 v}{9 D_{eff}[S]_S \nu}$	η_i
0.75	0.05	0.97	0.7	0.77	0.65

diffusional limitations. The overall effectiveness factor being around 0.6, in the absence of any diffusion limitations for sucrose an activity of 1.3 mol sucrose per second and liter beads would be obtained instead of the 0.8 mol per second and liter actually measured.

2. Collagen bound aspartate aminotransferase

Coulet et al. [10] covalently attached aspartate aminotransferase at the external surface of collagen films. In solution the kinetics of the two-substrate reaction between oxaloacetate and glutamate is of the ping-pong type with a Michaelis constant for oxaloacetate of 0.052 mM and for glutamate of 5 mM. The external transport coefficient for both substrates was estimated close to 10^{-3} cm $\cdot$ s^{-1} [11]. At 0.12 mM oxaloacetate and 20 mM glutamate an activity of $0.11 \cdot 10^{-9}$ mol $\cdot$ s^{-1} $\cdot$ cm^{-2} was obtained.

The procedure outlined in this study for a single substrate reaction following Michaelis-Menten kinetics can still be used with a two-substrate reaction by successively applying the method to each substrate. For glutamate first, $v/\mathcal{A}h$[S] is equal to 0.006, which, according to Fig. 3, indicates the absence of external diffusional limitations for glutamate ($\eta_e = 1$), whatever the value of of the normalized bulk glutamate concentration. For oxaloacetate, on the other hand, $v/\mathcal{A}h$[S] is equal to 0.92. Since the intrinsic rate expression of aspartate aminotransferase can be written as

$$v = \frac{V[\mathrm{Gl}][\mathrm{Ox}]}{K_{\mathrm{Ox}}[\mathrm{Gl}] + K_{\mathrm{Gl}}[\mathrm{Ox}] + [\mathrm{Ox}][\mathrm{Gl}]} = \frac{\dfrac{V[\mathrm{Gl}]}{K_{\mathrm{Gl}} + [\mathrm{Gl}]}[\mathrm{Ox}]}{\dfrac{K_{\mathrm{Ox}}[\mathrm{Gl}]}{K_{\mathrm{Gl}} + [\mathrm{Gl}]} + [\mathrm{Ox}]}$$

at a fixed glutamate concentration of 5 mM v obeys a Michaelis-Menten type of dependence on oxaloacetate concentration with a calculated pseudo Michaelis constant of 0.042 mM. The normalized oxaloacetate bulk concentration may thus be taken around 3, which gives on Fig. 3 an external effectiveness factor close to 0.2.

According to this simple method, only oxaloacetate diffusion limitations are affecting the collagen bound aspartate aminotransferase kinetics, and, in the absence of diffusion effects, a $0.55 \cdot 10^{-9}$ mol $\cdot$ cm^{-2} $\cdot$ s^{-1} activity would be measured.

Discussion

This study proposes two diagrams for a fast evaluation of both external and internal substrate diffusional effects on bound enzyme activity. The overall procedure involves only simple algebric calculations and mainly requires a prior estimation of the volume and external surface area of the porous matrix as well as of the substrate external transport coefficient and effective internal diffusivity. The precision on these parameters obviously determines the accuracy of the method.

The two sets of curves giving the external and internal effectiveness factors as a function of the measured activity have been calculated for Michaelis-

Menten kinetics. However, as illustrated in one of the examples, they can also be used to evaluate diffusion effects with two-substrate enzymic reactions. A similar approach is further applicable to reversible and product inhibited reactions provided the two graphs are recalculated taking into account the diffusion of both substrate and product.

Experimental results on immobilized enzyme kinetics are often published without great details on the system geometry. In view of the present quantitative analysis, it is then practically impossible to draw any conclusion on the possible interferences of diffusion phenomena on measured activities. It is thus hoped that this study will also contribute to emphasize the necessity, when dealing with immobilized enzyme kinetics, to specify the precise geometry and structure of the enzymic support as well as the hydrodynamic conditions in the bulk solution, in order to facilitate the discussion of experimental data.

Appendix I — *Values of the external transport coefficient*

Bound enzyme kinetics are usually investigated with particles or membranes in stirred solution or packed in a column. For most of these systems the external transport coefficient, h, has been experimentally determined. The following correlations of the literature can be used to estimate the value of h:

Spherical particles in stirred solution [12]

$$\left(\frac{hd_p}{D}\right)^2 = 4 + 1.21\left(\frac{d_p U}{D}\right)^{2/3} \tag{I-1}$$

where d_p is the particle diameter, D the substrate bulk diffusivity and U the relative velocity between the particles and the fluid. The velocity U can further be approximated [13] as

$$U = \frac{g d_p^2 \, \Delta\rho}{18\mu} \tag{I-2}$$

$\Delta\rho$ being the density difference between the particles and the fluid, g the gravitational acceleration (981 cm $\cdot$ s^{-2}) and μ the fluid viscosity. For example, with porous glass particles in water ($\Delta\rho$ = 0.25) and a substrate of diffusivity $5 \cdot 10^{-6}$ cm^2 $\cdot$ s^{-1}, h is approximately equal to 10^{-1} cm $\cdot$ s^{-1} for d_p = 1 μm, 10^{-2} cm $\cdot$ s^{-1} for d_p = 10 μm, and is close to $3.5 \cdot 10^{-3}$ cm $\cdot$ s^{-1} for d_p larger than 100 μm.

Particles packed in a column [14]

$$\frac{h}{U}\left(\frac{\mu}{\rho D}\right)^{2/3} = 2.5\left(\frac{d_p U \rho}{\mu}\right)^{-0.66} \tag{I-3}$$

where U is the average fluid velocity in the column and ρ the fluid density. With a fluid velocity of 1 cm $\cdot$ s^{-1} and a substrate of diffusivity $5 \cdot 10^{-6}$ cm^2 $\cdot$ s^{-1}, for instance, Eqn. I-3 yields an h value of $8 \cdot 10^{-2}$ cm $\cdot$ s^{-1} for d_p = 10 μm, $1.6 \cdot 10^{-2}$ cm $\cdot$ s^{-1} for d_p = 100 μm, and $3.6 \cdot 10^{-3}$ cm $\cdot$ s^{-1} for d_p = 1 mm.

Membranes in stirred solutions

At present no correlations seem to be available for pieces of membranes moving freely in a stirred solution. Only a few specific values, usually around 10^{-3} cm $\cdot$ s^{-1}, have been proposed [11,15].

Appendix II — *Calculations of effectiveness factors*

Internal effectiveness factor, η_i

The enzyme is assumed to be uniformly distributed inside a porous particle or membrane. The intrinsic kinetics of the bound enzyme, in the absence of diffusional limitations, is of the Michaelis-Menten type with a maximal activity V for the whole matrix and a Michaelis constant K_m. If $[S]_S$ is the substrate concentration at the external surface and v the measured enzymatic activity, the internal effectiveness factor is defined as:

$$\eta_i = \frac{v}{\dfrac{V[S]_S}{K_m + [S]_S}} \tag{II-1}$$

The determination of η_i as a function of the kinetic and diffusion parameters requires the calculation by numerical integration, first of the substrate concentration profile inside the matrix, second of the activity for the whole matrix, as detailed in previous studies [16,17].

External effectiveness factor, η_e

At steady state the rate of the enzymic reaction in the porous matrix is equal to the rate of substrate transport between the bulk solution and the external surface, i.e.:

$$v = \mathcal{A}h([S] - [S]_S) = \frac{\eta_i V[S]_S}{K_m + [S]_S} \tag{II-2}$$

$\mathcal{A}$ being the external surface area.

$[S]_S/K_m$, the normalized surface concentration, is thus the solution of the following rearranged equation:

$$\frac{\mathcal{A}hK_m}{\eta_i V}\left(\frac{[S]}{K_m} - \frac{[S]_S}{K_m}\right) = \frac{[S]_S/K_m}{1 + [S]_S/K_m} \tag{II-3}$$

and depends only on the value of $[S]/K_m$, the dimensionless bulk substrate concentration, and $(\eta_i V)/(\mathcal{A}hK_m)$, a dimensionless number reflecting the importance of diffusional limitations.

Since the overall effectiveness factor, η, is the product of both the external and internal effectiveness factors, $\eta_e\eta_i$, and represents the ratio of the measured rate, v, to v_{chem}, the rate in the absence of diffusional limitations, the external effectiveness factor, η_e, is given by:

$$\eta_e = \frac{v}{v_{chem}\,\eta_i} \tag{II-4}$$

According to Eqns II-1, η_e can be calculated as

$$\eta_e = \frac{\dfrac{v}{\eta_i V}}{\dfrac{v_{chem}}{V}} = \frac{\dfrac{[S]_S/K_m}{1 + [S]_S/K_m}}{\dfrac{[S]/K_m}{1 + [S]/K_m}} \qquad \text{(II-5)}$$

thus, like $[S]_S/K_m$ depends only on $[S]/K_m$ and $\eta_i V/\mathcal{A}h\ K_m$

The ratio of the actual rate to the maximum rate of external diffusion, on the other hand, is simply calculated by

$$\frac{v}{\mathcal{A}h[S]} = \frac{v}{\eta_i V}\frac{\eta_i V}{\mathcal{A}hK_m}\frac{K_m}{[S]} = \frac{[S]_S/K_m}{1 + [S]_S/K_m}\ \frac{\eta_i V}{\mathcal{A}hK_m}\ \frac{K_m}{[S]} \qquad \text{(II-6)}$$

Consequently, the curves in Fig. 3, representing η_e against $v/\mathcal{A}h\ K_m$ at different $[S]/K_m$, are obtained by solving Eqn. II-2 for varying values of $[S]/K_m$ and $\eta_i V/\mathcal{A}h\ K_m$ and reporting the calculated $[S]_S/K_m$ in Eqns. II-5 and II-6.

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