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Kinetic and Thermodynamic Aspects of Catalysis by Immobilised Enzymes

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The review deals with a quantitative analysis of the physicochemical characteristics of catalysis by immobilised enzymes. The role of factors such as inhibition by external and internal diffusion and the characteristics of diffusion in polymeric carriers, the nature of the carrier, the changes in the true kinetic parameters of enzymatic reactions after immobilisation, and steric limitation of the rates of reaction of immobilised enzymes with macromolecular substrates are considered. The bibliography includes 152 references.

CONTENTS

I.	Introduction	9
II.	The role of diffusion in catalysis by immobilised enzymes	9
III.	Effect of the nature of the carrier on the kinetics of reactions catalysed by immobilised enzymes	20
IV.	The change in the true kinetic parameters of enzymic reactions after immobilisation	22
V.	Steric rate limitation in reactions of immobilised enzymes with macromolecular substrates	22

I. INTRODUCTION

There has been an exponential growth in recent years of the number of studies on immobilised (fixed) enzymes, which has doubled in approximately two years. According to the data of the American Chemical Society[1], research in the field of immobilised enzymes was among the nine most intensely studied topics in chemistry in 1972.

Interest in immobilised enzymes can be explained by two fundamental causes. Firstly, the wide prospects which are opened up by the application of immobilised enzymes in technology and medicine. In this connection we may point out that the unique properties of biological catalysts have been for a long time attracting the attention of workers in different branches of sciences and engineering. These properties include primarily their high catalytic efficiency (i.e. the ability to ensure reaction rates greatly exceeding, sometimes by a factor of 10^{10}—10^{13}, those in the presence of non-biological catalysts), their insurpassable selectivity (not only as regards the type of reaction catalysed but also with respect to the structure of the substrate), and finally the high capacity of enzymes to respond to the regulating action of fine changes in the properties and specific composition of the medium. However, the attempts to use biological catalysts as catalytic or regulating elements in applied chemistry or physics have as a rule encountered a seemingly insurmountable obstacle, involving their exceptionally low resistance to the denaturing influence of the "medium" (the effects of heat and micro-organisms as the principal denaturing factors in protein systems *in vitro* must be mentioned in the first place). On the other hand, the first studies in the field of immobilised enzymes have already suggested a number of ways of improving their stability[2].

1. Covalent attachment of the enzyme to a water-insoluble (or water-soluble) polymeric carrier (such as cellulose, glass, polystyrene, polyaminoacids, etc.).
2. Covalent attachment of enzyme molecules to one another with the aid of an added polyfunctional reagent with formation of water-insoluble (or water-soluble) aggregates.
3. The incorporation of the enzyme in the three-dimensional network of a gel or polymer.
4. The adsorption of the enzyme on water-insoluble carriers (for example on ion-exchange resins).
5. The inclusion of the enzyme in semipermeable microcapsules or polymeric fibres.

Evidently as a result of immobilisation of this kind the enzyme acquires yet another property important for technology—it can be readily separated from the products of the catalysed reaction, which permits its repeated employment or its use under flow conditions[3].

In medical practice immobilisation facilitates the fixation of the enzymes (or other biologically important substances) in specific parts of the organism in addition to increasing its stability[4].

Immobilised enzymes can be regarded as extremely realistic models for biologically active systems acting *in vivo* within membranes or on their surface[2,5].

A number of reviews have already been published on immobilised enzymes[2,3,6-23]. Since a compilation of these reviews has been published in Russia[24], we shall not deal with the methodological aspects of the preparation of immobilised enzymes (see, for example, Crook et al.[10] and Weetall[16]) or the practical aspects of their use in science, engineering, and medicine[3,25]. Attention is concentrated on the kinetic and thermodynamic features which distinguish catalysis by immobilised enzymes from the behaviour of homogeneous (native) systems. These new specific features may include (a) the appearance of external and internal diffusion inhibition in catalysis, (b) factors associated with the distribution of substrates, inhibitors, hydrogen ions, and other effectors between the aqueous solution and the polymeric matrix (as a result of electrostatic or hydrophobic interaction, hydrogen bonds, etc.), and (c) the occurrence of steric hindrance in reactions with macromolecular substrates. In examining these aspects, we shall endeavour to achieve quantitative generalisations, which would make it possible to predict *a priori* in many cases the kinetic and thermodynamic properties of immobilised enzymic systems.

II. THE ROLE OF DIFFUSION IN CATALYSIS BY IMMOBILISED ENZYMES

The principal postulates formulated in the theory of heterogeneous catalysis are essentially applicable to

immobilised enzymes. The effect of diffusion on heterogeneous catalytic processes has been studied in extremely great detail for reactions of different kinetic orders[26-30]. Here we shall consider the influence of diffusion factors on an enzymic reaction whose rate v obeys the Michaelis–Menten equation:

$$v = \frac{V_{max} \cdot C}{K_m + C}, \quad (1)$$

where $V_{max} = k_{cat}[E]$, $[E]$, and C are the initial concentrations of the enzyme and substrate respectively, k_{cat} the catalytic first-order rate constant, and K_m the Michaelis constant. We shall bear in mind that the reacting enzyme is in an immobilised state, being, for example, bound covalently to a water-insoluble porous carrier or incorporated in a gel matrix or within a microcapsule. For the enzymic reaction to occur, it is evidently necessary in the first place for a substrate molecule in solution to approach the surface of the particle containing the immobilised enzymes and in the second place that it can diffuse within the latter. On the other hand, if the reaction in the surface layers of the particle is faster than the supply of the substrate from the solution to the surface, then a zone depleted in the substrate will arise round the particle after a time. As a result, the overall rate of the enzymic process will be determined by the rate of mass transfer of the substrate to the particle. By analogy with heterogeneous catalysis[26], we may say that in this case the process is controlled by external diffusion. On the other hand, we can imagine that the mass transfer of the substrate is faster than the enzymic reaction on the surface of the particle. Then another type of diffusion-dependent inhibition may arise, namely, if the size of the carrier particle is very great or its enzyme is very active, then almost the entire substrate reacts in the part of the carrier near the surface, while its bulk is depleted in the substrate. In such cases one usually says that the process is controlled by internal diffusion[26]. The effects of both diffusion factors on the effective kinetic parameters of an enzymic reaction are analysed below with the framework of the Michaelis equation [Eqn. (1)].

1. EXTERNAL DIFFUSION INHIBITION

Mass transfer takes place as a result of two different processes—convection and molecular diffusion. In liquids the transport of a substance as a result of convection is usually much faster than molecular diffusion[30] and therefore the latter process may be virtually disregarded. However, in examining heterogeneous systems (for example suspended solid particles) one must bear in mind that the boundary between the "phases" is directly adjoined by a layer of unstirred liquid within which the transport of molecules or ions proceeds exclusively via molecular diffusion. Consequently, when an enzymic reaction in which the substrate is consumed takes place on the surface of the particle, a steep reactant concentration gradient may arise within the unstirred layer, because molecular diffusion in liquids is very slow (with a diffusion coefficient of 10^{-5}–10^{-6} cm^2 s^{-1}).[30] As a result, the rate of mass transfer may be limited by the stage involving the heterogeneous catalytic process regardless of the rate of stirring.

We shall consider certain quantitative features of the process for a spherical particle assuming that the enzyme is immobilised either on the surface of a sphere or, if the carrier is porous, also within the sphere, but in the absence of internal diffusion inhibition. We shall assume further that the partition coefficient of the substrate between the solution and the carrier "phase" hardly differs from unity. The surface of the particle may be regarded as equally accessible[26]. Then the rate of supply of the substrate by diffusion j, referred to unit surface area of the particle is †

$$j = \beta (C_0 - C), \quad (2)$$

where C_0 and C are the substrate concentrations in the bulk of the solution and on the surface of the particle respectively, and β is the mass transfer coefficient.

a. In the special case where the enzymic reaction occurring on the surface of the particle follows first-order kinetics we have $K_m \gg C$ in Eqn. (1). Under these conditions, the steady state in the enzymic process is described by the following equation:

$$\beta (C_0 - C) = (V_{max}/K_m) \cdot C, \quad (3)$$

which reflects the fact that the specific rate of supply of the substrate to the surface of the particle with the immobilised enzyme (j) is equal to the rate of the enzymic reaction (v) referred to the unit surface area. From Eqn. (3), we have for the effective substrate concentration in the reaction zone

$$C = \frac{\beta \cdot C_0}{\beta + V_{max}/K_m}. \quad (4)$$

As a result, we conclude that the experimental steady-state rate of the enzymic reaction

$$v = (V_{max}/K_m) \cdot C = k_{eff} \cdot C_0 \quad (5)$$

is characterised by an effective first-order rate constant given by

$$k_{eff} = \frac{(V_{max}/K_m) \cdot \beta}{\beta + V_{max}/K_m} = \frac{1}{\frac{1}{\beta} + \frac{1}{V_{max}/K_m}}. \quad (6)$$

Two limiting cases are possible. When $\beta \gg V_{max}/K_m$, i.e. the mass transfer is much faster than the chemical reaction, we have $k_{eff} = V_{max}/K_m$. On the other hand, when $\beta \ll V_{max}/K_m$, i.e. the chemical reaction is much faster than the mass transfer, then $k_{eff} = \beta$. In the first case we shall say that the reaction proceeds under kinetic conditions and in the second case under diffusion conditions[31].

b. In the general case when the enzymic process is not of first order but of a fractional order (i.e. $K_m \sim C$) the existence of two process conditions (diffusion and kinetic) still remains in force despite the more cumbersome expressions for the reaction rate[26].

c. The second extreme case of the Michaelis equation [Eqn. (1)] when $K_m \ll C$, i.e. the enzyme is saturated by the substrate, constitutes an exception. Since an enzymic reaction is zero order with respect to substrate in the general case, evidently the process cannot in principle be controlled by diffusion and it proceeds under kinetic conditions.

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† This assertion is approximate. It has been shown[30] that the diffusion flux to the absorbing sphere in a moving liquid is not distributed uniformly on its surface. It is a maximum at the point where the liquid advances to the sphere and diminishes on its rear side.

Theoretical Estimation of the Limits of the Diffusion and Kinetic Regions

Under kinetic conditions, the reaction evidently proceeds in full conformity with the laws specified by its mechanism. The situation is different under diffusion conditions where the reaction kinetics cannot yield information about the chemical mechanism, since they are determined by mass transfer parameters alone. To make a choice between these process conditions, it is necessary to estimate β and compare it, for instance in case (a) (see above), with V_{max}/K_m. For this purpose, we shall show, following O'Neill[32], how this can be done after making certain assumptions. It follows from similarity theory[26] that

$$\beta = \frac{NuD}{d}, \qquad (7)$$

where Nu is the Nusselt number, D the diffusion coefficient of the substance transported, and d the diameter of the absorbing particle. For a laminar flow of the liquid around the spherical particle and low Reynolds numbers, we have[26,28] Nu = 2. As an approximation, we shall assume that this condition holds in our system‡. Then we have from Eqn. (7)

$$\beta = \frac{2D}{d}. \qquad (8)$$

This value constitutes the lower limit. In fact, in consequence of the stirring of the system, β can be higher[26]. However, in studies with enzymes stirring is not usually very fast and therefore this effect is not very significant.

Knowing D and d, it is possible to calculate β by Eqn. (8). By comparing it [case (a)] with V_{max}/K_m, one can in principle deduce the type of conditions governing the enzymic reaction [see Eqn. (6)]. However, V_{max}/K_m must be determined from experimental data, which are already distorted by the influence of diffusion and therefore we shall indicate another, more convenient approach. Having evaluated β, it is possible to calculate the maximum rate of mass transfer and to compare it with the experimental rate of the reaction catalysed on the immobilised enzyme. If these two quantities are similar, the implication is that the reaction proceeds under diffusion conditions. On the other hand, if the observed rate of the enzymic reaction is much lower than the maximum possible rate of mass transfer, the reaction evidently proceeds under kinetic conditions. This approach is valid for any kinetic order of the enzymic reaction and we shall consider it in greater detail.

The rate of mass transfer R to any catalyst particle, which is equal to the diffusion flux multiplied by the surface area of the particle A, is defined by the following expression in accordance with Eqns. (2) and (8):

$$R = jA = \frac{2D}{d} \cdot A(C_0 - C). \qquad (9)$$

Since $A = \pi d^2$ for a sphere, it follows that

$$R = 2\pi Dd(C_0 - C). \qquad (10)$$

‡ Empirical formulae are frequently used in the literature to determine β in various heterogeneous systems under different conditions[33-35]. Our approximation should hold at not unduly high flow rates and has the advantage that β can be readily estimated numerically. In certain studies on immobilised enzymes β is determined from other equations[35,36].

The diffusion coefficient of the substrate in the liquid D, entering into Eqn. (10), can be determined experimentally in a separate experiment or estimated from different empirical correlations, provided that the substrate concentration does not exceed 10 mole %.[37] On the other hand, if the particle containing the immobilised enzyme is not spherical, then d is usually assumed equal to the diameter of the sphere whose surface area is equal to that of the particle employed.

The maximum possible rate of mass transfer R_{max} follows from Eqn. (10) for $C_0 \gg C$:

$$R_{max} = 2\pi DdC_0, \qquad (11)$$

where R_{max} is usually expressed in mole per second per particle. According to the recommendations of the International Commission on Immobilised Enzymes, the rate of reaction should be expressed in micromoles of the substrate per minute per milligram of the dry immobilised enzyme preparation[38]. Then Eqn. (11) must be transformed into

$$R^{tot}_{max} = 7.2 \cdot 10^2 \cdot \frac{DC_0}{\rho d^2}, \qquad (12)$$

where ρ is the density of the immobilised enzyme preparation expressed in g cm^{-3}, D is expressed in cm^2 s^{-1}, d in cm, and C_0 is the concentration on the molar scale.

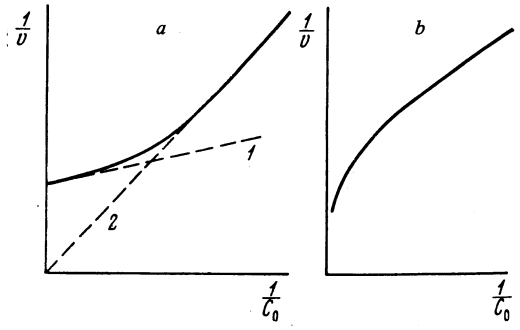


Figure 1. *a*) Theoretical variation of the rate of an enzymic reaction with substrate concentration in terms of the Lineweaver–Burk variables distorted by inhibition due to external diffusion; *b*) experimental curve in terms of the same variables[39,40] apparently corresponding to the presence of two kinetically different forms of the immobilised enzyme.

Certain Experimental Criteria

a. As mentioned above, at sufficiently high substrate concentrations (i.e. when the immobilised enzyme is saturated and the observed rate of reaction is of zero order with respect to the substrate), the enzymic process cannot in principle be controlled by the diffusion of the substrate and therefore the reaction proceeds under kinetic conditions. In its turn the probability of a transition of the reaction system to diffusion-dependent conditions increases with decrease of substrate concentration. This behaviour of the catalytic system is illustrated in Fig. 1, where the theoretical Lineweaver-Burk plot is presented for a reaction occurring at high substrate concentrations (dashed line *1*) under kinetic conditions, where

the experimental results can therefore be described with the aid of Eqn. (1), and at low substrate concentrations (dashed curve *2*) under diffusion conditions, where the variation of the experimental process velocity v with C_0 actually obeys Eqn. (12). On the other hand, if this behaviour of the $1/v-1/C_0$ plot is observed in experiments with an immobilised enzyme (i.e. the plot bends away from the usual linear Lineweaver–Burk relation), then we have the first indication that diffusion plays a significant role in the process. However, here attention should be drawn to the nature of the curvature. Thus a curved Lineweaver–Burk plot of another type, shown in Fig. 1*b*, was obtained in studies[39,40] on the catalytic properties of α-chymotrypsin and glucoamylase immobilised on cellulose derivatives. The authors also explain the observed curvature of the $1/v-1/C_0$ plot from the standpoint of diffusion-dependent inhibition. However, in this case such treatment of the results is incorrect, since it follows from theory that diffusion-dependent inhibition should be manifested particularly strongly at low substrate concentrations (Fig. 1*a*). However, the opposite behaviour actually follows from the experimental data (Fig. 1*b*), namely the process is accelerated at low substrate concentrations. Moreover, from the theoretical standpoint the process controlled by diffusion should follow first-order kinetics with respect to the substrate at low concentrations of the latter [Eqn. (12)] and this too is not observed in the experiment, since (Fig. 1*b*) extrapolation of the linear section of the $1/v-1/C_0$ curve, corresponding to low values of C_0, does not pass through the origin of coordinates. Therefore we believe that the curvature of the $1/v-1/C_0$ relation observed experimentally[39,40] (of the type of the curve in Fig. 1*b*) is probably due to the presence of several kinetically different forms of the immobilised enzyme.

b. Enzymic reactions usually have activation energies of the order of 10–30 kcal mole^{-1}. On the other hand, the rate of an enzymic process occurring under diffusion conditions should depend only slightly on temperature since the only temperature-sensitive parameter (D) in Eqn. (12) is associated with an activation energy of about 4–5 kcal mole^{-1} in water[41]. Consequently it is entirely valid to use the nature of the temperature variation of the reaction rate as a criterion for the determination of the limits of the kinetic and diffusion regions. In this connection one must, however, bear in mind that with increase of temperature the reaction undergoes a transition from kinetic to diffusion conditions [as follows, for example, from an analysis of Eqn. (6), bearing in mind the above differences between the temperature variation of β defined by Eqn. (8) and V_{max}/K_m].

c. Under diffusion conditions the reaction rate should evidently be independent of changes in the specific concentration of the immobilised enzyme [as follows, for example, from Eqns. (5) and (6)] assuming that under such conditions $V_{max}/K_m \gg \beta$. Moreover, if the reaction proceeds under diffusion conditions, its rate should not be affected by specific factors such as a change in pH or ionic strength and the addition of inhibitors or activators; in general these factors are associated with the enzymic mechanism and their influence can in principle be followed for the native catalyst. However, one should bear in mind that, for the same preparation of the immobilised enzyme, the reaction with a highly reactive substrate can proceed under diffusion conditions, while with a less reactive substrate kinetic conditions may obtain. All the postulates discussed in this section follow, for example, from an analysis of Eqns. (5) and (6) bearing in mind that the limits of the diffusion and kinetic regions are defined by the relative values of the parameters β and V_{max}/K_m.

d. Under kinetic conditions, the rate of reaction should be independent of the degree of comminution of the particles containing the immobilised enzyme (provided that there is no internal diffusion inhibition for the substrate). On the other hand, when the reaction proceeds under diffusion conditions its rate increases after comminution of the enzymic preparation, since the decrease in the particle diameter d leads to an increase of the diffusion parameter β [see Eqn. (8)].

e. The influence of diffusion factors may weaken when the rate of mass transfer of the substrate is accelerated by stirring. As already mentioned concerning Eqn. (7), this is because the parameter β depends in principle on the flow rate[30]. A very simple relation between the reaction rate and the rate of stirring can be arrived at from the standpoint of the concept of an unstirred layer, as a physically real feature, to which Fick's first law is applicable. [See, however, the objections to this approach raised by Levin[30] (p. 51) and Ioffe and Pis'men[27] (p. 113).] When the substrate concentration in the diffusion layer decreases linearly, we find that the specific rate of mass transfer $j = (C_0 - C)(D/\delta)$, where δ is the effective thickness of the stationary layer. Comparison of this expression with Eqn. (12) shows that the parameter β, which determines the type (diffusion or kinetic) of Eqn. (16) can be formulated as $\beta = D/\delta$. In its turn, it follows from hydrodynamics that the thickness of the diffusion layer diminishes with increase of the flow rate of the liquid u around the particle[30]:

$$\delta \propto u^{-n}, \qquad (13)$$

where n varies from 1/3 to 1 depending on the conditions (for example $n = 1/3$ for spherical particles or for the flow within a cylinder). This is true not only of an individual particle but also of layers of particles. Thus an increase in the rate of rotation of the stirrer in the reactor or even an increase in the flow rate of the substrate through the column should weaken diffusion-dependent control. In its turn, the dependence of the observed rate of the enzymic reaction on the rate of stirring or on the flow rate demonstrates a significant role of diffusion in this process. Presumably a significant increase in the rate of rotation of the stirrer can cause a transition from diffusion to kinetic conditions. However, the very fact that at low rates of rotation the observed reaction rate depends on stirring while at high rates of rotation such dependence is not observed, cannot alone serve as proof of a transition from diffusion to kinetic conditions, since, as shown in a study of heterogeneous reactions[42], at high rates of stirring the so called slip effect is observed, i.e. the stirrer ceases to drag the liquid with it and therefore the true flux velocities in the system do not change on further increase of the rate of rotation.

The Applications of Theoretical Postulates to the Analysis of Experimental Data

The predicted effects have been confirmed experimentally. It has been shown[39] that, with increase of the rate of stirring in a suspension of α-chymotrypsin bound covalently to KM-cellulose, the rate of enzymic hydrolysis of the ethyl ester of *N*-acetyl-L-tyrosine also increases. In its turn, the catalytic activity of columns with an immobilised enzyme rises with increase of the flow rate of

substrate solutions through them[35,36,40,43]. Fig. 2 illustrates this type of relation for the reaction of trypsin immobilised on porous glass with the *p*-nitroanilide on *N*-benzoyl-DL-arginine[35]. Experiment[40] also confirms the conclusion that there is a high probability that the reaction passes to diffusion conditions at low substrate concentrations: thus at low concentrations of starch (5%) the rate of its reaction with immobilised glucoamylase depends strongly on the flow rate through the column, while at high concentrations (30%) it is almost independent of the flow rate. It has been shown[44] that the reaction of trypsin bound covalently to the inner surface of a nylon tube with the ethyl ester of *N*-benzoyl-L-arginine is fully controlled by diffusion (even for $C_0 > 1000K_m$), the rate of reaction being proportional to the cube root of the flow rate of the substrate through the tube, as predicted by the theory [Eqn. (13)]. Bubbling of the stream of substrate with a gas, which improves the stirring within the tube, greatly increases the observed rate of the enzymic reaction.

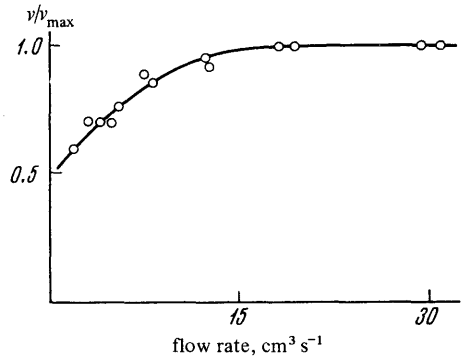


Figure 2. Dependence of the effective catalytic activity of a column filled with immobilised trypsin on the flow rate of the substrate (*p*-nitroaniline of *N*-benzoyl-DL-arginine) solution[35]; v = observed reaction rate at given flow rate Q; v_{max} = maximum reaction rate at fairly high values of Q.

Eqn. (12) can be used not only for qualitative but also for quantitative estimates of the role of external diffusion in catalysis by immobilised enzymes[32]. Thus the reaction of α-chymotrypsin immobilised on KM-cellulose and DEAE-cellulose with the ethyl ester of N-acetyl-L-tyrosine has been studied[39,45]. Table 1 presents the kinetic parameters of these reactions. In both cases the size of the particles containing the immobilised enzyme was about 40 μm and their density can be assumed to be 1 g cm^{-3}. The diffusion coefficient of the substrate, estimated with the aid of empirical correlations[46], will be assumed equal to 5×10^{-6} cm^2 s^{-1}. Then it follows from Eqn. (12) that $R^{tot}_{max} = 2.25 \times 10^2 C_0$. Consequently the maximum rate of mass transfer R^{tot}_{max} for α-chymotrypsin immobilised on DEAE-cellulose becomes lower than the calculated rate of reaction for $C_0 < 5 \times 10^{-2}$ M (as shown by the data in Table 1) and therefore diffusion should become a significant factor in the enzymic process. Indeed experiment shows[45] that the reaction is fully controlled by diffusion for $C_0 < 10^{-2}$ M and that the effect of diffusion is less significant for $C_0 > 10^{-2}$ M. Evidently the agreement between the theory and experiment is satisfactory. As was to be expected, the effect of diffusion disappears with increase of the rate of the enzymic reaction when the experiment is carried out at a pH far from the optimum (at pH < 5). In its turn, comminution of the particles containing the immobilised enzyme also lifts the diffusion-dependent inhibition[45]. The theoretical R^{tot}_{max} for α-chymotrypsin immobilised on KM-cellulose [Eqn. (12)] becomes equal to the theoretical values of ν (Table 1) for $C_0 \simeq 10^{-3}$ M. It has been shown experimentally[39] that at such substrate concentrations external diffusion does indeed play an appreciable role.

Table 1. Kinetic parameters of the hydrolysis of the ethyl ester of *N*-acetyl-L-tyrosine catalysed by immobilised α-chymotrypsin.

Carrier	V_{max}, μmole/min mg of preparation	K_m, 10^{-3} M
DEAE-Cellulose (Ref.45)	12	5
KM-Cellulose (Ref.39)	0.5	1

Enzymes are sometimes immobilised in membranes[47-50] or are grafted onto cellulose sheets[51,52]. On the surface of a membrane, there is also a stationary layer, which in some cases can lead to a transition of the reaction to diffusion conditions. Such a system has been analysed in detail by Katchalski and coworkers[48]. The thickness of the diffusion layer (about 50 μm) can be found from the kinetic properties of the system. This value agrees with the earlier determinations of δ from the variation of the permeabilities of various membranes with the efficiency of stirring[53].

We may note that the examples quoted are a sufficient indication that external diffusion factors can indeed play an important role in catalysis by immobilised enzymes. The theoretical relations determined make it possible in turn to outline procedures whereby the experimenter can cause a transition of the reaction from diffusion to kinetic conditions (for example by raising the substrate concentration or conversely by reducing the specific concentration of the immobilised enzyme, or by increasing the rate of stirring, by comminuting the particles containing the immobilised enzyme, etc.).

2. INTERNAL DIFFUSION INHIBITION

The reaction of an enzyme incorporated in the three-dimensional network of a gel or a polymer or joined chemically to a porous carrier can be sensitive to inhibition by internal diffusion, i.e. the diffusion of the substrate within the gel, polymer, or the carrier pores.

We shall consider this problem in relation to a membrane (for example, a polyacrylamide[50] or collodion[47-49] membrane) or a polymeric disc (with a diameter much greater than its thickness) containing an immobilised enzyme. The problem of internal diffusion in such systems was analysed by Katchalski and coworkers[49] for low substrate concentrations and by Laidler and Sundaram[7,54] in the general case.

Here and henceforth we shall base the treatment on Fick's simple diffusion model. If there is an interaction between the membrane and the diffusing substance (for example, an electrostatic interaction[55]), then Fick's first law must be written in the form

$$\frac{dm}{dt} = -D \cdot A \cdot \left[\frac{\partial C}{\partial x} + \frac{C}{kT} \cdot \frac{\partial U}{\partial x}\right],$$

where m and C are the amount and concentration of the substance transferred, t is the time, D the diffusion coefficient, A the area through which diffusion takes place, k the Boltzmann constant, T the absolute temperature, x the coordinate of the direction of diffusion, and U the potential energy of the diffusing species, which depends on its position relative to the membrane. The solution of this equation depends on the type of the function $U(x)$. For a simple electrostatic interaction, $U(x)$ is determined by Coulomb's law.

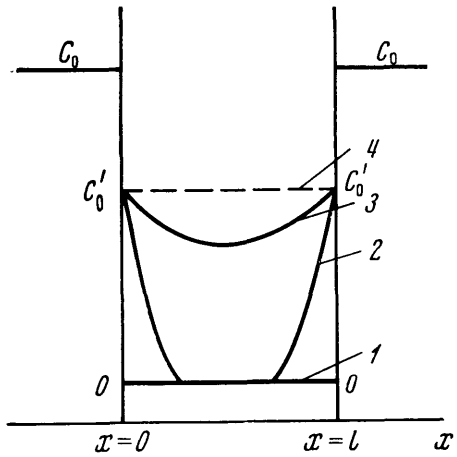


Figure 3. Diffusion of the substrate in the membrane (for explanation, see text): 1) distribution of the substrate for $t = 0$; 2) distribution of the substrate for $t > 0$; 3) steady state in the presence of enzymic reaction; 4) equilibrium state in the absence of enzymic reaction.

We place the membrane with a thickness l, on which the enzyme is immobilised, in a solution of the substrate at a concentration C_0 (Fig. 3).[7] At the initial instant ($t = 0$) the substrate concentration in the membrane is of course zero. Then it increases with time to the equilibrium value, which in the absence of an enzymic reaction is $C_0' = PC_0$, where P is the partition coefficient of the substrate between the membrane and the aqueous "phases" (dashed line in Fig. 3). On the other hand, when an enzymic reaction occurs in the membrane, consuming the substrate, then in the presence of internal diffusion inhibition the steady-state substrate concentration in the membrane may be less than C_0' and should depend on x, as shown in Fig. 3.

Time Required for the Attainment of the Steady State

For practical purposes, it is important to estimate the time required to establish the steady state where the rate of the diffusion flux becomes equal to the rate of the enzymic reaction and therefore the concentration of the substrate diffusing to the enzyme remains constant (steady-state value for C_0 = const.). In general an analytical solution of the problem is impossible and therefore it is necessary to resort to numerical methods. In this connection we shall analyse to a first approximation the case where there is no enzymic reaction in the membrane. Assuming that $P = 1$, the variation of the concentration C in the membrane with time t can be formulated thus[56]:

$$C = C_0\left[1 - \frac{2}{\pi}\sum_{n=1}^{\infty}\frac{1-\cos n\pi}{n} \cdot \sin\frac{n\pi x}{l} \cdot \exp\left(-\frac{Dn^2\pi^2 t}{l^2}\right)\right], \quad (14)$$

where D is the diffusion coefficient of the substrate in the membrane medium. For fairly long times, the sum of the series in Eqn. (14) is determined mainly by the first term ($n = 1$):

$$C = C_0\left[1 - \frac{4}{\pi} \cdot \sin\frac{\pi x}{l} \cdot \exp\left(-\frac{D\pi^2 t}{l^2}\right)\right]. \quad (15)$$

Naturally the substrate concentration is always a minimum in the centre of the membrane ($x = l/2$). It will be assumed that the time τ after the elapse of which the substrate concentration in the centre of the membrane is 90% of its initial concentration in the solution, i.e. $C = 0.9\,C_0$, corresponds to virtual attainment of equilibrium. Then we have from Eqn. (15)

$$\frac{4}{\pi} \cdot \exp\left(-\frac{D\pi^2\tau}{l^2}\right) = 0.9, \quad (16)$$

whence§

$$\tau = 0.035 \cdot \frac{l^2}{D}. \quad (17)$$

The time of the virtual attainment of the steady state can be readily estimated from Eqn. (17). The membrane thickness l can be measured. The diffusion coefficients of different (low-molecular-weight) substances in the membrane D may be assumed to differ little from the diffusion coefficients measured in water, when the polymer content in the gel is not too high (such membranes are in fact used for the immobilisation of enzymes[47-50]). Thus it is shown in Table 2 for sucrose as an example that the diffusion coefficient in a dilute gel is lower only by a factor of 2–3 compared with an aqueous medium. Ultimately, if it is assumed that $D \simeq 3 \times 10^{-6}$ cm^2 s^{-1}, which is typical for substances[57] with molecular weights up to 10^3, Eqn. (17) yields $\tau \simeq 2$ min for a membrane 1 mm thick.

On the other hand, when an enzymic reaction takes place in the membrane simultaneously with diffusion, the substrate concentration at the centre of the membrane may not reach values as high as in the absence of the reaction. Consequently the time required to attain the steady state with respect to the substrate in a membrane with an immobilised enzyme probably should not exceed the value of τ for the state of equilibrium of the membrane without the enzyme¶. Therefore τ found from Eqn. (17) can be regarded as the upper limit to the times of attainment of the diffusion steady state in enzymic membrane systems.

§ There is an arithmetical error in the derivation of Eqn. (16) from Eqn. (17) in publications by Laidler and Sundaram[7,54], which results in an incorrect expression for τ, the true value being exceeded by a factor of 7.3.

¶ Provided that of course the enzyme itself or changes induced by it in the membrane do not exhibit a significant influence on the diffusion coefficient of the substrate.

Determination of the Kinetic Parameters of an Enzymic Reaction in the Absence of Internal Diffusion Inhibition

On the other hand, if the reaction with an immobilised enzyme is slower than diffusion (because the enzyme is not very active or its specific concentration is low) so that the equilibrium distribution of the substrate between the aqueous solution and the carrier particles is not disturbed, the specific rate of reaction referred to unit volume of the carrier should be represented as follows according to Eqn. (1):

$$v_{sp.} = \frac{k'_{cat}[E]_M C'_0}{K'_m + C'_0}, \quad (18)$$

where $C'_0 = PC_0$, $[E]_m$ is the concentration of the enzyme in the bulk of the carrier, and k'_{cat} and K'_m are the true kinetic parameters of the enzymic process within the carrier, which may differ in principle from the corresponding parameters for the reaction in water if only because the enzyme can assume, as a result of being immobilised, a conformation different from that in water or because of the influence of the medium. Hence the overall rate of reaction, referred to the entire bulk of the carrier (v_{tot}, mole s^{-1}), can be formulated as follows:

$$v_{tot} = \frac{k'_{cat.} E C_0}{K_{m(obs.)} + C_0}, \quad (19)$$

where $K_{m(obs)} = K'_m/P$, E is the total amount of the enzyme in the carrier equal to $[E]_M Al$ for a membrane, A is the area of one side of the membrane, and l is its thickness. Eqn. (19) shows that the kinetic parameters k'_{cat} and K'_m can be found by the usual procedure used in enzyme kinetics for the analysis of the Michaelis equation, provided that P is determined in an independent experiment.

Table 2. Diffusion coefficients D of sucrose in different media.

Medium	$10^6 D$, $cm^2\ s^{-1}$	Refs.
Aqueous solution	5.0	58
9.5% polyacrylamide gel (95% acrylamide + 5% cross-linking agent[a])	2.5 ± 0.7	59
9.9% polyacrylamide gel (90% acrylamide + 10% cross-linking agent[a])	1.9 ± 0.7	59
7.2% polyacrylamide gel (99% acrylamide + 1% cross-linking agent[a])	3.5 ± 0.2	59
7% gelatin gel	1.5 ± 0.2	60
0.8% agar-agar gel	2.5 ± 0.5	61
Collodion membrane containing 90% of water	2.8	47

[a] *NN'*-Methylenebisacrylamide.

Effect of Internal Diffusion on the Effective Values of the Kinetic Parameters of an Enzymic Reaction in the Membrane

One can be by no means always certain that the occurrence of the enzymic reaction does not disturb the equilibrium in the distribution of the substrate between water and the carrier. In the general case a substrate concentration C at a specified point in the membrane (with the coordinate x, see Fig. 3) is defined by the equation for the steady-state:

$$D \cdot \frac{d^2C}{dx^2} = \frac{k_{cat.}[E]_M C}{K'_m + C}, \quad (20)$$

which in fact reflects the equality of the gradient of the diffusion flux through unit surface area, defined by Fick's second law, and the rate of the enzymic reaction. The problem consists of finding the form of the function $C(x)$. Generally speaking, Eqn. (20) can be solved only numerically with the exception of two cases.

a. If $C \ll K'_m$ (this is true, for example, at low substrate concentrations when $C'_0 = C_0 P \ll K'_m$), then

$$\frac{d^2C}{dx^2} = \alpha^2 C, \quad (21)$$

where

$$\alpha = \sqrt{\frac{k'_{cat}[E]_M}{K'_m D}}. \quad (22)$$

The solution of Eqn. (21) by Euler's method with the boundary conditions $x = 0$, $C = C'_0$, $x = l$, and $C = C'_0$ yields

$$C = C'_0 \cdot \frac{\sinh \alpha x + \sinh \alpha (l - x)}{\sinh \alpha x}. \quad (23)$$

The steady-state rate of consumption of the substrate in the system must be equal to the diffusion flux through both sides of the membrane, i.e.

$$v'_{tot.} = 2AD \frac{dC}{dx}\Big|_{x=0,l}, \quad (24)$$

where the substrate concentration gradient at the boundary of the membrane follows from Eqn. (23) and is given by

$$\frac{dC}{dx}\Big|_{x=0} = \frac{dC}{dx}\Big|_{x=l} = \pm \alpha C'_0 \cdot \frac{\cosh \alpha l - 1}{\sinh \alpha l}. \quad (25)$$

Then, after simple rearrangement, the overall rate of reaction, referred to the entire volume of the carrier (v'_{tot}) or its specific value (v'_{sp}) per unit volume carrier, can be formulated as follows on the basis of Eqn. (24) and (25):

$$v'_{sp} = \frac{v'_{tot}}{Al} = \frac{k'_{cat}[E]_M C'_0}{K'_m} \cdot F, \quad (26)$$

where

$$F = \frac{2}{\alpha l} \cdot \frac{\cosh \alpha l - 1}{\sinh \alpha l} \equiv \frac{\tanh \gamma}{\gamma}, \quad (27)$$

and $\gamma = \alpha l/2$. Evidently Eqn. (26) differs from Eqn. (18) for the rate of reaction in the absence of diffusion-dependent inhibition (subject to the condition that $C'_0 \ll K'_m$) only by a factor F, which in fact reflects the effect of internal diffusion. F is called the efficiency factor; its properties will be discussed in greater detail below. Here we shall only consider Fig. 4, where F is plotted against αl calculated on the basis of Eqn. (27). When $\alpha l \to 0$ {this holds for a very low enzyme activity $k'_{cat}[E]_M/K'_m$ or for a thin membrane}, we have $F \to 1$, as expected from general considerations. With increase of the thickness of the membrane l or with increase of the activity of the immobilised enzyme {$k'_{cat}[E]_M/K'_m$, see Eqn. (22)}, F falls, which indicates a growing influence of diffusion-dependent inhibition.

b. When $C \gg K'_m$ (this happens when C'_0 is so large that the steady-state substrate concentration at any point in the membrane is much greater than K'_m), we have from Eqn. (20)

$$\frac{d^2C}{dx^2} = \omega, \quad (28)$$

where $\omega = k'_{cat}[E]_M/D$. Subject to the boundary conditions $x = 0$, $C = C'_0$, $x = l$, and $C = C'_0$, the solution of Eqn. (28) becomes

$$C = C'_0 - \frac{1}{2}\omega x(l - x) . \quad (29)$$

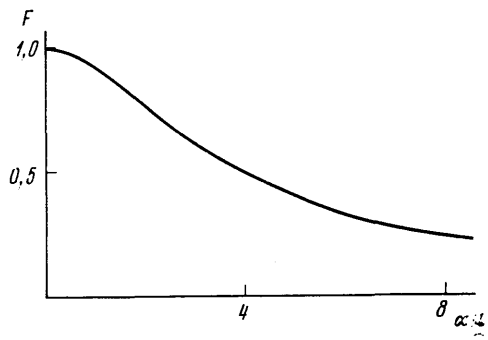


Figure 4. Theoretical variation of the F-factor with αl [Eqn.(27)].

As stated above, under steady-state conditions the overall rate of consumption of the substrate, referred to the entire volume of the carrier, must be equal to the diffusion flux through both sides of the membrane [see Eqn. (24)]. Since the substrate concentration gradient at the boundary of the membrane, derived from Eqn. (29), is

$$\left.\frac{dC}{dx}\right|_{x=0} = \left.\frac{dC}{dx}\right|_{x=l} = \pm\frac{1}{2}\omega l , \quad (30)$$

it follows that Eqn. (24) can be rewritten thus:

$$v'_{tot} = \omega ADl = k'_{cat}[E]_M Al . \quad (31)$$

Evidently the last expression is the same as Eqn. (18) (when $C'_0 \gg K'_m$), which holds for the rate of reaction in the absence of diffusion-dependent rate limitation. This implies that diffusion does not play any role in the enzymic reaction at fairly high substrate concentrations ($C \gg K'_m$).

c. We shall now consider the general case where $C \simeq K'_m$. As already stated, in the general case Eqn. (20) can be solved only numerically. However, the nature of the variation of the rate of the enzymic reaction v'_{tot} with substrate concentration in the solution C_0 can be represented qualitatively on the basis of Eqns. (26) and (31) characterising cases (a) and (b). If the rate of reaction obeys the Michaelis–Menten equation

$$v'_{tot} = \frac{k'_{cat\,(obs.)}EC_0}{K'_{m(obs.)} + C_0} , \quad (32)$$

over the entire experimental range of substrate concentrations, then on the basis of Eqn. (26) and (31) one may assume that

$$k'_{cat\,(obs.)} = k'_{cat} \text{ and } K'_{m\,(obs.)} = \frac{K'_m}{P \cdot F} . \quad (33)$$

Although the above reasoning is not entirely rigorous, the conclusions reached on its basis are satisfactorily confirmed by the results of the numerical integration of Eqn. (20).[7]

It is seen from Eqn. (33) that internal diffusion in no way affects the effective catalytic rate constant and influences only the observed Michaelis constant. The calculation based on Eqn. (27) shows that, when $\alpha l < 1$, F differs from unity by not more than 10%, i.e. $K'_{m(obs)} \simeq K'_m/P$, as predicted by Eqn. (33), and consequently diffusion does not have an appreciable influence on the reaction. On the other hand, when $\alpha l > 1$, F is significantly smaller than unity, i.e. $K'_{m(obs)} > K'_m/P$ and therefore diffusion of the substrate plays a significant role. Thus, in order to estimate beforehand the possible influence of internal diffusion on catalysis by an enzyme immobilised in a membrane, it is useful to calculate F [Eqn. (27)], on which the effective Michaelis constant depends [Eqn. (33)], and to compare it with unity. When $\alpha l > 3$, one can put approximately

$$F \approx \frac{2}{\alpha l} , \quad (34)$$

(see also Fig. 4). For an approximate estimate, it is useful to assume that k'_{cat} and K'_m, which determine the values of α [Eqn. (22)], differ little from the kinetic parameters of the reaction in water. A test of the theory carried out in Laidler's[50] and Katchalski's[49] laboratories fully confirmed this.

Certain Characteristics Distinguishing Rate Limitation by Internal Diffusion from Rate Limitation by External Diffusion

a. For very high values of αl, when $F \ll 1$ [Eqn. (4)] and therefore the reaction is significantly retarded by diffusion [Eqns. (32) and (33)], we have $K'_{m(obs)} \gg C_0$. In this case we obtain the following expression from Eqns. (22), (26), and (34) for the overall rate of the enzymic process referred to the entire volume of the carrier:

$$v'_{tot} = 2PC_0\sqrt{\frac{k'_{cat}[E]_M D}{K'_m}} . \quad (35)$$

It is seen from Eqn. (35) that, in contrast to rate-limitation by external diffusion, where the reaction rate under diffusion conditions is altogether independent of the catalytic properties of the enzyme [see, for example, Eqns. (5) and (6)], when the rate is limited by internal diffusion it is proportional to the square root of the enzyme concentration and enzyme activity.

b. In contrast to rate limitation by external diffusion, where the temperature variation of the reaction rate under diffusion conditions reflects only the activation energy governing the diffusion coefficient (see p. 12), when the rate is limited by internal diffusion [Eqn. (35)], the apparent activation energy ϵ_{app} depends on the temperature variation of not only the kinetic and diffusion parameters ($\sqrt{k'_{cat}/K'_m}$ and $\sqrt{D}$ respectively) but also of the partition coefficient P of the substrate between water and the carrier in the following way: $\epsilon_{app} = \frac{1}{2}(\epsilon + \epsilon_D + 2\Delta H_p)$, where ϵ is the activation energy for the enzymic reaction, ϵ_D the activation energy for the diffusion of the substrate, and ΔH_p the enthalpy of its transfer from water to the carrier.

c. It is interesting to note that the inhibition of the enzymic reaction diminishes the influence of internal diffusion in catalysis[62]. This follows from an analysis of Eqn. (34) bearing in mind that in the inhibition the values

of k'_{cat} and K'_m, which determine α[Eqn. (22)], are in fact effective quantities, which depend on the inhibitor concentration in the usual way.

Dependence of Internal Diffusion on the Geometrical Properties of the Particle

The influence of internal diffusion on the reaction between the substrate and the enzyme immobilised in the membrane was discussed above. However, the particles containing the immobilised enzyme usually have a spherical, cylindrical, or other shape. Naturally it is possible in principle to carry out a similar analysis in each of these instances[62,63]. However, we believe that it is far more useful to consider immediately a more universal case.

Suppose that we have a particle in which the enzyme molecules are distributed uniformly in the matrix (gel, polymer, or porous carrier). The substrate must diffuse into the particle (as before, external diffusion is regarded here as a factor which has been taken into account) and reacts there. The solution of this problem is analogous to that considered by Zel'dovich[64], Thiele[65], and Damköhler[66] for heterogeneous catalysis. They showed that the observed rate of the catalytic reaction controlled by diffusion is

$$v_{obs} = \eta v, \qquad (36)$$

where v is the rate of reaction in the absence of diffusion control, η the efficiency factor[65], which depends on the parameter Φ:

$$\Phi = a\sqrt{\frac{v}{C_0 D}} \qquad (37)$$

(Thiele's modulus), where a is the particle size (the radius for a sphere), C_0 the concentration of the reactant in the bulk phase, and D its diffusion coefficient within the catalyst particle. The specific form of the function η depends on the shape of the particle and the order of the reaction with respect to the substrate†. However, Aris[67] showed that the shape of the particles has only a slight influence on the function η. In all cases the curve relating η to Φ resembles closely the analogous curve for the relation between F and αl (Fig. 4). With increase of the reaction order from zero to third, the $\eta-\Phi$ curve in Fig. 4 is only a little displaced to the left and is slightly deformed. However, the following fact remains unaltered: when $\Phi < 1$, then η is close to unity and therefore diffusion is not important for the process; on the other hand, when $\Phi > 1$, then $\eta < 1$ and the reaction is retarded by diffusion. Therefore presumably the parameter αl, characterising the internal diffusion in the membrane, is a special case of Thiele's modulus. Consequently, in order to estimate beforehand the role of internal diffusion in the immobilised enzyme system[68], it is merely necessary to calculate Φ and compare the corresponding value of η with unity.

In the calculation of Φ [Eqn. (37)] account must be taken of the fact that the substrate diffusion coefficient in the porous particle (for example in porous glass)[28] is $D = D_{true}q$, where D_{true} is the true diffusion coefficient of the substrate in the liquid filling the pores of the particle and q is the fraction of the pore volume (i.e. the space available for diffusion) relative to the total volume of the particle. For example, in the diffusion of oxygen dissolved in water into the pores of porous glass[36] $D_{true} = 2.4 \times 10^{-5}$ cm^2 s^{-1} and $D = 0.93 \times 10^{-5}$ cm^2 s^{-1}, i.e. $q = 0.39$.

† For example, for a unimolecular reaction involving a plate, we have $\eta = (1/\Phi)\tanh\Phi$ and for a sphere we have $\eta = (1/\Phi)(1/\tanh 3\Phi - 1/3\Phi)$.[27,28]

Practical Application of Theoretical Relations

The proposed theory suggests ways of decreasing the rate limitation by internal diffusion in catalysis by mmobilised enzymes—see, for example, Eqns. (32)–(34) r (36)–(37). There are two main procedures: the irst, which is the most effective, involves a decrease of he particle size of the immobilised enzyme and the second nvolves a decrease of the specific enzyme concentration in the particle. As an example, Fig. 5 presents the experimental relation between η and Φ for glucose oxidase immobilised on porous glass[36], observed when the particle size is altered. It is found that η reaches about 0.95 only for a particle size of about 30 μm.

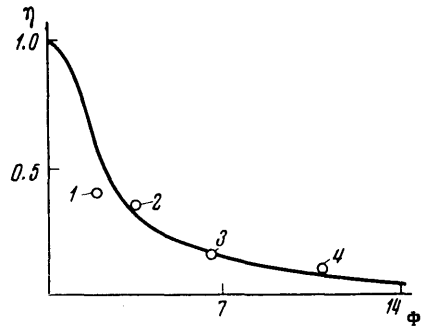


Figure 5. Variation of η with Φ. The curve was calculated theoretically and the circles represent experimental values for the given particle size (μm):[36] 1) 125–250; 2) 250–500; 3) 500–1000; 4) >1000.

With the aid of the theory described above, it is possible to predict the most favourable (from the standpoint of the absence of rate limitation by internal diffusion) immobilisation procedures. Thus analysis showed that[69], for a given particle size of the carrier and a constant amount of the deposited enzyme, Φ is smaller (and therefore η is closer to unity) when the enzyme is not distributed uniformly throughout the bulk of the carrier, but forms instead a surface film on a catalytically inert grain. This method for immobilising enzymes is in fact used successfully[70,71].

It is interesting to note that, as shown theoretically[72,73] and confirmed experimentally[74,75], in immobilised polyenzyme systems, where the reaction product of one enzyme is the substrate of another, the presence of rate limitation by internal diffusion can lead to an increase of the overall rate of reaction. This is because rate limitation by internal diffusion leads to the "concentration of the product" (i.e. of the substrate of the next reaction) in the bulk of the carrier relative to the equilibrium distribution.

3. DIFFUSION IN SIMPLE LIQUIDS AND POLYMERS

When enzymes are immobilised in polymeric carriers and also when they are enclosed in polymeric (usually polyamide) microcapsules[76-86] or in hollow fibres[87-89] there arises the problem of diffusion of the substrate through the polymeric medium.

According to the theory of Lieb and Stein[90-92], developed in recent years, the diffusional behaviour of non-electrolytes in simple liquids and in polymers can differ radically. In the diffusion in simple liquids the particle size of diffusing substances is as a rule greater than the molecular size of the diffusion medium, which therefore may be regarded as continuous and isotropic with respect to viscosity. On the other hand, in the diffusion of molecules into polymers, the molecular size of the diffusing substance is smaller than the molecular dimensions of the diffusion medium. Since diffusion in polymers proceeds via the motion of segments of polymeric molecules of the medium, the latter cannot be regarded as homogeneous and isotropic with respect to viscosity.

For a spherical diffusing species with a radius a in a simple liquid, the diffusion coefficient is defined by the Stokes–Einstein equation:

$$D = \frac{kT}{6\pi a \nu}, \qquad (38)$$

where k is the Boltzmann constant, T the absolute temperature, and ν the viscosity of the diffusion medium. If it is assumed that the diffusing molecules are spheres with a constant density, Eqn. (38) can be transformed into

$$D M^n = \text{const}, \qquad (39)$$

where M is the molecular weight of the diffusing substance and n a numerical coefficient equal to 1/3 for spherical molecules. This approximation is valid for many large protein molecules, although usually the molecules cannot be regarded as spherical and therefore n differs somewhat from 1/3. As an example, Table 3 presents the diffusion coefficients for various non-electrolytes in water[41], $n = 0.43$ follows from a plot of $\lg D$ against $\lg M$ on the basis of these data. Analysis of extensive data[91] has shown that in simple liquids n varies from 0.3 to 0.5, i.e. the diffusion coefficient depends only fairly slightly on the molecular weight of the diffusing substance.

Table 3. Diffusion of different non-electrolytes in water[41].

Diffusing substance	$10^6 D_{25°}$, cm^2 s^{-1}	Activation energy for diffusion ϵ_D, kcal mole^{-1}
Urea, $M = 60$	13.78	4.470
Glycine, $M = 75$	10.55	4.627
Alanine, $M = 89$	9.097	4.783
Glucose, $M = 180$	6.728	4.959
Cyclohepta-amylose, $M = 1134$	3.224	4.931
Bovine serum albumin, $M = 68000$	0.65	4.969

In polymers diffusion proceeds via "holes" formed at random as a result of the thermal motion of polymeric segments[93]. In order that the diffusing substance can pass through the holes, it must evidently have suitable dimensions. Diffusing species comprising very small species can pass through both small and large holes, while those with large species can only penetrate large holes. Since the formation of large holes requires more energy, their number is smaller than that of very small holes; this qualitative reasoning is confirmed by quantitative calculations[91]. Therefore the diffusion coefficients of small and large molecules in polymeric media should presumably differ sharply, i.e. n is large. Indeed, we have, for example, $n = 3.8$ for poly(methyl methacrylate) as a diffusion medium at 65°C.[90,94] Whereas in diffusion in the usual liquids doubling of the molecular weight leads to a decrease of D by a factor of about 1.4,[41] in diffusion in poly(methyl methacrylate) this leads to a 14-fold diminution of D! For different polymeric media, n varies from 2 to 8.[95-97]

Analysis of the effect of molecular shape on the diffusion coefficient showed that, for example, in water when the molecular shape deviates from the sphere, D diminishes and is correlated with the maximum change in the molecule[98]. On the other hand, in polymeric media the diffusion coefficients of substances are correlated with the smallest changes in their molecules (Table 4), which is also apparently associated with the "hole" mechanism of the diffusion process in polymers.

Table 4. Dependence of the diffusion coefficients of hydrocarbons in polyisobutylene on their molecular geometry[99].

Diffusing substance	n-Pentane	Isopentane	Neo-pentane
$10^9 D_{35°}$, cm^2 s^{-1}*	2.64	1.32	0.62

*D was determined by extrapolating the experimental data to zero concentration of the diffusing substance.

It follows from Table 3 and the literature[57,91,100] that, firstly, the diffusion coefficient depends only very slightly on temperature (for example, the activation energy for diffusion $\epsilon_D \simeq 4-5$ kcal mole^{-1} in water) and, secondly, ϵ_D is almost independent of the molecular weight of the diffusing substance. These findings are quite understandable, since the diffusion process in simple liquids is determined mainly by the properties of the diffusion medium and not by those of the diffusing substance itself[101] (see also the Stokes–Einstein equation). On the other hand, in diffusion in polymers the values of ϵ_D exceed significantly those characteristics of solutions[102] and furthermore, ϵ_D increases appreciably with increase of the molecular weight of the diffusing substance (see Table 5). This can also be accounted for within the framework of the "hole" concept of diffusion in polymers, since the formation of large holes, necessary for the diffusion of large molecules, requires more energy than the formation of very small holes. Thus, in contrast to the diffusion in liquids, in polymers D depends strongly on temperature.

Finally, we may note that at not unduly high concentrations of diffusing substances, their joint diffusion in simple liquids is independent. On the other hand, in diffusion in polymers the addition of small amounts of certain substances (called plasticisers) leads to a sharp increase of the diffusion coefficients of other substances[97,103,104], probably in consequence of the improvement of their solubility in the polymer[104]. For example, the addition of 2% of water to a specimen of poly(vinyl acetate) at 40°C leads to an increase of the diffusion coefficient of carbon tetrachloride by a factor of 75.[103]

The data quoted show that diffusion processes in simple liquids and in polymers are fundamentally different. Lieb and Stein[97] suggested that the diffusion in liquids be

assigned to the *s*-type (from the English word 'simple') and that in polymers to the *p*-type (from the English word 'polymer'). As we have already stated, diffusion processes of the *s*- and *p*-types differ significantly in terms of four characteristics, which are compiled in Table 6.

Table 5. Diffusion of different substances into a poly-(vinyl acetate) film[97].

Diffusing substance	$10^{12}D_{40°}$, $cm^2\ s^{-1}$*	ϵ_D, kcal $mole^{-1}$
Water	$1.2\cdot10^5$	15±1
Methanol	$1.5\cdot10^3$	21±2
Acetone	$1.3\cdot10^1$	39±3
1-Propanol	$4.8\cdot10^{-}$	41±4

*D was determined by extrapolating the experimental results to zero concentration of the diffusing substance.

The set of these four characteristics constitutes a criterion for the determination of the type of diffusion in the given medium. It was found[91,92] that non-mediated diffusion of non-electrolytes in biological membranes belongs fully to the *p*-type.

Table 6. Effect of various parameters on diffusion of *s*- and *p*-type[92].

Parameter	Diffusion of *s*-type	Diffusion of *p*-type
Molecular size of diffusing substance	Increase in size entails only a slight decrease of diffusion coefficient (n = 0.3–0.5)	Increase in size leads to a sharp decrease of diffusion coefficient (n = 2–8)
Molecular shape of diffusing substance	Diffusion coefficient is correlated mainly with the maximum dimension of the diffusing molecule	Diffusion coefficient is correlated mainly with the minimum dimension of diffusing molecule
Temperature	Increase of temperature leads to a slight increase of diffusion coefficient for both large and small molecules	Increase of temperature leads to an appreciable increase of diffusion coefficient—greater the greater the molecular size of diffusing substance
Plasticisers	Almost without effect	Their addition to polymeric medium leads to increase of the diffusion coefficients of other molecules

In catalysis by enzymes immobilised in polymeric matrices or microcapsules or in the diffusion of substrates (even low-molecular-weight substrates) through a polymeric medium, there is apparently also a possibility not only of *s*-type but also of *p*-type diffusion. For example, the results obtained by Mogenson and Vieth[105], who found that the permeability of a cellulose nitrate capsule to hydrogen peroxide exceeds the permeability of the same microcapsule to urea by a factor greater than twenty, can be explained only from this standpoint. In this connection we emphasise that in *p*-type diffusion one may expect a sharp dependence of the observed efficiency of catalysis on the molecular weight of the substrate and on temperature.

4. DEPENDENCE OF THE REACTION RATE CONSTANT ON THE DIFFUSION PROPERTIES OF THE MEDIUM

Even if account is taken of external and internal diffusion in an enzymic reaction, its rate may depend on the diffusion properties of the reaction medium. The problem of the effect of diffusion on the rate constants for chemical reactions was solved by Smoluchowski[106], Debye[107], and Noyes[108]. Evidently diffusion should not play any role in unimolecular reactions. For a bimolecular reaction between two uncharged species, the observed rate constant k_{obs} is given by the following general expression:

$$k_{obs} = \frac{k}{1 + \frac{k}{4\pi \cdot D_\Sigma \cdot \sigma}}, \qquad (40)$$

where k is the true reaction rate constant, D_Σ the sum of the diffusion coefficients of the reactants, and σ the equilibrium interaction distance. If $1 \gg k/4\pi D_\Sigma\sigma$, then $k_{obs} = k$ and diffusion is unimportant for the reaction; on the other hand, if $1 \ll k/4\pi D_\Sigma\sigma$, then $k_{obs} = 4\pi D_\Sigma\sigma$ (i.e. is independent of k)‡ and the reaction is fully controlled by diffusion. Eqn. (40) is valid, provided that the molecules do not interact when the distance between them (r) is greater than σ.

In general the Debye equation is valid:

$$k_{obs} = \frac{k}{1 + \frac{k\int_\sigma^\infty \exp\left(\frac{U}{kT}\right)\cdot\frac{dr}{r^2}}{4\pi D_\Sigma}}, \qquad (41)$$

where U is the energy of the interaction between the reactant species. If $U = 0$ when $r > \sigma$, Eqn. (41) becomes identical with Eqn. (40). The form of the function $U(r)$ is usually unknown and therefore Eqn. (41) cannot be put in an analytical form. However, if it is assumed that there is electrostatic Coulombic interaction between the species, then $U = z_1z_2e^2/\kappa\sigma$, where z_1e and z_2e are the charges of the reactant species (e is the electronic charge), and κ the dielectric constant of the medium. In this case we have the following expression for the rate constant for a diffusion-controlled reaction:

$$k_{obs} = 4\pi D_\Sigma \cdot \sigma \cdot \frac{\theta}{\exp\theta - 1}, \qquad (42)$$

where $\theta = z_1z_2e^2/\kappa\sigma kT$. Consequently, knowing the corresponding parameters of the medium and the reaction, it is possible to determine the limits of the region of diffusion control.

The formation of the Michaelis complex must be regarded as the fastest stage of enzymic reactions: the rate constants for this stage sometimes approach the values characteristic of diffusion-controlled processes even in water[109]. Therefore, in very viscous media, where D is exceptionally small, the reaction may be presumably fully diffusion-controlled[110] [it must be emphasised that under these conditions k_{obs} may be very small and k is proportional to D_Σ—see Eqn. (40)].

Consider the reaction of an enzyme and a substrate in the gel, assuming that the process is not controlled by diffusion in solution. With increase of the gel concentration, its pores diminish and a time must come when they

‡ This expression is valid to within the steric factor, since Eqns. (40) and (41) were derived on the assumption that each collision of the reactant leads to chemical reaction.

become comparable to the dimensions of the protein globule of the enzyme, which leads to a sharp fall of its diffusion coefficient (there is a transition from diffusion of s-type to p-type). The occurrence of this phenomenon has been established experimentally[111]. However, in a reaction with a low-molecular-weight substrate this does not lead to diffusion control even if the enzyme is completely "arrested", since Eqns. (40)—(42) include the sum of the diffusion coefficients and, furthermore, for low-molecular-weight substances D changes little with increase of the gel concentration even to very high values[59]. The situation is different when the substrate is a high-molecular-weight compound. Then an increase in the gel concentration ultimately drives the reaction towards diffusion-controlled conditions and, on further increase of the gel concentration (which entails a decrease of D), there should be a sharp decrease of the observed reaction rate. These effects were predicted and observed experimentally in our laboratory[111,112]. Fig. 6 presents data for the variation of the observed rate of reaction of trypsin with chymotrypsinogen ($M \simeq 25\,000$ for both proteins) in a polyacrylamide gel as a function of the gel concentration[112]. Evidently, with increase of the gel concentration to approximately 32%, the observed rate of reaction actually increases somewhat (which is related to its specific features) and then falls almost to zero on further increase of the concentration by only 2% owing to retardation by diffusion.

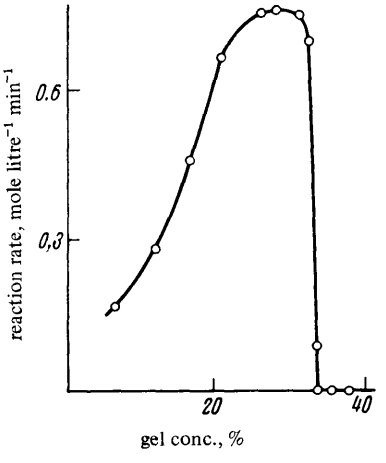


Figure 6. Variation of the observed rate of reaction ($\times 10^6$) of trypsin with chymotrypsinogen in polyacrylamide gel with its concentration[112].

In conclusion we may note that the rate of an enzymic reaction in a polymer may be controlled by diffusion even for a low-molecular-weight substrate, provided that its diffusion is of the p-type. In turn one should note that the retardation of enzymic reactions in polymeric (gel) media by diffusion may be used to investigate the fine structure of the polymeric matrix.

III. EFFECT OF THE NATURE OF THE CARRIER ON THE KINETICS OF REACTIONS CATALYSED BY IMMOBILISED ENZYMES

The main idea of this and the next two sections in the present review is that immobilisation of enzymes entails the actual appearance of a new type of specificity of the latter compared with the native biocatalyst. This phenomenon is due to the influence of the carrier on the catalytic process. In the first place one should note that the reaction system [consisting of the enzyme immobilised on (or in) the carrier placed in an aqueous solution] may be regarded as a two-phase system. The fact that the reaction components (substrates, cofactors, inhibitors, hydrogen ions, etc.) are uniformly distributed between the aqueous solution and the effective volume of the carrier (to which an adjacent layer of the solution should be assigned in the general case) is related precisely to the two-phase nature of the reaction system. Naturally such distribution influences the kinetics of the enzymic process. We shall consider this problem in somewhat greater detail.

1. pH EFFECTS

When the enzyme is immobilised on a charged carrier, one may expect that the concentration of hydrogen ions in the carrier phase will be smaller than in the solution if the carrier is charged positively and, conversely, will be greater for a negatively charged carrier. This follows from the quantitative theory developed by Goldstein et al.[113] Thus the ratio of the hydrogen ion concentrations in the carrier $[H^+]'$ and the solution $[H^+]$ is determined by the Boltzmann factor

$$P_{H^+} = \frac{[H^+]'}{[H^+]} = \exp\left(-\frac{e\psi}{kT}\right), \qquad (43)$$

where P_{H^+} is the partition coefficient, e the absolute electronic charge, ψ the electrostatic potential of the charged immobilised enzyme species, k the Boltzmann constant, and T the absolute temperature. As a result, the difference between the pH of the carrier and the solution is

$$\Delta pH \equiv pH' - pH = 0.43 \cdot \frac{e\psi}{kT}. \qquad (44)$$

The resulting pH shift can in turn influence the rate of the enzymic reaction, provided that the mechanism is associated with the dissociation of an ionogenic group in the enzyme or other reaction components (see the general theory of the pH effects in enzymic catalysis[114]). The dissociation constant of the ionogenic group BH^+ of the native enzyme $K = [B][H^+]/[BH^+]$ is by definition the value of $[H^+]$ for which the ionogenic group is half-neutralised. Consequently, on the basis of Eqn. (44), the pK shift due to the carrier is

$$\Delta pK \equiv pK' - pK = -0.43 \cdot \frac{e\psi}{kT}, \qquad (45)$$

where K' is the dissociation constant of the ionogenic group BH^+ in the enzyme joined to the charged carrier. It follows from Eqn. (45) that p$K' <$ pK for a positively charged carrier ($\psi > 0$) and, when the carrier is negatively charged ($\psi < 0$), then p$K' >$ pK.

Experiment confirms the above considerations. Thus at low ionic strengths the pH dependence of the catalytic activity of polyanionic trypsin derivatives is such that its optimum is displaced to the alkaline region and for polycationic trypsin derivatives it is displaced to the acid

region[13,113]. Similarly the pK of the imidazole group of the histidine-57 residue forming part of the active centre of α-chymotrypsin is 6.70 ± 0.02 for the native enzyme, 7.82 ± 0.05 for the polyanionic derivative of α-chymotrypsin, and 6.10 ± 0.05 for the polycationic derivative[115]. The list of similar factors can be extended. Thus when α-chymotrypsin[116,117], phosphatase[118], and urease[119] are immobilised on negatively charged carriers (bentonite or kaolin), the pH optimum shifts to the alkaline region. On the other hand, following immobilisation on positively charged DEAE-cellulose, the pH optima of aminoacylase[120] and invertase[121] are displaced to the acid region. Derivatives of other enzymes (papain[9], ficin[122], and subtilopeptidase A [9]) behave similarly.

The shifts of the reaction rate–pH curves described above become considerable only at a low ionic strength of the solution. This finding is explained by the fact that an increase in ionic strength as well as an increase in the dielectric constant decreases the electrostatic interaction between the charged species and hence the repulsion between the charged carrier and the effector (in the present case hydrogen ions)[123]. Table 7 lists the pK shifts for the ionogenic group controlling the catalytic activity of trypsin immobilised on a polyanionic carrier (a copolymer of ethylene and maleic acid) at different ionic strengths of the solution[113].

Table 7. Dependence of ΔpK and ψ on the ionic strength of the solution for trypsin immobilised on a polyanionic carrier[113].

Ionic strength, M	$\Delta pK \equiv pK' - pK$	Ψ, mV	Ionic strength, M	$\Delta pK \equiv pK' - pK$	Ψ, mV
0.006	−2.4	−150	0.2	−1.3	−80
0.010	−2.0	−120	1.0	−0.4	−30
0.035	−1.6	−100			

pH effects of this type occur also when the enzyme is immobilised on (or in) an uncharged carrier where one may also expect a distribution of hydrogen ions between the solution and the bulk of the carrier, namely $[H^+]' = P_{H^+}[H^+]$. Hence $\Delta pK = -\lg P_{H^+}$. This relation makes it possible to determine beforehand the sign and magnitude of the pH effects in the above systems, provided that the partition coefficient P_{H^+} is known.

The above elementary theory describes the shifts of the pH profiles not only for truly immobilised biocatalysts but also for water-soluble modified enzymes, for example for acetylated and succinylated derivatives of trypsin and α-chymotrypsin, in which the negative charge of the globule has been increased in consequence of the acylation of the amino-groups[13,17,113,124,125]. In certain cases the observed shift of the pH optimum of the reaction catalysed by the immobilised enzyme does not correspond to equilibrium and is due to the fact that there is not sufficient time for the diffusion of the acid or base formed in the reaction away from the carrier[47,49,126].

2. DISTRIBUTION OF THE SUBSTRATE BETWEEN THE CARRIER AND THE SOLUTION

A simple behaviour may be observed in the electrostatic interaction between the substrate and the carrier. Indeed, if the carrier and substrate are both charged, the substrate concentration C_0' on (or in) the carrier is defined by the following relation:

$$P = \frac{C_0'}{C_0} = \exp\left(-\frac{ze\psi}{kT}\right), \qquad (46)$$

analogous to Eqn. (43), where P is the partition coefficient, C_0 the substrate concentration in the solution, and ze the charge of the substrate. Then the rate of the reaction in the carrier phase catalysed by the immobilised enzyme can be represented by Eqn. (19), in which the physical significance of the coefficient P is defined by Eqn. (46). Consequently the effective Michaelis constant observed experimentally is given by the relation $K_{m(obs)} = K_m' \exp(ze\psi/kT)$. Evidently, when the charges of the carrier and the substrate are of the same sign (ze and ψ are of the same sign), $K_{m(obs)} > K_m'$; on the other hand, when they are of different sign (ze and ψ are of different signs), $K_{m(obs)} < K_m'$§. It follows from Eqn. (19) that the electrostatic interaction between the substrate and the carrier is not manifested when $C_0 \gg K_{m(obs)}$.

Table 8. The observed Michaelis constants for different immobilised enzymes[7].

Enzyme	Carrier	Charge of carrier	Substrate	Charge of substrate	$K_{m(obs)}$, M	Refs.
ATP : creatine phosphotransferase	None		ATP	−	6.5×10^{-4}	
	p-Aminobenzylcellulose	0	ATP	−	8.0×10^{-4}	127
	KM-Cellulose	−	ATP	−	7.0×10^{-3}	
Ficin	None		EEBA[a]	+	2.0×10^{-2}	
	KM-Cellulose	−	EEBA	+	2.0×10^{-3}	122
Trypsin	None		BAA[b]	+	6.8×10^{-3}	
	Ethylene–maleic acid copolymer	−	BAA	+	2.0×10^{-4}	113
Papain	None		EEBA	+	1.9×10^{-2}	
	pp-Aminophenylanaline-L-leucine copolymer	0	EEBA	+	1.9×10^{-2}	128
Bromelain	None		EEBA	+	0.11	
	KM-Cellulose	−	EEBA	+	0.049	129

[a] EEBA = ethyl ester of *N*-benzoyl-L-arginine.

[b] BAA = *N*-benzoyl-L-argininamide.

§ One should note that there is an error in the fundamental communication of Goldstein et al.[113], which was unfortunately subsequently transferred to other papers by the same workers and also to certain reviews[8,9]. After deriving correctly Eqn. (44), the authors[113] then adopt as the pH shift between the medium of the carrier and the solution the observed shift of the pH profile of the catalytic activity of the enzyme, which evidently is of the opposite sign. As a result negatively charged carriers would have to be characterised by a positive potential ψ! On the basis of this erroneous postulate, the authors quote an incorrect equation for the distribution of the charged substrate between the charged carrier and the solution [cf. Eqn. (46)]:

$$C' = C_0 \cdot \exp\left(\frac{ze\psi}{kT}\right).$$

We may note that this error is by no means purely terminological, since the last equation and the equation for the pH shift between the carrier and the solution [Eqn. (44)], quoted by Goldstein et al.[113], are mutually exclusive.

Table 8 presents the values of $K_{m(obs)}$ for certain reactions of charged substrates with enzymes immobilised on the charged carrier. Evidently in many systems the electrostatic interactions between the substrate and the carrier may result in a difference between $K_{m(obs)}$ and K'_m greater than by an order of magnitude. As was to be expected, at high ionic strengths of the solution the above effects become insignificant. Thus $K_m = 6.9 \times 10^{-6}$ M was found for the reaction of trypsin with the ethyl ester of *N*-benzoyl-L-arginine in solution; $K_{m(obs)} = 0.2 \times 10^{-6}$ M at an ionic strength of 0.04 M and $K_{m(obs)} = 5.2 \times 10^{-6}$ M at an ionic strength of 0.50 M for trypsin immobilised on a copolymer of ethylene and maleic acid[113].

The interaction between the substrate and the carrier can in principle be other than electrostatic. In the general case, when P cannot be predicted theoretically, it can be measured in a separate experiment (using a carrier without the enzyme). For example, in the case of a hydrophobic substrate $P > 1$ may be expected for a hydrophobic carrier and $P < 1$ for a hydrophilic matrix. In the reaction with a hydrophilic substrate the opposite situation may obtain. The nature of the distribution (the value of P) has in turn a corresponding effect on the observed Michaelis constant [Eqn. (19)]. As an example we may point to the reaction of immobilised cholinesterase with benzoylcholine (a hydrophobic substrate), for which the observed Michaelis constant decreases approximately by a factor of ten on passing from a hydrophilic to a hydrophobic matrix[130]. It has been shown[131] that, in the adsorption of lipase on a hydrophobic carrier (siliconised glass), the rate of its reaction with tripropionin [glycerol tripropionate (Ed. of Translation)] increases sharply owing to the concentration of the hydrophobic substrate at the surface of the carrier.

3. DISTRIBUTION OF INHIBITORS AND COFACTORS BETWEEN THE CARRIER AND SOLUTION

When the enzyme reacts with an inhibitor or a cofactor, their influence on the reaction rate is known to be determined[114] by the product $K_G[G]_b$, where $[G]_b$ is the concentration of the inhibitor or the cofactor and K_G is the constant for its binding to the enzyme. For immobilised enzymes, we have the analogous product $K'_G[G]'_b$, where $[G]'_b$ is the concentration of the effector on (or in) the carrier and K'_G is the constant for the binding of the immobilised enzyme to the effector. However, in a kinetic experiment it is possible to find only the effective factor $K_{G(obs)}[G]_b$, where $K_{G(obs)} = K'_G P_G$ and $P_G = [G]'_b/[G]_b$ is the partition coefficient. Evidently all that has been said above about the distribution of the substrate between the carrier and the solution applies to the partition coefficient P_G.

As an example, we may mention β-fructofuranosidase immobilised on polyaminostyrene (a hydrophobic carrier), where the observed constant for inhibition by aniline is higher by a factor of three compared with the reaction of the native enzyme[132].

IV. THE CHANGE IN THE TRUE KINETIC PARAMETERS OF ENZYMIC REACTIONS AFTER IMMOBILISATION

The true kinetic parameters should be understood as quantities uncomplicated by the influence of diffusion factors or the distribution of the reactants between the carrier phase and the solution. There are at least two main causes responsible for the change in the true kinetic parameters after immobilisation. Firstly, the immobilised enzyme can have a conformation different from that of the native enzyme. This type of explanation can be frequently encountered in the literature. However, in practice it can be neither confirmed nor disproved experimentally. There are at present no reliable experimental data indicating the existence of effects of this kind. The second cause is a possible change in the medium in which the enzymic reaction takes place after immobilisation. In certain instances the effect of the medium on the enzymic process can be satisfactorily accounted for within the framework of the influence of the dielectric constant on the ionic strength, particularly for reactions between charged species[133,134].

According to Laurent's concept[135], when neutral polymers are introduced into the solution the chemical potentials of the macromolecules in it change owing to their exclusion from part of the volume of the solvent. Theoretical analysis (which was confirmed experimentally) showed that this phenomenon can lead to the decrease of the observed Michaelis constant (or of the observed constant for the inhibition by the protein). Possibly similar phenomena occur also when enzymes are immobilised.

V. STERIC RATE LIMITATION IN REACTIONS OF IMMOBILISED ENZYMES WITH MACROMOLECULAR SUBSTRATES

Immobilised enzymes (particularly in porous carriers) as a rule retain a high catalytic activity with respect to low-molecular-weight substrates but in many cases lose at the same time the capacity to catalyse reactions involving high-molecular-weight compounds. Thus it has been found[136] that trypsin immobilised in silica gel reacts with the ethyl ester of *N*-benzoyl-L-arginine almost like the native enzyme but does not hydrolyse casein at all. Water-insoluble derivatives of polytyrosyltrypsin and polytyrosylpapain[138] cleave casein at rates amounting to only 15 or 30% respectively of the values which might have been expected on the basis of their intrinsic activities with respect to the low-molecular-weight substrate. Similar results were obtained also for KM-cellulose derivatives of ficin[43,122], bromelain[139], trypsin[140], α-chymotrypsin[137,140], and also for polyalcohol, polyanionic, and polycationic derivatives of papain[8] and subtilopeptidase A.[9] In all these cases the decrease of protolytic activity is probably related to steric hindrance of the approach of the protein substrate to the immobilised enzyme[13,14,17,137]. The same steric factors are also responsible for another finding, namely that immobilised trypsin is 92% inhibited by the comparatively low-molecular-weight pancreatic protein trypsin inhibitor and is not inhibited at all by the more bulky soya bean inhibitor[141].

Certain quantitative relations have been traced[142]. Suppose that χ is the specific activity of pronase immobilised on a water-insoluble copolymer of *p*-aminophenylalanine and leucine, referred to the specific activity of the native enzyme. It was found that $\chi^{1/3}$ varies linearly with $M^{1/2}$, where M is the molecular weight of the substrate. The authors[142] drew attention therefore to data[143,144] for the distribution of various proteins between the aqueous solution and Sephadex. It was shown for this system that not all the solvent which has diffused into Sephadex is accessible to proteins and that only a fraction $N < 1$ is

available, the relation between N and M being satisfactorily described by the equation

$$\sqrt[3]{N} = \text{const}_1 - \text{const}_2 \cdot \sqrt{M} \quad . \qquad (47)$$

Therefore the authors[140] suggest that in the immobilised enzyme system which they describe a similar effect may occur, i.e. only a fraction of the volume of the porous particle containing the immobilised enzyme is accessible to protein substrates, its value depending on M in the way indicated by Eqn. (47).

Steric hindrance in reactions with immobilised enzymes may be combined with electrostatic interaction between high-molecular-weight substrates and the charged carrier. Trypsin immobilised on an anionic carrier cleaves only ten peptide bonds in pepsinogen (and also in reduced carboxymethylated pepsinogen), while the native enzyme cleaves fifteen such bonds[145]. The same trypsin derivative cleaves myosin approximately fifty times more slowly than the native enzyme and at different peptide bonds[146,147]. Native deoxyribonuclease and the enzyme immobilised on glass cleave DNA into different oligonucleotides[148]. Similar effects were also noted in other studies[149-151].

Summarising, one may conclude on the basis of the data presented in Sections III–V that the substrate specificity of the immobilised enzyme can depend strongly on the nature of the carrier and hence may differ significantly from the specificity of the native biocatalyst.

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Only some kinetic and thermodynamic features of catalysis by immobilised enzymes have been explained in the present review from physicochemical standpoints. Many problems remain outside the present discussion. This is because there is so far no quantitative theory in many fields of study involving catalysis by immobilised enzymes. The most important of these are as follows.

1. The study of the dependence of the catalytic activity and substrate specificity of immobilised enzymes on the nature of the carrier (particularly in relation to the individual stages of enzymic catalysis). It is necessary to set up criteria for the choice of the optimal carrier for the given enzyme system.
2. The explanation of the causes of the stabilisation of enzymes on immobilisation. Only the first steps have been taken in this field[152].
3. The development of methods for the immobilisation of enzymes with cofactors and of polyenzyme systems.
4. More attention should be paid in terms of technology to the possibility of the application of "naturally" immobilised systems, in particular micro-organisms.
5. Problems associated with the choice of the optimal reactor for the given process.

In studies on immobilised enzymes efforts should be concentrated on the solution of the above problems.

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