Acetylcholine Esterase – Dynamic Behaviour with Flow Calorimetry*

F. MALÍK and V. ŠTEFUCA**

Department of Chemical and Biochemical Engineering, Faculty of Chemical and Food Technology, Slovak University of Technology, SK-812 37 Bratislava
e-mail: malik@chelin.chtf.stuba.sk, stefuca@cvt.stuba.sk

Received 28 March 2002

The application of enzyme flow calorimetry for the monitoring of hysteresis behaviour of immobilized enzyme was investigated. The hysteresis of immobilized acetylcholine esterase induced by combination of mass transfer and substrate inhibition was considered. Theoretical analysis of the hysteresis was based on mathematical modelling using kinetic and diffusion parameters from literature. The influence of Thiele modulus and the ratio of substrate inhibition and Michaelis constant (K_i/K_m) on the size and region of hysteresis was determined. It was shown that by increasing the value of Thiele modulus or K_i/K_m, the region of hysteresis was shifted towards higher substrate concentrations. On the basis of the simulation results it was concluded that a commercial preparation of acetylcholine esterase should give rise to hysteresis at value of Thiele modulus higher than 150. The considered particle diameter should be in the range from 0.1 to 1.0 mm.

Enzyme reactions taking part in metabolic pathways, regulation processes, or in *in vitro* conditions often exhibit complicated dynamic behaviour in terms of the relation between the reaction rate and reaction. It can be induced by kinetic nonlinearities resulting from allosteric interactions, by autocatalytic mechanisms, or by combination of enzyme reaction with mass transfer conditions. This can lead to one of typical effects, the so-called hysteresis, when a retardation of the evolution between reaction rate and concentration of substrate or other compounds is observed. When the system is repeatedly wiggled back and forth (the concentration is cycled up and down), a hysteresis loop can appear in the reaction rate—concentration diagram.

It has been suggested that hysteresis effects in biological systems are adequate to account for short-term memory [1]. Such "memory" effect in *in vitro* conditions was observed with urease-albumin membrane [2] and the existence of hysteresis was explained simply by coupling the enzyme kinetics and diffusion transport. It was also shown that in some cases the changes in conformation of enzyme molecule may give rise to hysteresis [3]. In the work [4] there were developed techniques for preparation of synthetic enzyme membranes for pH electrode coating that provided a new tool for investigation of this kind of hysteresis. The authors used papain membrane-coated electrode and demonstrated hysteresis loop formed by the dependence between external and internal pH measured experimentally when the external pH was progressively changed. Hysteresis was explained by autocatalytic effect of the enzyme reaction. Afterwards the pH electrode coated by the membrane with immobilized uricase was studied [4, 5]. Hysteresis loop was observed when the bulk substrate concentration was progressively increased and decreased and it was explained by combination of the substrate inhibitory effect with diffusion limitation in the membrane. Later on this observation was confirmed by mathematical modelling [6]. Similar behaviour was observed in a membrane reactor system with rabbit muscle phosphofructokinase, the enzyme strongly inhibited by substrate ATP [7].

From the above introduction it follows that these phenomena can be observed if mass transfer restrictions are combined either with autocatalytic effect of pH change due to the enzyme reaction or with substrate inhibition. At the same time, the immobilized enzyme has to be sufficiently stable during the experimental run. The latter condition is often incompatible with the pH-induced mechanism, as an extreme local pH change can have a suicide effect on enzyme. Such effect was observed in the case of immobilized urease where it was impossible to close experimentally the hysteresis loop due to pH inactivation of the enzyme [8]. Therefore, the substrate inhibition principle seems to be a more suitable alternative for the experimental system construction. For the present work acetyl-

*Presented at the 29th International Conference of the Slovak Society of Chemical Engineering, Tatranské Matliare, 27—31 May 2002.

**The author to whom the correspondence should be addressed.

choline esterase (AChE) was chosen as a model enzyme that is strongly inhibited by its substrate, acetylcholine.

The experimental investigation of dynamic behaviour of AChE requires a rapid method of measurement of the enzyme reaction rate. Generally, the AChE reaction was followed by several indirect or direct methods, *e.g.* by pH change registration [9], spectrophotometry [10], automated pH-stat method [11], titrimetry [12]. Most of the methods suffer from one or more drawbacks like sensitivity, specificity, reproducibility, or complexity that can be avoided by flow calorimetry. The flow calorimetry was previously used for direct monitoring of the course of reactions catalyzed by immobilized enzymes [13]. The principle of the method is registration of the temperature change induced by the heat of the enzyme reaction released in the thermostated column with an immobilized biocatalyst that operates as a packed bed reactor. The aim of this work was to estimate the possibility to follow hysteresis behaviour of AChE immobilized in spherical particles by mathematical modelling.

THEORETICAL

Mathematical modelling of processes in the flow calorimeter filled with particles with immobilized biocatalyst is based on material and heat balances of the calorimetric column. The column can be described as a continuous packed bed reactor [13]. Balance equations were derived from the following assumptions: the reactor is differential, interstitial velocity of flow is high enough to prevent the effect of external diffusion, heat loss through the reactor wall is negligible so that the reactor is considered to be adiabatic [13]. Then, the heat balance of the reactor results in a linear relation between overall substrate concentration change in the column Δc_S, and the temperature change, ΔT, measured by flow calorimetry

$$\Delta T = \alpha(-\Delta c_S) \tag{1}$$

where α is a constant parameter. As a differential bed of biocatalyst is used, it can be written

$$-\Delta c_S = v_{obs} t_r \tag{2}$$

where t_r is the residence time in the reactor and v_{obs} is the reaction rate and it is directly proportional to the overall particle reaction rate, v_p

$$v_{obs} \propto v_p = \frac{A_p}{V_p} D_e \left(\frac{dc_S}{dr}\right)_{r=R_p} \tag{3}$$

where A_p, V_p, and R_p are the particle surface, volume, and radius, respectively. D_e is the effective diffusion coefficient of substrate and r is the particle radial coordinate. It follows from eqns (*1*—*3*) that ΔT is proportional to the substrate concentration gradient on the particle surface.

$$\Delta T \propto \left(\frac{dc_S}{dr}\right)_{r=R_p} \tag{4}$$

The following part shows the application of eqn (*4*) for testing the hysteresis behaviour of immobilized enzyme particles by mathematical modelling keeping in mind that the theoretical results are to be verified experimentally by the flow calorimetry. It has already been shown that in a system with a membrane-bound enzyme strongly inhibited by substrate the multiplicity of steady states can lead to hysteresis revealed in the dependence between the reaction rate and bulk substrate concentration [4]. For a spherical particle the reaction rate calculation requires solving particle balance equation for substrate

$$\varepsilon_p \frac{\partial c_S}{\partial t} = D_e \left(\frac{\partial^2 c_S}{\partial r^2} + \frac{2}{r}\frac{\partial c_S}{\partial r}\right) - \frac{V_m c_S}{K_m + c_S + \frac{c_S^2}{K_i}} \tag{5}$$

Kinetic parameters in eqn (*5*) are: K_m – Michaelis constant, K_i – substrate inhibition constant, V_m – maximum reaction rate. ε_p is particle porosity. Eqn (*5*) was combined with the initial condition

$$t = 0 \quad 0 \le r \le R_p \quad c_S = 0 \tag{6}$$

and boundary conditions

$$\begin{aligned} t > 0 \quad r = 0 \qquad & \frac{\partial c_S}{\partial r} = 0 \\ r = R_p \quad & c_S = c_{S0} \end{aligned} \tag{7}$$

where c_{S0} is the substrate concentration on the particle surface. After introducing dimensionless quantities

$$\begin{aligned} c = \frac{c_S}{K_m} \quad x = \frac{r}{R_p} \quad \tau = \frac{t D_e}{R_p^2} \\ \Phi = R_p \sqrt{\frac{V_m}{K_m D_e}} \quad \beta = \frac{K_i}{K_m} \end{aligned} \tag{8}$$

eqn (*5*) was rewritten into a dimensionless form

$$\frac{\partial c}{\partial \tau} = \left(\frac{\partial^2 c}{\partial x^2} + \frac{2}{x}\frac{\partial c}{\partial x}\right) - \Phi^2 \frac{c}{1 + c + \frac{c^2}{\beta}} \tag{9}$$

with the initial and boundary conditions

$$\begin{aligned} \tau = 0 \quad 0 \le x \le 1 \quad & c = 0 \\ \tau > 0 \quad x = 0 \qquad & \frac{\partial c}{\partial x} = 0 \\ x = 1 \qquad & c = c_0 \end{aligned} \tag{10}$$

By integration of eqn (*9*) up to steady-state solution, the substrate concentration gradient at the particle surface was calculated and used as a quantity that directly determines the evolution of the reaction rate hence the temperature change in the calorimeter, as shown above. Hysteresis loops were constructed by mathematical simulation of experiments where the bulk concentration was changed step-by-step and after each change, eqn (*9*) was integrated until the steady-state solution while the reached particle concentration profile was introduced in the next integration as the initial condition. The simulations were made by ATHENA Visual Workbench (Stewart and Associates, Madison, Wisconsin, USA).

The influence of mass transfer on the overall reaction rate was analyzed *via* the effectiveness factor, η, defined as

$$\eta = \frac{v_\mathrm{p}}{v(c_{\mathrm{S}0})} \qquad (11)$$

where $v(c_{\mathrm{S}0})$ represents the rate which would be obtained with no diffusion limitation in the biocatalyst particle

$$v(c_{\mathrm{S}0}) = \frac{V_\mathrm{m} c_{\mathrm{S}0}}{c_{\mathrm{S}0} + K_\mathrm{m} + \dfrac{c_{\mathrm{S}0}^2}{K_\mathrm{i}}} \qquad (12)$$

Combining eqns (*3*), (*11*), and (*12*) and using dimensionless parameters (*8*), the effectiveness factor can be expressed as

$$\eta = \frac{3(\mathrm{d}c/\mathrm{d}x)_{x=1}}{\Phi^2 c_0/(1 + c_0 + c_0^2/\beta)} \qquad (13)$$

RESULTS AND DISCUSSION

Although it is not complicated to predict the hysteresis behaviour of immobilized enzyme systems mathematically, its experimental confirmation is rare. Therefore the main motivation of the present work was to open the possibility for such experimental study. A mathematical simulation approach was applied to determine the conditions in which the hysteresis behaviour of acetylcholine esterase immobilized in porous particles can be investigated by flow calorimetry. Initially, Thiele modulus, Φ, and $\beta = K_\mathrm{i}/K_\mathrm{m}$ were tested in relation to the occurrence and size of hysteresis loop. Figs. 1 and 2 show that at certain parameter values hysteresis can be observed. With increasing value of Thiele modulus or β, the region of hysteresis shifts towards higher substrate concentrations. The crucial parameter conditioning the possibility of experimental detection of hysteresis loop is the loop width taken as the substrate concentration range within which the loop is situated. Fig. 3 shows the dependence of the loop width on β and Φ. It is evident from the data that the width is more sensitive to Φ than to β. This is advantageous from the practical point of view because the Thiele modulus can more easily be adjusted (by the particle diameter or enzyme concentration) than the enzyme kinetics expressed by parameter β.

The hysteresis loop corresponds to a series of mul-

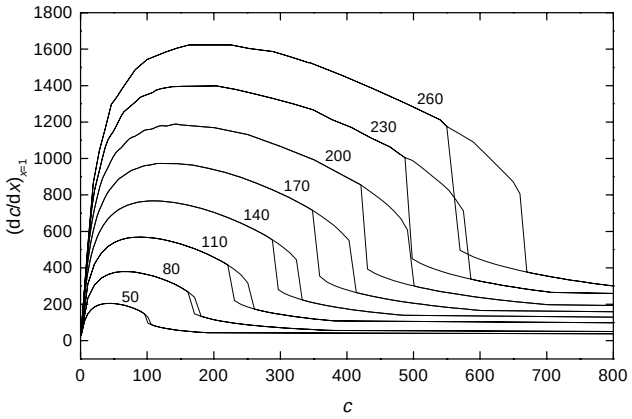

Fig. 1. Influence of different Thiele modulus on the hysteresis pattern at constant $\beta = 10$.

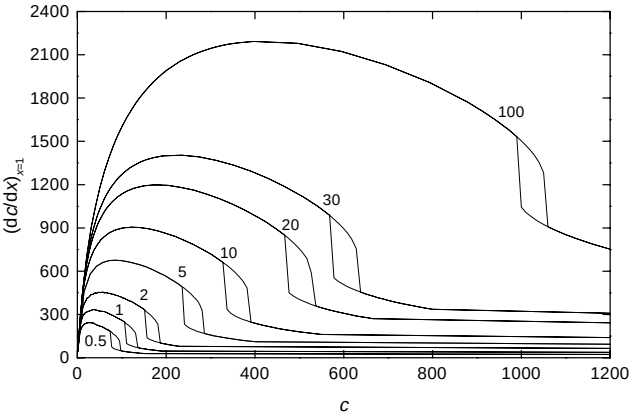

Fig. 2. Influence of different values of β on the hysteresis pattern at constant value of Thiele modulus of 160.

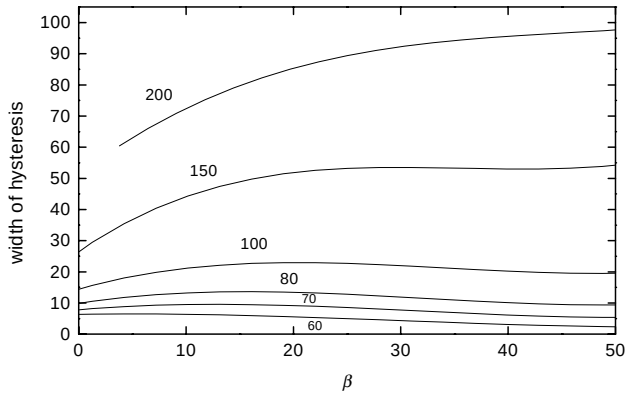

Fig. 3. Influence of β and Thiele modulus (parameter of the lines) on the width of hysteresis loop. The width is defined as the difference between substrate concentrations on the left and right side of the loop.

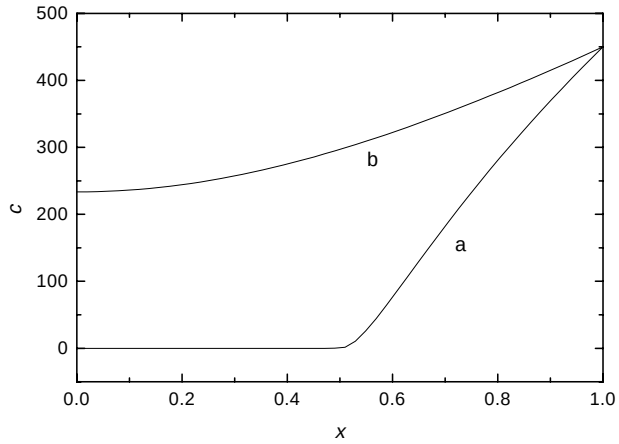

Fig. 4. Dimensionless concentration profiles in particle for the same surface dimensionless concentration and different concentration trends in hysteresis loop: a – increasing concentration, b – decreasing concentration.

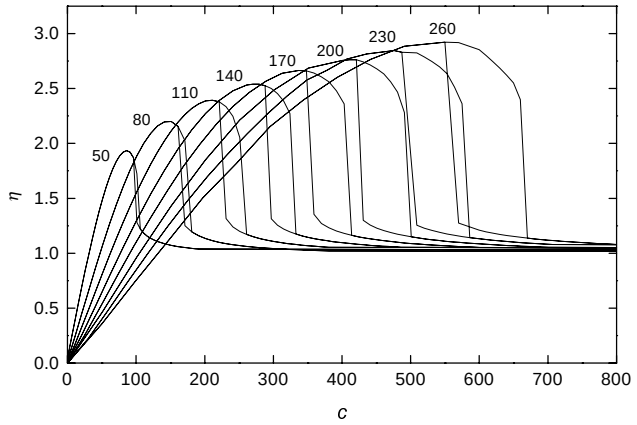

Fig. 5. Effectiveness factor characteristics at various Thiele modulus and constant $\beta = 10$.

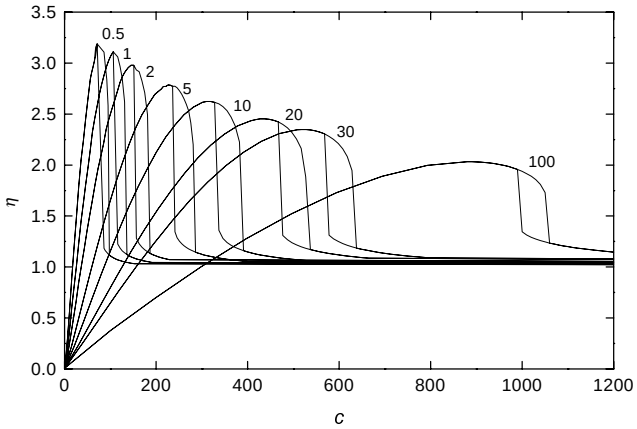

Fig. 6. Effectiveness factor characteristics at various values of β and constant value of Thiele modulus of 160.

tiple steady-state solutions. The multiplicity of reaction rates achieved at these states corresponds to different substrate concentration profiles. The concentration profiles in Fig. 4 calculated for the same substrate concentration and opposite points of hysteresis loop show that when going up and down with the substrate concentration the reaction rate does not have the same value. When the substrate concentration is gradually increased, the concentration profile is sharper and the reaction rate is higher than in the inversed case. Interestingly, the direction of evolution of bulk substrate concentration influences the shape of concentration profile, as well. The profile corresponding to the successive concentration increase (line a) is more typical for the region of substrate inhibition than the profile at the inverse situation (line b). These differences demonstrate the mentioned "memory" effect in the reaction—diffusion systems and it can be said that the concentration profile reflects the system history.

The hysteresis occurrence can be demonstrated also in terms of steady-state effectiveness factor values calculated for the above-simulated experiments shown in Figs. 5 and 6. The regions of hysteresis are situated at the effectiveness factor value exceeding unity. Such high values are caused by a damping effect of mass transfer limitation on substrate inhibition at higher bulk substrate concentrations. It can be concluded that a strong mass transfer limitation must be introduced for hysteresis to develop. Under such conditions, hysteresis can be explained as the result of variable damping effect of mass transfer limitation on the substrate inhibition.

Application of Simulation Results to Immobilized Acetylcholine Esterase

The necessity to prepare immobilized enzyme particles with a precise diameter and enzyme concentration led us to consider alginate entrapment for this purpose. For simulations of experiments considering an immobilized AChE, the value of the effective diffusion coefficient of substrate, acetylcholine, in alginate particles is needed. A value of 6.05×10^{-10} m^2 s^{-1} was determined by a sorption method described previously [19]. This value together with the published data on AChE reviewed in Table 1 was used for simulations. Taking the kinetic parameters K_i and K_m, the region of hysteresis for each enzyme simulation was estimated. The experimentally observable region was assigned to the substrate concentration where hysteresis emerged with the minimum width of 5 mmol dm^{-3}. According to the calculations, commercial preparations of the most widely used eel AChE should give rise to the hysteresis at values of Thiele modulus higher than 150. This minimal value of Thiele modulus can be achieved by adjusting the particle diameter in the range from 0.1 to 1.0 mm and by the mass concentration of immobilized enzyme from 0 to 10 mg cm^{-3} (considering activities of commercial preparations).

Table 1. Simulation of Hysteresis Behaviour on the Basis of Published Information about Kinetic Properties of Acetylcholine Esterase

Type of enzyme	Form	Substrate[a]	Temperature / °C	Buffer	pH	K_m / mmol dm^{-3}	K_i / mmol dm^{-3}	K_i/K_m	c_S^b / mmol dm^{-3}	Φ^c	Ref.
									Simulation results		
Human	Soluble	AtCh	25	20 mM-sodium phosphate, 0,02 % Triton X-100	7	0.058	18	310.3	130	>210	[14]
Human	Soluble	M7A	25	20 mM-sodium phosphate, 0.02 % Triton X-100	7	0.015	0.9	60.0	33	>400	[14]
Electric eel	Soluble	ACh	25	0.02—0.05 M-sodium phosphate, 0.1 M-NaCl, 0.01 M-$MgCl_2$	7	0.092	30	326.1	181	>190	[15]
Electric eel	Immobilized	AtCh	–	1—2 mM-Tris maleate/phosphate	6	0.13	50	384.6	239	>165	[16]
Electric eel	Soluble	AtCh	23	0.05 M-sodium/potassium phosphate, 0.1 M-KCl	7.4	0.087	11.22	129.0	99	>150	[17]
Fetal bovine serum	Soluble	AtCh	25	0.05 M-sodium phosphate	8	0.187	11.3	60.4	80	>90	[18]

a) M7A – 7-acetoxy-*N*-methylquinolinium iodide, ACh – acetylcholine, AtCh – acetylthiocholine. *b*) Calculated approximate substrate concentration around which hysteresis occurred; *c*) calculated minimal value of Thiele modulus with the occurrence of hysteresis loop of the width of 5 mol m^{-3} of substrate concentration.

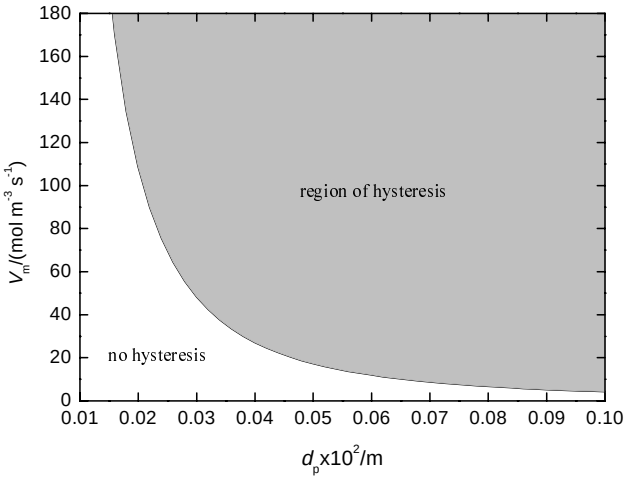

Fig. 7. Plot of evaluation of eqn (7) for the value of Thiele modulus 150 with values of parameters in workable range, where d is particle diameter; the line corresponds to the extracted product of the effective diffusion coefficient, $D_e = 6.05 \times 10^{-10}$ m^2 s^{-1} and Michaelis constant $K_m = 0.08$ mmol dm^{-3}. The space above the line corresponds to the higher values of Thiele modulus.

Both these ranges are experimentally achievable and applicable for the flow calorimeter. The result that is not less important than the latter conditions is that the hysteresis falls into the substrate concentration regions below the solubility limit.

Based on the above results, a diagram was constructed (Fig. 7) showing two regions depending on the parameters that can be experimentally adjusted – particle diameter and maximum reaction rate, the quantity that depends on the total enzyme activity bound in the particle. The line separating the region of hysteresis from the region where hysteresis cannot be observed was calculated from eqn (7) defining the Thiele modulus. The region below the line corresponds to the situation when the Thiele modulus is lower than 150. The diagram can be used as a rapid guideline for the design of real particles with immobilized AChE.

The necessary criterion of applicability of the flow calorimetry for the reaction monitoring is a sufficiently high thermal effect. The instrument used by our research group registers temperature changes, ΔT, from millikelvins up to one kelvin. The temperature change expected for immobilized AChE was calculated from the data introduced in Table 1 for the enzyme from electric eel, specifically, $\Phi = 160$, $K_m = 0.087$ mol m^{-3}, $K_i = 11.22$ mol m^{-3}. Other chosen values were $R_p = 2.5 \times 10^{-4}$ m, $V_m = 86$ mol m^{-3} s^{-1}, $c = 1000$, $\eta = 1.5$ (a representative value taken from Fig. 5). The approximate molar reaction enthalpy for acetylcholine hydrolysis was 4 kJ mol^{-1} [20]. Upon physicochemical conditions characterizing the calorimetric measurement, the temperature change calculated from the heat balance in the column packed with immobilized AChE would be around 13 mK. This value falls to the range of temperature changes that can be measured with a sufficient precision.

Taking into account the substrate concentration range, temperature changes, and Thiele modulus de-

termined by mathematical simulations, it can be concluded that the hysteresis could be detected by the flow calorimetry, and the future plan will be the experimental confirmation of the introduced results.

Acknowledgements. This study was supported by the Slovak Grant Agency for Science VEGA (Grant No. 1/7343/20).

SYMBOLS

A_p	particle surface area	m^2
c	dimensionless substrate concentration	
c_S	substrate concentration	mol m^{-3}
c_{S0}	substrate concentration at particle surface	mol m^{-3}
D_e	particle effective diffusion coefficient	m^2 s^{-1}
K_m	Michaelis constant	mol m^{-3}
K_i	substrate inhibition constant	mol m^{-3}
r	particle radial coordinate	m
R_p	particle diameter	m
t	time	s
t_r	residence time in packed bed	s
v_{obs}	observed reaction rate	mol m^{-3} s^{-1}
v_p	overall particle reaction rate	mol m^{-3} s^{-1}
V_m	maximum reaction rate	mol m^{-3} s^{-1}
V_p	particle volume	m^3
x	dimensionless particle radial coordinate	

Greek Letters

α	constant in eqn (*1*)	K mol^{-1} dm^3
β	dimensionless kinetic parameter defined by eqn (*8*)	
ε_p	particle porosity	
Φ	Thiele modulus	
η	effectiveness factor	
τ	dimensionless time	

REFERENCES

1. Kernevez, J. P., Joly, G., Duban, M. C., Bunow, B., and Thomas, D., *J. Math. Biol. 7*, 41 (1979).
2. Thomas, D., Barbotin, J. N., David, A., Hervagault, J. F., and Romette, J. L., *Proc. Natl. Acad. Sci. U.S.A. 74*, 12, 5314 (1977).
3. Selkov, E. E. and Dynnik, S. N., *Biofizika 21*, 2, 214 (1976).
4. Naparstek, A., Romette, J. L., Kernevez, J. P., and Thomas, D., *Nature 249*, 490 (1974).
5. Naparstek, A., Thomas, D., and Caplan, S. R., *Biochim. Biophys. Acta 323*, 643 (1973).
6. Kernevez, J. P., *Analysis and Control of Immobilized Enzyme Systems.* (Thomas, D. J. P., Editor.) P. 199. North-Holland Publishing Company, Amsterdam, 1976.
7. Hervagault, J. F., Duban, M. C., Kernevez, J. P., and Thomas, D., *Proc. Natl. Acad. Sci. U.S.A. 80*, 5455 (1983).
8. Štefuca, V., Kohutovičová, E., and Polakovič, M., *2nd European Symposium on Biochemical Engineering Science*, Porto, 16—19 September 1998, p. 295 in abstract proceedings. Poster presentation.
9. Michel, H. O., *J. Lab. Clin. Med. 34*, 1564 (1949).
10. Ellman, G. L., Courtney, K. D., Andres, V., and Featherstone, R. M., *Biochem. Pharmacol. 7*, 88 (1961).
11. Nabb, D. P. and Whitfield, F., *Arch. Environ. Health 15*, 147 (1967).
12. Lawler, H. C., *J. Biol. Chem. 238*, 132 (1963).
13. Štefuca, V. and Gemeiner, P., *Advances in Biochemical Engineering/Biotechnology.* (Scheper, T., Editor.) P. 71. Springer-Verlag, Berlin, 1999.
14. Rosenberry, T. L., Mallender, W. D., Thomas, P. J., and Szegletes, T., *Chem.-Biol. Interact. 119—120*, 85 (1999).
15. Wilson, I. B. and Harrison, M. A., *J. Biol. Chem. 236*, 2292 (1961).
16. Barbotin, J. N. and Friboulet, A., *Colloids Surf. 10*, 359 (1984).
17. Bardsley, W. G. and Leff, P., *Biochem. J. 187*, 739 (1980).
18. Saxena, A., Hur, R., and Doctor, B. P., *Biochemistry 37*, 145 (1998).
19. Gemeiner, P., Kurrilová, Ľ., Markovič, O., Malovíková, A., Uhrín, D., Ilavský, M., Štefuca, V., Polakovič, M., and Báleš, V., *Biotechnol. Appl. Biochem. 13*, 69 (1991).
20. Das, Y. T., Brown, H. D., and Chattopadhyay, S. K., *Biophys. Chem. 23*, 105 (1985).