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## On the Activation Process of Pepsinogen

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The activation process of pepsinogen was followed by the appearance of peptic activity and the conformational changes measured by difference spectrophotometry. The activation process of pepsinogen followed by peptic activity exhibited characteristic features ; for instance, it showed a clear induction period at low temperature and no sensitivity for a competitive inhibitor isoamyl alcohol. The conformational changes accompanied by the activation to appear behind the appearance of the peptic activity and showed several distinguishable stages. Furthermore, it has been well known that the activation of pepsinogen took place by acidification of the pepsinogen solution without any assistance of other proteolytic enzyme and that the activation mechanism depended upon the pH value of medium used for the activation. Thus, the activation mechanism of pepsinogen has been considered to be of very complex.

In the present experiment, the activation process was simulated by analog computer with postulating several schemes in order to classify the plausible schemes which can explain the observed characteristic features in the activation process of pepsinogen. Then, kinetic parameters in most probable schemes were determined by digital computer, using a nonlinear programming technique.

### INTRODUCTION

Similar to other proteolytic zymogens (Singer *et al.*, 1968; Wright *et al.*, 1968), pepsinogen has been known to release a peptide segment during its conversion to the active pepsin molecule (Herriott, 1962; van Vanakis and Herriott, 1957 ; Ong and Perlmann, 1968). The mode of activation, however, has been recognized to be different from those of most proteolytic zymogens ; pepsinogen converts spontaneously or autocatalytically in acid medium without any assistance of other active proteolytic enzyme, while most of proteolytic zymogens needs the assistance of active proteolytic enzyme to release a peptide segment in the process of their conversions to active enzymes.

Such a unique process of the activation of pepsinogen has attracted the interests of many investigators and the studies were focused on the molecular mechanism by which pepsinogen converts to the active pepsin (Bovey and Yanari, 1960). The early kinetic study of the pepsinogen activation was made by Herriott (1938). He reported that at pH 4.6 the rate of the activation had the second-order rate constant and the activation proceeded autocatalytically, i. e., the produced active pepsin accelerated the rate of activation. Additional experi-

mental results supporting the activation mechanism proposed by Herriott have been reported by several investigators (Varandani and Shlamowitz, 1963; Wang and Edelman, 1971). Contrary to above, it has been reported that the monomolecular activation of pepsinogen also occurred under certain conditions (Busting and Conway-Jacobs, 1971; Al-Janabi *et al.*, 1972). Busting and Conway-Jacobs observed that at pH 1.8 and 37°C, and at pH 3.0 and 23°C, added pepsin to the pepsinogen solution did not accelerate the rate of the activation of pepsinogen. Thus, the experimental results obtained so far indicated that there are at least two types of the activation mechanism; one is observable on the activation in low pH region and the other in a considerably high pH region. Al-Janabi *et al.* (1972) studied the effect of pH value on the activation of pepsinogen and found that at pH 4 bimolecular activation of pepsinogen with the pepsin molecule and intermolecular activation between the pepsinogen molecules (second-order rate constant) were simultaneously taken place, and below pH 3 intramolecular activation (first-order rate constant) was predominantly occurred. The intramolecular activation was also demonstrated by Busting and Conway-Jacobs (1971). They studied the activation of Sepharose-bound pepsinogen and found that the potential activity of Sepharose-bound pepsinogen was independent of the average amount of pepsinogen bound per gram of Sepharose. This may suggest that the activation process is not caused by neighboring molecules mutually activating.

The activation process has been followed by two main techniques : One is based upon the appearance of peptic activity due to the pepsinogen-pepsin conversion, and the other based upon the conformational change of the enzyme molecule due to the conversion. The appearance of peptic activity was followed by the chemical (for example, Busting and Conway-Jacobs, 1971) or physico-chemical (for example, Al-Janabi *et al.*, 1972) estimation of hydrolytic extent of a substrate which was concomitantly incubated with pepsinogen on the activation. The conformational change due to the conversion has been followed by spectrophotometry, such as UV-spectrum (McPhie, 1972) or fluorometry (Wang and Edelman, 1971), and chemical method such as quantitative analysis of newly formed N-terminal amino acid of the pepsin molecule (Funatsu *et al.*, 1971).

It has been recognized that the change in the fluorescence spectrum on the activation process was detectable prior to the increase in the peptic activity (Bohak, 1973) and that the change in UV-spectrum became observable behind the appearance of the peptic activity.

In general, the activation process followed by the appearance of peptic activity may show considerably simple features, because each activation step not directly connecting to the activity will be rounded and in most extreme case, the activation process represents only a limited step in the successive activation steps. In contrary, the activation process followed by conformational change seems to show detailed features of the conversion. In fact, the activation process followed by physico-chemical method such as spectrophotometry has been known to exhibit a complex pattern. This indicates that the all events included in the pepsinogen-pepsin conversion are very complex and that it may be difficult task to establish a clear scheme or a detailed molecular mechanism in the activation of pepsinogen.

In previous reports, the authors reported that (1) the pepsinogen molecule does not contain peptide bonds susceptible to 0.1-0.01 M hydrogen ion (below pH 2), (2) competitive inhibitor isoamyl alcohol (Neumann and Shinitzky, 1971) did not delayed the appearance of peptic activity but the formation of new N-terminal of the pepsin molecule and (3) from the experimental results, it was concluded that the inert pepsinogen molecule first forms the active site in its molecule without the release of peptide segment on the activation process by acidification of the zymogen solution, and following steps proceed successively with triggering by the intermolecular or intramolecular hydrolytic action of the formed active site (Funatsu et *al.*, 1971; Funatsu et *al.*, 1972).

Nevertheless the comprehensive studies on kinetics of the activation process have been done, the molecular mechanism of the activation seems to remain unsolved. The present article deals mainly with the kinetical analysis, computer simulation and parameter estimation, of the activation process of pepsinogen at low pH and low temperature, directing to accumulate the information about the molecular mechanism of the activation.

## EXPERIMENTAL

### Materials

Pepsinogen was isolated from swine fundic mucosa by the method of Vanakis and Herriott (1956) and purified by DEAE-cellulose column chromatography according to the report of Ryle (1960). Hemoglobin was isolated from horse blood and recrystallized several times before use. The substrate carbobenz-oxy glycyl-L-phenylalanine was synthesized by carbodiimide coupling method. Isoamyl alcohol and other reagents were of reagent grade and used without further purification.

### Methods

#### *Activation of pepsinogen*

To aqueous solution of pepsinogen at concentration of **0.02-0.25 %** preincubated at given temperature, 0.3 N hydrochloric acid was added to desired pH value to start the activation.

#### *Measurement of peptic activity*

One ml of incubation mixture (final concentration: **0.02-0.04 %**) was added to 2 ml of 0.6 % hemoglobin solution at pH 2.5 and 15°C. After 1 min incubation, 2 ml of 0.44 M trichloroacetic acid was added. The optical density at 275 nm of the supernatant was measured and this value was used as the relative peptic activity.

#### *Difference spectrum*

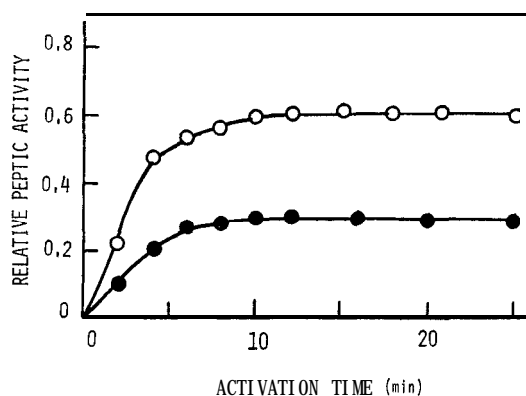
A Beckman DB spectrophotometer with thermostatable cell holder was used throughout the experiment. In reference cell, 3 ml of 0.25 % aqueous solution of pepsinogen and **0.43** ml of water were filled. In sample cell, 3 ml of 0.25 %

aqueous solution of pepsinogen was placed and the activation was started by addition of 0.43 ml of 0.3 N hydrochloric acid. The blue-shift difference spectrum of this system was punctually recorded in the range from 260 to 320nm.

## RESULTS

### Time-course of activation at 15°C

The time-course of the activation of pepsinogen at pH 2.2 and 15°C followed by the appearance of peptic activity is shown in Fig. 1. The activity attained a certain maximum value within 10 min independently of the initial concentration of pepsinogen. The data in Fig. 1 were not corrected for the activation during 1 min incubation on the assay of the activity at the same pH and same temperature.



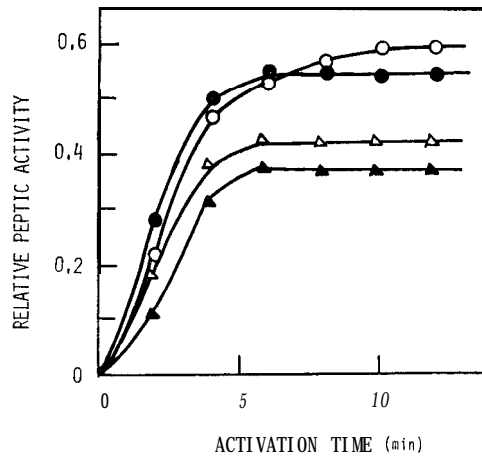
**Fig. 1.** Activation of pepsinogen incubated at 15°C and pH 2.2. The concentrations of pepsinogen solution were 0.02 % (●) and 0.04 % (○).

### Effect of isoamyl alcohol

The effects of isoamyl alcohol that has been known to be a competitive inhibitor of pepsin action are shown in Fig. 2. Although the presence of isoamyl alcohol did not change the time needed for the full activation, it inhibited the peptic activity in proportion to the inhibitor concentration. The inhibitor at low concentration such as 0.63 % accelerated rather the rate of the activation process, in contrast to the observation of Neumann and Shinitzky (1971) that aliphatic normal alcohols inhibited the activation of pepsinogen when they were incubated previously with pepsinogen around pH 8 and above 30°C. The mode of the effect of isoamyl alcohol may be an important factor for the postulation of the activation scheme.

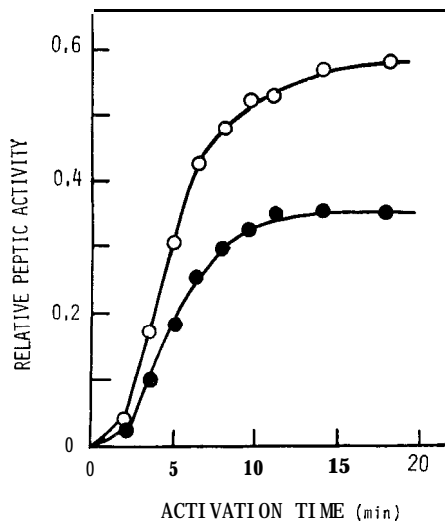
### Activation at low temperature

In activation at 15°C, the detailed features of the early stages of the activation were not observed because of a great rate of the activation reaction. The activation at pH 2.5 and near 0°C (in ice bath) showed a clear induction period



**Fig. 2.** Activation of pepsinogen incubated at 15°C and pH 2.2 in the presence of inhibitor isoamyl alcohol. The concentration of pepsinogen solution was 0.04 % and inhibitor concentrations were 0 % (○), 0.63 % (●), 1.25 % (△) and 2.5 % (▲).

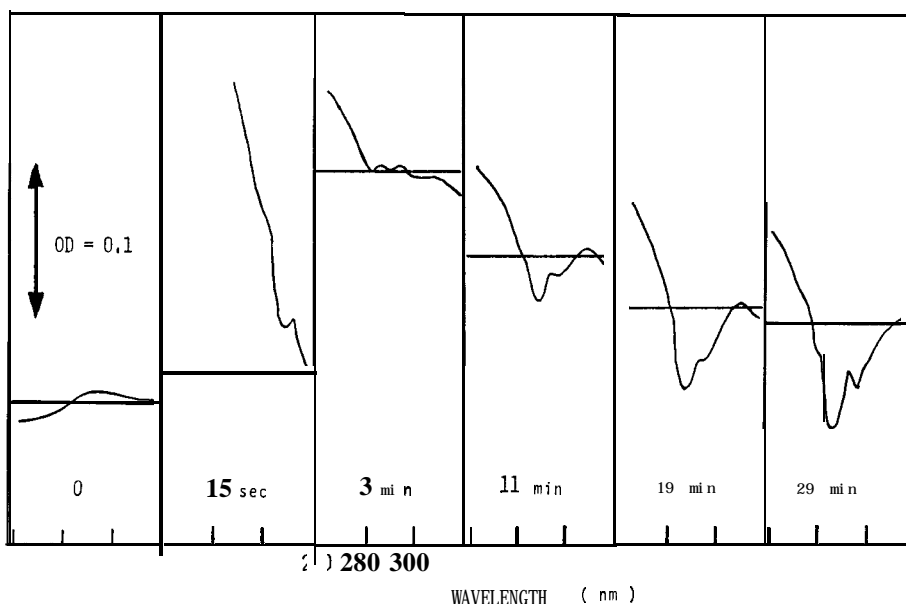
in the early stage as shown in Fig. 3. Since the curves in Fig. 3 were also not corrected for the additional activation occurring during the incubation on peptic activity assay, the true induction period must be much longer than that seen in the curves in Fig. 3. Thus, the appearance of induction period appears to be most distinctive features of the activation of pepsinogen.



**Fig. 3.** Activation of pepsinogen incubated at 0°C and pH 2.5. The concentrations of pepsinogen solution were 0.02 % (●) and 0.04 % (○).

### Difference spectrum in activation process

The difference spectrum in the activation process at 4°C and pH 2.5 is shown in Fig. 4. Initial base-line was recorded with placing 0.25 % pepsinogen solution in both reference and sample cells. The difference spectrum was suddenly changed to irregular one by the addition of 0.3 N hydrochloric acid to aqueous solution of pepsinogen in the cell of sample compartment, and simultaneously the base-line shifted greatly upward. After 3 min incubation, a great change in the difference spectrum ceased and the shape recovered normal. Thereafter, the base-line lowered gradually and trough of negative peaks increased with time. After 10 min incubation, the difference spectra showed a typical pattern of the blue-shift with the main negative peak at 287 nm. After 40 min incubation, the base-line leveled down to that recorded at the zero time.

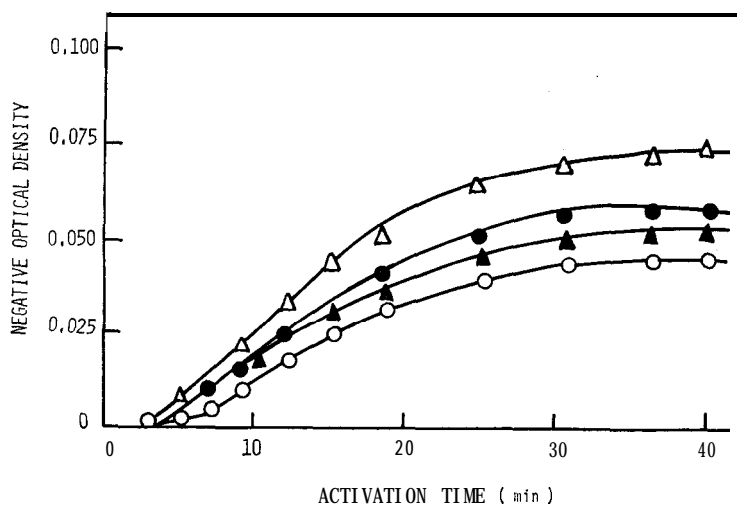


**Fig. 4.** Difference spectra in the activation process of pepsinogen at 4°C and pH 2.5.

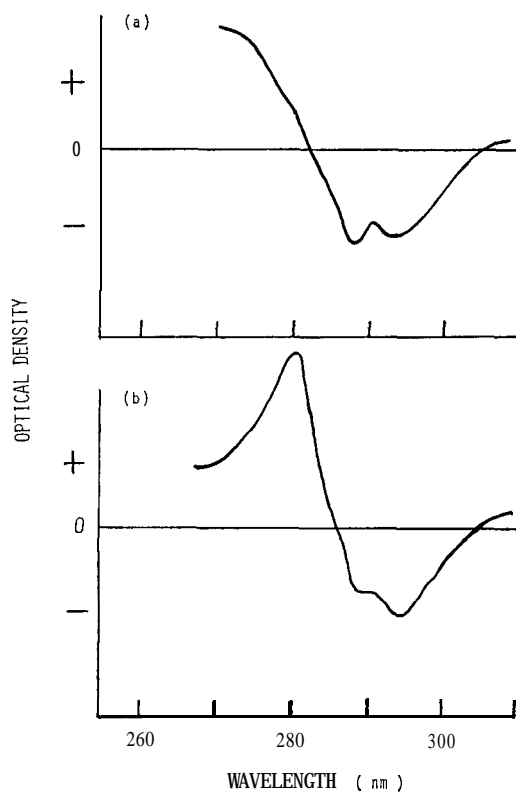
The negative intensities of peaks at 287 and 293 nm measured from each base-line are plotted as a function of incubation time in Fig. 5, except for those observed in the early stage of incubation. Similar plottings are done for the activation system which contained 1.5 % isoamyl alcohol. In this case, the intensity of negative peak decreased considerably. All curves in Fig. 5 show a lag time of induction period of about 4 min.

### Difference spectrum in the presence of synthetic substrate

Typical pattern of difference spectrum observed in the activation process of pepsinogen, in the presence of synthetic substrate carbobenzoxy glycyl-L-phenyl-alanine (0.012 %), is shown in Fig. 6. The activation was carried out at 15°C



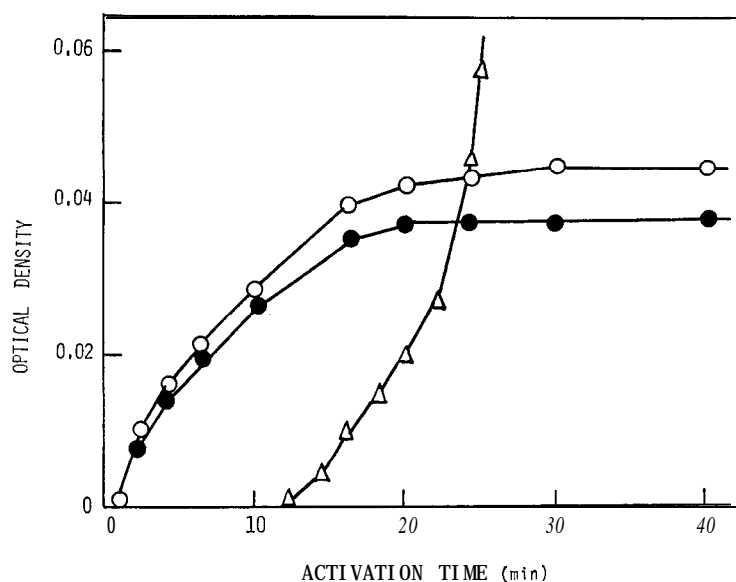
**Fig. 5.** Change in the intensity of difference spectrum in the activation process.  $\triangle$ : Intensity at 287 nm without inhibitor,  $\blacktriangle$ : Intensity at 293 nm without inhibitor,  $\circ$ : Intensity at 287 nm in the presence of 1.5 % inhibitor and  $\bullet$ : Intensity at 293 nm in the presence of 1.5 % inhibitor. Activation was conducted at 4°C and pH 2.5



**Fig. 6.** Difference spectrum in the absence (a) and in the presence (b) of substrate. Incubated at 15°C and pH 2.2 for 30 min.

and pH

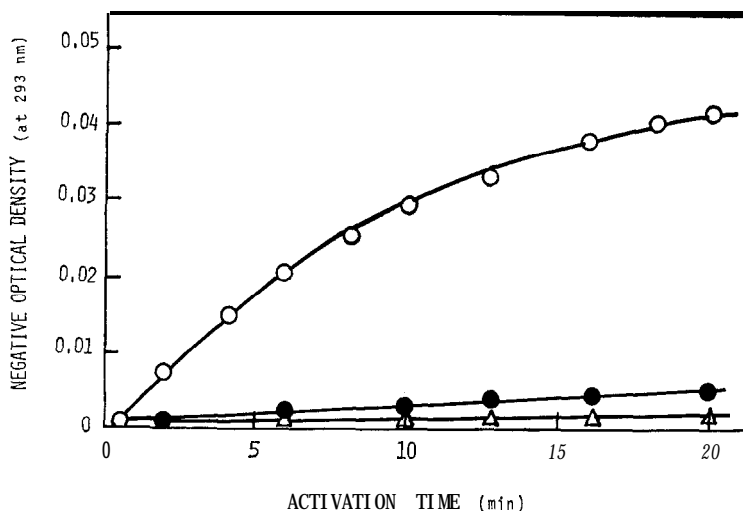
2            8            0



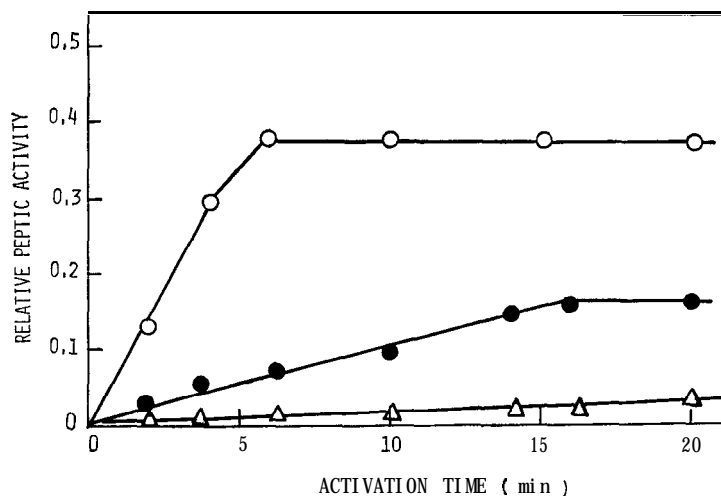
**Fig. 7.** Effect of substrate on activation. ○: Negative intensity at 293 nm in the absence of substrate, ●: Negative intensity at 293 nm in the presence of substrate and △: Positive intensity at 280 nm in the presence of substrate. Incubated at 15°C and pH 2.2.

#### pH-Dependence of activation

The pH-dependence of the negative intensity (optical density) of the difference spectrum,  $-\Delta OD$ , in the activation process is shown in Fig. 8. After 20 min incubation, the negative intensity at 293 nm,  $-\Delta OD_{293}$ , at pH 4.7 and 5.0 are 15 and 5 % respectively of that observed at pH 2.2. The activity in the activation process shows a similar pH-dependence as shown in Fig. 9. After 20



**Fig. 8.** pH-Dependence of activation followed by difference spectrum. ○ : pH 2.2, ● : pH 4.6 and ▲ : pH 5.0. Incubated at 15°C.



**Fig. 9.** Effect of pH on activation followed by activity at 15°C. ○ : pH 2.8, ● : pH 3.6 and △ : pH 5.0.

min incubation, the activities measured at pH 5.0 is about 10 % of that observed at pH 2.8. However, the activation followed by the appearance of the activity was completed after 6 min incubation at pH 2.8 and 15 min at pH 3.6. The peptic activity was measured with the substrate solution at the same pH value as that of medium in the activation.

#### Activation and conformational change

The appearance of the peptic activity and the intensities at the peaks in the

difference spectra are summarized in Fig. 10, in order to make easily the comparison between them. The activation process may roughly be divided into six stages. A-stage shows neither the appearance of the activity nor the visible intensity of difference spectrum; induction period of 3 min is observed. At the end of B-stage, the activation followed by the peptic activity is nearly completed, although the difference spectrum is gradually increasing with the incubation time. C-stage is corresponding to that of rapid increase in intensity of the difference spectrum, where the activation followed by peptic activity becomes complete. The synthetic substrate present in the incubation mixture may be decomposed gradually in the D-stage. At E-stage, the change in the intensity of difference spectrum becomes moderate, and contrarily the intensity of positive peak as 280 nm due to the decomposition of the substrate becomes great. There are little changes in the difference spectrum at the F-stage. All activation events finished at the F-stage. The following changes would be arisen from the autolysis of the formed pepsin molecule on an elongated incubation.

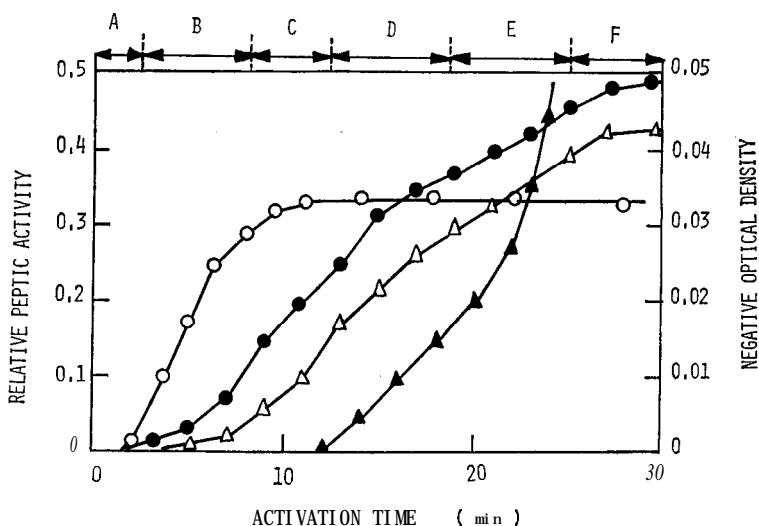


Fig. 10. Comparison between appearances of activity and difference spectrum. For measurement of activity, activation was carried out at 0°C and pH 2.5, and for the difference spectrum, activation was carried out at 4°C and pH 2.5, ○: Activity, ●: Negative intensity at 293 in the absence of inhibitor, △: Negative intensity at 293 nm in the presence of inhibitor and ▲: Positive intensity at 280 nm in the presence of substrate.

## SIMULATION

### Assumed schemes for activation process

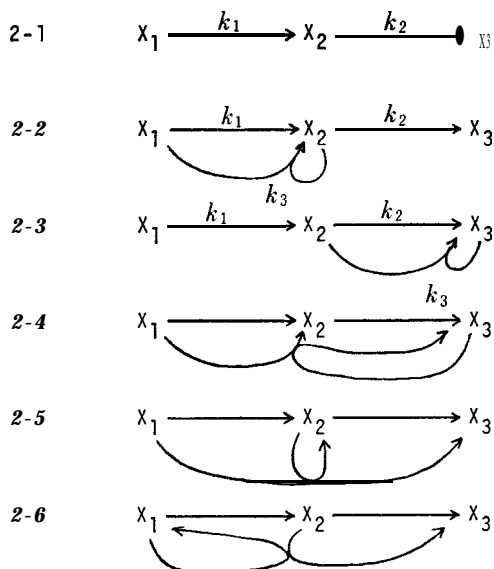
From experimental results obtained so far, the characteristic features of the activation process of pepsinogen can be summarized as follows: (1) The activation process followed by the appearance of peptic activity preceded that followed by spectrophotometry and that followed by the detection of newly formed

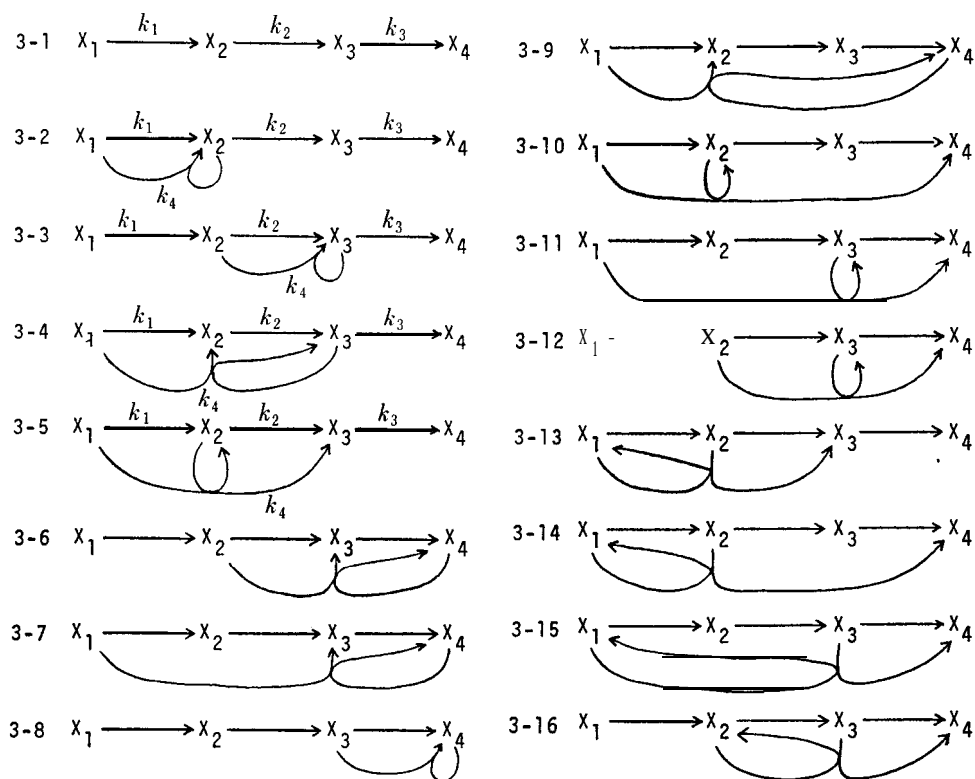
N-terminal of the pepsin molecule. This would indicate that some of the intermediates in the activation process show the peptic activity. (2) There was distinctive induction period in the activation reaction at low temperature, whereas it disappeared in the activation at considerably high temperature such as 15°C. (3) Although, under the present experimental conditions, the autocatalytic activation in which the product pepsin accelerates the rate of activation was not observed, the activation curves exhibited typical self-multiplicative mode. This fact suggests that the multiplication conducted by an intermediate would be taken into consideration on the scheme for the activation. (4) Isoamyl alcohol which has been known as a competitive inhibitor for the peptic activity did not affect the rate of activation except that low concentration of the alcohol rather accelerated the activation to a small extent.

A scheme of the activation, which can explain above findings and kinetic behaviors described in Introduction, seems to be not simple one. However, it is not likely that only very specific scheme can explain basically such a complexity, i. e., there must be a possibility that some types of scheme will be able to fit in qualitatively the real activation process of pepsinogen.

Simulation of the activation process of pepsinogen was conducted with intent to search the basic types of scheme which are qualitatively fitting to the real activation process and to design the succeeding experiments on the molecular mechanism of the activation of pepsinogen. Assumed schemes for simulation are listed in Tables 1, 2 and 3. In tables,  $X_1$  represents the pepsinogen molecule,  $X$  with largest suffix number represents the pepsin molecule and others intermediates. The loop means autocatalytic process. The  $X$  with its own loop potentiates only the coupled reaction similarly to the action of grid-voltage in an

**Table 1.** Assumed two-step schemes for simulation. Loop on one intermediate means that there is no mass change in respect with that intermediate.



**Table 2.** Assumed three-step schemes for simulation.

amplifier circuit. In Table 3, asterisk indicates dimeric intermediate, I represents isoamyl alcohol and XI the inhibitor-pepsin complex or inhibitor-intermediate complex. Scheme I-4 is dissolved into two schemes for simplicity. In these schemes, it is assumed that product  $X_m$  and intermediate  $X_{m-1}$  are to have mutually the same peptic activity and that in the reaction of  $X_i \rightarrow X_m$  the main conformational change which reflects on spectrophotometric behavior is observable, where suffix *m* means the largest number in each scheme.

#### Simulation with analog computer

Simulation with analog computer (A Hitachi analog computer Model ALS-220) was carried out to see whether each assumed scheme is basically fitting to the observed time-course of the real activation process or not.

#### Differential equation

Prior to drawing the set-up diagram, it is necessary to derive the differential equations corresponding to each scheme. For example, differential equations corresponding to Scheme 3-10 may be derived in nonlinear simultaneous form as follows :

**I-1**

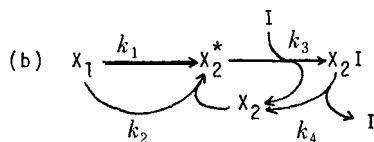
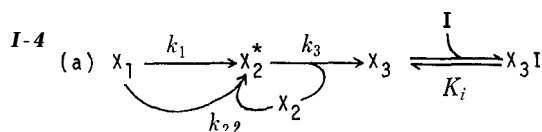
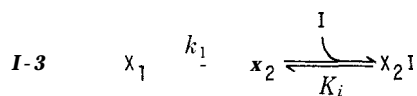
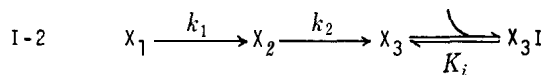
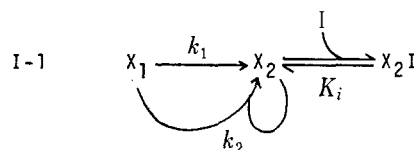
$x_1 \xrightarrow{k_1} x_2 \xrightleftharpoons[K_i]{1} x_2$

**I-2**

$x_1 \xrightarrow{k_1} x_2 \xrightarrow{k_2} x_3 \xrightleftharpoons[K_i]{1} x_3$

**I-3**

$x_1 \xrightarrow{k_1} x_2 \xrightleftharpoons[K_i]{1} x_2$



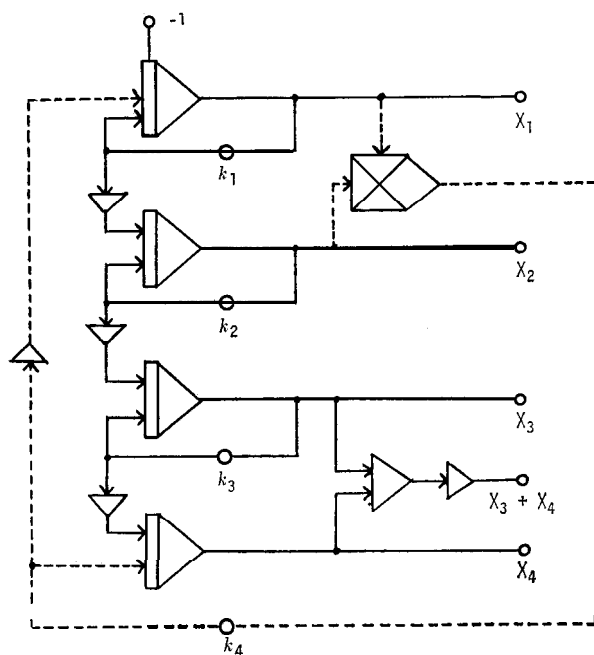
$$\begin{aligned}\dot{X}_1 &= -k_1 X_1 - k_4 X_1 X_2 \\ \dot{X}_2 &= k_1 X_1 - k_2 X_2 \\ \dot{X}_3 &= k_2 X_2 - k_3 X_3 \\ \dot{X}_4 &= k_3 X_3 + k_4 X_1 X_2\end{aligned}\quad (1)$$

Initial conditions are  $X_1(0) = 1$ ,  $X_2(0) = X_3(0) = X_4(0) = 0$ . The initial concentration of pepsinogen was reduced to unity. The corresponding integral forms are written as:

$$\left. \begin{aligned} X_1 &= 1 - k_1 \int_0^t X_1 dt - k_4 \int_0^t X_1 X_2 dt \\ X_2 &= k_1 \int_0^t X_1 dt - k_2 \int_0^t X_2 dt \\ X_3 &= k_2 \int_0^t X_2 dt - k_3 \int_0^t X_3 dt \\ X_4 &= k_3 \int_0^t X_3 dt + k_4 \int_0^t X_1 X_2 dt \end{aligned} \right| \quad (2)$$

**Set-up diagram**

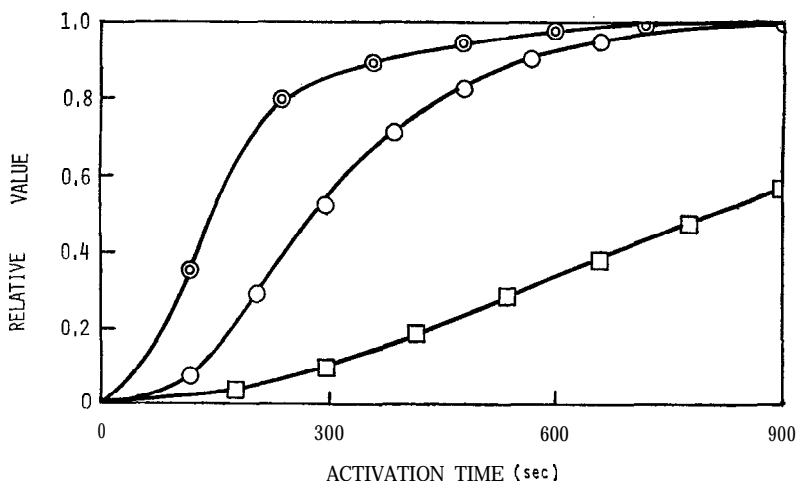
Set-up diagram directly applicable for patching on the computer is easily drawn from the integral forms. Set-up diagram for Scheme 3-10 can be easily obtained from the equation (2) as shown in Fig. 11. The output  $X_1$  represents the amount of remained pepsinogen. The sum of  $X_3$  and  $X_4$  represents the peptic activity and change in  $X_4$  corresponds to the conformational change or the amount of formed pepsin molecule in the activation process. The  $k_1, k_2, k_3$  and  $k_4$  are readings of potentiometers and are the rate constants of the steps of Scheme 3-10. In computation, the initial concentration of pepsinogen was reduced to the initial value  $X_1(0) = 1$  by scale transform.



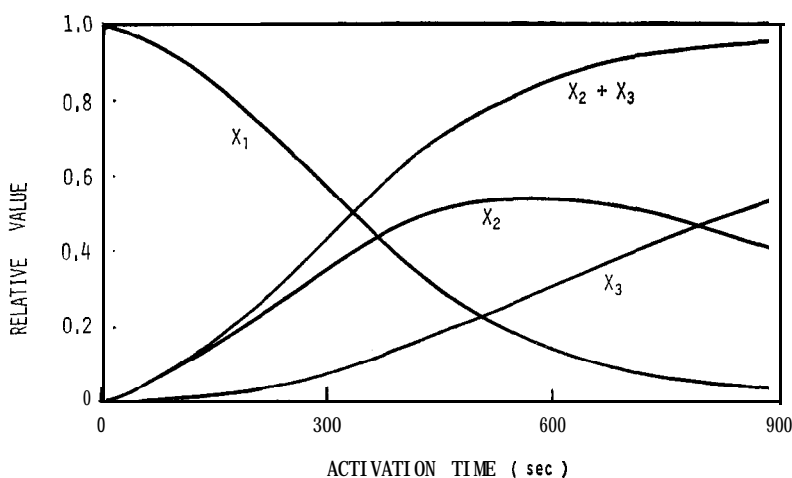
**Fig. 11.** Set-up diagram for analog simulation (Scheme 3-10).

**Results of simulation**

The set-up diagram corresponding to each scheme was drawn and computation was performed with changing the values of potentiometers until the values of  $X_1 + X_2$  (activity) and  $X_4$  (conformational change) fit in best with the experimental results transcribed in Fig. 12. Figs. 13 to 17 show the examples of the scheme which can basically explain the real activation process of pepsinogen. Two-step scheme with autocatalysis by  $X_2$  intermediate is one of the plausible scheme as shown in Fig. 13. Direct pathway  $X_1 \rightarrow X_2$  has a small rate constant and probably causes the induction period in an early stage of the activation. Simple three-step scheme also fits to experimental results as shown in Fig. 14, whereas simple two-step scheme fails to explain clearly the induction period. Three-step scheme with multiplicative loop at first or second step is also a pos-



**Fig. 12.** Transcribed experimental results. ○: Activity appearance at 0°C, ⊙: Activity appearance at 15°C and □: Negative intensity at 293 nm at 4°C.



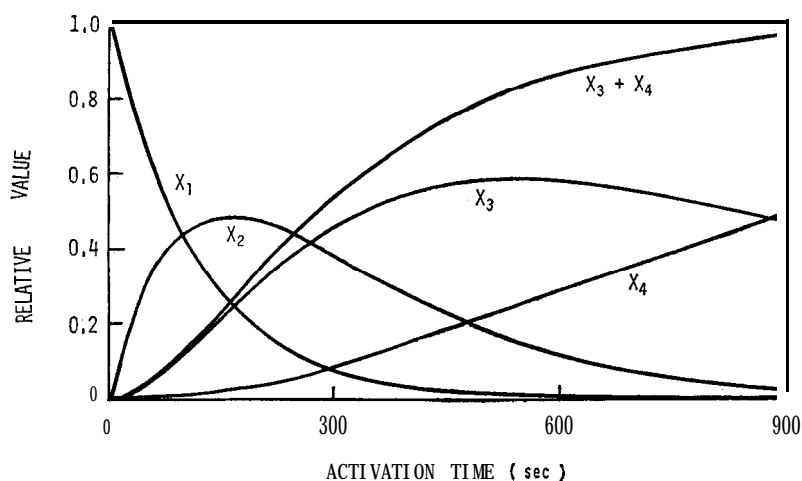
**Fig. 13.** Simulated time-course for Scheme 2-3.

sible one as shown in Figs. 15 and 16, while that with the loop at third step cannot be. Three-step scheme with complicated loop may be possible one as shown in Fig. 17. In this case, direct conversion of  $X_2$  to  $X_3$  is not necessarily required, though in practice the step of  $X_2 \rightarrow X_3$  would have, at least, a small rate constant. Three-step scheme with the loop at third step cannot fit to the experimental results if the rate constant of the looped step is assumed to be  $1.0 \times 10^{-1} \text{ M}^{-1} \text{ sec}^{-1}$  (see Fig. 13). The lowering of the rate constant at looped step tends to approach to the experimental results as shown in Figs. 19 and 20. However, since this scheme can be reduced to simple three-step scheme if small rate constant of the looped step is chosen, this scheme appears to be not plausible.

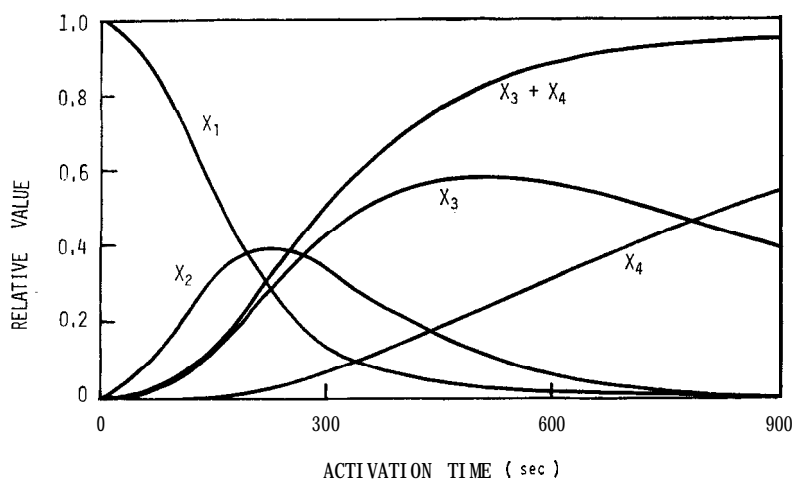
one. The kinetic parameters of basically plausible schemes thus determined are listed in Table 4.

**Table 4.** Kinetic parameters of assumed schemes at 0°C and pH 2.5.

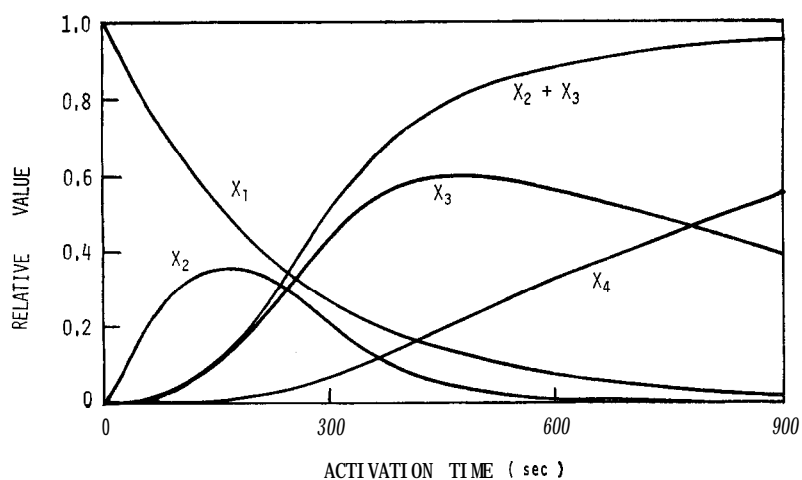
Schem	$k_1(\text{sec}^{-1})10^{-3}$	$k_2(\text{sec}^{-1})10^{-3}$	$k_3(\text{sec}^{-1})10^{-3}$	$k_2(\text{M}^{-1}\text{sec}^{-1})10^{-3}$	$k_4(\text{M}^{-1}\text{sec}^{-1})10^{-3}$
2-2	0.70		1.7	8.0	—
3-1	8.9	6.9	1.7		2.6
3-2	1.4	2.3	1.6	—	2.54
3-3	2.5	5.9	1.7	—	10
3-4			1.6	—	29.0
3-5	0.1				



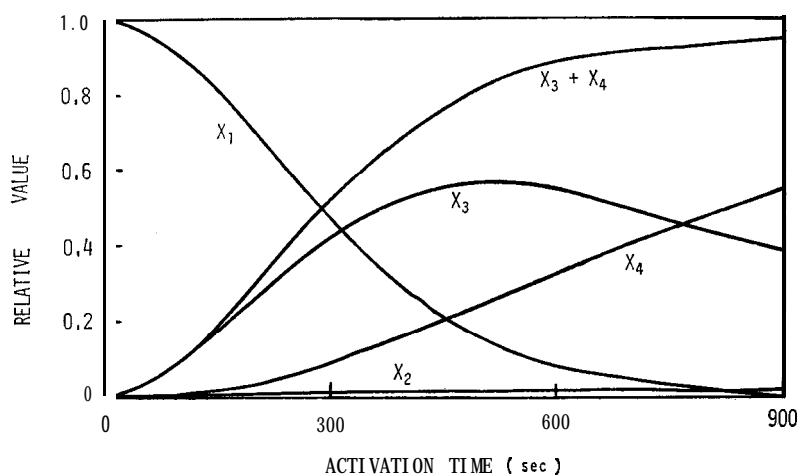
**Fig. 14.** Simulated time-course for Scheme 3-1.



**Fig. 15** Simulated time-course for Scheme 3-2.



**Fig. 16.** Simulated time-course for Scheme 3-3.



**Fig. 17.** Simulated time-course for Scheme 3-5.

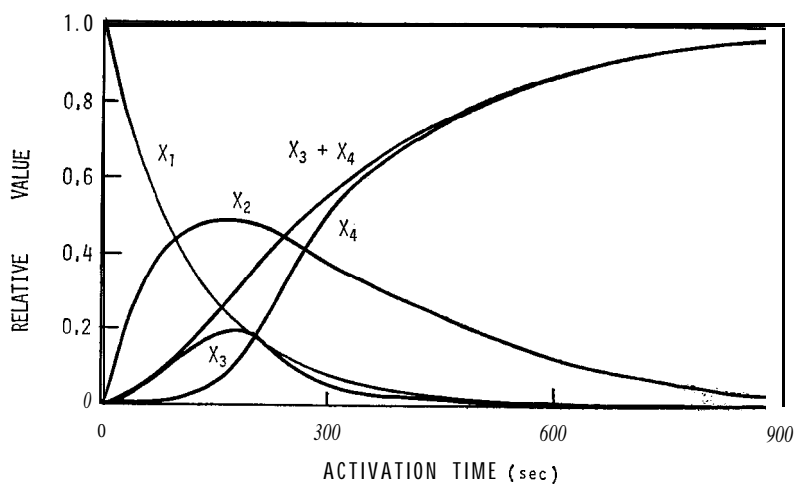


Fig. 18. Simulated time-course for Scheme 3-8 with  $k_4=1.0 \times 10^{-1} \text{M}^{-1} \text{sec}^{-1}$ .

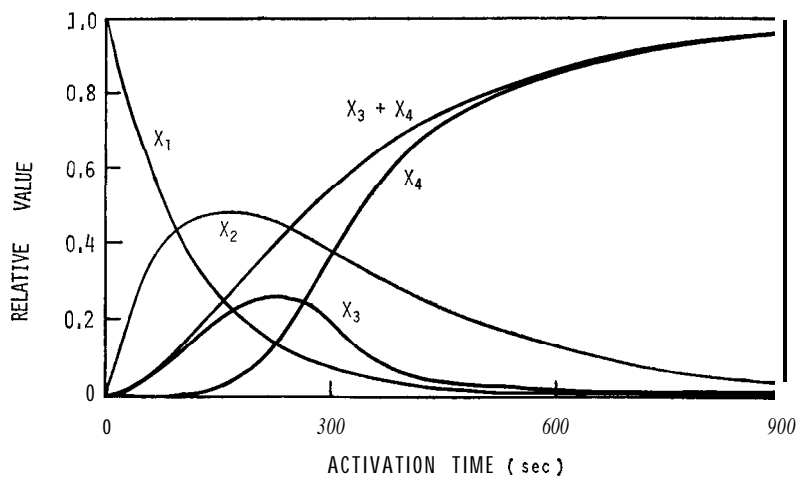
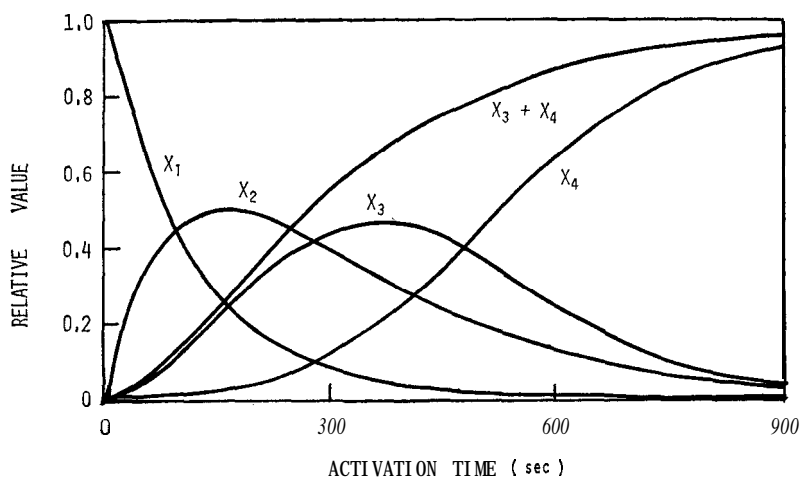


Fig. 19. Simulated time-course for Scheme 3-8 with  $k_4=5.0 \times 10^{-2} \text{M}^{-1} \text{sec}^{-1}$ .



**Fig. 20.** Simulated time-course for Scheme 3-8 with  $k_4=1.0 \times 10^{-2} \text{ M}^{-1} \text{ sec}^{-1}$ .

### Simulation with digital computer

The time-course of the activation of pepsinogen in the presence of isoamyl alcohol, followed by the appearance of peptic activity, was simulated by digital computer (FACOM 230-60) using assumed Schemes I-1, -2 and -3 listed in Table 3.

#### *Solution of differential equation*

The differential equation for Scheme I-1 may be written as:

$$\begin{aligned}
 \dot{X} &= k_1(X_1(0) - X) + k_2(X_1(0) - X) \frac{X}{1 + K_1 I} \\
 &= -\frac{k_2}{(1 + K_1 I)} (X - X_1(0)) \left\{ X + \frac{k_1}{k_2} (1 + K_1 I) \right\} \\
 &= -\frac{k_2}{(1 + K_1 I)} (X - \alpha) (X + \beta)
 \end{aligned} \tag{3}$$

where  $K_1$  is association constant of the system of enzyme + inhibitor-z complex,  $X_1(0)$  is the initial concentration of pepsinogen,  $X/(1 + K_1 I)$  represents the amount of free pepsin or relative peptic activity,  $X = X_2 + X_2 I$ ,  $\alpha = X_1(0)$  and  $\beta = \frac{k_1}{k_2} (1 + K_1 I)$ . Solving the equation (3),

$$X = \frac{\alpha \beta \left[ 1 - \exp \left\{ -(\alpha + \beta) \frac{k_2 t}{(1 + K_1 I)} \right\} \right]}{\beta + \alpha \exp \left\{ -(\alpha + \beta) \frac{k_2 t}{(1 + K_1 I)} \right\}} \tag{4}$$

is obtained. The ratio  $R(t)$  of free pepsin to  $X_1(0)$  is

$$R(t) = \frac{X}{X_1(0)(1+K_1I)} = \frac{k_1 \left[ 1 - \exp\left\{ -\frac{k_1(\alpha+\beta)t}{\beta} \right\} \right]}{k_2 \left[ \beta + \alpha \exp\left\{ -\frac{k_1(\alpha+\beta)t}{\beta} \right\} \right]} \quad (5)$$

The changes in the ratio with the reaction time are written as:

$$\dot{R}(t) = \frac{k_1 \frac{(\alpha+\beta)^2}{\beta} \exp(-A(t))}{k_2 \beta^2 + 2\alpha\beta \exp(-A(t)) + \alpha^2 \exp(-2A(t))} \quad (6)$$

$$\ddot{R}(t) = \frac{k_1 \frac{-(\alpha+\beta)^3 k_1^2 \exp(-A(t)) \left[ 1 - \frac{\alpha^2}{\beta^2} \exp(-2A(t)) \right]}{[\beta + \alpha \exp(-A(t))]^3}} \quad (7)$$

where  $A(t) = \{k_1(\alpha+\beta)t\}/\beta$ .

Putting  $\ddot{R}(t) = 0$ ,

$$1 - \frac{\alpha^2}{\beta^2} \exp\left\{ -\frac{2k_1(\alpha+\beta)t}{\beta} \right\} = 0 \quad (8)$$

is obtained. By rearrangement with  $t$ ,

$$t_{inf} = \frac{\beta}{k_1(\alpha+\beta)} \ln\left(\frac{\alpha}{\beta}\right) \quad (9)$$

is obtained. The equation (9) gives the time  $t_{inf}$  corresponding to the inflection point of the activation curves.

For Scheme I-2, the ratio of free pepsin to the initial concentration of pepsinogen is written as:

$$\begin{aligned} R(t) &= \frac{X}{X_1(0)(1+K_1I)} \\ &= \frac{1}{(1+K_1I)} \left\{ 1 + \frac{k_2}{k_1-k_2} \exp(-k_1t) + \frac{k_1}{k_1-k_2} \exp(-k_2t) \right\} \end{aligned} \quad (10)$$

and the inflection time  $t_{inf}$  is written as:

$$t_{inf} = \frac{1}{k_1-k_2} \ln\left(\frac{k_1}{k_2}\right) \quad (11)$$

where  $X = X_1 + X_2I$ .

For Scheme I-3, the ratio may be written as:

$$R(t) = \frac{1}{1+K_1I} \left\{ 1 - \exp(-k_1t) \right\} \quad (12)$$

### Results of simulation

The equations (5), (10) and (12) were numerically calculated using  $X_1(0) = 9.75 \times 10^{-6} M$  and  $K_1 = 100$  by changing the rate constants. The  $K_1$  value at 0°C was calculated from the data in the report of Tang (1965). For Scheme I-1 and the equation (5), the calculated curves most resembled to the experimental data

at 0°C are shown in Fig. 21. The values of  $k_1$  and  $k_2$  were  $5 \times 10^{-4} \text{sec}^{-1}$  and  $1 \times 10^{-3} \text{M}^{-1} \text{sec}^{-1}$  respectively. The inflection time of curves shifted toward longer time according to the increase in the inhibitor concentration. The inflection time may be considered as a measure of the time needed for the full appearance of the activity. The elongation of inflection time with increase in the inhibitor concentration is inconsistent with the experimental results. If the value of the ratio  $k_1/k_2$  increases beyond the  $X_1(0)$  value according to the rise of the activation temperature, the curve will lose its inflection point and becomes to have the shape similar to the experimental curves shown in Fig. 1. Thus, the equation (5) may be able to explain qualitatively the difference in the curve-shape at different temperature and the induction period of the activation process, though it failed to explain the effect of isoamyl alcohol, especially at low concentration on the activation. For Scheme I-2 and the equation (10), the calculated curves most fitting to the experimental one are shown in Fig. 22. The values of  $k_1$  and  $k_2$  were  $5 \times 10^{-3} \text{sec}^{-1}$  and  $6 \times 10^{-3} \text{sec}^{-1}$ , respectively. The shape of the curves are independent of the combination of kinetic parameters and of the incubation temperature, differing from the experimental findings. The calculated curves with equation (12) derived from the Scheme I-3 show the hyperbolic shape without the inflection point as shown in Fig. 23. These curves resemble quite well to the experimental ones at 15°C, but do not to the activation curves at 0°C which exhibit the clear inflection point and the induction period.

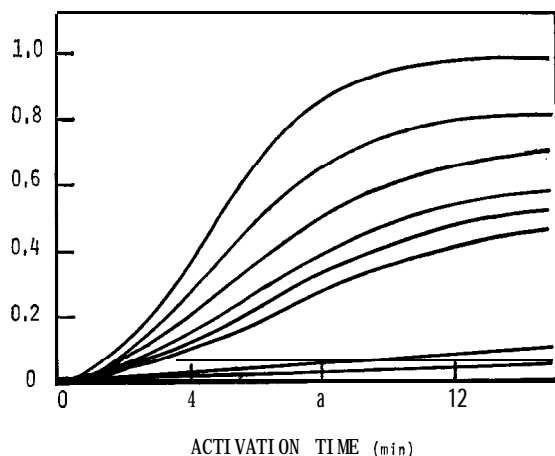
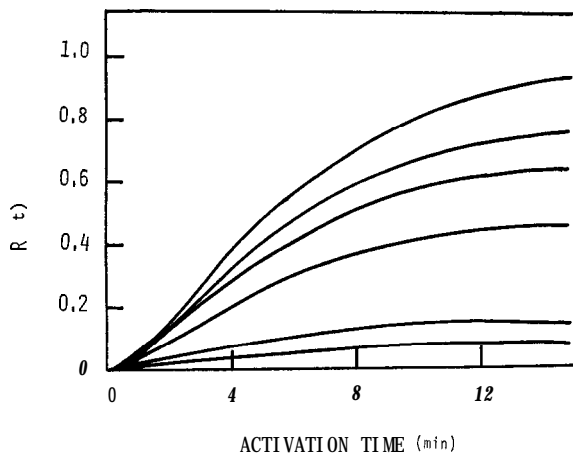


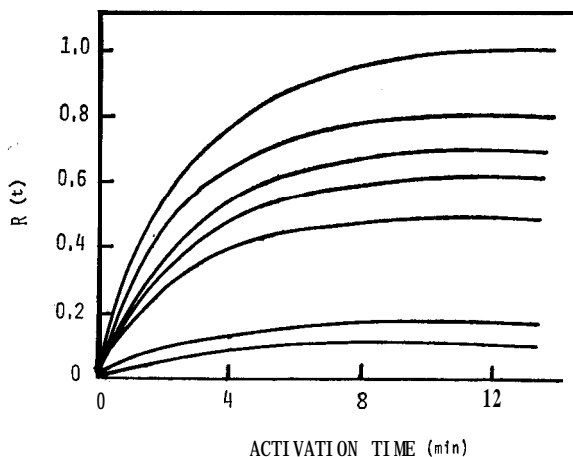
Fig. 21. Time-course of activation calculated according to Scheme I-1 and equation (5) assuming that  $k_1=5 \times 10^{-4}$ ,  $k_2=1 \times 10^{-3}$ ,  $X_1(0)=9.75 \times 10^{-6}$  and  $K_i=100$ . The concentrations of inhibitor were, from top to bottom, 0, 0.002, 0.004, 0.006, 0.008, 0.01, 0.05 and 0.1 M, respectively.

#### ESTIMATION OF KINETIC PARAMETERS

The precise estimation of kinetic parameters in a plausible scheme, which can explain the several characteristic features of the real activation process of pepsinogen near pH 2.5 and 0°C, was made by digital computer using an optimiza-



**Fig. 22.** Time-course of activation calculated according to Scheme I-2 and equation (10) assuming that  $k_1=5\times 10^{-3}$ ,  $k_2=6\times 10^{-3}$ ,  $X_1(0)=9.75\times 10^{-6}$  and  $K_i=100$ . The concentrations of inhibitor were, from top to bottom, 0, 0.002, 0.004, 0.01, 0.05 and 0.1 M, respectively.



**Fig. 23.** Time-course of activation calculated according to Scheme I-3 and equation (12) assuming that  $k_1=6.5\times 10^3$ ,  $X_1(0)$  and  $K_i$  were the same as those in Fig.22. The concentrations of inhibitor were, from top to bottom, 0, 0.002, 0.004, 0.006, 0.01, 0.05 and 0.1 M, respectively.

tion technique consisted of a numerical solution of nonlinear differential equation and an optimal decision of a revised parameter for iterative computation.

#### Optimization technique

In the optimization technique for parameter estimation, first the assumed rate equation is calculated by arbitrarily chosen parameters and the calculated result is compared with the experimental results to observe the deviation and the parameters are revised on the basis of the observed deviation. The calcula-

tions are iterated to approach to optimal parameters. The optimization technique for parameter estimation may be divided into four procedures ; (1) representation and formulation of the reaction system, (2) discriminations of convergency toward optimal parameters and formulation of performance index for the convergency of the revised parameters, (3) revision of parameters to maximize or minimize the performance index under certain constraints, (4) iteration with revised parameters to reach to optimal parameters.

(1) A nonlinear differential equation as rate equation is generally obtained from the assumed scheme. It has the form of

$$\dot{X}_i = f(k_1, \dots, k_n, X_1, \dots, X_m) \quad (13)$$

where  $X_i (i=1, \dots, m)$  represents the concentration of  $i$ -species and  $k_j (j=1, \dots, n)$  the kinetic parameter of  $j$ -th step.

(2) The performance index is composed of the experimental results and the equation (13), and should involve the all kinetic parameters. The performance index affects the convergency of revised parameters to the optimal ones. It is difficult to solve analytically the equation (13) because of its nonlinearity. The direct integration of the equation (13) gives the following forms:

$$\begin{aligned} X_{iT} &= \int_0^T f(k_1, \dots, k_n, X_1, \dots, X_m) dt \\ &= \sum_{j=1}^n k_j \int_0^T f_j(X_1, \dots, X_m) dt \end{aligned} \quad (14)$$

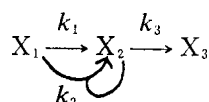
where  $X_{iT}$  represents the concentration of  $i$ -species at time  $T$ . The equation (14) should be solved in order that  $X_{iT}$  is represented by a linear equation containing only parameters  $k_j$ . For this purpose, the equation (13) is solved by numerical calculation and  $X_{iT}$  is obtained by the numerical integration with calculated values. The square of difference in the calculated concentration  $X_{iT}$  and the experimental values is defined as the performance index by multiplying sometimes a certain weight.

(3) A suitable constraint is imposed on the performance index.

(4) A revised parameter is decided and the above procedures are iterated until the optimal parameters which are satisfying the convergency qualification are obtained.

### Computation

The practical procedure of computation may be explained as follows using Scheme 2-2 as an example :



The experimentally measured values are the appeared activity and the conformational change (or amount of pepsin). These are defined as

$$\left. \begin{aligned} A(L) &= X_2(L) + X_3(L) \\ C(L) &= X_3(L) \end{aligned} \right\} \quad (15)$$

where  $A$  and  $C$  represent the activity and conformational change respectively and  $L$  represents the reaction time. The differential equations derived from the above scheme are

$$\begin{aligned} \dot{X}_1 &= -k_1 X_1 - k_2 X_1 X_2 \\ \dot{X}_2 &= k_1 X_1 + k_2 X_1 X_2 - k_3 X_2 \\ \dot{X}_3 &= k_3 X_2 \end{aligned} \quad I \quad (16)$$

with initial conditions of  $X_1(0)=1$ ,  $X_2(0)=X_3(0)=0$ . The direct integration of the equation (16) gives

$$\left. \begin{aligned} X_1 &= 1 - k_1 \int_0^T X_1 dt - k_2 \int_0^T X_1 X_2 dt \\ X_2 &= k_1 \int_0^T X_1 dt + k_2 \int_0^T X_1 X_2 dt - k_3 \int_0^T X_2 dt \\ X_3 &= k_3 \int_0^T X_2 dt \end{aligned} \right\} \quad (17)$$

and the concentration of  $X_i (i=1, 2, 3)$  can be represented by linear equation with  $k_j (j=1, 2, 3)$ .

First the equation (16) was numerically solved by Runge-Kutta-Gill method using initially assumed parameters (arbitrarily chosen). The obtained  $X_i (i=1, 2, 3)$  was used for the numerical integration by trapezoidal rule in the right terms of the equation (17). Such a roundabout is necessary to represent  $X_i (i=1, 2, 3)$  by the linear equation with parameter  $k_j (j=1, 2, 3)$ , because the performance index must contain all parameters and it should be differentiated by  $k$ , to obtain revised parameters. The performance index was defined by

$$F = \sum_{L, T=1}^{L, T} \left\{ (A(L) - X_2(T) - X_3(T))^2 + (C(L) - X_3(T))^2 \right\} \quad (18)$$

where  $X_i(T)$  is the calculated value of  $X_i$  at time  $T$ . Substitution of the equation (18) by equation (17) gives an second-order equation with the parameter  $k$ . The optimization technique was applied to minimize the performance index  $F$  under non-negative constraint.

Let the Lagrange function  $L$  be defined by

$$L = F - \lambda_1(k_1 - \alpha) - \lambda_2(k_2 - \beta) - \lambda_3(k_3 - \gamma) \quad (19)$$

$$k_1 - \alpha \geq 0, \quad k_2 - \beta \geq 0, \quad k_3 - \gamma \geq 0 \quad (20)$$

where  $\lambda_i \geq 0 (i=1, 2, 3)$  is Lagrange multiplier and  $\alpha, \beta$  and  $\gamma$  are small non-negative number of order of  $10^{-5}$ . On taking derivative of  $L$  with respect to  $k_j$ ,

$$\left. \begin{aligned} \frac{\partial L}{\partial k_1} = \frac{\partial F}{\partial k_1} - \lambda_1 &= 0 \\ \frac{\partial L}{\partial k_2} = \frac{\partial F}{\partial k_2} - \lambda_2 &= 0 \\ \frac{\partial L}{\partial k_3} = \frac{\partial F}{\partial k_3} - \lambda_3 &= 0 \end{aligned} \right\} \quad (21)$$

are obtained. On the other hand, from Kuhn-Tucker's constraint qualification, the following relations are derived:

$$\left. \begin{aligned} g_1 = \lambda_1(k_1 - \alpha) &= 0 \\ g_2 = \lambda_2(k_2 - \beta) &= 0 \\ g_3 = \lambda_3(k_3 - \gamma) &= 0 \end{aligned} \right\} \quad (22)$$

Depending on the values of initially assumed or revised parameters, eight cases are taken into consideration:

Case 1.

$$k_1 > \alpha, \quad k_2 > \beta, \quad k_3 > \gamma;$$

From the equation (22),  $\lambda_1 = \lambda_2 = \lambda_3 = 0$  is obtained and from the equation (21),

$$\frac{\partial F}{\partial k_1} = \frac{\partial F}{\partial k_2} = \frac{\partial F}{\partial k_3} = 0$$

are obtained.

Case 2.

$$k_1 \leq \alpha, \quad k_2 > \beta, \quad k_3 > \gamma;$$

$$k_1 = \alpha, \quad \lambda_1 > 0, \quad \lambda_2 = \lambda_3 = 0$$

$$\frac{\partial F}{\partial k_1} - \lambda_1 = 0, \quad \frac{\partial F}{\partial k_2} = \frac{\partial F}{\partial k_3} = 0$$

Case 3.

$$k_1 > \alpha, \quad k_2 \leq \beta, \quad k_3 > \gamma;$$

$$k_2 = \beta, \quad \lambda_2 > 0, \quad \lambda_1 = \lambda_3 = 0$$

$$\frac{\partial F}{\partial k_2} - \lambda_2 = 0, \quad \frac{\partial F}{\partial k_1} = \frac{\partial F}{\partial k_3} = 0$$

Case 4.

$$k_1 > \alpha, \quad k_2 > \beta, \quad k_3 \leq \gamma;$$

$$k_3 = \gamma, \quad \lambda_3 > 0, \quad \lambda_1 = \lambda_2 = 0$$

$$\frac{\partial F}{\partial k_3} - \lambda_3 = 0, \quad \frac{\partial F}{\partial k_1} = \frac{\partial F}{\partial k_2} = 0$$

Case 5.

$$k_1 \leq \alpha, k_2 \leq \beta, k_3 > \gamma;$$

$$k_1 = \alpha, k_2 = \beta, \lambda_1 > 0, \lambda_2 > 0, \lambda_3 = 0$$

$$\frac{\partial F}{\partial k_1} - \lambda_1 = 0, \quad \frac{\partial F}{\partial k_2} - \lambda_2 = 0, \quad \frac{\partial F}{\partial k_3} = 0$$

Case 6.

$$k_1 \leq \alpha, k_2 > \beta, k_3 \leq \gamma;$$

$$k_1 = \alpha, k_3 = \gamma, \lambda_1 > 0, \lambda_3 > 0, \lambda_2 = 0$$

$$\frac{\partial F}{\partial k_1} - \lambda_1 = 0, \quad \frac{\partial F}{\partial k_3} - \lambda_3 = 0, \quad \frac{\partial F}{\partial k_2} = 0$$

Case 7.

$$k_1 > \alpha, k_2 \leq \beta, k_3 \leq \gamma;$$

$$k_2 = \beta, k_3 = \gamma, \lambda_2 > 0, \lambda_3 > 0, \lambda_1 = 0$$

$$\frac{\partial F}{\partial k_2} - \lambda_2 = 0, \quad \frac{\partial F}{\partial k_3} - \lambda_3 = 0, \quad \frac{\partial F}{\partial k_1} = 0$$

Case 8.

$$k_1 \leq \alpha, k_2 \leq \beta, k_3 \leq \gamma;$$

$$\lambda_1 > 0, \lambda_2 > 0, \lambda_3 > 0,$$

$$\frac{\partial F}{\partial k_1} - \lambda_1 = 0, \quad \frac{\partial F}{\partial k_2} - \lambda_2 = 0, \quad \frac{\partial F}{\partial k_3} - \lambda_3 = 0$$

From each case, the revised parameters are obtained. For example, in the case 2, the revised value of  $k_1$  is fixed on the boundary value  $\alpha$  and  $k_2$  and  $k_3$  are calculated from  $\frac{\partial F}{\partial k_2} = \frac{\partial F}{\partial k_3} = 0$ , that is,  $k_2$  or  $k_3$  value is revised so that the derivative  $F$  in the form of the equation (18) at each step of iteration with respect to  $k_2$  or  $k_3$  becomes zero. The computation is iterated with revised  $k_j$  ( $j=1, 2, 3$ ), until the calculated  $F$  value falls below  $3.79 \times 10^{-2}$  or  $\delta = \frac{k_{jn} - k_{j,n+1}}{k_{j,n}}$  becomes smaller than  $10^{-5}$ . The suffix  $n$  and  $n+1$  represent the number of iteration.

The flow chart of computation is shown in Fig. 24. The rate constants  $k_{10}$ ,  $k_{20}$ , and  $k_{30}$  are initially assumed constants (arbitrarily chosen), and  $k_1$ ,  $k_{21}$  and  $k_{31}$  are first revised constants. The iteration was made until the conditions in rhombus are satisfied, where  $\epsilon$  is  $\delta k_{j0}$  ( $j=1, 2, 3$ ). The time-courses of the activation calculated by computed final  $k_j$  are graphed as shown in Fig. 25. For Scheme 2-2, computed rate constants were  $k_1 = 7.81 \times 10^{-4} \text{ sec}^{-1}$ ,  $k_2 = 7.96 \times 10^{-1} M^{-1} \text{ sec}^{-1}$  and  $k_3 = 2.77 \times 10^{-1} \text{ sec}^{-1}$  (at  $0^\circ\text{C}$  and pH 2.5) with  $F$  value of  $3.972 \times 10^{-2}$ . These values are qualitatively in agreement with those listed in Table 4.

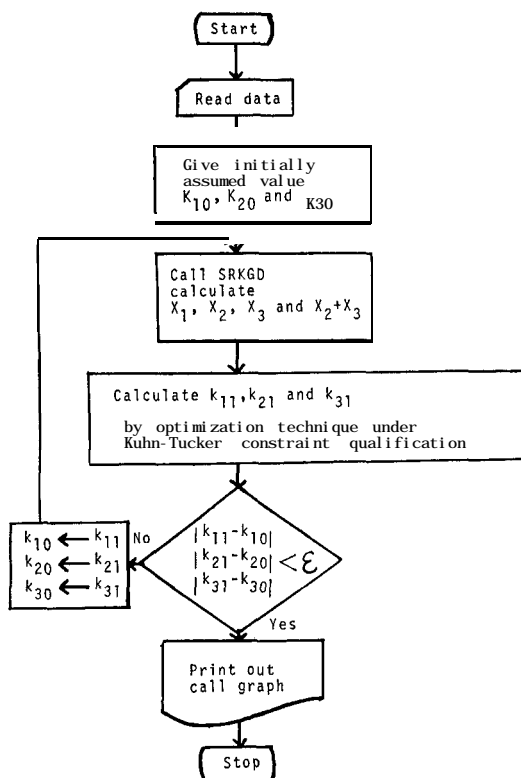


Fig. 24. Flow chart for parameter estimation.

## DISCUSSION

As far as the activation of pepsinogen was followed by the appearance of the peptic activity, two distinctive features were observable as described already. The presence of the competitive inhibitor isoamyl alcohol did not delay the time which was necessary for the full conversion of the pepsinogen to pepsin that is either in free or combined form with inhibitor. This means that the formed pepsin molecule did not accelerate the activation process under the present experimental conditions, although it accelerates the conversion of pepsinogen to pepsin at high pH medium (Wang and Edelman, 1971). The fact that low concentration of the inhibitor accelerates rather the activation may be important factor for establishing the mechanism of the activation. The activation process showed an induction period of about 3 min at 0°C, which disappeared in the activation at a room temperature (15°C). The reaction with induction period has usually been known to have complicated scheme. In the activation of pepsinogen, a conformational change which seems not to accompany the chemical events, must occur first and this might be one of the factors causing the induction period.

In contrary to the appearance of the peptic activity, the conformational change showed complex pattern. The specific and remarkable changes in the

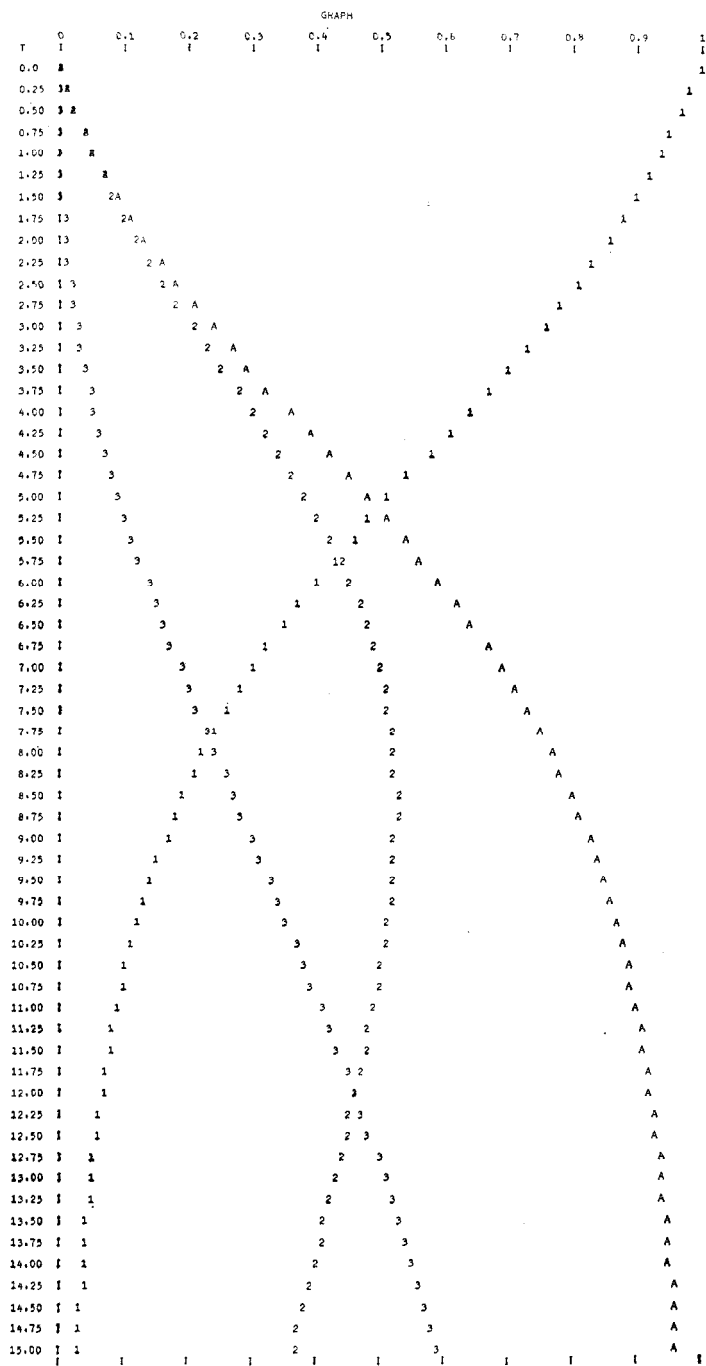


Fig. 25. Time-course for Scheme 2-3 plotted by optimal parameters.

shape and height of the base-line of the difference spectrum (Fig. 4) were observed within 3 min after the acidification of the pepsinogen solution. This seems to be caused by a turbidity of pepsinogen solution due to the acidification, because the height of the base-line recovered the original level by an elongated incubation. These changes observable in the early stages of the activation of pepsinogen may not be reflection of the conformational change of pepsinogen molecule necessary to form the active site in its own molecule. The conformational change accompanying the formation of the active site may not cover a large region of polypeptide main chain, because there were no detectable difference spectrum in early stages. The pepsinogen molecule contains 6 tryptophan residues and 16 tyrosine residues (Arnon and Perlmann, 1963). If there were large conformational changes in the early stages, it could be detected by difference spectrophotometry (Donovan, 1969). It is, therefore, concluded that the conformational change accompanying the formation of the active site in the early stages in the activation must be one very specific and restricted to small region.

The difference spectrum observable clearly after 10 min incubation was a type of blue-shift. This may mean that some aromatic residues were exposed from an interior of the molecule to more hydrophilic region (surface of molecule) on the activation. Although the plot of  $-\Delta$  OD vs the incubation time indicated that overall conformational changes could be divided into six stages, the practical meaning or phenomenological definition of each stage was not completely established.

The pH-dependence of the appearance of the peptic activity and the pH-dependence of changes in the value of  $-\Delta$  OD showed nearly the same profile. This fact indicates that the conformational change observed by difference spectrophotometry is indeed accompanied by the release of peptide segment from the zymogen molecule in the activation process.

The appearance of the peptic activity began after a lag period of about 3 min and finished after 8 min incubation. Contrary to a rapid activation, the conformational change followed by the value of  $-\Delta$  OD completed after 30 min incubation with lag time of about 6 min.

The difference spectrophotometry can only detect a part of the conformational changes accompanying the formation of the active site of pepsinogen molecule and the formation of the active pepsin molecule. When aromatic amino acid residues were not included in a conformational change, the difference spectrophotometry may not detect such a change. The content of aromatic amino acid in the pepsinogen molecule seems to be of average of proteins. This suggests that the conformational change including a considerably wide region must be detected by the difference spectrophotometry. Reversely, the conformational change not detectable by difference spectrophotometry must be restricted to a relatively small and very specific region.

The simulation with computer was adopted to obtain the fundamental schemes explaining the basic features of the appearance of the peptic activity and conformational change in the real activation process of pepsinogen molecule. One-step scheme with a loop (positive feedback) may explain the induction period of the activation but not the delayed conformational changes during the activa-

tion. Therefore, two- and three-step schemes with or without the loop were postulated for the simulation of the activation process as shown in Tables 1 and 2. More complicated schemes may, of course, be postulated. Practically, those schemes were ignored because of the difficulty of computation of their kinetic parameters. Since the main conformational change took place behind the appearance of the peptic activity, it should be assumed that at least one intermediate not released the peptide segment exhibits the peptic activity. Thus, in the simulation, an intermediate adjacent to the pepsin molecule was assumed to have same peptic activity as the pepsin molecule. Furthermore, the main conformational change was assumed to be due to the formation of the pepsin molecule from nearest intermediate.

As a result of analog simulation, it was found that four types of schemes can qualitatively explain the activation process of pepsinogen under the present experimental conditions; (1) two-step scheme with the loop (2-2), (2) three-step scheme without the loop (3-1), (3) three-step scheme with the loop (3-2, -3 and -4) and (4) three-step with loop and zero rate constant (3-5). Other postulated schemes failed to represent the real activation process.

It is understandable that isoamyl alcohol did not affect the activation, since this substance has been known as a competitive inhibitor of peptic activity of the pepsin molecule. The effect of isoamyl alcohol on the appearance of the peptic activity in the activation process was simulated by digital computer using three postulated schemes. As expected, all of them cannot explain the acceleration effect of isoamyl alcohol, especially at low concentration. It has been reported, in addition, that isoamyl alcohol decreased the rate of the formation of new N-terminal (Leu) of pepsin molecule (Funatsu et al., 1972). For explaining such effects of isoamyl alcohol, Scheme I-4 in Table 3, for example, was postulated. This scheme involves the active dimeric intermediate  $X_2^*$ . However, neither simulation nor estimation of kinetic parameters was performed because of its complexity. The existence of such intermediates in the real activation process should, therefore, be demonstrated in succeeding experiments.

If the computation technique is properly established, the estimation of kinetic parameters by digital computer will be powerful tool for the analysis of complicated reaction of which the detection of intermediate receives several experimental limitations. In the present experiment, the parameters were clearly determined by optimization technique, because a simple scheme was postulated and computed as an example. For more complicated scheme, much sophisticated performance index and more suitable method for obtaining the revised parameter in the iteration may be required.

In the present article, the authors clarified only the possible schemes which can explain the experimental findings and pointed out implicitly that if a scheme postulated by investigator's wish explained the experimental results, it is not probable to decide that scheme as a true one.

For establishing more precise scheme, it may be absolutely necessary that different activation patterns caused by the different conditions of the activation medium are taken into consideration and that more experiments under various conditions should be carried out in succeeding studies.

## REFERENCES

- Al-Janabi, J., J. A. Hartsuck and J. Tang 1972 Kinetic and Mechanism of Pepsinogen Activation. *J. Biol. Chem.*, 247: 4628
- Arnon, R. and G. E. Perlmann 1963 The Amino Acid Composition of Chromatographically Purified Pepsinogen. *J. Biol. Chem.*, 238: 653
- Bohak, Z. 1973 Kinetics of Conversion of Chicken Pepsinogen to Chicken Pepsin. *Eur. J. Biochem.*, 32: 547
- Bovey, F. A. and S. S. Yanary 1960 Pepsin. In "The Enzymes," Vol. 4, ed. by P. D. Boyer, H. Lard and K. Myrbaeck, Academic Press, New York, p. 63
- Busting, M. and A. Conway-Jacobs 1971 Intramolecular Activation of Porcine Pepsinogen. *J. Biol. Chem.*, 246: 615
- Donovan, J. W. 1969 Ultraviolet Absorption. In "Physical Principle and Techniques in Protein Chemistry," ed. by S. J. Leach, Academic Pres, New York, p. 101
- Funatsu, M., Y. Harada, K. Hayashi and B. Jirgenson 1971 Studies on the Mechanism of Activation of Pepsinogen Part I. Conformational Change in the Process of Activation in Acid Solution. *Agr. Biol. Chem.*, 35: 566
- Funatsu, M., Y. Harada, K. Hayashi and T. Kaneda 1972 Studies on the Mechanism of Activation of Pepsinogen Part II. Effects of Substrate and Competitive Inhibitor on the Activation of Pepsinogen. *Agr. Biol. Chem.*, 36: 305
- Herriott, R. M. 1938 Kinetics of the Formation of Pepsin from Swine Pepsinogen and Identification of an Intermediate Compound. *J. Gen. Physiol.*, 22: 65
- Herriott, R. M. 1962 Pepsinogen and Pepsin. *J. Gen. Physiol.*, 45: Suppl. 1, 57
- McPhie, P. 1972 Spectrophotometric Investigation of the Pepsinogen-Pepsin Conversion. *J. Biol. Chem.*, 247: 4277
- Neumann, H. and M. Shinitzky 1971 Reversible Inactivation of the Potential Pepsin Activity of Pepsinogen by Alcohols. *Biochemistry*, 10: 4335
- Ong, Y. B. and G. E. Perlmann 1968 The Amino-terminal Sequence of Porcine Pepsinogen. *J. Biol. Chem.*, 243 : 6104
- Ryle, A. P. 1960 Parapepsinogen II. The Zymogen of Parapepsin II. *Biochem. J.*, 75: 145
- Singer, P. B., D. M. Blow, B. M. Mathews and R. Henderson 1968 Structure of Crystalline  $\alpha$ -Chymotrypsin II. A Preliminary Report Including a Hypothesis for the Activation Mechanism. *J. Mol. Biol.*, 35: 143
- Tang, J. 1965 Competitive Inhibitor of Pepsin by Aliphatic Alcohols. *J. Biol. Chem.*, 240: 3810
- Vanakis, H. V. and R. M. Herriott 1956 Structural Change Associated with the Conversion of Pepsinogen to Pepsin II. The N-Terminal Amino Acid Residues of Pepsin and Pepsinogen ; Amino Acid Composition of Pepsinogen. *Biochim. Biophys. Acta*, 23: 600
- Varandani, P. T. and M. Shlamowitz 1963 The influence of salts on the conversion of pepsinogen to pepsin. *Biochim. Biophys. Acta*, 77: 496
- Wang, J. L. and G. M. Edelman 1971 Fluorescent Probes for Conformational States of Proteins IV. The Pepsinogen-Pepsin Conversion. *J. Biol. Chem.*, 246 : 1.185
- Wright, H. T., J. Kraut and P. E. Wilcox 1968 Comparison of  $\pi$ -, S- and  $\gamma$  -Chymotrypsin Family with  $\alpha$ -Chymotrypsin. *J. Mol. Biol.*, 37 : 363