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Determination of Intrinsic Properties of Immobilized Enzymes

1. Kinetic Studies on Sepharose–Staphylococcal Nuclease in the Absence of Diffusional Limitations

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Abstract

Staphylococcal nuclease has been insolubilized, directly through its amino groups, on CNBr-activated Sepharose 2B. For kinetic studies, a small substrate (thymidine 5'-(*p*-nitrophenyl phosphate) 3'-phosphate) has been used to measure the hydrolytic activity. With this system the absence of diffusional limitations has been proven. Eadie-Hofstee analysis of the data has been employed to determine the intrinsic kinetic constants of the insolubilized enzyme. The k_{cat}–pH and K_M–pH profiles and the activation energies are similar for the soluble and for the insolubilized nuclease. At the same time conditions are established in which a stirred batch reactor containing particles of insolubilized nuclease behaves as an open system.

Index Entries: Immobilized enzymes; kinetics, of sepharose-staphylococcal nuclease; sepharose-staphylococcal nuclease; staphylococcal nuclease immobilized on sepharose; diffusional effects, on sepharose-staphylococcal nuclease.

Introduction

The intrinsic kinetic properties of an insolubilized enzyme are determined by the properties of the native enzyme and by the changes that occur in the protein molecule as a consequence of the insolubilization process. These changes depend on the type of support and the method of binding of enzyme and

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support. Frequently, the measured kinetic properties are not those intrinsic to insolubilized enzyme derivatives. Other factors such as steric, diffusional, and electrostatic effects can play an important role on the observed kinetic behavior (*1*).

Knowledge of those intrinsic kinetic properties is very important. It is the key to the catalytic potential of that type of insolubilized enzyme derivative, compared to the potential of the native enzyme, and provides the basis for further studies on the steric, diffusional, and electrostatic effects.

In our laboratory we are working on the hydrolysis of nucleic acids by staphylococcal nuclease covalently bound to sepharose gels. These gels are highly porous supports and their nearly complete lack of charged groups (information from Pharmacia Fine Chemicals, Uppsala, Sweden) precludes electrostatic effects. Micrococcal nuclease, an extracellular phosphodiesterase from *Staphylococcus aureus,* is a well-known enzyme (*2*) that interestingly hydrolyzes either DNA or RNA. In the hydrolysis of nucleic acids by sepharose–nuclease, the effect of steric and diffusional limitations is very important (*3*). In this paper we present a study of the intrinsic kinetic properties of the sepharose–nuclease derivatives using a model substrate, thymidine 5′-(*p*-nitrophenyl phosphate) 3′-phosphate (NPpdTp). As support for the enzyme we have employed sepharose 2B, the largest pore sepharose commercially available. Thus, from the small size of the substrate and the great size of the support, steric and diffusional effects will be eliminated or minimized. The sepharose–nuclease derivatives were obtained by covalent binding between CNBr-activated sepharose 2B and the amine groups of the protein. This type of linking was found best, in terms of activity and stability, among several others tried (*4*). Since, as we demonstrate below, the hydrolysis of NPpdTp by these insolubilized nuclease derivatives is not affected by diffusional limitations, the quantification of the intrinsic kinetic properties of the insolubilized nuclease is straightforward.

The enzyme has 23 lysines plus the amino-terminal alanine (*2*). In theory, then, a heterogeneous population of molecules of insoluble nuclease can be obtained, so that the kinetic data would be an overall representation of many enzyme molecules differently modified. Furthermore, because of the high number of amino groups in the protein, it would be reasonable to expect multiple point attachment of the nuclease to the Sepharose.

Kinetics

Rate of Product Formation in a Stirred Batch Reactor (SBR)

In the absence of diffusional limitations, if the insolubilized enzyme follows Michaelian kinetics and if $[S]$, free substrate concentration, can be replaced by S_0, initial known substrate concentration, then the initial rate of reaction per unit volume of insolubilized enzyme particle, v_p, will be

$$v_p = k'_{cat}\ E_{gel} S_0 / (K'_M + S_0) \qquad (1)$$

where E_{gel} is the concentration of enzyme within the support particle and k'_{cat} and K'_M are the intrinsic kinetic constants of the insolubilized enzyme.

In an SBR of volume V_R that contains a volume V_d of insolubilized enzyme derivative, the rate of product formation will be the sum of the rates of formation in all the particles of insolubilized enzyme. The initial rate of product formation per unit volume of reactor will be

$$v_R = \Sigma v_p V_{Pi} / V_R = v_p \Sigma V_{Pi} / V_R = v_p V_d / V_R = v_p \gamma \tag{2}$$

V_{Pi} being the volume of each single particle and $\gamma = V_d / V_R < 1$. For a certain insolubilized derivative, the v_R/γ ratio is always constant and numerically equal to v_p.

To analyze the experimental results any of the classical plots of enzyme kinetics could be helpful. However, we have chosen the Eadie-Hofstee representation because, as will be discussed in the companion paper (*5*), it is the most useful for the case of mixed diffusion- and reaction-controlled kinetics presented there.

Replacing v_p by v_R/γ, Eq. (1) can be rearranged as follows

$$v_R/\gamma / E_{gel} S_o = (k'_{cat}/K'_M) - (1/K'_M)\,[(v_R/\gamma)/E_{gel}] \tag{3}$$

Relevance of the γ Factor

Our reactor, an SBR, is a 2-cm pathlength spectrophotometer curvette provided with a magnetic stirrer. The γ factor allows us to obtain the reaction rates in the insolubilized enzyme particles from results of product formation rates in the SBR. Now we will discuss the conditions for correct replacement of $[S]$ by S_o in order to apply Eq. (1). It will then emerge that ν factor plays another important role.

If, in an SBR, we consider that when the steady state is reached the amount of product formed is negligible we can state the following mass balance

$$S_o V_R = [S] V_R + [ES] V_d \tag{4}$$

If we substitute $[ES]$, concentration of enzyme–substrate complex in the insolubilized enzyme particles, by its maximal value, E_{gel},

$$[S] = S_o - \gamma E_{gel} \tag{5}$$

Then the correct replacement of $[S]$ by S_o will be expressed by the condition $S_o \gg \gamma E_{gel}$. From here stems the importance of the γ factor: its value will determine whether the Michaelis-Menten equation applies to the SBR. (a) For γ values near 1, this condition is the same as that required for the usual case of closed reactors containing soluble enzymes, i.e., enzyme concentration must be much lower than substrate concentration. For those closed systems, Cha (*6*) has calculated the large error resulting from replacing $[S]$ by S_o when enzyme and substrate concentrations are of the same order of magnitude; in these cases the depletion of free substrate by binding to the enzyme must be taken into account. (b) If $\gamma \ll 1$, S_o can be substituted for $[S]$ even when S_o and E_{gel} are similar. In this case the behavior of the SBR is similar to that described by Engasser and Horvath (*7*) for the case of continuous-flow stirred reactors, i.e.,

when the steady state is reached, the SBR behaves like an open system in a stationary non-equilibrium state. Evidently, during the course of the reaction in the SBR the amount of product formed becomes important and $[S]$ decreases, whereas this is not the case in the flow reactors.

The variation of γ does not modify the kinetic conditions in the assays (substrate and enzyme concentrations); hence its value can be decreased as much as the experimental device allows. In most cases we will be able to work in open system conditions.

With respect to Cha's calculations, we can say that when applying Eq. (1) to an insolubilized enzyme derivative of concentration E_{gel} contained in a SBR, the errors introduced by neglecting the depletion of free substrate are the same as in the case of a soluble enzyme of concentration $E_o = \gamma E_{gel}$, contained in a closed vessel, and much smaller than in the case of a soluble enzyme of concentration $E_o = E_{gel}$. In this paper we also present kinetic data obtained with derivatives having high enzyme concentration and the results confirm these assumptions.

Materials and Methods

Materials

Sepharose 2B (bead size, 60–250 μm) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden; CNBr from E. Merck, Darmstadt, West Germany; micrococcal nuclease (EC.3.1.4.7, mw 16,800) from Worthington Biochemical Corporation, Freehold, New Jersey 07728; and thymidine 5'-(*p*-nitrophenyl phosphate) 3'-phosphate (NPpdTp, mw 523), from Ash Stevens, Inc., 5861 John C. Lodge Freeway, Detroit, MI 48202. All other reagents were from commercial sources.

Methods

Sepharose beads were normally used as purchased from Pharmacia. To reduce their size they were occasionally manually homogenized in a Potter-Elvehjelm glass homogenizer. Deionized water was used throughout, and glassware used for handling soluble enzyme was siliconized.

CNBr activation of Sepharose 2B was carried out according to the latter method of Porath et al. [procedure A of (*8*)], which takes advantage of the very high buffer capacity of alkaline phosphate solutions, rendering pH control unnecessary.

Preparation of insolubilized derivatives. Nuclease was insolubilized through its amino groups directly on the CNBr-activated Sepharose 2B (Sepharose—(NH_2) nuclease) as previously reported (*4*). The amount of enzyme insolubilized was obtained from the difference between the activity of the soluble enzyme added to the suspension of activated Sepharose beads and the activity recovered in the filtrate and washings. In control experiments with unactivated Sepharose, the activity of the enzyme in the filtrate remained at 100%.

Enzyme assays. Initial activity of the soluble and insoluble enzyme toward NPpdTp was measured by following graphically the increase in absorbance at 330 nm at 25° C (*9*), using a Zeiss PMQII spectrophotometer equipped with a 2-cm pathlength cuvette with magnetic stirrer. The stirrer speed, determined with a tachometer, was usually fixed at its maximal setting (2500 rpm). Reaction velocities were calculated using a molar extinction coefficient for the *p*-nitrophenyl phosphate at 330 nm of 4260 M^{-1} cm^{-1} (*9*). Measurements of activity were made at pH 8.8 (25 m*M* Tris-HCl buffer) and pH 9.5 (50 m*M* glycine–NaOH buffer). [The pH values of these two buffers decrease with temperature in the interval 6–30° C; therefore, pH adjustment (made at room temperature, approx. 25° C) was set at a higher or lower value for temperatures higher or lower than 25° C.] The amount of insolubilized derivative in the assays was always maintained in the region of linearity between activity and amount of derivative. In this region the statement enunciated in the Introduction, v_R/γ = constant = v_p, is correct.

Results

Table 1 lists the properties of the three derivatives studied, obtained using sepharose 2B as support. The physical characteristics of the sepharose 2B beads (R, mean radius; r_p, pore radius) were determined as described in the accompanying paper (*5*). E_{gel} is the concentration of insoluble enzyme in the gel (calculated in μg of nuclease insolubilized per mL of gel; in Table 1 it is expressed in μM).

Diffusion Tests

In the absence of external diffusion the reaction rate, v_R/γ, must be independent of the relative motion of the solid particles and the liquid, i.e., independent of the stirring speed. At two concentrations of substrate (6 and 150 μM, figure not shown) the hydrolysis reaction was unaffected by the speed of the stirring device, showing that even at 500 rpm diffusion of the substrate to the external surface of the support beads was not affecting the rate of product formation. However, for the sake of comparison with other

TABLE 1
Properties of the Insolubilized Derivatives[a]

Derivative	$\bar{R}$, μm	r_p, nm	E_{gel}, μM
N-Se 2a	50	86	2.8
N-Se $2a_H$	9	86	2.8
N-Se $2b_H$	9	86	15

[a] $\bar{R}$, mean radius; r_p, pore radius; E_{gel}, concentration of the insoluble enzyme in the gel.

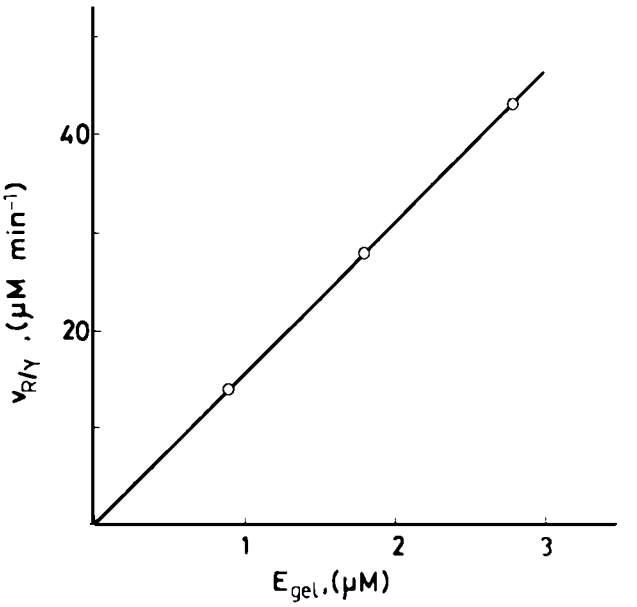

FIG. 1. Rate of hydrolysis as a function of enzyme concentration in the agarose. Substrate concentration: 150 μM. Assay conditions: 25 mM tris-HCl buffer (pH 8.8), 10 mM $CaCl_2$; 25°C.

experiments with high molecular weight substrates (*3, 10*), the stirring speed was routinely fixed at its maximal setting—2500 rpm.

In the absence of internal diffusion the reaction rate must be independent of the particle radius, but directly proportional to the enzyme concentration in the insoluble derivative. As is shown in the accompanying paper (*5*), internal diffusional limitations depend on k'_{cat}/K'_M and on β ($= K'_M/S_o$). For a given value of β, diffusional resistances increase with k'_{cat}/K'_M. Figure 1 shows the initial velocities obtained as a function of enzyme concentration in the agarose. Under those conditions internal diffusion across the porous network does not influence the rate. Since we have found that the value of k'_{cat}/K'_M is highest at pH 8.8 (see below), the absence of diffusional problems obtained at pH 8.8 can be extended, at the same value of β, to other pH values.

Effect of Substrate Concentration

At NPpdTp concentrations below 200 μM native nuclease follows Michaelian kinetics, but at higher concentrations a strong inhibition is observed (*11*). Hence, our experiments with the insolubilized nuclease were carried out at the optimal Ca^{2+} concentration, 10 mM (*12*), and at substrate concentrations of 3–200 μM. (At concentrations over 200 μM, inhibition was also observed.) The results obtained at pH 8.8 and 9.5 [most of the detailed study of soluble staphylococcal nuclease has been done at these pH values (*2*)], appear in Figs. 2 and 3 as Eadie-Hofstee plots according to Eq. (5). Figure 2 shows that, in the 26–200 μM substrate concentration range, we obtain the same values with the derivatives N-Se 2a and N-Se $2a_H$, which differ greatly in

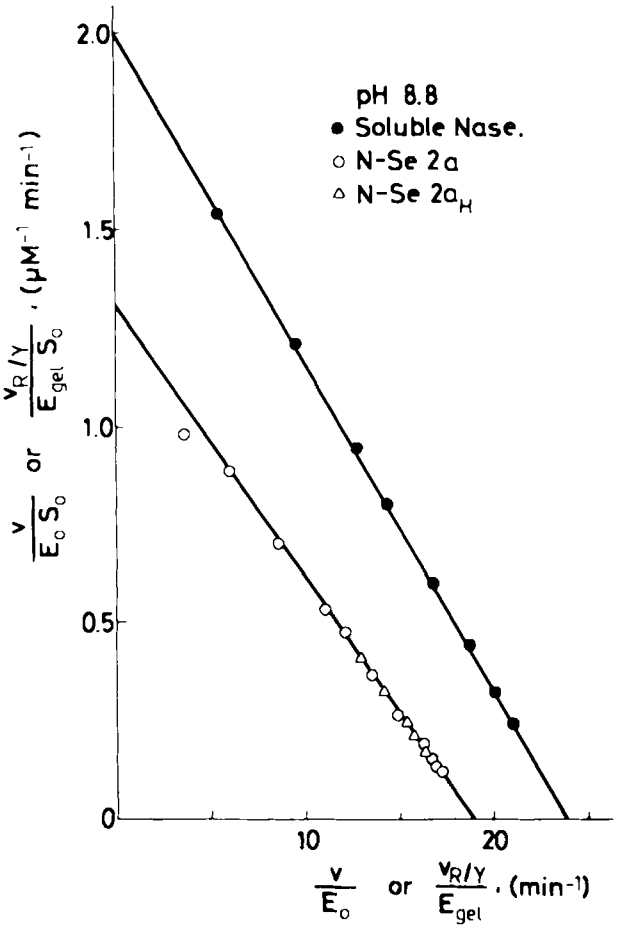

FIG. 2. Eadie-Hofstee plots at pH 8.8 for derivatives N-Se 2a and N-Se 2a$_H$. Assay conditions were as in Fig. 1. The behavior of the soluble nuclease is also included: in this case v/E_oS_o is represented vs v/E_o (v = initial rate of hydrolysis by the soluble enzyme; E_o = enzyme concentration).

particle size. This means that there are no diffusional limitations. From those plots the kinetic constants for the native (k_{cat} and K_M) and insolubilized (k'_{cat} and K'_M) enzyme were evaluated. We found the following values: For the soluble enzyme, 24 min^{-1} and 12 μM (at pH 8.8) and 64 min^{-1} and 35 μM (at pH 9.5); and for the insoluble derivatives, 19 min^{-1} and 14.7 μM (at pH 8.8), and 42 min^{-1} and 56 μM (at pH 9.5), respectively. These values and the ones obtained at pH 10 and 10.8 (Eadie-Hofstee plots not shown) are represented in Fig. 4 as a function of pH. The profiles k_{cat}-pH and K_M-pH are similar for both soluble and insolubilized nuclease.

However, at lower substrate concentrations (Figs. 2 and 3), the experimental values do not exhibit the linearity characteristic of Michaelian kinetics. In this region the internal diffusion of substrate controls the overall rate, as we shall see in the accompanying paper (*5*).

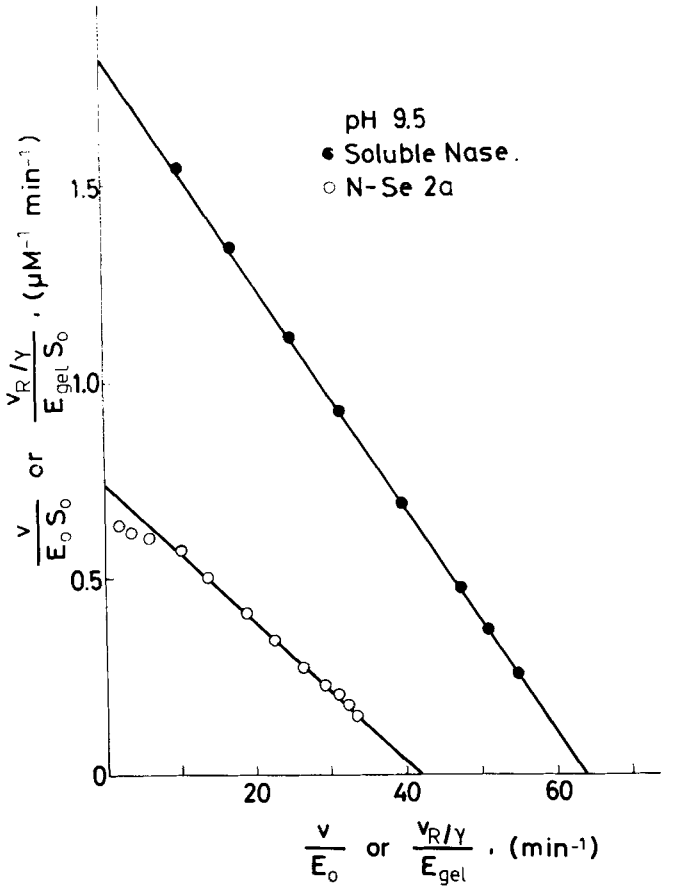

FIG. 3. Eadie-Hofstee plots at pH 9.5. Assay conditions: 50 m*M* glycine–NaOH buffer, 10 m*M* $CaCl_2$; 25°C.

Kinetics at Very High Enzyme Concentration in the Derivative

These experiments—at pH 8.8—were carried out with derivative N-Se $2b_H$, having an enzyme concentration (15 μM) similar to the K'_M value (14.7 μM). (The support beads were previously homogenized to preclude mass-transfer resistances in the resulting derivative.) Figure 5 presents the results obtained at values of S_o from 3.3 to 150 μM (β ranging from 4.5 to 0.1). We can see that even at low substrate concentrations the experimental points follow Eq. (1). Taking into account the depletion of free substrate by binding, Fig. 5 also includes the theoretical representations of Cha (*6*) for a soluble enzyme at concentrations $E_o = \gamma E_{gel}$ and $E_o = E_{gel}$.

Temperature Dependence

Studying the variation of the kinetic constants k'_{cat} and K'_M in the interval 6–30°C we found that K'_M values were very similar in that interval. Therefore, for a substrate concentration of 200 μM, several times greater than the K'_M

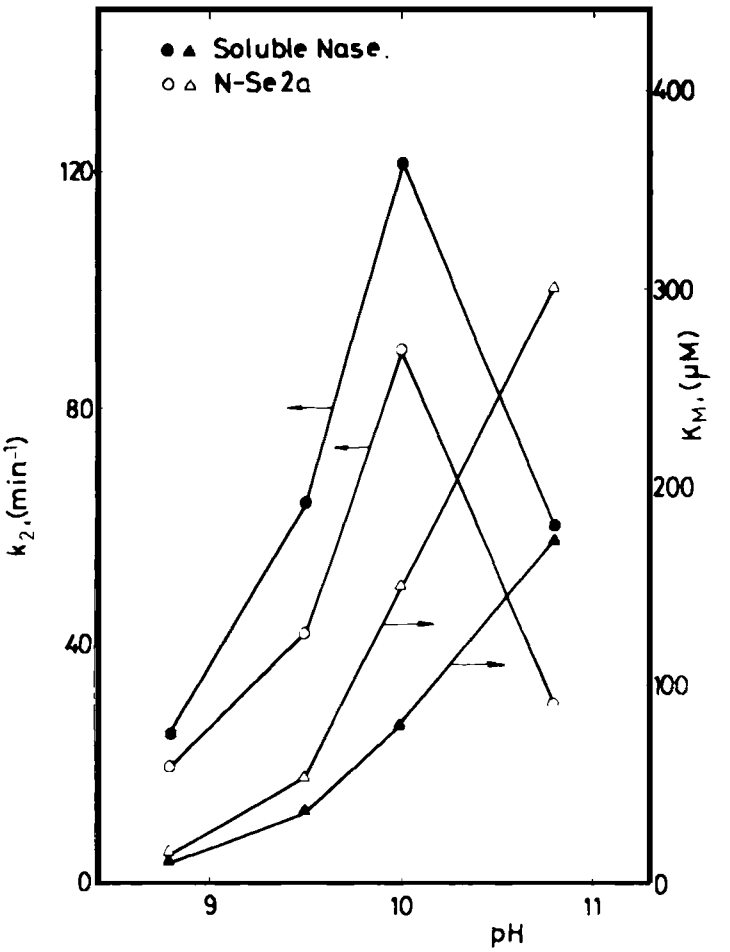

FIG. 4. k_{cat} (circles) and K_M (triangles) values of soluble (closed symbols) and insoluble (open symbols) nuclease as a function of pH.

values, it is possible to calculate the activation energies, E_a, by plotting the logarithm of the reaction rate as a function of $1/T$ (Fig. 6). At pH 8.8 the E_a values were 7.6 and 6.2 kcal/mol (31.8 and 25.9 kJ/mol), for the soluble and insoluble enzyme, respectively, whereas at pH 9.5 the corresponding values were 11.6 and 10 kcal/mol (48.5 and 41.9 kJ/mol), respectively.

Discussion

As we have seen above, the estimation of the kinetic constants of an insolubilized enzyme is a straightforward operation if one selects nondiffusional conditions. The values obtained for the sepharose 2B-(NH_2) nuclease derivatives are not very different from the corresponding values for the native nuclease, and confirm the high retention of activity when this

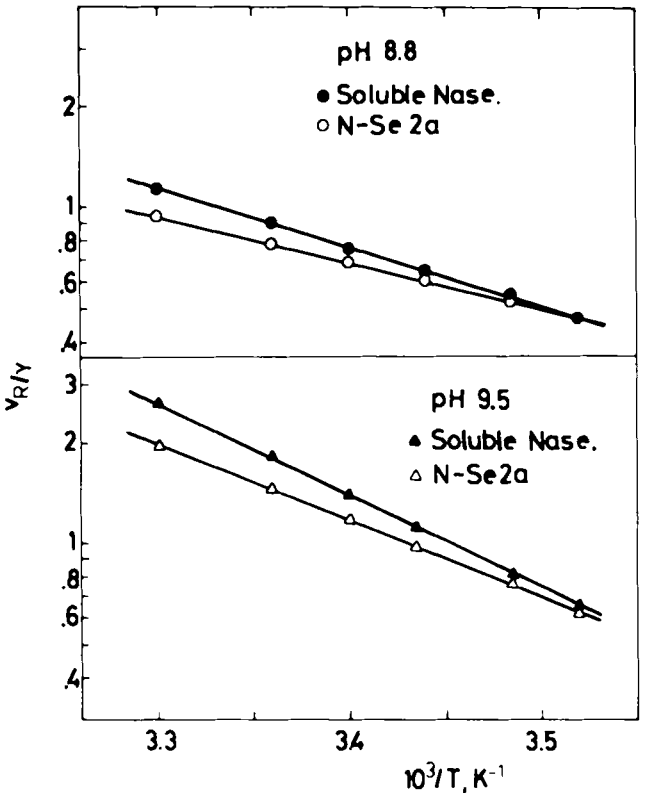

FIG. 5. Eadie-Hofstee plot for derivative N-Se $2b_H$. The value of ν was 0.002. Assay conditions: as in Fig. 1. In the figure are also indicated the lines (.-.-.-) corresponding to values of β from 8 to 0.125.

enzyme is immobilized to sepharose 4B (*4*). The pattern observed in the plots k_{cat}-pH and K_M-pH, equal for the soluble and the insoluble enzyme, is what one would expect in the case of an electrically neutral matrix (*1*). The activation energies are also similar in both cases.

The behavior of the insolubilized derivatives was found to be similar to that of the native enzyme, indicating that, upon insolubilization, the nuclease had not undergone substantial structural changes. On the other hand, the thermal and storage stability of the sepharose-(NH_2) nuclease was greatly enhanced (*4*). Both phenomena may be explained in view of the large number of enzyme groups able to bind to the support [23 lysines plus the amino-terminal Ala (*2*)] Thus, it would be possible to obtain a multipoint attachment that stabilizes the enzyme in a three-dimensional structure little distorted with respect to its native conformation, resulting in high retention of activity.

The data obtained with high concentrations of enzyme in the insoluble derivatives, confirm the hypothesis put forward in the Introduction that closed stirred vessels containing immobilized enzyme particles behave like open systems. Our experimental data fit Eq. (1) in a similar way to soluble enzyme of concentration $E_o = \gamma E_{gel}$, but this is not the case for $E_o = E_{gel}$. These conclusions will also be valid for other batch systems in which the immobilized

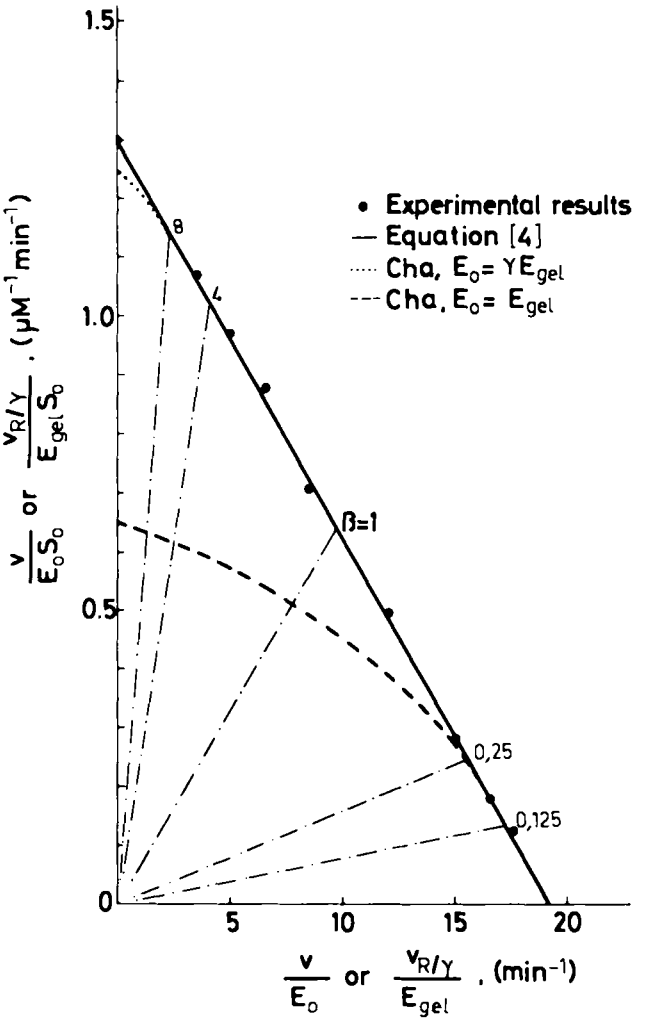

FIG. 6. Arrhenius plots for native and insoluble nuclease at two different pHs. Substrate concentration: 200 μM.

enzyme represents an open system owing to other types of immobilization[i.e., microencapsulation, enzymic assays "in situ" (*13*)].

The diffusional effects and the mixed kinetics enzymic reaction-internal diffusion are presented in the accompanying paper (*5*).

Nomenclature

E_{gel} Enzyme concentration in the immobilized derivative.

E_o Enzyme (soluble) concentration.

$[ES]$ Enzyme-substrate complex concentration in the immobilized derivative.

$[S]$ Free substrate concentration when steady state is reached.

S_o Initial substrate concentration.
k'_{cat} Catalytic constant for immobilized enzyme.
K'_M Michaelis constant for immobilized enzyme.
E_a Activation energy.
T Temperature, K.
v_p Reaction rate per unit volume in the immobilized enzyme particles.
v_R Initial rate of product appearance per unit volume of reactor.
V_p Volume of an immobilized enzyme particle.
V_d Total volume of the immobilized enzyme particles suspended in the reactor.
V_R Total volume of reaction mixture.
$\beta = K'_M / S_o$
$\gamma = V_d / V_R$

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