APPLICABILITY OF THE PSEUDO-STEADY STATE ASSUMPTION TO THE IRREVERSIBLE MICHAELIS-MENTEN REACTION

HARUO ISHIKAWA, KATSUHIKO OKADA AND HARUO HIKITA
Department of Chemical Engineering, University of Osaka Prefecture, Sakai 591

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The applicability of the pseudo-steady state assumption to an enzyme-substrate complex was examined for an irreversible single-substrate reaction which obeys the Michaelis–Menten mechanism by using computer simulation. The pseudo-steady state solutions of the time courses of the substrate and product were compared with the exact solutions calculated by using a complete set of rate equations under various conditions. The maximum relative errors of the pseudo-steady state solutions to the exact solutions could be correlated well only in terms of a dimensionless parameter, which the authors designate ψ_M and define by the ratio of the pseudo-steady state solution of the intermediate complex at $t=0$ to the initial substrate concentration. The simulations showed that if $\psi_M \leqq 0.05$, the pseudo-steady state solutions are accurate within a tolerable error of 5% and therefore the pseudo-state assumption holds.

Introduction

In the theoretical formulation of enzyme reaction rates in terms of the concentrations of substrates and other substances such as products and inhibitors, it is usual to use the pseudo-steady state (PSS) assumption. Rate equations derived in this way are useful in understanding the effects of substrate, product and inhibitor concentrations and of the kinetic parameters on the reaction rates, and are also useful in the experimental determination of the kinetic parameters. They are especially useful in the design and control of batch reactors in which the time courses of the components in the reaction must be calculated.

Applications of enzymes in various forms in which the enzyme concentrations are relatively high compared with those of substrates are increasing in industry and clinical diagnoses. Two examples are: (1) enzymatic amplification techniques[2] for the determination of the concentration of substrates which exist in tissue at a very low level, and (2) *in-situ* cofactor recycling[7,8] which is essential for the effective utilization of cofactor-requiring enzymes. In these examples, however, the PSS assumption, where the concentrations of enzyme-substrate complexes are assumed to remain constant, does not always hold. Therefore, the conditions under which the PSS assumption may hold should be known, and considerable research has been devoted to clarifying these conditions.

For an irreversible single-substrate reaction, Laidler[4] studied qualitatively the conditions under which the Michaelis–Menten rate equation derived on the basis of the PSS assumption is valid and suggested that it is accurate if any one of the following inequalities holds: $[A]_0 \gg [E]_0$, $[E]_0 \gg [A]_0$, $K_m \gg [E]_0$ or $K_m \gg [A]_0$, where $[A]_0$, $[E]_0$ and K_m express the initial substrate concentration, the total enzyme concentration and the Michaelis constant, respectively. The first and third criteria shown above were also proposed by Bartha.[1] Lim[5] compared the time courses of the substrate calculated by using the integrated Michaelis–Menten equation, i.e., the PSS solution, with the exact time courses calculated by using the complete set of elementary rate equations, and concluded that the PSS solution is accurate when the ratio $[A]_0/[E]_0$ of the initial substrate concentration to the total enzyme concentration is greater than 100. Sakamoto[9] obtained a similar conclusion. Wong[13] mapped the range of the $[E]_0$ and $[A]_0$ in which the PSS assumption holds.

For a reaction which follows the reversible Michaelis–Menten mechanism, the applicability of the PSS assumption has also been discussed.[6,10–12]

As briefly reviewed above, previous investigations have focused on the individual effects of terms such as the ratio $[A]_0/[E]_0$, $K_m/[E]_0$ or others on the applicability of the PSS assumption to the single-substrate system in which the reaction follows the

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irreversible or the reversible Michaelis–Menten mechanism. The combined effect of $[A]_0$, $[E]_0$ and K_m on the applicability of the PSS assumption to the reaction systems mentioned above or to any other reaction systems have not yet been studied. Furthermore, the extent of the validity of the PSS assumption for a given reaction system has never been studied quantitatively.

In this paper, for an irreversible single-substrate reaction which obeys the Michaelis–Menten mechanism, a new parameter by which the errors of the PSS solutions of the time courses of substrate and product can be correlated is introduced to determine the conditions under which the PSS assumption is valid.

1. Theoretical Analysis

The reaction under consideration is the single-substrate reaction in a batch reactor shown below.

$$E+A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E+P \tag{1}$$

Since Eq. (1) involves four species, the four rate equations can be written as

$$d[E]/dt = -k_1[A][E]+(k_2+k_3)[EA] \tag{2}$$

$$d[A]/dt = -k_1[A][E]+k_2[EA] \tag{3}$$

$$d[EA]/dt = k_1[A][E]-(k_2+k_3)[EA] \tag{4}$$

$$d[P]/dt = k_3[EA] \tag{5}$$

with the initial conditions:

$$t=0;\quad [A]=[A]_0,\quad [E]=[E]_0,\quad [EA]=[P]=0 \tag{6}$$

The conservation equations of the enzyme and the substrate are given by:

$$[E]_0=[E]+[EA] \tag{7}$$

$$[A]_0=[A]+[EA]+[P] \tag{8}$$

of the four rate equations above, only two are independent. By choosing any two of the four rate equations with the exception of the combination of Eqs. (2) and (4), and combining them with Eqs. (6) through (8), one can calculate the exact time courses of the components A, P, E, and EA.

When the PSS assumption is made for the enzyme-substrate complex EA, one can set $d[EA]/dt=0$. By equating the right-hand side of Eq. (4) to zero and eliminating [E] from the resulting equation using Eq. (7), we obtain the following equation.

$$[EA]=[E]_0[A]/\{K_m+[A]\} \tag{9}$$

Substitution of this relation into Eqs. (3) and (5) yields

$$v=-d[A]/dt=d[P]/dt=V_m[A]/\{K_m+[A]\} \tag{10}$$

where V_m and K_m are the maximum reaction rate and the Michaelis constant, respectively, and are defined by

$$V_m=k_3[E]_0 \tag{11}$$

$$K_m=(k_2+k_3)/k_1 \tag{12}$$

Equation (10) is the so called "Michaelis–Menten" equation, and can be readily integrated with the initial condition ($t=0$; $[A]=[A]_0$) as

$$V_m t = K_m \ln\{[A]_0/[A]\}+[A]_0-[A] \tag{13}$$

From this equation, [A] can be known at any time, and hence [EA] from Eq. (9), [E] from Eq. (7) and [P] from Eq. (14) below.

It is well known that reactions catalyzed by α-chymotrypsin [EC 3.4.21.1] obey the Michaelis–Menten kinetics relatively well. A number of α-chymotrypsin-catalyzed reaction systems have been investigated, among which the hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester was chosen for examining the validity of Eq. (13) because all the kinetic constants have been measured. In this reaction, substrate A and product P represent *N*-acetyl-L-phenylalanine ethyl ester and *N*-acetyl-L-phenylalanine, respectively. The kinetic constants were given as $k_1=10^7\ l/(\mathrm{mol\cdot s})$, $k_2=1.2\times10^4\ \mathrm{s}^{-1}$ and $k_3=107\ \mathrm{s}^{-1}$ at pH 7.0 and 298 K based on the kinetic study of Hammond and Gutfreund.[3] The only method[5,9] for examining whether the PSS assumption holds or not is to compare the PSS solution of the time course of the substrate or product with their exact solution. **Figure 1** shows a comparison of the $[A]/[A]_0$ values calculated by using Eq. (13) under the condition of $[E]_0=10^{-3}\ \mathrm{mol}/l$ ($K_m/[E]_0=1.21$) with the numerical solutions calculated by using the complete set of two rate equations and the conservation equations. The figure indicates that the PSS solution, Eq. (13), is accurate when $[A]_0/[E]_0\geqq100$. This result coincides with that obtained by Lim[5] and also confirms Laidler's first criterion ($[A]_0\gg[E]_0$). However, a similar comparison shown in **Fig. 2**, made at an enzyme concentration of $[E]_0=1.5\times10^{-5}\ \mathrm{mol}/l$ ($K_m/[E]_0=80.7$), suggests that the PSS solution is accurate even for lower values of $[A]_0/[E]_0$ such as 20. These two figures revealed that the conditions under which the PSS assumption holds cannot be defined simply by examining the magnitude of the term $[A]_0/[E]_0$. Therefore, it is necessary to introduce a new parameter to better define the conditions.

Equation (10) states that the rate of decrease in [A] is equal to the rate of increase in [P] and that these rates are independent of the rate of increase or decrease in [EA]. This means that when the PSS assumption is made, the mass balance equation for

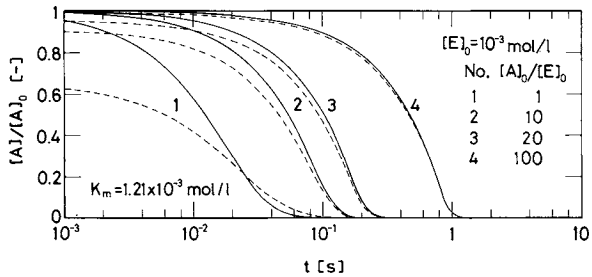

Fig. 1. Hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester by α-chymotrypsin, in which the reaction occurs by the Michaelis–Menten mechanism. Comparison of PSS solutions of the time course of substrate (——) with exact solutions (– – – –)

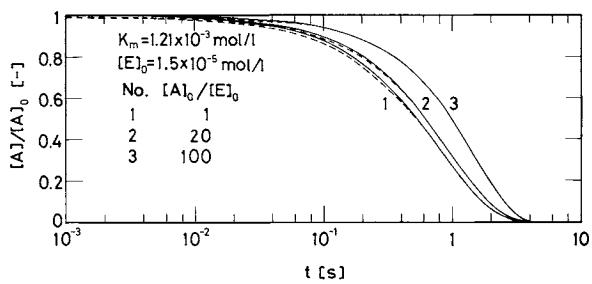

Fig. 2. Hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester by α-chymotrypsin. Comparison of PSS solutions of the time course of substrate (——) with exact solutions (– – – –)

the substrate, Eq. (8), is reduced to

$$[A]_0 = [A] + [P] \tag{14}$$

Therefore, the error which has arisen from neglecting the EA term in the conservation equation becomes considerable when the value of [EA] cannot be neglected compared with the total value of [A] and [P] or the value of $[A]_0$.

The values of [EA] vary essentially with time as shown in **Fig. 3**, in which the values of $[EA]/[A]_0$ are plotted against time with $[A]_0/[E]_0$ as a parameter. As can be seen in the figure, the values of $[EA]/[A]_0$ calculated by using Eqs. (9) and (13) are low and remain almost constant for a considerably long period when the values of $[A]_0/[E]_0$ are large. These constant values are given by

$$[EA]_{P0}/[A]_0 = [E]_0/\{K_m + [A]_0\} \tag{15}$$

Furthermore, when the value of $[A]_0/[E]_0$ is large, the $[EA]/[A]_0$ values calculated by Eqs. (9) and (13) agree well with its exact values except for the initial short periods which are not shown in the figure.

Accordingly, we introduce the new parameter ψ_M, defined by

$$\psi_M = [EA]_{P0}/[A]_0 = [E]_0/\{K_m + [A]_0\} \tag{16}$$

ψ_M is a function of $[A]_0$, $[E]_0$ and K_m, and therefore its value can be evaluated with the values available at $t=0$. When the value of ψ_M is small, the mass balance

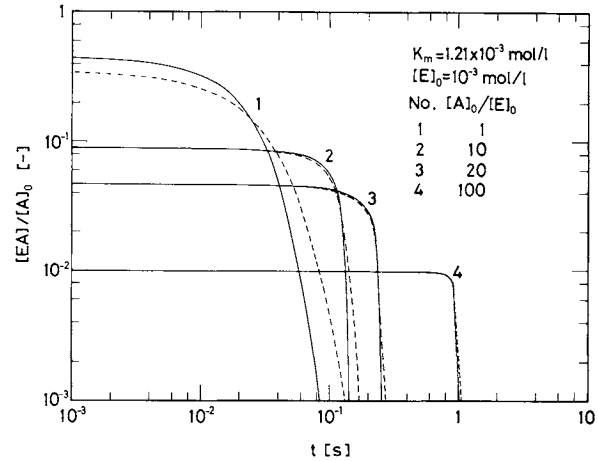

Fig. 3. Hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester by α-chymotrypsin. Comparison of PSS solutions of the time course of the enzyme-substrate complex (——) with exact solutions (– – – –)

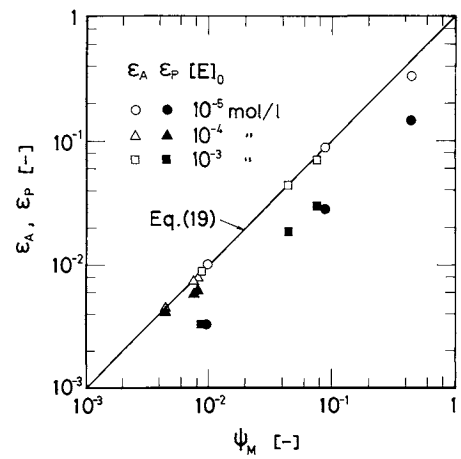

Fig. 4. Hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester by α-chymotrypsin. Maximum relative errors, ε_A and ε_P, are plotted against values of ψ_M

equation of A given by Eq. (14) is sufficiently accurate, as mentioned above. Therefore, under this condition, the PSS assumption is acceptable for deriving the rates of the enzyme reactions.

2. Correlation and Discussion of Errors in the PSS Solutions

For the hydrolysis of *N*-acetyl-L-phenylalanine ethyl ester by α-chymotrypsin, the time courses of A and P in a batch reactor were calculated by using Eqs. (13) and (14) (the PSS solutions), and the complete set of the rate and conservation equations (exact solutions) under conditions in which $[A]_0$ ranges from 10^{-5} to 10^{-1} mol/*l* and $[E]_0$ from 10^{-5} to 10^{-3} mol/*l*. These results are compared in **Fig. 4** as plots of ε_A and ε_P against ψ_M, where ε_A and ε_P are the maximum relative errors for substrate A and product P, respectively, and are defined by

$$\varepsilon_A = \{|[A]_{pss} - [A]_{exact}|/[A]_0\}_{max} \tag{17}$$

Table 1. Rate constants and concentrations of substrate and enzyme used for additional calculations

Series	Key	k_1 $l/(\text{mol}\cdot\text{s})$	k_2 s^{-1}	k_3 s^{-1}	K_m mol/l	k_2/k_3 —	$[A]_0$ mol/l	$[E]_0$ mol/l	$[E]_0/[A]_0$ —
10	▽	10^6	10^2	10	1.1×10^{-4}	10	1×10^{-4}–5×10^{-3}	1×10^{-6}–1×10^{-3}	0.001–1
20	◇	10^6	10	10^2	1.1×10^{-4}	10^{-1}	1×10^{-4}–5×10^{-3}	1×10^{-6}–1×10^{-3}	0.01 –1
30	◈	10^5	10	10^2	1.1×10^{-3}	10^{-1}	1×10^{-4}–1×10^{-2}	1×10^{-6}–1×10^{-3}	0.01 –1
40	⊖	10^5	10^2	10	1.1×10^{-3}	10	1×10^{-4}–5×10^{-3}	1×10^{-6}–1×10^{-3}	0.01 –1
50	○, ●	10^6	10^3	10	1.01×10^{-3}	10^2	1×10^{-4}–7×10^{-3}	1×10^{-6}–1×10^{-3}	0.01 –1
60	⊠	10^6	10	10^3	1.01×10^{-3}	10^{-2}	1×10^{-4}–5×10^{-3}	1×10^{-6}–1×10^{-3}	0.01 –1
70	⊙	10^6	10	1	1.1×10^{-5}	10	1×10^{-4}–1×10^{-3}	1×10^{-5}–1×10^{-4}	0.01 –1
80	△, ▲	10^5	10^3	10	1.01×10^{-2}	10^2	1×10^{-4}–1×10^{-3}	1×10^{-6}–1×10^{-3}	0.01 –3
90	□, ■	10^7	10^3	10	1.01×10^{-4}	10^2	1×10^{-4}–1×10^{-3}	1×10^{-6}–1×10^{-3}	0.01 –1

$$\varepsilon_P=\{|[P]_{pss}-[P]_{exact}|/[A]_0\}_{max} \quad (18)$$

The subscripts 'pss' and 'exact' following [A] and [P] express the value corresponding to the PSS solution and to the exact solution, respectively. The figure shows that the maximum relative errors for substrate A, ε_A, can be correlated well only in terms of ψ_M as

$$\varepsilon_A=\psi_M \quad (19)$$

and that the value of ε_P is much lower than that of ε_A for the same value of ψ_M.

If $\psi_M\leqq0.01$, in which ψ_M is evaluated by using the initial condition, the PSS solutions are accurate within an error of 1% and therefore the PSS assumption is acceptable over the whole time course of the reaction. To make this conclusion more certain, further calculations were carried out for reactions with the kinetic constants listed in **Table 1**. The values of the kinetic constants of series 10 are almost the same as those used by Lim,[5] and the values for the other series were modified from them. They are all also almost the same as those used by Sakamoto.[9] **Figures 5** and **6** show the maximum relative errors ε_A and ε_P, respectively. These figures show that, in all the series except 50, 80 and 90, the values of ε_A can be correlated well with Eq. (19), and that some values of ε_A and all the values of ε_P are well below the line expressing Eq. (19). For the series 50, 80 and 90 in which the value of k_2/k_3, 10^2, is the largest in Table 1, two sets of values of ε_A, (○, ●), (△, ▲) and (□, ■), are plotted. The plotting of these two sets of values and the significance of the keys can be explained as follows. In many cases, the PSS and the exact solutions of the time course of the substrate cross each other in the course of the reaction, as typically shown by the set of curves in example 1 in Fig. 1. The curve of the PSS solution is higher than that of the exact solution until they intersect, and thereafter their positions are reversed. In the three series described above, the maximum relative error ε_A often appears after the time course curves $[A]/[A]_0$ cross each other. Parameter ψ_M was defined above to take into account the error caused by neglecting the EA term in the conservation equation of substrate A. The reaction begins with this error at its greatest, because the concentration of the complex EA calculated by Eq. (9) is highest at $t=0$, whereas the exact or actual complex concentration is zero at $t=0$. This means that the errors involved in the PSS solutions of the time course of the substrate and the product during

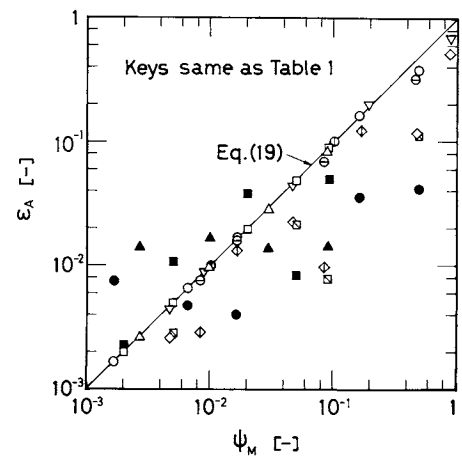

Fig. 5. Reactions for which the rate constants are given in Table 1. Maximum relative errors ε_A are plotted against values of ψ_M

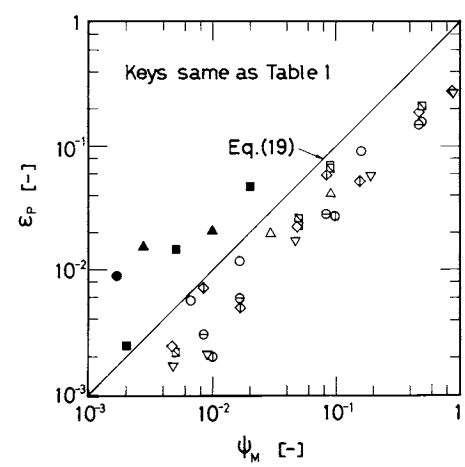

Fig. 6. Reactions for which the rate constants are given in Table 1. Maximum relative errors ε_P are plotted against values of ψ_M

the early stage of the reaction can be correlated well with parameter ψ_M, but after the early stage they cannot be correlated with ψ_M. The blank keys (○, △, □) in Fig. 5 express the highest values of the relative error ε_A in the region between time $t=0$ and the time when the two curves intersect each other. The solid keys (●, ▲, ■) show the highest relative error ε_A during the latter part of the reaction after the two curves intersect each other, and they are the maximum relative errors in the whole region of the reaction curve when the value of ψ_M is small. As can be seen in Fig. 5, the values of ε_A shown by the blank keys can be correlated well with Eq. (19), and those shown by the solid keys cannot be correlated with Eq. (19). Furthermore, in Fig. 6, the maximum relative errors ε_P in the series 50, 80 and 90 are shown by the solid keys when the relative error ε_A is at its maximum during the latter part of the reaction after the two curves cross each other, and by the blank keys when the relative error ε_A is at its maximum during the time region between $t=0$ and the time when the two curves cross each other. As can be seen in Figs. 5 and 6, the values of neither ε_A nor ε_P in these series can be correlated with Eq. (19) if the relative error ε_A is at its maximum in the latter part of reaction after the crossing of the two curves, and in many cases the values of ε_P are greater than those of ε_A at the same ψ_M values. However, neither exceeds 0.05 (5%) when $\psi_M \leqq 0.05$.

The results of the additional calculations can be summarized as follows: (1) When $k_2/k_3 \leqq 10$, the values of ε_A can be correlated with Eq. (19) and those of ε_P are well below the line given by Eq. (19). Therefore, if $\psi_M \leqq 0.01$, both the PSS solutions of A and P are accurate within an error of 1% in the whole time course of the reaction. (2) When $k_2/k_3 > 10$, the PSS solutions of both A and P are accurate within an error of 5% if $\psi_M \leqq 0.05$.

The above results show that the PSS assumption holds irrespective of the individual values of $[A]_0/[E]_0$ or $K_m/[E]_0$ if $\psi_M \leqq 0.05$ for $k_2/k_3 > 10$ or $\psi_M \leqq 0.01$ for $k_2/k_3 \leqq 10$. However, the criterion $\psi_M \leqq 0.05$ should be used when the individual kinetic constants such as k_2 and k_3 are not available. Since the values of ψ_M can be calculated by using the Michaelis constant and the total enzyme and the initial substrate concentrations, one can judge beforehand whether the PSS assumption will be valid for a reaction in a batch reactor under the given condition, or can easily confirm the validity of the PSS assumption afterward by using the kinetic parameters determined when their values need to be measured.

For the same reaction system, Lim[5] revealed that the PSS assumption holds when $[A]_0/[E]_0 > 100$, and this was later confirmed by Sakamoto.[9] However, from the results in the present work we can conclude that the condition defined by Lim is correct but not limiting. Laidler[4] suggested that the PSS assumption will be valid if any one of the following inequalities holds: $[A]_0 \gg [E]_0$, $[E]_0 \gg [A]_0$, $K_m \gg [E]_0$ or $K_m \gg [A]_0$. The first and third criteria were also proposed by Bartha.[1] The first criterion is substantially the same as that revealed by Lim, and the third is compatible with the conclusions in the present work. However, the conditions under which the PSS assumption holds cannot be determined by using the second or fourth criterion. For example, when $[E]_0 \gg [A]_0$, the extent of A which exists as a form of the enzyme-substrate complex EA is considerable compared to the amount of free A species and therefore the PSS assumption is not valid, but the inequality $K_m \gg [E]_0$ holds at the same time.

The results obtained in this work have already been successfully extended to some other reaction systems such as reactions which require cofactors, but this research is not within the scope of the present paper.

Nomenclature

A	=	substrate concentration	[mol/l]
E	=	enzyme concentration	[mol/l]
EA	=	concentration of enzyme-substrate complex	[mol/l]
K_m	=	Michaelis constant defined by Eq. (12)	[mol/l]
k_1	=	second-order reaction rate constant	[l/(mol·s)]
k_2, k_3	=	first-order reaction rate constant	[s^{-1}]
P	=	product concentration	[mol/l]
t	=	reaction time	[s]
V_m	=	maximum reaction rate defined by Eq. (11)	[mol/(l·s)]
v	=	reaction rate	[mol/(l·s)]
ε_A	=	maximum relative error for substrate A	[—]
ε_P	=	maximum relative error for product P	[—]
ψ_M	=	dimensionless parameter defined by Eq. (16)	[—]

〈Subscripts〉

A	=	substrate A
exact	=	exact solution
P	=	product P
pss	=	PSS solution
0	=	initial value or total value

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ANALYSIS OF WATER ENTRAINMENT INTO DISPERSED W/O EMULSION DROPS

TAKAAKI OHTAKE, TADASHI HANO AND KAZUMI TAKAGI
Department of Environmental Chemistry and Engineering, Oita University, Oita 870-11
FUMIYUKI NAKASHIO
Department of Organic Synthesis, Kyushu University, Fukuoka 812

Key Words: Mixing, Liquid Surfactant Membrane, W/O Emulsion, (W/O)/W Emulsion, Water Entrainment, Drop Diameter, Stirred Tank

The effects of operating conditions on mechanical water entrainment into W/O emulsion drops in a (W/O)/W emulsion system were studied in a stirred tank in the absence of permeation due to osmotic pressure.

The water entrainment was influenced by surfactant concentration, water volume fraction in W/O emulsion, inner water drop size, salt concentration in the external water phase, and agitation speed in the stirred tank. There was a satisfactory correlation between the extent of water entrainment and the weight of surfactant per unit interfacial area. These observations suggested that water entrainment proceeded as a result of additional emulsification at the drop surface. This idea was confirmed by examining the water entrainment in an oil–water dispersion system where water entrainment occurred by emulsification. The effects of the operating conditions on water entrainment in the oil–water dispersion system were quite similar to those in the (W/O)/W emulsion system. In addition, the volume of water entrained per unit surface area of dispersed oil drops was in fair agreement with that in the (W/O)/W emulsion system. These results supported the proposed mechanism of water entrainment.

Introduction

In extraction processes with liquid surfactant membrane where W/O emulsion is dispersed into aqueous solution, the lowering of the extract concentration in the inner aqueous phase results in the decrease of process efficiency. This problem occurs mainly by the permeation and mechanical entrainment of the external water. The former is caused by the concentration difference between internal and external aqueous solutions, and has been discussed in detail.[2,6,7] The mechanical entrainment, however, is still not completely elucidated because of its complex mechanism.[4,5,10]

Recently, Nakashio *et al.*[6,7] investigated this problem quantitatively under conditions of no permeation. Fujinawa *et al.*[2,3] studied the same subject for systems where mechanical entrainment and permeation proceeded simultaneously, and proposed operating conditions which suppressed entrainment. They also showed that increasing the emulsion viscosity was effective in reducing the entrainment. Although the effects of various operating and emulsion-preparing conditions were examined in these papers, it is still obscure how drop surface contributes to water entrainment.

In the present study, the effects of operating conditions on mechanical entrainment were examined, using emulsions of various compositions. The extent of water entrainment was related to surfactant concentration per unit surface area, taking into account the contribution of additional emulsification at the drop surface. To establish the entrainment mechanism, the entrainment in an oil–water dispersion system was studied and compared with water entrainment in a W/O emulsion–water dispersion system, termed "(W/O)/W emulsion" in this paper. A mechanism of mechanical water entrainment was proposed on the basis of these experimental findings.

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