Simulation for Activation Process of Pepsinogen

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The activation of pepsinogen to pepsin was investigated by a combination of experiments and computations. An algorithm was at first made for the present study.
According to the established algorithm, experiments and computations were performed.
In the experiments, the spectral changes in the early stage of the activation were
measured by stopped flow technique, and the changes in the amounts of pepsinogen
and pepsin during the activation were followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Furthermore, the sensitivity of the molecular species
involved in the activation process to alkaline solution was measured to characterize
intermediate components. The time-course in the early stage exhibited an exponential
profile. The existences of dimeric intermediate and the other intermediates were
evidenced by gel electrophoresis and alkali treatment, respectively. The activation
process showed the dependence to the initial concentration of pepsinogen, indicating
that the activation process is essentially nonlinear reaction. Computer simulations
were performed with many postulated basic schemes, and Scheme 8 was eventually
selected as the most reasonable one. In Scheme 8, the first step is a conformational
change with a rate constant k_1 which is a function of the first power of hydrogen ion
concentration of the medium. The second step consists of two steps; an intramolecular (or unimolecular) reaction accompanied with the release of peptides and an
intermolecular (or bimolecular) reaction in which X_2 and X_3 combine mutually into
dimeric intermediate X_5. X_5 cleaves to two molecules of X_3 with releasing the peptides.
The third step is reversible one with pK **3.1.** The conversion of X_3 to X_4 is accompanied with the spectral change and the uptake of hydrogen ion from the medium.
X_3 and X_4 are conformational isomers, and have same molecular weight. They
exhibit the same peptic activity in acidic medium.

INTRODUCTION

The first study of the activation of pepsinogen was performed by Herriott
(1938a) using crystallized preparation from swine. He reported that at pH 4.6
pepsinogen would be activated by the produced pepsin by an autocatalytic
mechanism involving a bimolecular reaction. Furthermore, it was confirmed
that the rate of the activation was enhanced with increase in the initial concentration of pepsinogen (Herriott, 1938b). These findings suggested that the
initial event of the activation may be caused by contaminated pepsin molecule
in the pepsinogen preparation. Alternately, the possibility that pepsinogen

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converts to pepsin by itself, at least, in the initial stage had been sought by many investigators.   Since pepsinogen was further purified with column chromatography by Ryle (1960) and Rajagopalan *et al.* (1966), many studies were performed to prove whether pepsinogen activates by a self activation mechanism with the unimolecular reaction or not.   Bustin and Conway-Jacobs (1971) revealed that the pepsin molecule is not essential for the activation by the experiment on the activation under the coexistence of excess of the substrate and by the activation of sepharose-bound pepsinogen.   Thus, the activation mechanism seemed to include both the self activation mechanism with the intramolecular reaction and the autocatalytic mechanism with the intermolecular reaction.

Bustin and Conway-Jacobs (1971) also indicatd that exogenously added pepsin did not enhance the activation rate at pH 1.8 and 37°C, and at pH 3.0 and 23°C. Funatsu *et al.* (1972) suggested the self activation reaction by the experiment on the activation in the presence of pepsin inhibitor isoamyl alcohol or in the presence of excess synthetic substrate.   Rajagopalan *et al. (1966)* reported that the chromatographic patterns of peptides released during the activation were different each other according to the pH value of the medium used.   Al-Janabi et *al. (1972)* suggested that the activation between pH 1 and 3 occurs by the self activation mechanism, since the half time of the activation did not change with decrease in the initial concentration of pepsinogen to one tenth, and at pH 4.0 occurs by the mixed mechanism with the intra- and intermolecular reactions. McPhie (1972) reported the same conclusion as that of Al-Janabi *et al.* on the basis of the experimental results obtained by difference spectrophotometry.

The goals of the present study are to reveal kinetically the mechanism of such a complex activation process of pepsinogen and to establish a generally applicable algorithm for the analysis of complex chemical reactions.   Particularly, a computer technique was introduced under the combination with experiments to estimate the values of parameters (rate constants) by an optimization method and to demonstrate the most reasonable model scheme by simulation method.

MATERIALS AND METHODS

Materials

Pepsinogen was isolated from swine fundic mucosa by the method of Vunakis and Herriott (1956) and purified by DEAE-cellulose column chromatography according to the method of Ryle (1960).   Pepsinogen thus prepared was dialyzed against 0.1 M ammonium bicarbonate buffer, pH 8.1 for 3 days to inactivate a small amount of contaminating pepsin.

Activation of pepsinogen

Pepsinogen (2mg) was dissolved in 2.5 ml of deionized water. An equal volume of HCl solution at varied pH was added to the pepsinogen solution to maintain the desired pH value for the activation.   In some cases, 0.1 M citrate buffer was used instead of HCl solution.   During the activation, the temperature of the solution was kept at 0°C or 15°C. At appropriate time

intervals, an aliquot was taken out and mixed with the same volume of 0.1 M phosphate buffer, pH 7.0 to cease the activation. When citrate buffer was used as the activation medium, the adjustment of pH to 7.0 was made by addition of NaOH solution. The extents of the activation were estimated by measuring the amounts of remaining pepsinogen and formed pepsin after the mixture was separated by gel electrophoresis.

Gel electrophoresis

Gel electrophoresis was carried out according to the method of Weber and Osborn (1969) with 10 % acrylamide in 0.1 M phosphate buffer, pH 7.0. The current was 8 mA per gel column. Protein in the gel column was directly detected by reading optical density at 280nm against 320 nm using a Shimazu double-beam scanner and a Gilford spectrophotometer.

Alkali treatment

Pepsinogen (0.04 %, 1 ml) was activated in 0.025 M citrate buffer, pH 2.3 at 0°C for various times (O-15 min). The activation was stopped by adding alkaline solution (2ml) to the expected pH, and the mixture was kept at 37°C for various times. Then, the remaining pepsinogen in the mixture (3 ml) was completely activated to pepsin by adding 2 ml of 0.1 M HCl at 0°C for 20 min. The peptic activity was measured with 1 ml of 1.8 % casein solution at pH 1.8, 37" C for 6 min. The reaction was stopped by adding 2 ml of 0.44 M trichloro-acetic acid solution. After the centrifugation, the absorption of the supernatant was measured at 275 nm.

Algorithm for study of mechanism

A procedure was designed to investigate not only the mechanism for the pepsinogen activation, but also that for general, complicated chemical reaction. The flow diagram of the procedure is shown in Diagram 1. (1) Collection of the experimental data which represent the characteristics of the activation, especially the nonlinearity. **(2)** Basic model schemes are built to explain the experimental data. (3) Simultaneous differential equation is derived from the schemes. (4) Computer simulations are performed to expect the character-istic features of the selected scheme with changing the values of kinetic param-eters. (5) Judgement and discussion are done for appropriateness of the selected model schemes. If the scheme is reasonable, then proceeds to (6), otherwise go back to (2). (6) To select the most reasonable one from the schemes qualified at above steps, experimental projects are designed on the basis of the results of computer simulation. (7) According to the experimental projects, several experiments are carried out to collect the desired data. (8) The second judgement is done. If the scheme does not explain the experimental data, then the scheme is revised at step (10) and go to (3), otherwise go to (9). (9) Here the most reasonable scheme will be obtained.

Computation

A digital computer (FACOM 230-60) and an analog computer (Hitachi Model ALS-220) were used for simulation and optimization.

Beforehand to analyze the activation process of pepsinogen, the following
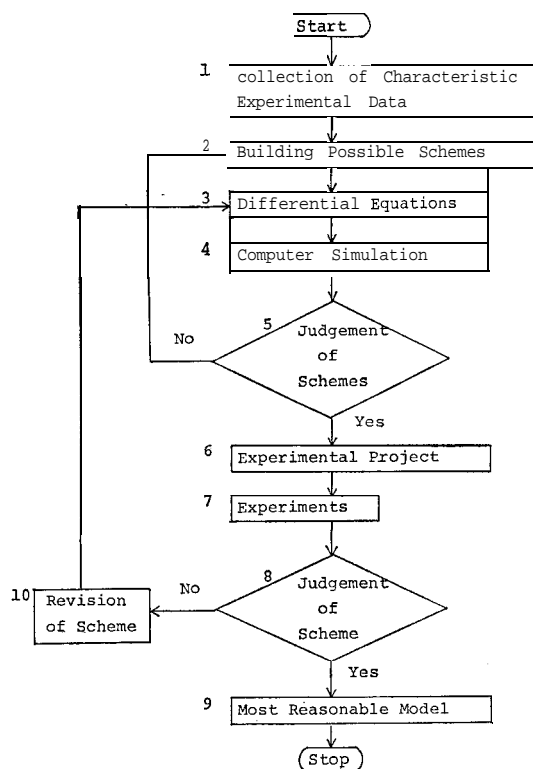
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Diagram 1.   Flow diagram of procedure of study.

assumption was made; the activation would occur with only one mechanism and the various observations on the activation process would be realized from varied experimental conditions used.

The simulation is to describe the behaviors and the characteristics of a certain system by computation instead of experiments.   In the present study, the simulation was performed to expect the characteristics of the postulated schemes, to obtain the kinetic parameters and to design the experimental project mainly by analog computer which is useful for solving differential equations. Graphic display unit attached to the digital computer is also useful for the simulation of the schemes, since interruption and instruction are possible during the execution of computation (Koga *et al.,* 1974), though it is a weak point that the program becomes very complex.   The computed results were directly read on the cathode ray tube or recorded by an XY-plotter.

In order to obtain the kinetic parameters fitting well in the experimental data, an optimization method was used.   The flow diagram of the procedure for the optimization is shown in Diagram 2.   (1) Experimental data are fed. (2) Initially assumed values are given to the rate constants.   (3) The differential equations derived from the scheme are solved by a numerical method. (4) The objective function and the revised parameters are calculated by an optimization
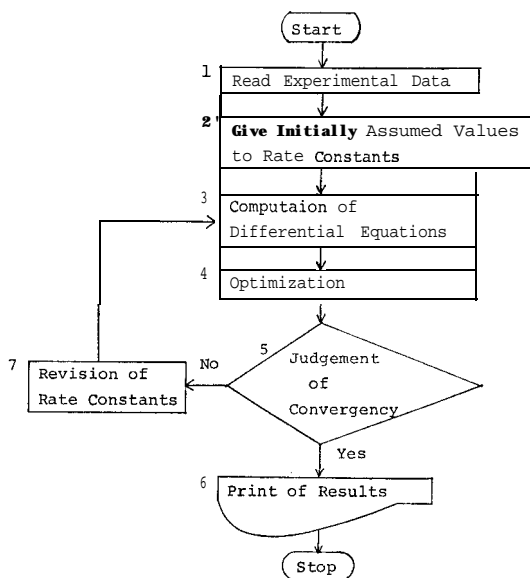
Diagram 2. Flow diagram of optimization method.

method. (5) The convergency of the parameters is judged. If the obtained parameters are reasonable, then the results are printed at step (6), otherwise next calculation is done with the revised parameters at step (7) and go to (3).

For the optimization method, a kind of self consistent method with Kuhn-Tucker's constraint qualification was used. In general, the simultaneous differential equations are derived from the scheme as,

$$\frac{dX_i}{dt} = f(k_1, \ldots k_n, X_1, \cdots, X_i) \tag{1}$$

where X, *(i = 1, $\cdots$, m)* is the concentration of i-th molecular species and *k,* *(j =* 1,.... *n)* is the rate constant. As can be seen from $E_q(1)$, the differential equations are represented as a first order function of *k,*. On the other hand, the objective function, *F,* is represented by,

$$F = \sum_i \sum (X_i(t) - Y_i(t))^2 \tag{2}$$

where $X_i(t)$ is the calculated concentration of i-th species at time *t* and *Y,(t)* is the experimentally measured concentration of i-th species at time *t*. In this case, the optimization is to minimize the objective function so as to satisfy the following partial differerential equation with respect to *k, (j = 1, $\cdots$, n)*.

$$\frac{\partial F}{\partial k_j} \quad 0 \tag{3}$$

To solve E_q (3) with respect to k_j, the objective function must be represented as a function of *k,*. For this purpose, E_q (1) is integrated and X,(t) is represented as a function of *k,* as follows,

$$X_j(t) = \int_0^t f(k_1, \cdots, k_n, X_1, \cdots, X_m)dt \tag{4}$$

Thus, F in the E_q *(2)* can be represented as a function of k,, by substituting E_q (4) into X_i (t) of E_q (2). The rate constant k, should not be negative. Therefore, Kuhn-Tucker's constraint qualification was introduced. That is, if some of the revised rate constants were negative, these rate constants are arbitrarily replaced by small positive values, and only the remaining rate constants are determined in order to minimize the objective function. The judgement on the convergency of the revised parameters is performed to monitor the objective function (Hayashi et al., 1973).

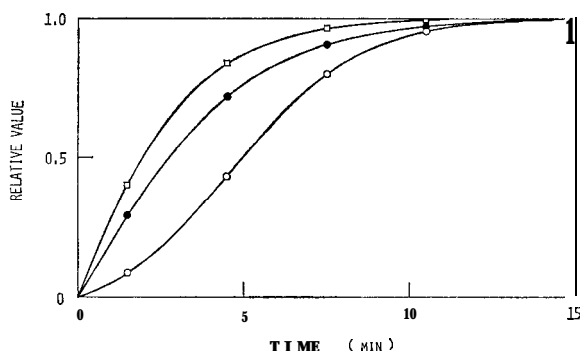## RESULTS AND DISCUSSIONS

Selection of schemes

According to the procedure in Diagram 1, at first the characteristic experimental data of the activation were collected: (1) The time-course of the activation at 0°C and pH **2.5** showed the induction or lag phase (Hayashi *et al., 1973).* (**2**) The induction was not seen at higher temperature above 15°C and low pH (Hayashi *et al.*, 1973; Al-Janabi *et al.,* 1972). (**3**) After the activation followed by the appearance of the peptic activity has completed, the change in UV spectrum was still observable (Hayashi et *al.,* 1973). The characteristic induction may be dependent on the values of the rate constants which are controlled by the experimental conditions. The UV spectral change was considered to be derived from the conformational change of the produced pepsin, since the released peptides do not contain amino acid residues which can absorb UV light (Rajagopalan et al., 1966; Ong and Perlman, 1968).

$$1' \qquad X_1 \xrightarrow{\; k_1 \;} X_2$$
$$2' \qquad X_1 \xrightarrow{\; k_1 \;} X_2$$
$$\underset{k_2}{\overset{\frown}{}}$$
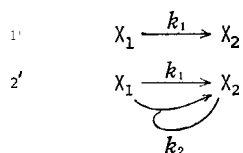
Basic schemes which are required to be as simple as possible because of the simplicity of computer simulation, were constructed to explain the above characteristics. The time-course based on Scheme 1' (one-step activation) showed an exponential curve, but not the induction. However, Scheme 2' which contains a looped step can explain the characteristic induction. The inflection time of the activation, t_{inf}, may be defined as a measure of the existence of the induction. The inflection time based on Scheme 2' may be represented as a function of rate constants and the initial concentration of pepsinogen.

$$t_{inf} = \ln \frac{k_2 X_1(0)}{k_1} \bigg/ (k_2 X_1(0) + k_1) \tag{5}$$

where X,(O) is the initial concentration of pepsinogen, X_1, and k_1 and k_2 are

Fig. 1. Simulated time-course with Scheme 1-2. $\square k_1 = 0.005$, $k_2X_1(0) = 0.0033$. ● $k_1 = 0.0033$, $k_2X_1(0) = 0.0033$. ○ $k_1 = 0.0007$, $k_2X_1(0) = O.\ 008$. Units of parameters are sec^{-1}. Value on vertical axis indicate fraction of formed pepsin.

rate constants. If $k_2X_1(0)$ is larger than k_1, t_{inf} is positive, and the induction may be seen, otherwise, the induction may not be seen, As shown in Fig. 1, it is clear that the appearance of the induction depends, as expected, on a combination of the rate constants and the initial concentration. Consequently, it is concluded that the looped step such as in Scheme $2'$ is a possible one for explaining the characteristic induction. On the other hand, in a sequential re-action which is composed of several first-order reactions, the induction is not seen until three-step reaction. Therefore, the induction might be explained by a looped step or sequential reaction containing more than two steps.

On taking account of the UV spectral change, at least two steps seem to be necessary for the activation process. As a result, two-step and three-step reactions with or without the looped step have been investigated (Hayashi *et al.*, 1973). In two-step reactions, only Scheme 1 could explain the induction and the UV spectral change. Sixteen schemes were constructed for the three-step reaction. However, only five schemes were considered to be reasonable for the

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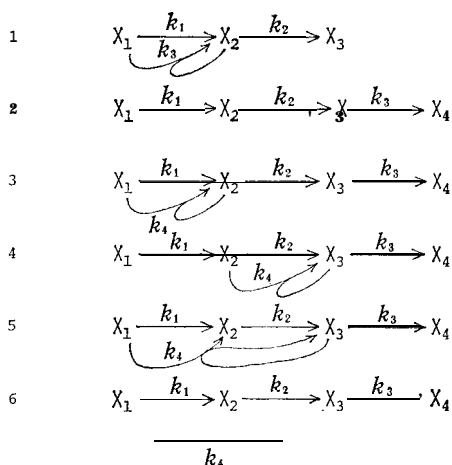
Table 1. Kinetic parameters of schemes.

Scheme	$k_1(\text{sec}^{-1})10^{-3}$	$k_2(\text{sec}^{-1})10^{-3}$	$k_3(\text{sec}^{-1})10^{-3}$	$k_3X_1(0)$ or $k_4X_1(0)(\text{sec}^{-1})\ 10^{-2}$
1				
2	a. 0.7815 9	4.7 1.77	1.7	0.796
3		6.9		–
4	4.4 1.2	2.3	1.6 1.7	2.15 2.54
5	2.1	5.9	1.7	5.10
6	0.1	0	1.6	29.0

activation. The rate constants for each reasonable scheme were obtained by
the optimization method as shown in Table 1. After all, six model schemes
were selected for representing the activation process.

Revision of model schemes

To select or to revise the most reasonable scheme from the above postu-
lated schemes (1-6), more specific experimental data which are able to discrimi-
nate the schemes one another are required. To specify the most useful data
to be measured experimentally, the behaviors of each of the postulated schemes
were simulated. As a result, the experiments were designed to observe the
change in UV absorption spectrum at a very early stage of the activation, the
effect of initial pepsinogen concentration on the activation rate and the pH
dependence of the activation process (Koga and Hayashi, 1976).

According to the experimental data thus obtained, Scheme 4 was selected
as the most reasonable one. Since a dimeric intermediate was evidenced by gel
electrophoresis which was used to estimate the extents of the activation, Scheme
4 was further revised to Schemes 7-l and 7-2.   Finally, Scheme 7-2 was excluded,
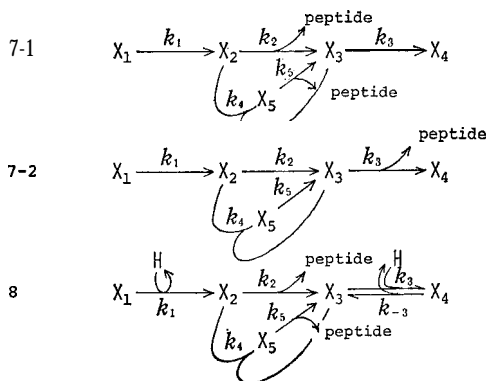
because this was not able to explain the UV spectral change in the late stage
of the activation, and only Scheme 7-1 was examined as the most reasonable
one in the following study.

pH Dependence

Computer simulation of Scheme **7-1** to expect the sensitivity of the activation rate to the values of parameters showed that only the parameter k_1 at the first step altered the activation rate markedly. The first step was assumed to correspond to the conformational change of pepsinogen molecule to frame the active site. Since the conformational change is sensitive to pH value of the activation medium, the effect of pH on the rate was represented by taking k_1 in Scheme 7-l as a function of the first power of hydrogen ion concentration, that is $k_1 = f(H)$.

In a narrow range of pH values (2.5–3.0), the computed time-course with Scheme 7-l was in good agreement with the experimental data. However, the real activation time-course obtained at pH 1.5 (adjusted with HCl) was considerably different from that computed with Scheme 7-l (top figure in Fig. 2). This
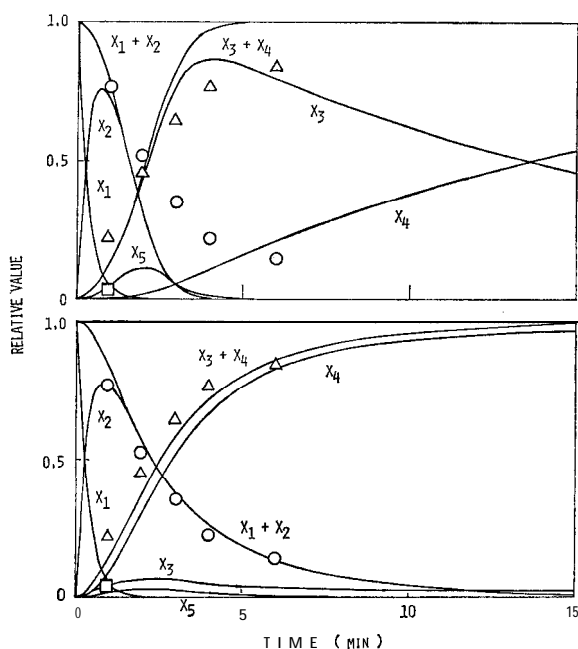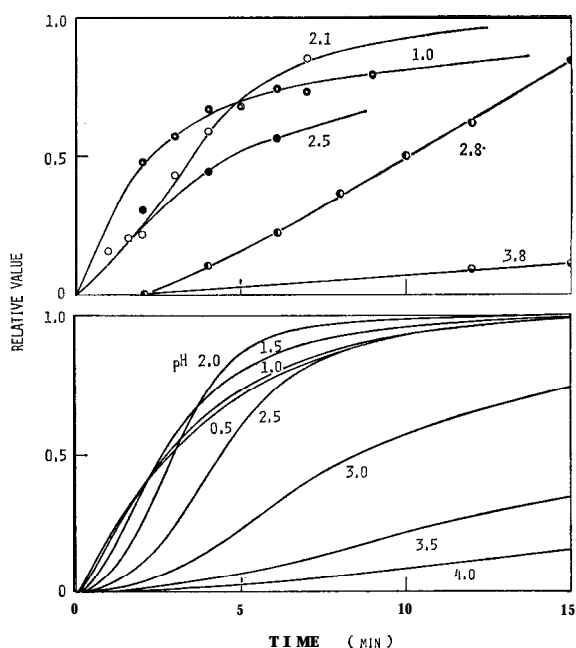

Fig. 2. Comparison of experimental data with simulated time-courses. Top, simulation with Scheme 7-1; bottom, simulation with Scheme 8. Symbols show experimental data by gel electrophoresis at pH 1.5 (HCl), 0°C and initial concentration of pepsinogen, IC_1, of 0.2 %. o pepsinogen, △ pepsin, ◻ dimeric intermediate. Solid lines are simulated time-courses with Scheme 7-l (top) assuming that $k_1 = 0.0526$, $k_2 = 0.0038$, $k_3 = 0.001$, $IC_1 k_4 = 0.0422$ and $k_5 = 0.05$, and with Scheme 8 (bottom) assuming that $k_1 = 0.0526$, $k_2 = 0.0038$, $k_3 = 0.0663$, $IC_1 k_4 = \mathbf{0.0422}$, $k_5 = 0.05$ and $k_{-3} = 0.0016$. Units of parameters are sec^{-1}.

fact means that Scheme 7-l should further be revised to explain the real time-course at very low pH values. Bohak (1973) reported that the activation process required the uptake of proton from medium by a group with pK 3.1 in the

active site of pepsin.   Thus, Scheme 7-1 was revised to Scheme 8 by inserting
a reversible protonation step between X_3 and X_4, the latter being a protonated
species of pepsin. The time-courses (solid line) in the bottom figure in Fig. 2 (cited
from Koga and Hayashi, 1976) were computed ones with Scheme 8.   The good
fit indicates that Scheme 8 is more reasonable model for the activation process
than Scheme 7-l. Furthermore, to explain the validity of Scheme 8, computer
simulation was performed with respect to pH dependence of the activation. As
shown in the bottom figure in Fig. 3, the computed time-courses of X,+X, at

Fig. 3.   Comparison of experimental data with simulated time-courses with Scheme
8 for pH dependence. Top, experimentally obtained time-courses ; bottom, simulated
time-courses. Activation was carried out at 0°C and $IC_1 = 0.2\%$. Parameter values
are assuming to be ; $k_1 = 1.66(H), k_2 = 0.0038, k_3 = 1.26\times10^3(H)k_{-3}, IC_1k_4 = 0.0422,$
$k_5 = 0.5$ and $k_{-3} = 0.0016$.   Units of parameters are sec^{-1}. Value on line indicates
pH value.

various pH's exhibit a complicated nonlinearity: that is, the time-courses below
pH 2.0 cross each other. The experimental results by gel electrophoresis (the
top figure in Fig. 3) also show the same type of nonlinearity. These character-
istic features have also been observed by Harboe et al. (1974). Simulation of
Scheme 8 with respect to pH value also suggested that the optimum pH may
fluctuate according to the estimation methods.   As shown in Fig. 4, the optimum
pH changes with the estimating time (bottom figure) or with the extents ($\%$)
of the activation (top figure). In the top figure in Fig. 4, the reciprocal of the
activation time required to attain to the specified extent is taken as the velocity
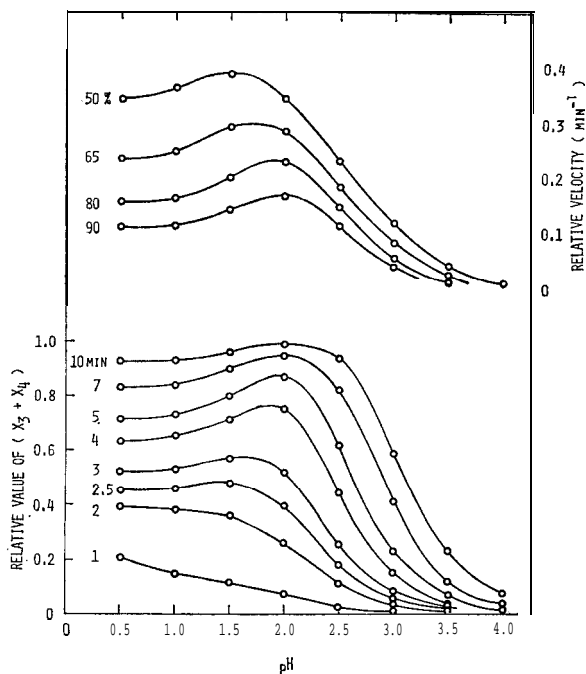of the activation.   The bell shaped pH-rate profile has been observed by Herriott

Fig. 4. Simulation with Scheme 8 for pH dependence of activation. Parameters are the same as those in Fig. 3. Top, relative velocities (reciprocal of time required for attaining of $X_3 + X_4$ to specified percentage): bottom, amount of $X_3 + X_4$ at various activation times,

(1938b). In addition to the simulated results described above, Scheme 8 could cover all behaviors produced by Scheme 7-1.

Effect of citrate buffer

Both citrate buffer and HCl were used for adjusting pH value of the activation medium. The rate constants, k_1 and k_2, obtained at pH's 2.5 and 3.0 (citrate buffer) were larger than those obtained at pH's 1.5 and 2.85 (HCl). The rate enhancement in citrate buffer seems to relate with the salt effect on the activation reported by Herriott (1938a), Varandani and Schlamowitz (1963), Seijffers et al. (1964) and McPhie (1972).

Temperature dependence

To investigate whether Scheme 8 can explain the disappearance of the induction in the activation time-course at higher temperature or not, experiment and simulation were further performed at 15°C, pH's 2.2 and 3.0 (citrate buffer), and the initial concentration of 0.2 %. The results are shown in Fig. 5. As can be seen in the figure, Scheme 8 exhibits a good fit between experimental result and computer simulation even at higher temperature. Although the induction in the simulated time-course of X_4 is still slightly seen, it could not be experimentally confirmed. In addition, the computer simulations suggested that only rate constants, k_1 and k_2, were strongly dependent on the activation temperature.
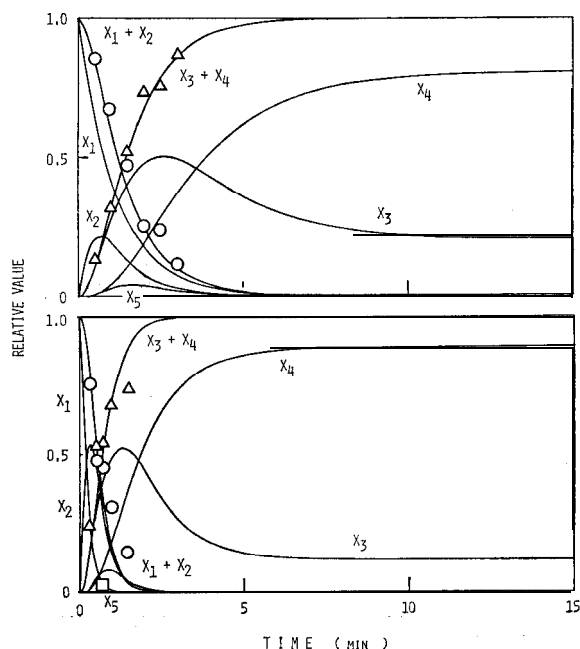
Fig. 5.   Temperature dependence of activation time-course. Symbols are experimentally obtained data in 0.05 M citrate buffer, pH 3.0 (top) and pH 2.2 (bottom) at 15°C and $IC_1 = 0.2$ %. ● pepsinogen, △ pepsin, ▫ dimeric intermediate.   Solid lines are simulated time-course with Scheme 8 assuming that $k_1 = 14.4(H), k_2 = 0.03$, $k_3 = 1.26 \times 10^3 (H) k_{-3}, IC_1 k_4 = 0.042$, $k_5 = 0.05$ and $k_{-3} = 0.0016$.   Units of parameters are sec^{-1}.

Simulation for effect of added pepsin

It was reported by Herriott (1938b) and McPhie (1972) that pepsin accelerated the activation of pepsinogen.   Contrarily, Bustin and Conway-Jacobs (1971) experimentally denied that effect.   These contradictory findings would be considered to be derived from the difference in the experimental conditions used. Therefore, computer simulation was performed with Scheme 8 assuming that added pepsin is in the form of X_3 which is predominant above pH 4. As shown in Fig. 6, the acceleration effect was slightly seen by addition of pepsin (final conc., 0.1 %) at 0°C, pH 2.85 (HCl) and at the initial pepsinogen concentration of 0.2 %. The relation between the activation rate and the amount of added pepsin is shown in Fig. 7.   It was suggested by computer simulation that pepsin would accelerate the activation rate even at low pH value.   However, it would be difficult to observe experimentally the acceleration effect at low pH region, because a great rate of self activation may shade such the slight effect.

Sensitive intermediate to alkaline solution

McPhie (1972, 1974) has suggested the existence of the active pepsinogen, which is able to return reversibly to pepsinogen at neutral pH region, as judged by photometric measurement. To characterize the intermediates including the
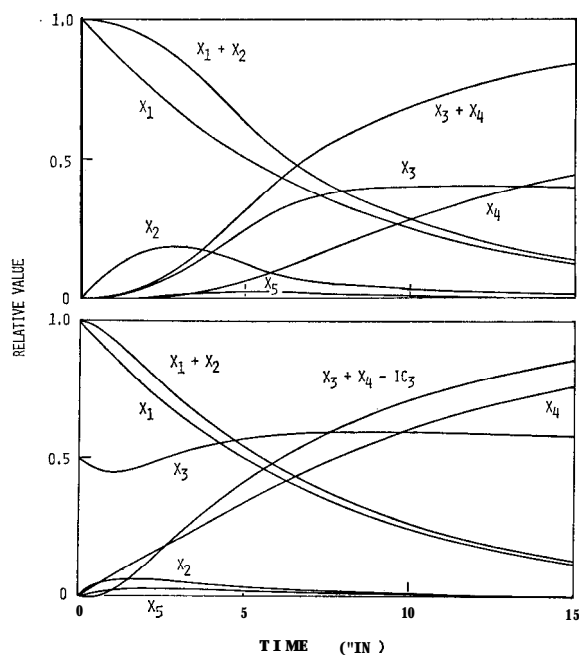
Fig. 6.   Simulation with Scheme 8 for effect of added pepsin species X_3 on activation. Conditions for activation are assumed to be pH 2.85 (HCl), 0°C and $IC_1 = 0.2$ % (top) or initial concentrations of X_1 and X_3 arc 0.2 and 0.1% respectively (bottom). $k_1 = 0.0023$, $k_2 = 0.0038$, $k_3 = 0.0028$, $IC_1 k_4 = 0.0422$, $k_5 = 0.05$ and $k_{-3} = 0.0016$. IC_3, initial concentration of X_3.
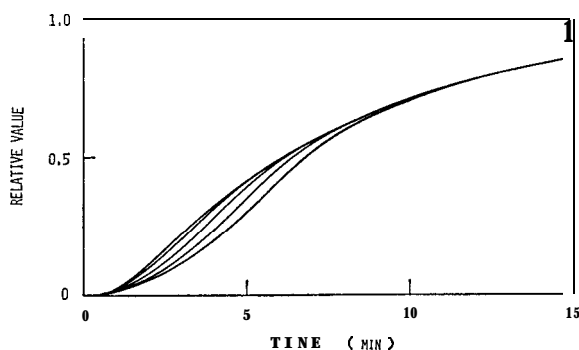

Fig. 7.   Simulation with Scheme 8 for effect of initial concentration of X_3 on activation. Solid line indicates time-course of $X_3 + X_4$. IC_3 are from top to bottom, 0.5, 0.3, 0.2, 0.1 and 0. IC_3 is defined as ratio to IC_1.

active pepsinogen, alkali-denaturation experiments were carried out. After pepsinogen was activated to several extents at 0°C, pH 2.5, the activation mixture was brought to desired pH by addition of alkaline solution and the remaining peptic activity was measured in acidic medium.   Figure 8 shows the denaturation time-
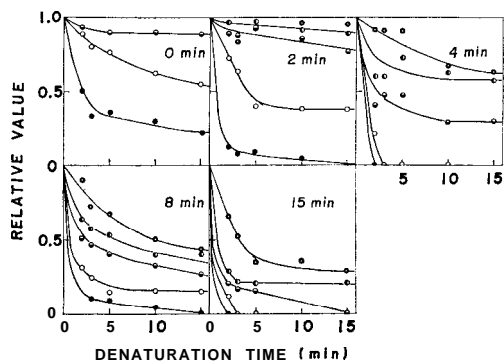
Fig. 8. Denaturation time-course in alkaline solutions at various pH's. Activation was carried out at 0°C, pH 2.5 (0.025 M citrate buffer) for 0 to 15 min. Denaturation was carried out at 37°C. Remaining peptic activity (vertical axis) was measured with 1.8 % casein solution, at pH 1.8, 37°C for 6 min. Time indicated in each figure is activation time. ⊜ pH 7.5, ⊙ pH 8.0, ⊖ pH 8.5, ○ pH 9.0, . pH 9.5.
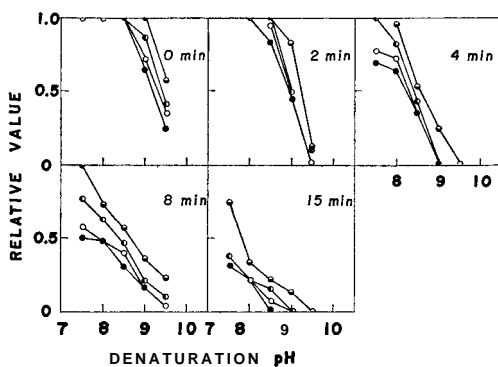
Fig. 9. Denaturation in alkaline solution. Experimental conditions are the same as those in Fig. 8. Denaturation times are, ⊖ 2 min, ⊙ 5 min, ○ 10 min, . 15 min. Times indicated in figures are activation time and vertical axis is remaining peptic activity.

course at various pH's. The data in Fig. 8 were rearranged, being shown in Fig. 9 which indicates pH dependece of denaturation at each specified activation time. The denaturations of pepsinogen and pepsin had been already investigated by Herriott (1938a) and Northrop (1930). However, the denaturation time-course was not reported yet. As shown in Fig. 9, pepsinogen was denatured above pH 9.0 and was stable below pH 8.5. Pepsin solution obtained by 15 min activation was denatured above pH 8.5. These results were in good agreement with data by Herriott and Northrop. At 4 min activation, the denaturation was markedly caused at pH 9.0 as compared with that at 8 min activation. However, below pH 8.5, the denaturation proceeded in parallel with the activation time. Therefore, there would be intermediates which exhibit different sensitivity to alkaline solutions between pH's 8.5 and 9.0. Sanny *et al.* (1975) reported

that a molecular species which corresponds to the active pepsinogen suggested by McPhie (1972, 1974) would be a complex of pepsin and the released peptides, which dissociates to pepsin and the peptides and then is denatured at pH 8.5. The present results suggest further that another intermediate might be formed during the activation process. It is reasonable to assume therefore that in Scheme 8, X_2 is the complex of pepsin and the peptides or active pepsinogen, and X_3 is the intermediate evidenced in this study, since X_2 has the same molecular weight as pepsinogen and X_3 is more basic and unstable than X_4. This assumption should be confirmed by following investigations.

As described above, Scheme 8 was selected as the most reasonable scheme at the present time for representing the activation process of pepsinogen. The characteristic behaviors of the activation process, for instance different kinetic reaction orders observed below pH 3 and near pH 4 respectively (Herriott. 1938a; Al-Janabi *et al.,* 1972; Bustin and Conway-Jacobs, 1972; McPhie, 1972), can be consistently explained by only Scheme 8. Since Scheme 8 is involving the looped step between X_2 and X_3, being essentially nonlinear scheme, all nonlinearities in the activation process such as the initial concentration dependence can be also easily explained by this scheme.

In the first step, pepsinogen X_1 converts to X_2 with assistance of hydrogen ion, being accompanied with UV spectral change (conformational change to form the active site). X_2 has the same molecular weight as pepsinogen and is presumed to be the complex of pepsin and the fragmented peptides which may release at the next step (Sanny *et al.,* 1975). This intermediate, called the active pepsinogen (McPhie, 1972, 1974), might have potentially the peptic activity. In the second step, X_2 converts to X_3 by the intramolecular and intermolecular reactions. In this step, the peptides are released. X_3 can bind the released peptides to form the complex which may not be distinguished photometrically from the pepsinogen molecule (Seijffers et *al.,* 1964 ; McPhie 1972 ; Anderson and Harthill, 1973; Harboe et *al.,* 1974; Sanny *et al.,* 1975). X_5 is the dimeric intermediate consisting of X_2 and X_3, and changes rapidly to two molecules of X_3 with releasing the peptides. This X_5 complex may correspond to a complex of an enzyme and its substrate.

The third step between X_3 and X_4 is reversible with pK 3.1 (Bohak, 1973). X_3 and X_4 have the same peptic activity in acidic medium and are conformational isomers. X_3 is more basic and unstable than X_4. X_3 converts to X_4 being accompanied with the UV spectral change and the uptake of hydrogen ion. Since there are two ways for the conversion of X_2 to X_3 and for the release of the peptides, different N-terminal products (Sanny *et al.,* 1975) or different active products (Rajagopalan *et al.,* 1966) are expected. X_3 or X_4 would have several N-terminal species: Ile-Gly N-terminal product would be derived from an intramolecular reaction and the other N-terminal product would be derived from intermolecular reaction. Sanny *et al.,* (1975) also suggested that the molecular species with other N-terminals are formed more at pH 3.2 than pH 2.2. This finding is also explainable by Scheme 8.

The algorithm was made for the present study. According to the algorithm, experiments and computer simulations were performed. A computer simulation

was very useful to design the experimental project and to expect the character-
istic behavior of model scheme. The optimization method used here was validly
adoptable for the decision of parameter values fewer than four in a model
scheme. For schemes containing more than five parameters, it was found that
the computer simulation was more preferable than the optimization method for
the decision of kinetic parameters.

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