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STUDIES ON THE KINETICS OF ENZYMIC REACTIONS, I and II

I. The Mechanism of the Degradation of Amylose by Action of Bacterial α-Amylase*

By Jiro Osugi

Introduction

The mechanism of the degradation of high polymers can be elucidated by comparing the observed value with the theoretical value when the degradation can be treated by a kinetical or statistical theory.

On the degradation of natural or synthetic chain-like polymers by action of acid or base, theoretical treatments have been performed by assuming that all the linkages can be split at equal probability or perfectly at random[1].

The present report concerns on the degradation of amylose by action of bac. α-amylase. To elucidate the kinetical behavior of the degradation, the relation between the change of weight average degree of polymerization obtained from the viscosity change and the amount increased of reducing end is examined in Part I, and the rate of the degradation is considered in Part II.

Materials and Experimentals

The amylose used was extracted from purified soluble or potato starch by the method of hot water extraction[2]. The purities of amylose solutions thus prepared were found to be above 95% from the potentiometric measurements after Rundle[3], and the degrees of polymerization of the amylose used were relatively low ($P = 100 \sim 200$).

Highly purified bacterial amylase kindly furnished by Dr. Hukumoto[4] belongs to pure α-amylase and the other feeble activities contained were destroyed by heating. (Maltase was not contained.) The results obtained were also confirmed by crystalline bac. α-amylase which was lately prepared by Dr. Hukumoto and his co-workers.

The rates of amylose degradation were measured at a fixed interval of time from the change of viscosity, the amount of reducing end and the absorption of amylose-iodine complex. The viscosity was measured by means of a Ostwald viscosimeter

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* *Proc. Japan Acad.*, 27, 241 (1951), comm. by S. Horiba, M. J. A., May 16, 1951

1) W. Kuhn et al., *Ber.*, 63, 1503, 1510 (1930)
H. Mark et al., *Ber.*, 62, 1103 (1929), *Trans. Farad. Soc.*, 36, 611 (1940)
Alf af Ekenstam, *Ber.*, 69, 553 (1936)

2) K. H. Meyer et al., *Helv. Chim. Acta.*, 23, 845, 854 (1940), 24, 378 (1941)

3) F. L. Bates, D. Frenchand and R. E. Rundle, *J. Am. Chem. Soc.*, 65, 142 (1943)

4) J. Hukumoto, *J. Agric. Chem. (Japan)*, 19, 487, 689, 789, 853 (1943), 20, 23, 121 (1944)

in the thermostat at 25°C. The amount of reducing end was satisfactorily determined by a photometric method using 3, 5-dinitrosalicylic acid[5]. The absorption of amylose-iodine complex was measured by a photometric method.

Experimental Results

The general features of the experimental results are illustrated in Fig. 1, which

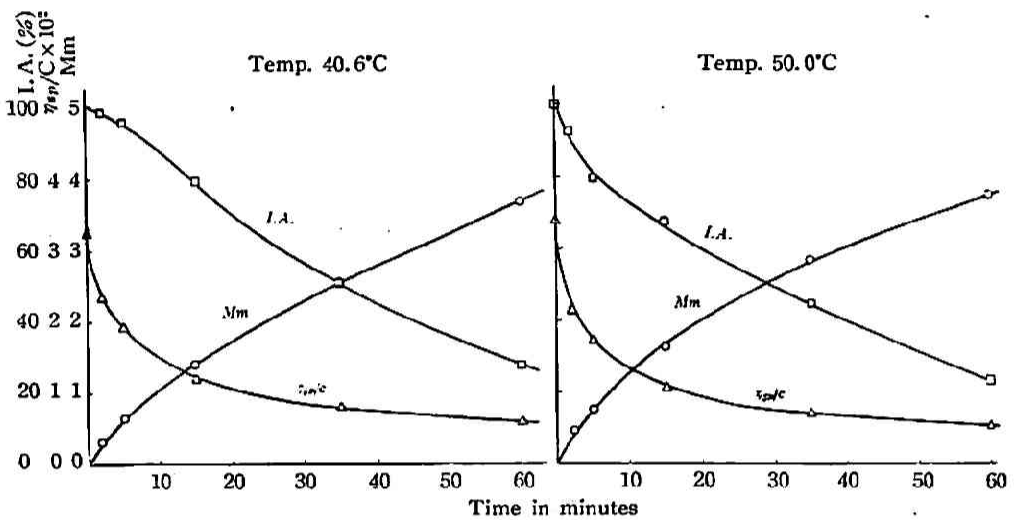

Fig. 1 Processes of amylose degradation by action of bac. α-amylase

shows the rate of amylose degradation by action of bac. α-amylase. In the figures, the abscissa is the time in minutes and the ordinates show intrinsic viscosity η_{SP}/C (l/g), the increased amount of reducing end Mm (mg maltose per cc) and the absorption percentage of amylose-iodine complex I. A.

These measurements were performed under various experimental conditions. The effects obtained under different conditions were examined by comparing with control, and the mechanism and the kinetics of the amylose degradation considered.

Considerations

The intrinsic viscosities of the amylose solutions prepared were found to be constant irrespective of the concentrations. As Staudinger's viscosity equation can be applied to such a chain-like polymer as amylose[6], we can calculate the weight average degrees of polymerization P from the measurements of viscosity.

$$\eta_{SP}/C = KP, \qquad (1)$$

5) J. B. Summer, *J. Biol. Chem.*, 47, 5 (1921), G. N. Smith and C. Stocker, *Arch. Biochem.*, **21**, 95 (1949)

6) L. H. Lampitt, C. H. F. Fuller and N. Goldenberg, *J. Soc. Chem. Ind.*, **66**, 417 (1947), **67**, 38, 41 (1948)

where C is the concentration of an amylose solution (g/*l*), and K is constant. The value of K was estimated to be 2.31×10^{-4} by the end group determination. The exact value of K is not required in the present research from the reason mentioned below.

The degradation of high polymers has been studied theoretically and experimentally to elucidate the mechanism of the degradation reaction by various investigators. The basic assumption of the theoretical treatments is that the linkages of high polymers with homogenious degrees of polymerization can be split at random or at equal probability. If the assumption that a chain-like polymer, such as amylose, can be split at random is valid, we can obtain the relation between the bond split which can be calculated theoretically from the weight average degrees of polymerization and the bond split which can be measured from the amount increased of reducing end, and conclude whether the assumption is valid or not.

Theoretical treatments of the degradation of which Kuhn and his co-workers[7] get the start, give the numbers of the bond split S when the initial degrees of polymerization N decrease to P degrees by random splitting. The relations between S, N and P which can be obtained by the theory of statistics, or combinations in consideration of the probability at which the (P − 1) bonds do not split, or of the way in which a chain with N degrees splits into S + 1 groups, differ according to the investigators who take different bases of the treatments.

The statistical relation obtained by E. W. Montroll and R. Simha[8] is

$$P = \frac{\left(\frac{S}{N}\right)^2 (N+1) + 2\left(1 - \frac{S}{N}\right)\left[\left(1 - \frac{S}{N}\right)^{N+1} + \frac{S}{N}(N+1) - 1\right]}{\left(\frac{S}{N}\right)^2 (N+1)}. \qquad (2)$$

I. Sakurada and S. Okamura[9] improved the statistical treatment and obtained the following equation:

$$\frac{P}{N} = \frac{2}{S^2}\left(S - 1 + \frac{1}{e^S}\right). \qquad (3)$$

From the theory of combinations, W. H. Durfee and Z. I. Kertesz[10] obtained the following simple equation:

$$P = \frac{2N - S}{S + 2} \quad \text{or} \quad S \fallingdotseq 2\left(\frac{N}{P} - 1\right). \qquad (4)$$

In these equations, P is the weight average degrees of polymerization.

Alf af Ekenstam[11] considered that when a high polymer with N degrees of poly-

7) W. Kuhn et al., *Ber.*, **63**, 1503, 1510 (1930)
8) E. W. Montroll and R. Simha, *J. Chem. Phys.*, **8**, 721 (1940)
9) I. Sakurada and S. Okamura, *J. Soc. Chem. Ind. (Japan)*, **45**, 1101 (1942)
10) W. H. Durfee and Z. I. Kertesz, *J. Am. Chem. Soc.*, **62**, 1196 (1940)
11) Alf af Ekenstam, *Ber.*, **69**, 553 (1936)

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merization degraded to P′ degrees, one molecule would be split to N/P′ molecules, and so N/P′ was equal to S+1 where S was the numbers of the bond split:

$$S = \frac{N}{P'} - 1, \qquad (5)$$

in which P′ represented apparently the number average degrees of polymerization.

It is tedius to calculate S from the arbitary values of N and P from Eq. (2) or (3) and as the relations between N/P and S are shown in Fig. 2, the values calculated from Eq. (2) or (3) are proportional to those from Eq. (4) in the range of larger S. In case of inhomogenious materials, such as amylose extracted by the hot water method, the weight average agrees of polymerization tend to be approximately twice as large as the number average degrees, so by comparing Eq. (4) with (5) it is reasonable to employ Eq. (4) in the present research[12]. By Eq. (4), the values of S are independent of the K in Eq. (1). Table 1 shows an example of calculation of S from Eq. (4).

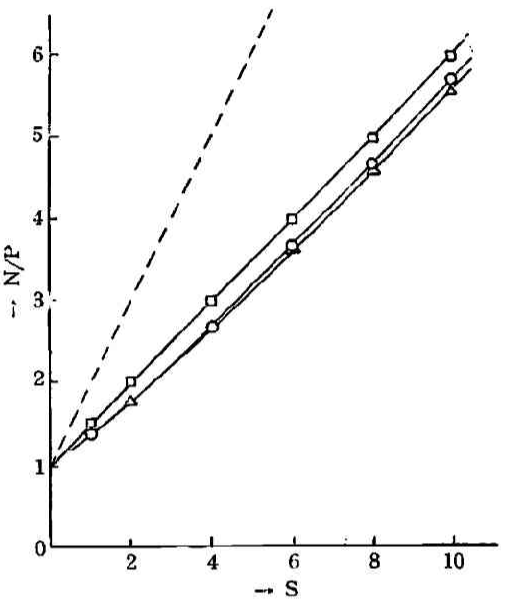

Fig. 2 Relations between N/P and S
□ Durfee and Kertesz
△ Sakurada and Okamura
○ Montroll and Simha (N=200)
··· Alf af Ekenstam

When one α-1,4 glucosidic linkage in amylose is split, one reducing end is produced. If the assumption that the α-1,4 glucosidic linkages are split at random is valid, the values of S calculated must be proportional to the amounts increased of reducing end Mm. As seen in Fig. 3 which shows the relation between S and Mm, a satisfactorily linear relation is obtained, so we can conclude that the mechanism of

Table 1

Time (minutes)	η_R	η_{SP}	η_{SP}/C	P	S
0	1.353	0.353	0.0335	145.0	0
2	1.246	0.246	0.0234	101.3	0.86
5	1.202	0.202	0.0191	82.7	1.50
15	1.127	0.127	0.0120	51.9	3.58
35	1.085	0.085	0.0081	35.0	6.28
60	1.064	0.064	0.0060	26.0	9.17

(C = 10.5 g/l)

12) S. Matsumoto, *Chem. High Polymer (Japan)*, 6, 36, 77 (1949)

the degradation of amylose by action of bac. α-amylase is perfectly random splitting of α-1,4 glucosidic linkages in amylose. This conclusion is also supported by the experiments of Caldwell[13] and Swanson[14].

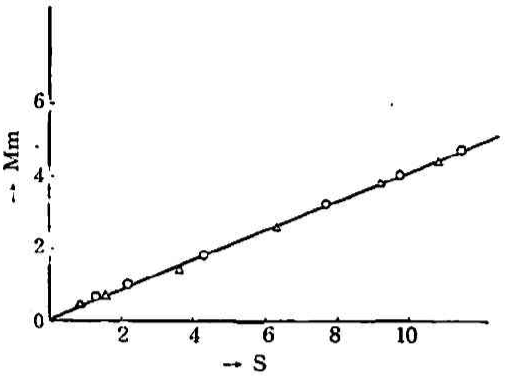

Fig. 3 Relation between Mm and S
△ 40.6°C, ○ 50.0°C

The rate of the degradation of the amylose solution which contained amylopectin (20%) was measured, and the values of S were calculated from the viscosity data and compared with the increases of reducing end Mm. The relation between S and Mm deviates from the linear relation obtained on the control as shown in Fig. 4. This fact can be reasonably understood in considering that the α-1,6 glucosidic linkages in branched amylopectin are not split by action of bac. α-amylase[14].

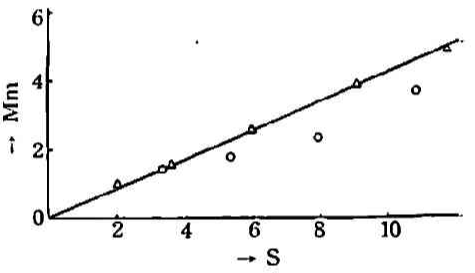

Fig. 4 Relation between Mm and S
△ control. ○ amylopectin added

The relation between S and Mm obtained from the measurement of amylose degradation caused by action of a mixed enzyme containing bac. α-amylase and β-amylase extracted from ungerminated barley, is shown in Fig. 5. The deviation from the linear relation is remarkable in the initial stage of the degradation. This suggests that the action of β-amylase differs from that of bac. α-amylase[14].

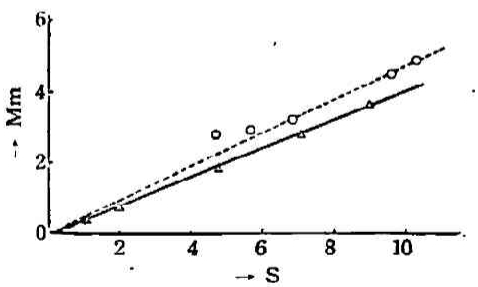

Fig. 5 Relation between Mm and S
△ control, ○ β-amylase added

The linear relation between S and Mm holds only in such a case of random splitting as the amylose degradation by action of bac. α-amylase.

The absorption of amylose-iodine complex is chiefly due to the blue components which are higher than 30 degrees of polymerization in the degraded amylose[15]. The percentage of the fraction of the components which are higher than 30 degrees is expected to correspond to the observed percentage of absorption.

As we can obtain the values of N, P and S from the viscosity data, the distri-

13) R. B. Alfin and M. L. Caldwell, *J. Am. Chem. Soc.*, **71**, 128 (1949)
14) M. A. Swanson, *J. Biol. Chem.*, **172**, 805 (1948)
15) M. A. Swanson, *J. Biol. Chem.*, **172**, 825 (1948)

bution of the components with various degrees of polymerization can be calculated from the equations[16]. In order to obtain the fraction m_c of the components which are higher than optical C degrees of polymerization, the equation obtained by S. Okamura[17] assuming random splitting, is favourable in the present case. The fraction m_c is given by the following equation:

$$m_C = \left\{1 + \left(1 - \frac{C}{N}\right)\frac{SC}{N}\right\} e^{-\frac{SC}{N}}. \qquad (6)$$

The percentages of the fractions m_c calculated assuming $C = 30 \sim 40$ are found to nearly coincide with the absorption percentages of amylose-iodine complex measured. The values calculated as $C = 35$ nearly conform with the observed percentages I. A. as shown in Fig. 6.

The absorption of amylose-iodine complex is mainly attributable to the fraction of the components which are higher than about 35 degrees in the degraded amylose, and the mechanism of random splitting is also confirmed by this fact.

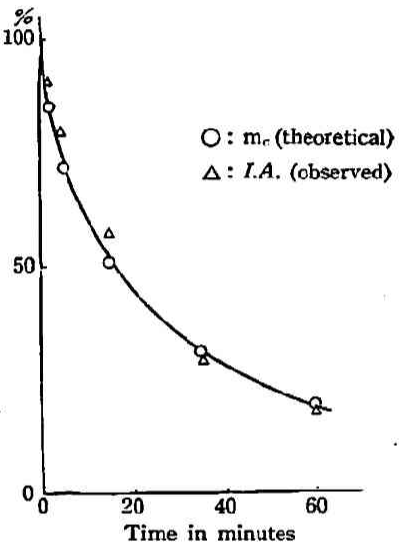

Fig. 6 Absorption of amylose-iodine complex

II. Kinetics of the Degradation of Amylose by Action of Bacterial α-Amylase*

The rate of the degradation of starch by action of amylase was represented by a 1st order rate equation at the initial stage[18], and the rate constant of the equation was used to indicate the activity of amylase[19]. The kinetical studies of Sjöberg and Erikson[20], Hanes[21] or Schwimmer[22] showed that the rate of the degradation followed the simple rate equation of Michaelis and Menten[23], but there were objections[24] to

* *Proc. Japan Acad.*, 27, 245 (1951) comm. by S. Horiba, M. J. A., May 16, 1951.

16) H. Donstal and H. Mark, *Trans. Farad. Soc.*, 33, 350 (1937)
E. W. Montroll and R. Simha, *J. Chem. Phys.*, 8, 721 (1940)
H. Mark and R. Simha, *Trans. Farad. Soc.*, 36, 611 (1940)
Herden, *Nature*, 163, 139 (1949)

17) S. Okamura, *J. Soc. Chem. Ind. (Japan)*, 45, 1111 (1942)

18) R. Willstätter, E. Waldschmidt-Leitz and A. R. F. Hesse, *Z. Physiol. Chem.*, 126, 143 (1923)

19) J. Blom, A. Bak and B. Brase, *Z. Physiol. Chem.*, 250, 104 (1937)

20) K. Sjöberg and E. Erikson, *Z. Physiol. Chem.*, 139, 118 (1924)

21) C. S. Hanes, *Biochem. J.*, 26, 1406 (1932)

22) S. Schwimmer, *J. Biol. Chem.*, 186, 181 (1950)

23) L. Michaelis and M. L. Menten, *Biochem. Z.*, 49, 333 (1913)

24) G. S. Eadie, *Biochem. J.*, 20, 1016 (1926)

their results. Most of the kinetical studies on the degradation of starch were performed, employing soluble starch and amylase, both of which consisted of more than one component.

The present report concerns to kinetical consideration of the degradation of amylose by action of bac. α-amylase and of the effects of the product and salts on the degradation.

The rate of the degradation is usually represented by the change of reducing end with time, so the rate equation can be derived from the increase of reducing end Mm or the number of bond split S. According to the analysis of the curves of the Mm $-$ t relation shown in Fig. 1 in the previous paper or the S $-$ t relation the following equation holds between k_a and v calculated from the values of Mm or S.

$$m k_a = n + v, \tag{7}$$

where

$$k_a = \frac{1}{t} \ln \frac{a}{a - x} \quad \text{and} \quad v = \frac{x}{t},$$

in which a is the initial concentration of amylose or the initial degree of polymerization, x is the increase of reducing end Mm or the number of bond split S, and t is time in minutes. The m and n are constants. The results of calculation from the viscosity data are similar to and more accurate than those from reducing end measurements, so the consideration described below is based on the former. The linear relation between k_a and v is shown in Figs. 7 and 8. Eq. (7) is the catalytic rate equation

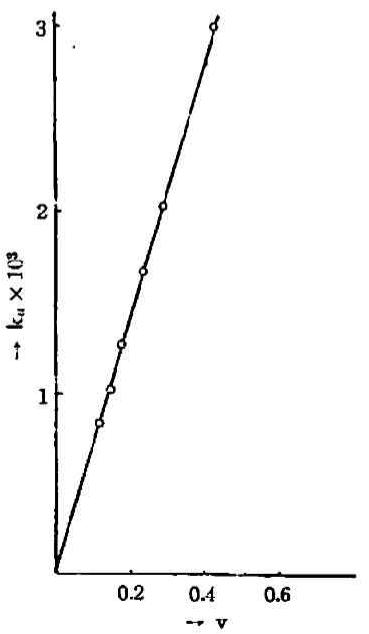

Fig. 7 Relation between k_a and v (calculated from S)

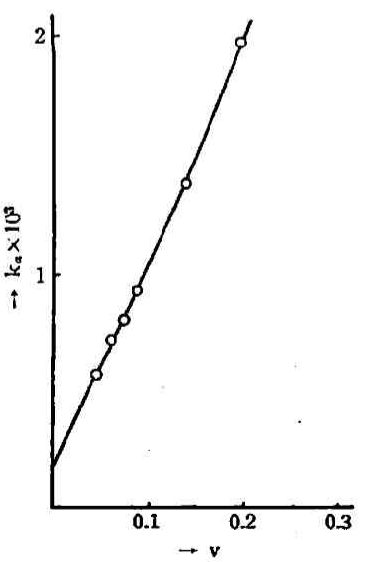

Fig. 8 Relation between k_a and v (calulated from Mm)

which attends on moderate retardation by the product.

Assuming the intermediate substrate-enzyme complex after Michaelis and Menten[23], the processes of the degradation may be considered as follows:

$$[G-G]+[E] \longrightarrow [EG-G], \quad k_1 \quad \text{(i)}$$
$$[EG-G] \longrightarrow [E]+[G-G], \quad k_2 \quad \text{(ii)}$$
$$[EG-G] \longrightarrow [E]+[G]+[G], \quad k_3 \quad \text{(iii)}$$
$$[G]+[E] \longrightarrow [EG], \quad k_4 \quad \text{(iv)}$$
$$[EG] \longrightarrow [E]+[G], \quad k_5 \quad \text{(v)}$$

where $[G-G]$ indicates substrate, $[G]$ product, $[E]$ enzyme, and $[EG-G]$ and $[EG]$ intermediate complexes. k_1, k_2, etc. are the respective rate constants.

By assuming the stationary concentration of the intermediate complexes, we can derive the following rate equation:

$$V = \frac{k_3 \dfrac{k_1}{k_2+k_3}[G-G][E]}{1+\dfrac{k_1}{k_2+k_3}[G-G]+\dfrac{k_4}{k_5}[G]},$$

or

$$\frac{dx}{dt} = \frac{k_3 \dfrac{k_1}{k_2+k_3}(a-x)[E]}{1+\dfrac{k_1}{k_2+k_3}(a-x)+\dfrac{k_4}{k_5}(x)}.$$

By the integration, we obtain

$$\frac{1+aK_B}{K_B-K_A}k_a = \frac{k_3K_A}{K_B-K_A}[E]+v, \tag{8}$$

where

$$K_A = \frac{k_1}{k_2+k_3} \quad \text{and} \quad K_B = \frac{k_4}{k_5}. \tag{9}$$

Eq. (8) is coincident to Eq. (7) experimentally obtained, where

$$m = \frac{1+aK_B}{K_B-K_A} \quad \text{and} \quad n = \frac{k_3K_A}{K_B-K_A}[E]. \tag{10}$$

The calculation from the experimental data shows $m>0$, $n>0$, so we obtain $K_B>K_A$. From the consideration of the rate constants in Eq. (9), we find that $[EG]$ is more stable than $[EG-G]$ and the retardation of the rate results from the complex formation between enzyme and product*.

* The consideration on the mechanism of the retardation differs from the description in *Proc. Japan Acad.*, and the present consideration is more justified, taking into consideration the meaning that x is equal to S.

It is shown in the Mm − S relation (Fig. 3) mentioned above that the mechanism of the degradation is not changed by the elevation of temperature, but the rate of the degradation is accelerated by temperature elevation and the constants in the rate equation change with temperature. The changes of m and n in Eq. (7) are shown in Table 2. These values were calculated from the values of N and S.

Table 2

Temp. (°C)	m	$n \times 10^3$	E (kcal)
35	157.8	5.26	16.10
40	155.5	8.01	
50	148.8	6.32	12.30
40	148.5	3.42	
50	344.0	5.28	13.95
40	341.6	2.60	15.87
32	341.5	1.33	

We find that m's are nearly constant, but n's are changed with temperature. If we assume that K_A, K_B and [E] (activity of enzyme) do not change remarkably with temperature, the temperature coefficients of n will give the activation energy of the rate determining step (iii) of the degradation processes. The values of the activation energy are shown in Table 2.

To confirm the retardation of the reaction rate by the products mentioned above, we compared the degradation of the amylose solution to which 0.01 M glucose or maltose was added with control. As shown in Fig. 9, the rates of the degradation are retarded by the addition of glucose or maltose. Eq. (7) also holds in this case, and the $k_\alpha - v$ relations are parallel to each other. The values of m and n in Eq. (7) are shown in Table 3, i. e. the values of m are nearly constant, but those of n are changed. We find from Eq. (10) that the activities of bac. α-amylase are decreased by the addition of glucose or maltose. The stability of the product-enzyme complex [E G] mentioned above is considered to be related to the decrease of the activity of enzyme.

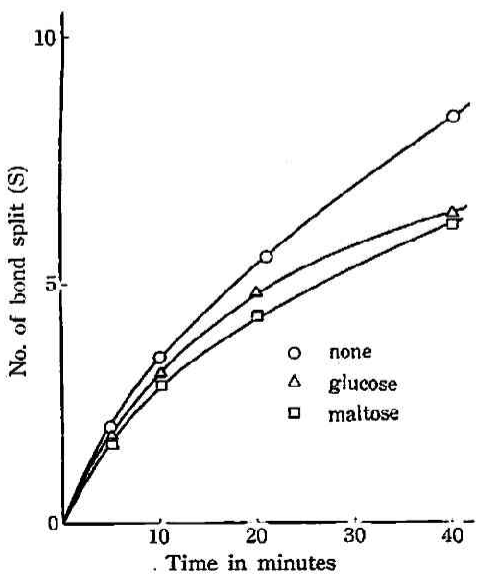

Fig. 9 Effect of glucose or maltose

Table 3

Material added	m	n
none	161.6	0.0091
glucose	160.4	0.0046
maltose	160.5	0.0049

To examine the effect of the addition of salts, the experiments in which 0.01 N LiCl, NaCl or $NaNO_3$ was added to enzyme solutions were performed. The rates of the degradation are accelerated by the addition of these salts, as shown in Fig. 10. The linear relations between k_α and v in Eq. (7) also hold in this case and are parallel to each other. The calculated values of m and n in Eq. (7) are shown in Table 4. The values of m are approxi-

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mately constant, and those of n are changed. From this fact and Eq. (10) the activities of bac. α-amylase are thought to be increased by the addition of salts.

Table 4

Salt added	m	n
none	348.4	0.0006
LiCl	348.5	0.0015
NaCl	348.0	0.0018
none	184.3	0.0007
NaCl	184.5	0.0012
$NaNO_3$	184.4	0.0015

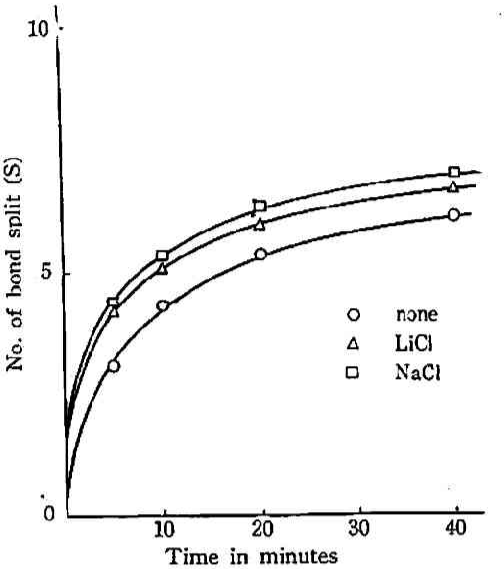

Fig. 10 Effect of salts

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