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Effect of Enzyme Microheterogeneity and Distribution of Initial States on First-Order Deactivation and on Conversion in a Fixed-Bed Reactor

Scientific Note

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INTRODUCTION

A major constraint on the use of enzymes in biotechnological processes is deactivation. Enzyme deactivation can be a result of physical factors like thermal or stress effects or chemical, like poisoning, or pH changes. This paper discusses the phenomena of thermal deactivation of enzymes.

Traditional enzyme deactivation models assume samples to be made up of a group of identical molecules. This approach tends to ignore the differences between the individual enzyme molecules. Enzymes are complex polypeptides with secondary and tertiary structural and spatial characteristics that in turn depend on the environment, source, and history of the enzyme sample.

The concept that a sample of any protein is made up of molecules with identical structure and spatial configuration is difficult to accept. Instead, protein and enzyme samples should be considered to be microhet-

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erogeneous, that is, a sample of an enzyme consists of a large number of closely related molecules.

Microheterogeneity refers to the property of certain proteins and enzymes to show molecular variation, even in a purified state, when scrutinized by high-resolution immunological, biochemical, or biophysical techniques (*1,2*). The prefix "micro" is used to exclude large variations and subpopulations, such as isoenzymes, from the discussion. Colvin et al. (*3*) suggest that microheterogeneity is an inherent property of proteins, and that even with a high degree of purification, the protein will represent a population of variable but closely related members.

Colvin et al. (*3*) further expressed the view that it seems more correct to describe the native protein, not in terms of a finite number of definite chemical entities, but as a population of closely related individuals that differ either discretely or continuously in a number of properties.

Microheterogeneity has been demonstrated in several protein and enzyme systems. Colvin et al. (*3*) have tabulated some of these, including lysozyme, chymotrypsin, and ribonuclease. Doi and Nishida (*4*) show that human lecithin cholesterol acyltransferase shows microheterogeneity with a common core polypeptide, associated with varying sialic acid content.

On studying horse serum γ-globulin, Cann (*5*) was able to detect a difference in the sedimentation coefficients of pseudoglobulin and euglobulin within a given preparation of γ-globulin. Moreover, within each of these subfractions there was a continuous distribution of sedimentation coefficients, with the standard deviation being about 5% of the mean. More recently, Cann and Fink (*6*) have studied the effects of microheterogeneity on the sedimentation behavior of self-associating proteins.

Another commonly known protein system showing microheterogeneity are the plasma albumins. Foster (*7*) and Foster et al. (*8*) show protein heterogeneity with respect to solubility, and transition between two forms N and F at different pH.

Microheterogeneity occurs in varying degrees, depending on preparation and state of the enzyme. Thus, a large degree of microheterogeneity can be expected in immobilized enzymes, as each enzyme molecule experiences differences in binding and in the microenvironment. Semipurified and industrial enzymes can also be expected to have a large amount of microheterogeneity.

Some studies of enzyme deactivation have considered the presence of microheterogeneity. Kawamura et al. (*9*) have investigated the thermal stability of free and immobilized α-chymotrypsin. Stokrova and Sponar (*10*) used similar concepts for a thermally denaturing enzyme.

More recently, Agarwal (*11*) has proposed a model for the denaturation of enzymes based on heterogeneous enzyme subpopulations with nonuniform initial activities and non zero final activities.

Investigations into the effects of microheterogeneity on enzyme deactivation in our laboratory have concentrated on understanding the im-

plications of this phenomena on thermal deactivation. In an earlier paper (*12*) we presented a simple model to describe the behavior of a microheterogeneous enzyme undergoing first-order thermal deactivation. This paper presents the model and its implications, in brief, and then goes on to examine the behavior of a microheterogeneous enzyme in a fixed-bed reactor.

The nature of deactivation of an enzyme affects its performance in a chemical reactor. Sadana (*13*) discussed the effects of immobilized enzyme deactivations in fixed- and fluid-bed reactors. The presence of microheterogeneity can play a major part in the way an enzyme deactivates, and, consequently, on the design of enzyme reactors. These factors are also examined.

NOMENCLATURE

$[E]$, $[E_{li}]$, $[E_{2i}]$	Enzyme concentration of the E, E_{li}, and E_{2i} states, respectively (g/cm^3).
E, E_o	Total enzyme activities, at time, t, and at time, $t = 0$, respectively, $E = \beta[E]$, and so on.
f	Distribution function.
h	Planck's constant.
k	First-order deactivation rate constant (min^{-1}).
k_i	First-order deactivation rate constant for the ith reaction (min^{-1}).
k_{avg}	Average (first-order) deactivation rate constant (min^{-1}).
k_B	Boltzmann constant (erg/molecule K).
k_r	Reaction velocity constant.
k'_r	k_r/V_r.
K_{mA}	Michaelis-Menten constant for substrate S_A (g/cm^3).
K'_{mA}	Apparent Michaelis-Menten constant, K_{mA}/S_{A_o}.
L	Reactor length (cm).
N	Number of reactions.
r_A	Rate of reaction.
R	Gas constant (cal/gmol K).
S_A	Substrate A concentration (g/cm^3).
S_{A_o}	Substrate A concentration at $t = 0$ (g/cm^3).
S_v	Space velocity in reactor (min^{-1}).
t	Time (min).
t_{ed}	Time for total (or partial) deactivation of enzyme in the reactor, also run time for reactor (min).
t_L	Space time in reactor (min).
T	Absolute temperature (K).

U_L	Velocity of liquid substrate in reactor (cm/sec).
x	Normalized axial distance, z/L.
y_A	Normalized concentration of substrate A, S_A/S_{A_o}.
z	Axial distance in reactor, cm.
Greek Letters	
α	Normalized activity of the enzyme sample, *see* Eqs. [7] and [8].
β_1, β_2	Specific activities of the E_1 and E_2 states, respectively (units/g).
γ	Reaction parameter, *see* Eq. [12].
δ	Deviation from first-order kinetics, *see* Eq. [9].
ϵ	Activation energy of the deactivation reaction (kcal/gmol).
ϵ_o	Mean activation energy of the deactivation reaction (kcal/gmol).
Δ	Difference in conversion between a microheterogeneous and homogeneous enzyme sample, *see* Eq. [17].
$\bar{\eta}_A$	Time-averaged conversion of substrate A.
θ	Normalized time, t/t_{ed}.
ψ	*See* Eq. [13].
Subscripts	
1,2	Enzyme states.
i	ith reaction.

Simple Model for Deactivation of an Enzyme Displaying Microheterogeneity in the Deactivation Activation Energy

Consider a simple case of an enzyme form E_1 with a specific activity, β_1, deactivating into another form, E_2 with a specific activity, β_2.

$$\begin{array}{ccc} \beta_1 & & \beta_2 \\ & \xrightarrow{k_{avg}} & \\ E_1 & & E_2 \end{array} \tag{1}$$

The enzyme displays microheterogeneity in the activation energy (and, hence, rate) of the first-order thermal deactivation process.

We can treat the microheterogeneous enzyme as being made up of a large number of subpopulations, (say, N); each form inactivates with a parallel deactivation reaction; each enzyme form E_{1i} having a constant deactivation activation energy, ϵ_i, and a first-order rate constant, k_i. Thus, the ith reaction is

$$E_{1i} \xrightarrow{k_i} E_{2i} \qquad (i = 1, 2, \ldots\ldots N) \tag{2}$$

where k_i and the corresponding activation energy, ϵ_i, are constant. From the general reaction rate theory we have

$$k_i = k_B T/h \exp(-\epsilon_i/RT) \tag{3}$$

where k_B is the Boltzmann constant, h, is the Planck's constant, R, is the gas constant, and T is the temperature.

The rate of loss of E_{1i} is given by

$$-d(E_{1i})/dt = k_i(E_{1i}) \tag{4}$$

Here (E_{1i}) is the concentration of the E_{1i} form.

The distribution of the activation energy for the deactivation reaction can be given by a function f. Thus

$$(E_{1i}) = f(\epsilon_i) \tag{5}$$

that is the concentration of the enzyme form E_{1i} with the activation energy, ϵ_i, is given by the distribution function, f.

For microheterogeneous systems a fairly continuous distribution of the activation energy can be assumed. We assume a normal Gaussian distribution for our model. Thus

$$(E) = f(\epsilon) = (\beta_1/\sqrt{2\pi}\,\sigma) \exp\left[-\frac{(\epsilon - \epsilon_o)^2}{2\sigma^2}\right] \tag{6}$$

where σ is the standard deviation, and ϵ_o is the average activation energy corresponding to k_{avg}, the average first-order deactivation rate constant.

Using the normal Gaussian distribution of the activation energies yields the following normalized activity-time expression.

$$\alpha(t) = \beta_2/\beta_1 + (1 - \beta_2/\beta_1) \int e^{-k_B T/h \exp(-\epsilon/RT)\, t} \exp[-(\epsilon - \epsilon_o)^2/2\sigma^2] d\epsilon \Big/ \int \exp[-(\epsilon - \epsilon_o)^2/2\sigma^2] d\epsilon \tag{7}$$

where the integration limits are ideally from zero to infinity. For all practical purposes, however, the integration limits can be taken from $\epsilon_o - 4\sigma$ to $\epsilon_o + 4\sigma$. This covers 99.9% of the activation energy spread exhibited by the enzyme sample.

Equation [7] describes the deactivation characteristics of a microheterogeneous enzyme. Similar activity–time relationships may be derived for other activation energy distributions.

For comparison, the activity–time expression of a homogeneous enzyme with a constant activation energy, ϵ_o, and the first-order deactivation rate constant k_{avg}, is given by

$$\begin{aligned} \alpha(t) &= \beta_2/\beta_1 + (1 - \beta_2/\beta_1) \exp(-k_{avg} t) \\ &= \beta_2/\beta_1 + (1 - \beta_2/\beta_1) \exp[-k_B T/h \exp(-\epsilon_o/RT) t] \end{aligned} \tag{8}$$

As expected, Eq. [7] reduces to Eq. [8], the classical first-order deactivation, when we consider the standard deviation, σ, to be zero, which corresponds to a homogeneous enzyme sample.

Implications of Microheterogeneity

The above model can be used to study the deactivation behavior of a microheterogeneous enzyme sample.

As an example, we use the deactivation kinetics data for immobilized glucose oxidase reported by Malikkides and Weiland (*14*). They reported a ln (k_{avg}) value equal to 131.6 ± 9.1 min^{-1}, and an average activation energy equal to 90.5 ± 6.3 kcal/gmol. The final deactivated form had no specific activity, and hence β_2 equals zero. The activity–time data for glucose oxidase (*14*) exhibited the classic straight-line plot when the 1*n* activity was plotted against time. Thus, we assume that the enzymes were behaving as "on-off" switches, and that the enzymes are not undergoing a continuous change in character until they become totally inactive.

To describe quantitatively the effect of microheterogeneity, a term, δ, the time-averaged deviation from first-order kinetics is defined as

$$\delta(t) = 1/t \int_0^{\text{time, } t} \left| \alpha(t)_{\text{heterogeneous enzyme}}/\alpha(t)_{\text{homogeneous enzyme}} - 1.0 \right| dt \quad (9)$$

This term gives the average deviation from first-order homogeneous behavior over a period of time, t.

In our model, the extent of microheterogeneity is characterized by the standard deviation, σ, of the activation energy spread. For a sample of immobilized glucose oxidase, a spread of $\sigma = 2$ kcal/gmol gives a deviation of more than 13% from first-order homogeneous behavior over the first 80 min of deactivation and goes to as high as 34% over 160 min. Figure 1 shows the deviation from homogeneous behavior as a function of the extent of microheterogeneity. Figure 2 shows the normalized activity-time profiles for microheterogeneous enzyme samples, as compared to a homogeneous enzyme.

A standard deviation of just 2 kcal/gmol is quite small indeed. This corresponds to 95% of the enzyme molecules having their activation energy, ϵ, in the range of 8 kcal/gmol about the average, ϵ_o ($\epsilon_o \pm 2\sigma$). This is in the order of experimental error in measurement of the average activation energy, and represents a spread of less than 9% of ϵ_o.

Thus, a small degree of microheterogeneity can cause a substantial and noticeable change in the deactivation behavior of an enzyme sample. Microheterogeneity makes the deactivation have a higher apparent order of reaction.

Microheterogeneity gives an enzyme system more stability and a higher apparent activation energy. The less stable molecules in an en-

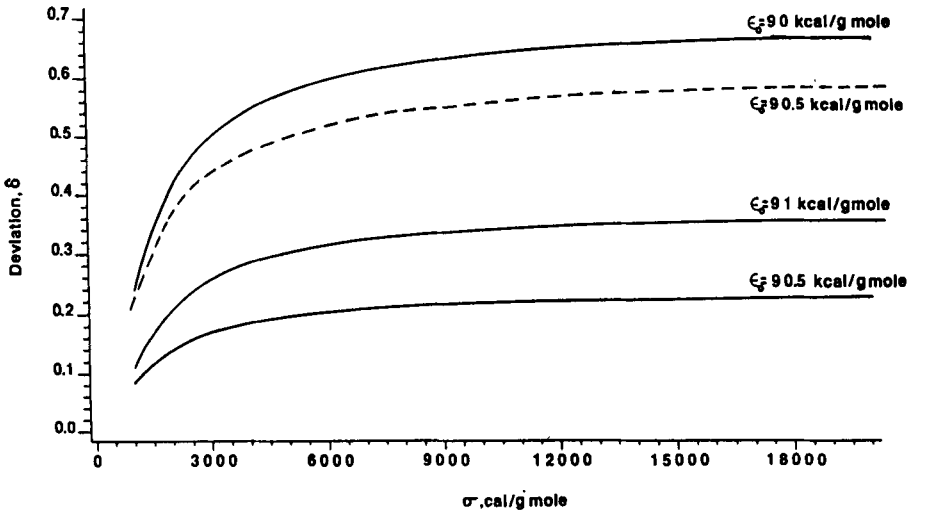

Fig. 1. Deviation from first-order kinetics, δ, as a function of the extent of microheterogeneity, σ, for different ϵ_o values.

ln k_{avg} = 131.86

T = 334K (immobilized glucose oxidase (14)

Time period for which deviation has been calculated (—) 80 min; (---) 160 min.

zyme sample deactivate at a faster rate, giving increased stability and changing the distribution of activation energy with time.

We talk of enzyme samples, rather than enzymes in our model, as the extent of microheterogeneity, for any enzyme varies from sample to sample, depending on the method of preparation, purification, storage, and modifications of the enzyme sample. The microheterogeneity would, of course, be also dependent on the chemical nature of the enzyme itself.

THEORY

Deactivation of Microheterogeneous Enzymes in Reactors

Our analysis has been developed for a diffusion-free isothermal reactor. We assume that the enzyme is available in a solid, presumably immobilized state. The continuity equation for an isothermal reaction occurring under diffusion-free conditions in a plug-flow reactor is given by

$$\partial y_A/\partial t + U_L\, \partial y_A/\partial z = - r_A(y_A,t) \tag{10}$$

where r_A is the reaction rate, U_L is the liquid substrate velocity, and t is the reaction time. This basic relation can be used to develop equations for the two reactor types.

Fixed-Bed Reactor

We assume a single step catalysis of the substrate S_A, following simple Michaelis-Menten kinetics. Thus

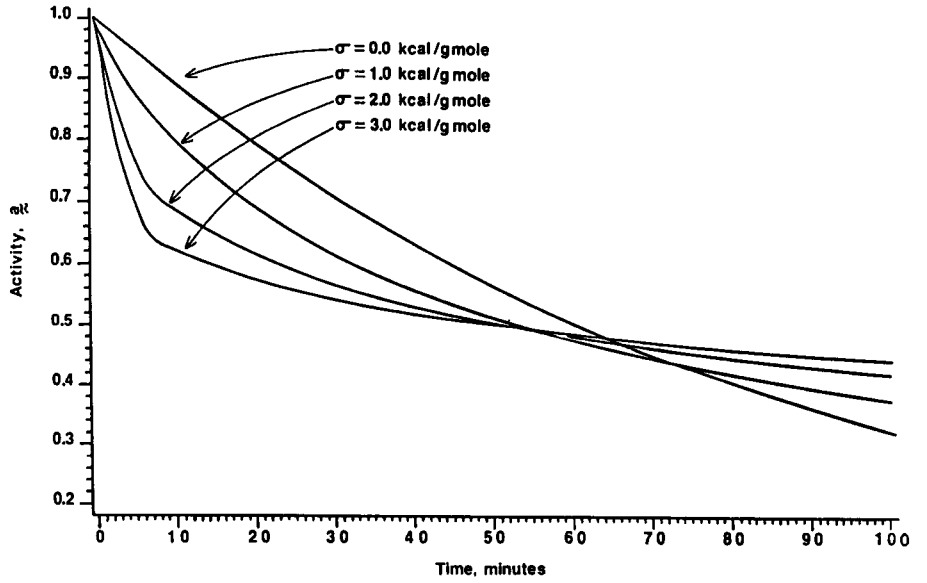

Fig. 2. Normalized activity curve at various levels of microheterogeneity, σ, in immobilized glucose oxidase (14).
ϵ_o = 90.5 kcal/gmol
ln k_{avg} = 131.86
T = 334K

$$r_A(S_A,t) = k_r' E S_A / K_{mA} + S_A \tag{11}$$

where $E = E_o\,\alpha(t)$. The activity expression $\alpha(t)$ is given by Eqs. [7] and [8] for heterogeneous and homogeneous enzymes, respectively. $k_r' = k_r/V_r$, where k_r is the reaction rate constant, V_r is the reactor volume, E is the enzyme activity at time t, and K_{mA} is the Michaelis-Menten constant. We can simplify the equation using dimensionless time $\theta = t/t_{ed}$, and dimensionless length $x = z/L$.

$$\psi\,\partial y_A/\partial\theta + \partial y_A/\partial x = -\gamma\alpha'(\theta)\,y_A/K'_{mA} + y_A = -\gamma\alpha(t)\,y_A/K'_{mA} + y_A \tag{12}$$

where:

$$\gamma = k_r' E_o/S_{A_o}\,(L/U_L) \cong k_r' E_o/S_{A_o} S_v$$

is the reaction parameter, S_v is the space velocity assuming no density changes, and $K'_{mA} = K_{mA}/S_{A_o}$. Note that

$$\alpha(t) = \beta_2/\beta_1 + (1 - \beta_2/\beta_1)\exp[-k_{avg}t_{ed}(t/t_{ed})] = \beta_2/\beta_1 + (1 - \beta_2/\beta_1)\exp[-k_{avg}t_{ed}\theta] = \alpha'(\theta)$$

and

$$\psi = (L/U_L\,t_{ed}) = t_L/t_{ed} \tag{13}$$

where t_L, the space time, is the liquid transit time, or the time taken for the substrate molecules to pass through the bed. For most operating conditions t_L is much less than the enzyme deactivation time, t_{ed}. Therefore, we take $\psi = 0$. Equation [12] can then be written as

$$-dy_A/dx = \gamma\alpha'(\theta)\, y_A/K'_{mA} + y_A \tag{14}$$

and, we can solve this equation with the boundary condition, at the reactor inlet, $y_A(0) = 1$, to get

$$K'_{mA} \ln y_A + y_A - 1 + \gamma\, \alpha'(\theta)\, x = 0 \tag{15}$$

The time-averaged conversion at the reactor outlet, i.e., $x = 1$, for a fixed-bed reactor is

$$\bar{\eta}_A = 1 - \int_0^1 y_A d\theta \tag{16}$$

Note that the time-averaging has been carried out over the reactor age 0 to t_{ed}. For the top limit, $\theta = t_{ed}/t_{ed} = 1$.

Parameteric Studies and Reactor Behavior

Conversion in the fixed-bed reactor depends on parameter, γ, the reaction parameter, K'_{mA}, the apparent Michaelis-Menten constant, and the nature of deactivation, expressed by the function $\alpha'(\theta)$.

The normalized activity given by $\alpha'(\theta)$ is dependent on ϵ_o, the average activation energy of deactivation, as well as σ, a measure of the degree of microheterogeneity. For homogeneous enzymes $\sigma = 0$.

Exit conversions are also dependent on t_{ed}, the time for which the enzyme is utilized in the reactor. In practice, t_{ed} is usually the time after which the remaining activity of the enzyme is too low for it to be productive.

Of these parameters, K'_{mA} and ϵ_o are usually fixed by properties of the enzyme being used and the substrate concentration. K'_{mA} can change as a function of time and temperature. Isothermal conditions exist. For this analysis K'_{mA} is also assumed to be time-invariant. γ, the reactor parameter, is assumed to be the only variable parameter and can be conveniently varied by changing the space velocity. σ is dependent on the microheterogeneity of the enzyme sample being used.

The exit conversion $\bar{\eta}_A$ can be numerically calculated using Eqs. [15] and [16] for the fixed bed reactor. $\alpha'(\theta)$, the normalized activity function, is evaluated at different times using Eq. [7] for microheterogeneous enzymes, and Eq. [8] for homogeneous enzyme samples. Figure 3 shows the relationship between conversion and the reactor parameter, γ. As can be seen, the exit conversion increases with increasing amounts of microheterogeneity (increasing σ). A small change in microheterogeneity (σ = 2 kcal/gmol) produces a significant change in conversion. Conversion sensitivity decreases for increasing σ values. Figure 4 plots the difference

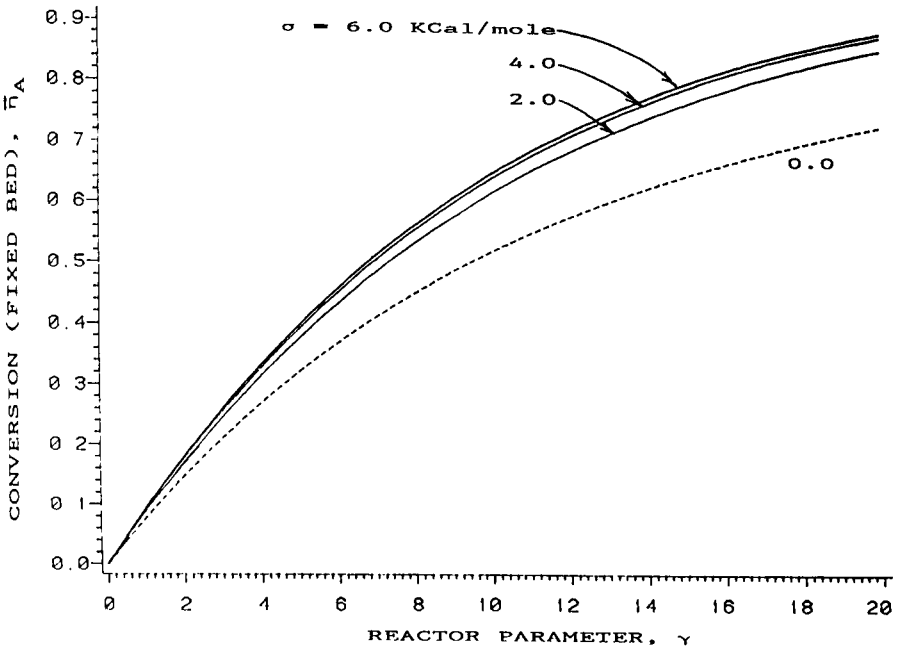

Fig. 3. Effect of different degrees of microheterogeneity, σ, and reactor parameter, γ, on time-averaged conversion, $\bar{\eta}_A$, in a fixed-bed reactor.
$K'_{mA} = 4.0$
$\epsilon_o = 90.5$ kcal/gmol
$\ln k_{avg} = 131.86$
$T = 334$K
(Values used are representative of deactivation of immobilized glucose oxidase)(14).

in conversion between a heterogeneous enzyme sample and a homogeneous enzyme sample

$$\Delta = \bar{\eta}_{A,\ \text{heterogeneous}} - \bar{\eta}_{A,\ \text{homogeneous}} \tag{17}$$

as a function of the reactor parameter, γ, for different values of ϵ_o. The plot shows that conversion is rather sensitive to the average activation energy, ϵ_o. In general, when the average activation energy decreases, the microheterogeneous enzyme sample gives a higher conversion. For example, for about a one percent change in the average activation energy at the reactor parameter (γ) value equal to six there is about a twenty percent difference in Δ.

The value of ϵ_o, the average activation energy of deactivation, controls the rate of deactivation. As ϵ_o decreases, the rate of deactivation increases. In this context, the value of t_{ed}, time allowed before the enzyme is replaced, becomes important. If t_{ed} is kept short, and the enzyme is replaced before the stabilizing effects of heterogeneity take effect, the performance of homogeneous samples will be superior to that of the heterogeneous enzyme. For example, an examination of Fig. 2 shows that until a time of about 60–70 min, the microheterogeneous enzyme is

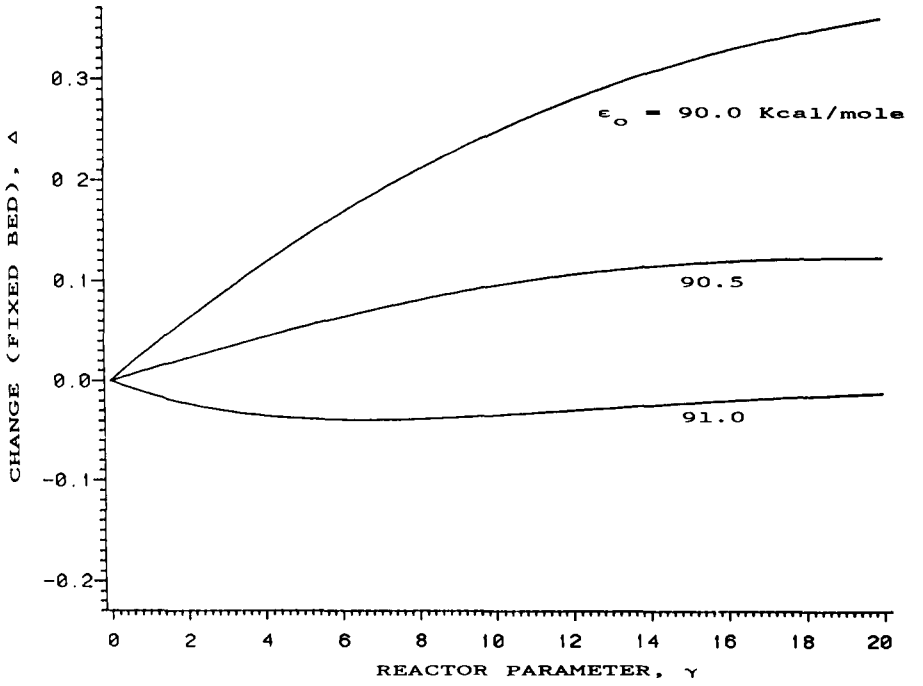

Fig. 4. Δ, difference in time-averaged conversion using microheterogeneous and homogeneous samples, for different ϵ_0 values in a fixed-bed reactor.
K'_{mA} = 4.0
σ = 2 kcal/gmol
T = 334K
ln k_{avg} = 131.86

deactivating faster than the homogeneous enzyme. If t_{ed} was chosen in this region, the performance of the homogeneous enzyme will obviously be better. If t_{ed} is greater than about 70 min, when the microheterogeneous enzyme population displays stability, the heterogeneous sample would do better in a reactor. This is also a function of the initial enzyme distribution that one selects.

DISCUSSION AND CONCLUSIONS

The presence of microheterogeneity in enzyme systems is an important parameter in their thermal deactivation behavior. Microheterogeneity causes the deactivation to appear to be of higher order, and more complex, for enzymes that we assume are following simple first-order thermal deactivation, like "on-off" switches. Microheterogeneity also gives, in general, enzyme systems more stability over a period of time, especially if the initial rapid deactivation period is over.

Many higher order deactivations may be empirically modeled by microheterogeneity of the enzyme sample rather than because of complex deactivation systems, as is commonly believed. Complex reactions, if ex-

perimentally observed, would, of course, play a part in deciding the deactivation kinetics of an enzyme.

Not much experimental data is presently available about the magnitude and nature of microheterogeneity. This is an important area for future research, since even a relatively small amount of microheterogeneity can significantly alter enzyme deactivation behavior.

In a fixed-fed reactor, microheterogeneity, in the enzyme sample being used, gives higher conversions compared to homogeneous enzyme samples, provided t_{ed} is large enough to take advantage of the stabilizing effects of microheterogeneity.

Caution needs to be exercised in the use of the models presented. These models are simple representations of the phenomena of microheterogeneity and give a feel for the effects of microheterogeneity, rather than equations for curve fitting. The models should be based on physical measurements, and not fitted values, of enzyme sample properties such as σ and ϵ_0.

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