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<https://doi.org/10.5109/22895>

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出版情報：九州大学大学院農学研究院紀要. 20 (3/4), pp.125-141, 1976-10. Kyushu University

バージョン：

権利関係：

## Simulation for Activation Process of Pepsinogen

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(Received May 4, 1976)

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 indicated 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 Shimadzu 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 (0-15 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 trichloroacetic 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 characteristic features of the selected scheme with changing the values of kinetic parameters. (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

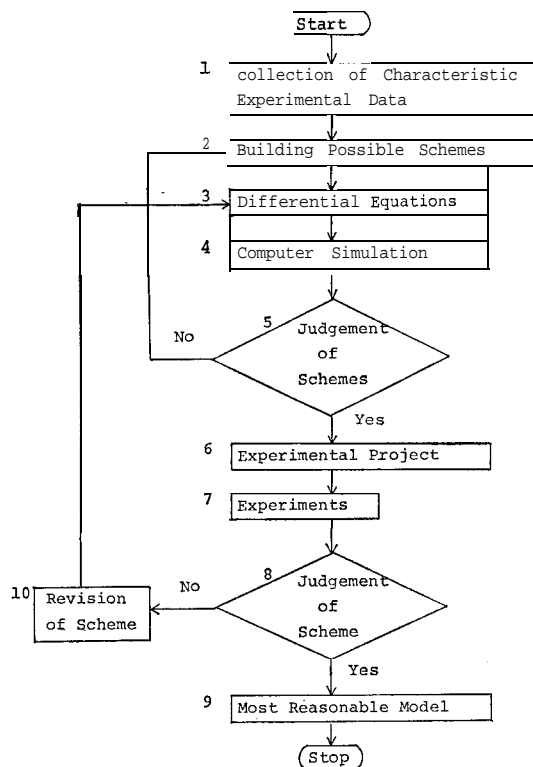
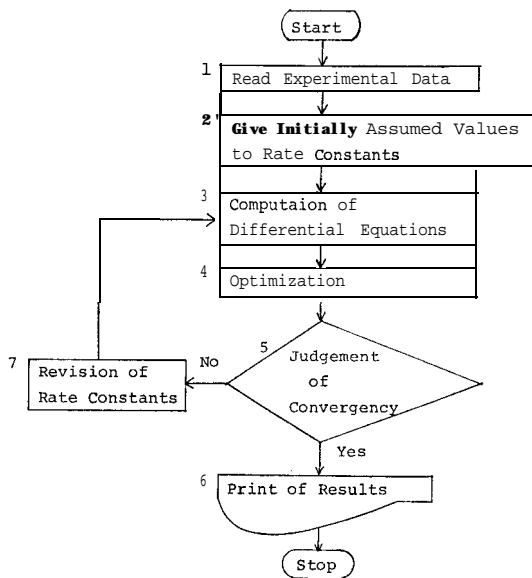


Diagram 1. Flow diagram of procedure of study.

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,

$$\frac{dX_i}{dt} = f(k_1, \dots, k_n, X_1, \dots, X_m) \quad (1)$$

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

$$F = \sum_i \sum_t (X_i(t) - Y_i(t))^2 \quad (2)$$

where  $X_i(t)$  is the calculated concentration of  $i$ -th species at time  $t$  and  $Y_i(t)$  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 differential equation with respect to  $k_j$  ( $j = 1, \dots, n$ ).

$$\frac{\partial F}{\partial k_j} = 0 \quad (3)$$

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









**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 form 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 7-1 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 7-1 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 7-1 (top figure in Fig. 2). This

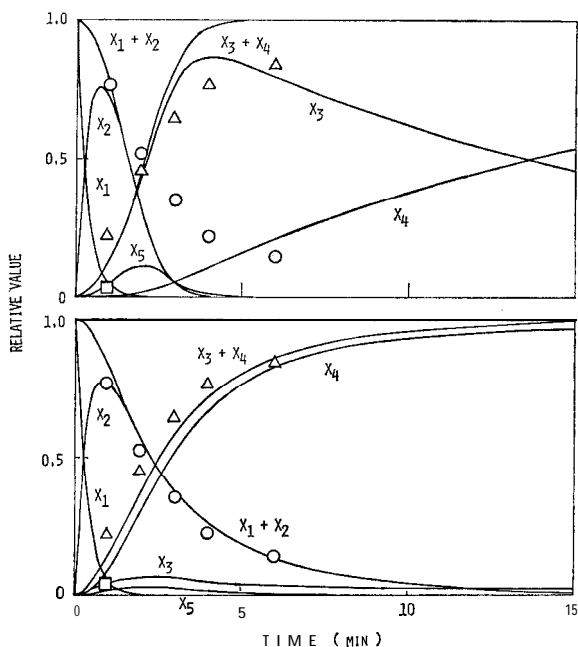
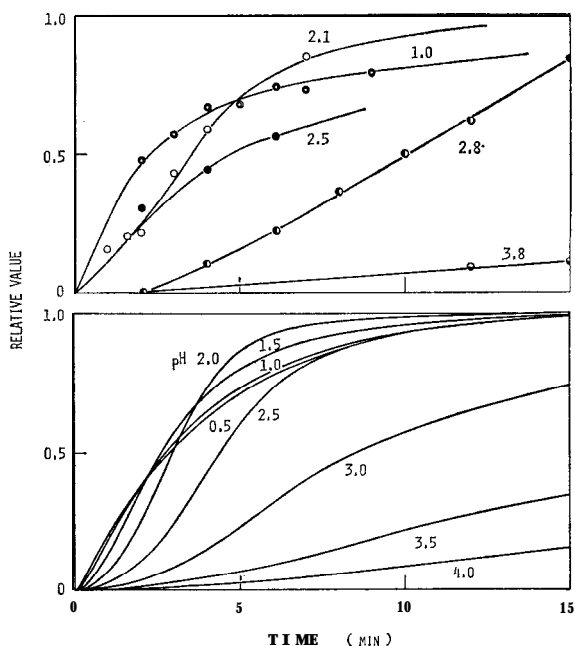


Fig. 2. Comparison of experimental data with simulated time-courses. Top, simulation with Scheme 7-1; bottom, simulation with Scheme 8. Symbols show experimental data by gel electrophoresis at pH 1.5 (HCl), 0°C and initial concentration of pepsinogen,  $IC_1$ , of 0.2 % pepsinogen,  $\Delta$  pepsin,  $\square$  dimeric intermediate. Solid lines are simulated time-courses with Scheme 7-1 (top) assuming that  $k_1 = 0.0526$ ,  $k_2 = 0.0038$ ,  $k_3 = 0.001$ ,  $IC_1k_4 = 0.0422$  and  $k_5 = 0.05$ , and with Scheme 8 (bottom) assuming that  $k_1 = 0.0526$ ,  $k_2 = 0.0038$ ,  $k_3 = 0.0663$ ,  $IC_1k_4 = 0.0422$ ,  $k_5 = 0.05$  and  $k_{-3} = 0.0016$ . Units of parameters are  $\text{sec}^{-1}$ .

fact means that Scheme 7-1 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 7-1. 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  $X_3+X_4$  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  $0^\circ\text{C}$  and  $IC_1 \approx 0.2\%$ . Parameter values are assuming to be ;  $k_1 = 1.66(\text{H})$ ,  $k_2 = 0.0038$ ,  $k_3 = 1.26 \times 10^3(\text{H})$ ,  $k_{-3}$ ,  $IC_1 k_4 = 0.0422$ ,  $k_5 = 0.5$  and  $k_{-5} = 0.0016$ . Units of parameters are  $\text{sec}^{-1}$ . 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 characteristic 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

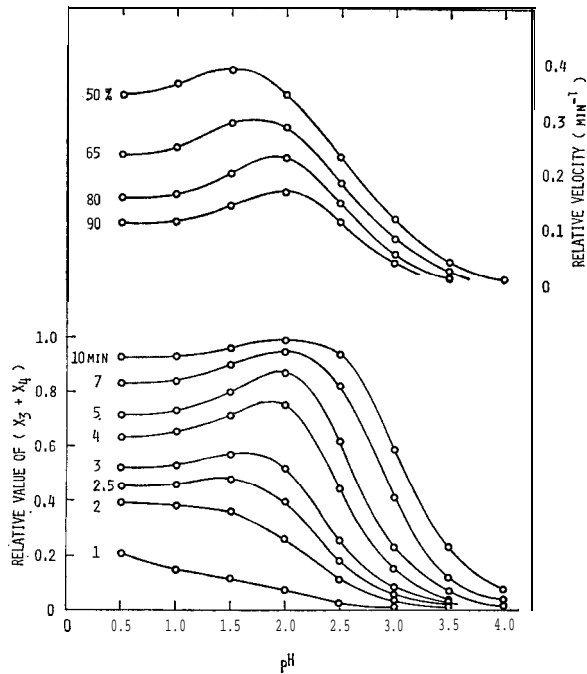


Fig. 4. Simulation with Scheme 8 for pH dependence of activation. Parameters are the same as those in Fig. 3. Top, relative velocities (reciprocal of time required for attaining of  $X_3+X_4$  to specified percentage): bottom, amount of  $X_3+X_4$  at various activation times,

(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.

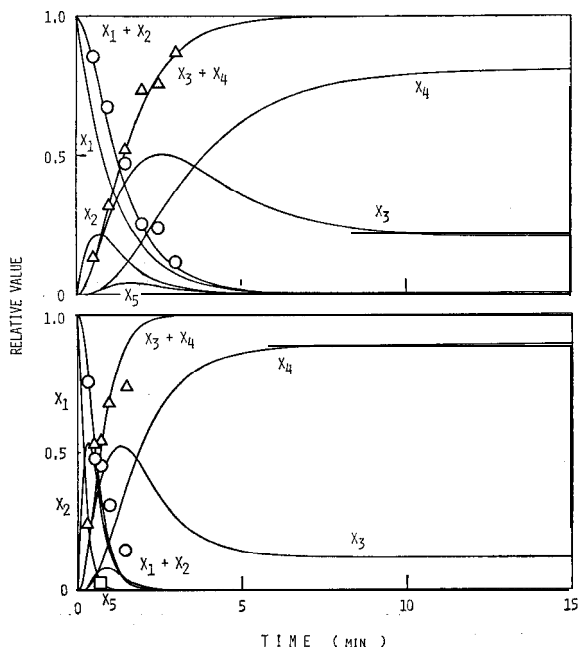


Fig. 5. Temperature dependence of activation time-course. Symbols are experimentally obtained data in 0.05 M citrate buffer, pH 3.0 (top) and pH 2.2 (bottom) at 15°C and  $IC_1 = 0.2\%$ .  $\circ$  pepsinogen,  $\triangle$  pepsin,  $\square$  dimeric intermediate. Solid lines are simulated time-course with Scheme 8 assuming that  $k_1 = 14.4(\text{H})$ ,  $k_2 = 0.03$ ,  $k_3 = 1.26 \times 10^3(\text{H})k_{-3}$ ,  $IC_1 k_4 = 0.042$ ,  $k_5 = 0.05$  and  $k_{-3} = 0.0016$ . Units of parameters are  $\text{sec}^{-1}$ .

#### 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

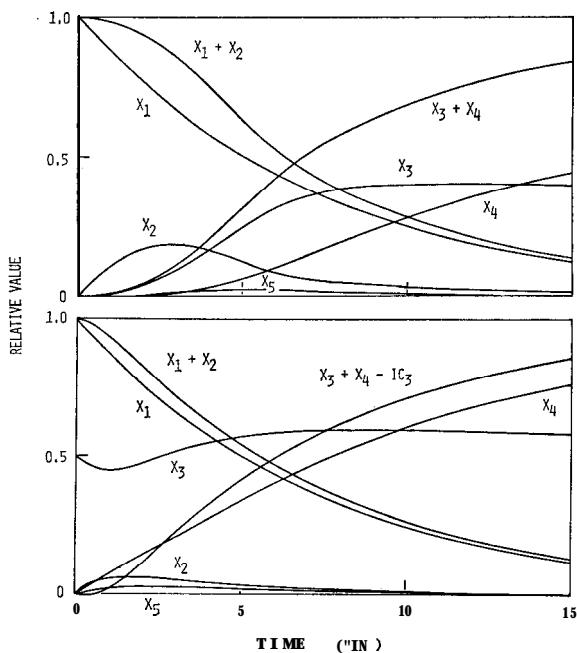


Fig. 6. Simulation with Scheme 8 for effect of added pepsin species  $X_3$  on activation. Conditions for activation are assumed to be pH 2.85 (HCl),  $0^\circ\text{C}$  and  $IC_1 = 0.2\%$  (top) or initial concentrations of  $X_1$  and  $X_3$  are 0.2 and 0.1% respectively (bottom).  $k_1 = 0.0023$ ,  $k_2 = 0.0038$ ,  $k_3 = 0.0023$ ,  $IC_1 k_4 = 0.0422$ ,  $k_5 = 0.05$  and  $k_{-3} = 0.0016$ .  $IC_3$ , initial concentration of  $X_3$ .

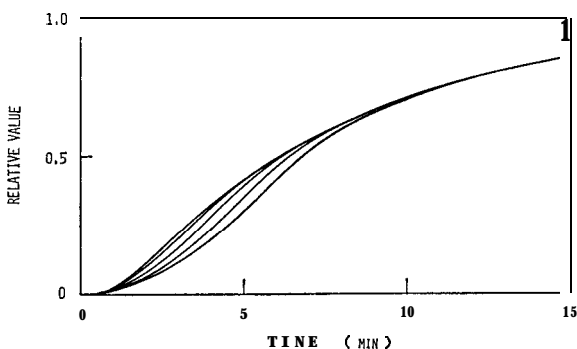
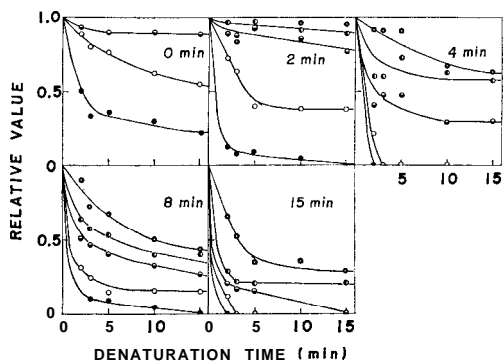
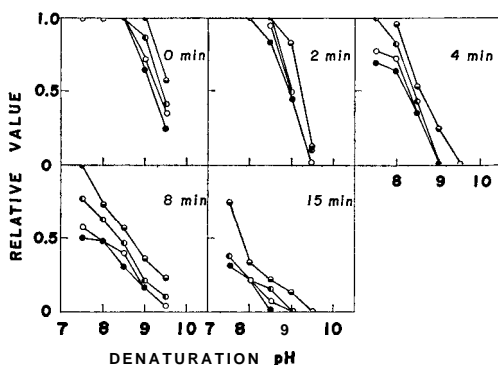


Fig. 7. Simulation with Scheme 8 for effect of initial concentration of  $X_3$  on activation. Solid line indicates time-course of  $X_3+X_4$ .  $IC_3$  are from top to bottom, 0.5, 0.3, 0.2, 0.1 and 0.  $IC_3$  is defined as ratio to  $IC_1$ .

active pepsinogen, alkali-denaturation experiments were carried out. After pepsinogen was activated to several extents at  $0^\circ\text{C}$ , 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 dependence 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  $X_1$ . 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).  $X_3$  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  $X_3$  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 characteristic 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.

The authors wish to thank Dr. T. Torikata, Mrs. T. Yoshizumi, Mr. M. Tanabe and Mr. Y. Shibuya for their technical assistance.

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