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Studies on Lysozyme Catalyzed Transglycosylation

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The lysozyme catalyzed transglycosylation with glycol chitin or related oligosaccharides as substrate and *p*-nitrophenyl β-glycoside of 2-acetamido-2-deoxy-D-glucopyranoside as acceptor was studied in order to obtain basic information which is expected to be available for the elucidation of the mechanism of lysozyme catalyzed reaction.
In the present experiments, the general features of the transglycosylation, the effects of additives, the total transglycosylation by gel-filtration method and the abilities of several sugars as acceptor were examined and the exact comparison between the hydrolysis and the transglycosylation was made. Some subjects related to the mechanism of lysozyme catalysis were also discussed briefly.

INTRODUCTION

It has been well recognized that most carbohydrolases catalyze transglycosylation as well as hydrolysis of glycosidic linkages. In the case of lysozyme catalyzed reaction, it had been vaguely recognized that lysozyme could catalyze a reaction other than the hydrolysis of glycosidic linkages, owing to the observation that the appearance of reducing power in the hydrolysis of polysaccharides of 2-acetamido-2-deoxy-D-glucopyranose (NAG) showed frequently an irregular pattern depending upon the conditions used for the enzymatic reaction (for example ; Hayashi *et al.*, 1964). The first clear-cut observations on a transglycosylation in a lysozyme catalyzed reaction were reported by Kravchenko (1967), Rupley (1967) and Rupley *et al.* (1967). Kravchenko has reported that the digestion of disaccharide (NAG), by lysozyme was first observable after an incubation for a long period (about 7 hr) and it was remarkably accelerated by the addition of a small amount of tetrasaccharide $(NAG)_4$. This observation was explained by the transglycosylation catalyzed by lysozyme. Rupley *et al.* observed that when $(NAG)_4$ was incubated with lysozyme in the presence of ^{14}C NAG, the radioactive residue was incorporated into the oligosaccharides of NAG at the reducing end. This was most reliable evidence of transglycosylation catalyzed by lysozyme. Thereafter, the transglycosylation was intensively investigated in many laboratories in connection with the mode of binding of the substrate on the active site of lysozyme and with the mechanism of the lysozyme catalysis.

Abbreviations NAG :2-Acetamido-2-deoxy-D-glucopyranose, (NAG), :n-Saccharide of NAG with p-glycosidic linkage.

Pollock et *al.* (1967) studied the capability of various saccharides as acceptor in the transglycosylation and estimated the free energy change in the acceptor binding at the subsites E and F.

Raftery and Rand-Meir (1968) used p-nitrophenyl glycosides as acceptor and (NAG), as substrate, and measured the incorporation and the release of *p*-nitrophenol moiety of the acceptor. They found that p-nitrophenyl moiety of the acceptor was incorporated into various oligosaccharides produced by lysozyme catalysis and that the amount of released p-nitrophenol was largest in the case where *p*-nitrophenyl β-2-deoxy-D-glucopyranoside was used as the acceptor. From these results, the mechanism of lysozyme catalysis was discussed in detail.

Rupley *et al.* (1968) studied the efficiency of transglycosylation with various alcohols or thiol compounds as acceptor, and concluded that the intermediate in a lysozyme catalyzed reaction is a saccharide fragment with the residue in the form of C, carbnium ion at the reducing end.

Lysozyme is one of enzymes of which the reaction mechanisms have been well understood due to the establishment of their three-dimensional structures. However, there remains several problems to be elucidated to demonstrate the fine mechanism of lysozyme catalysis. Those are, for instance, the structure and stabilization mechanism of the intermediate, the mode of stabilization of the catalytic group Glu 35 in conjugate base form, the role of Asp *52* in carboxylate ion form and so on.

It has been well known that the efficiency of a lysozyme catalyzed transglycosylation is exceedingly larger than that of the hydrolysis, on the molar basis of acceptor and water molecules in the reaction system (Chipman, 1971). There might be a special mechanism or molecular device providing the high efficiency of the transglycosylation, because it takes more complicated pathway, such as release of broken fragment of the substrate from the subsites E and F, and the binding of the acceptor molecule at the same subsites, than the case of the hydrolysis with the simple attack of the water molecule. If an origin of such a high efficiency of the transglycosylation is elucidated in comparison with the efficiency of the hydrolysis, it may be expected that more fine mechanism of lysozyme catalysis could be discussed.

The present paper deals mainly with experimental results on the transglycosylation in a reaction system consisted of lysozyme, glycol chitin or oligosaccharides of NAG as the substrate and p-nitrophenyl NAG as the acceptor.

EXPERIMENTAL

Materials

Hen's egg-white lysozyme was a five recrystallized preparation. 6-O-Carboxymethyl chitin (CM-chitin) and partially deacetylated chitosan were prepared according to the methods reported in a previous paper (Hayashi *et al., 1969).* Iodine-oxidized lysozyme (IO-lysozyme) was prepared according to the method reported by Hartdegen and Rupley (1967), and the esterification of lysozyme (E-lysozyme) was carried out by the method of Fraenkel-Conrat (1950).

p-Nitrophenyl NAG was synthesized according to the method of Leaback and Weissmann (1963). Oligosacchrides of NAG were prepared from a partial

hydrolyzate of chitin by the method of Rupley (1964) with detailed advice from his laboratory. 2-Benzamido-2-deoxy-D-glucopyranose was synthesized by the method of Konstas *et al. (1959).* Methyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Methyl β-NAG) and its anomer (Methyl a-NAG) were prepared from NAG in absolute methanol using Amberlite IR ***120*** (H-form) as a catalyst and fractionated on a charcoal-celite column (Zilliken ***et al.***, 1955).

Ovalbumin was three-times recrystallized before use. Casein was Hammerstein preparation and gelatin was the commercial preparation. The other reagents were of analytical grade.

Preparation of glycol chitin

Five grams of purified and pulverized chitin was soaked in 42 % sodium hydroxide solution for 3 hr at 30°C under reduced pressure. The swollen chitin (alkali chitin) was collected on a glass-filter and sodium hydroxide solution was removed as completely as possible with suction pump. Crushed ice (-20°C, 3.5 times weight of the alkali chitin) was mixed with the alkali chitin in a stainless-steel beaker and the mixture was kneaded by a stainless-steel spoon for about 30 min while keeping the temperature below -10°C. To the resulting paste was added a 42 % sodium hydroxide solution necessary to increase the concentration of sodium hydroxide in the paste to 14 %. Thus prepared paste or viscous syrup was kept in ice bath, 5 g of ethylene oxide was added and mixed well to distribute uniformly. The reaction mixture was allowed to stand for 1 hr in ice bath and the same amount of ethylene oxide was added. After standing for another 1 hr in ice bath, the viscous mixture was brought to room temperature and allowed to stand for ***2*** hr. To the resulting mixture, ethanol was added to 90 %, and the precipitate was washed several times with 90 % ethanol to remove sodium hydroxide. The precipitate was dissolved in about 500 ml of water and neutralized by acetic acid, dialyzed against several changes of water. Finally it was lyophilized.

Modification of lysozyme with water-soluble carbodiimide (WSCD-lysozyme)

To a solution containing 200 mg of lysozyme and 500 mg of glycinamide at pH 4.7 and 4°C, total 200 mg of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide was added in five portions while keeping the pH value of the reaction mixture at 4.7 by addition of N hydrochloric acid. After converting the excess carbodiimide to the corresponding urea by addition of N acetic acid, the solution was dialyzed against water and lyophilized. The prepared sample contained two molar excess of glycine residues per mole of lysozyme, i. e. two carboxyl groups were modified to amide.

Modification of lysozyme with Woodward's reagent K (WR-lysozyme)

To 20 ml of a 1 % lysozyme solution at pH 6.5 and 4°C, total 40 mg of Woodward's reagent K was added in three portions at the intervals of 30 min. Ater final addition of the reagent, the reaction mixture was allowed to stand for 2 hr. The reaction mixture was dialyzed against water and lyophilized. Spectrophotometric estimation, assuming that the molar extinction coefficient of the reagent is 14500, showed that modified lysozyme contained two reagents per mole lysozyme.

Hydrolysis of p-nitrophenyl NAG

The reaction mixture containing lysozyme (2-10 x 10^{-4} M), glycol chitin (l-6 x 10^{-3} M*) and p-nitrophenyl NAG (0.1-5 x 10^{-3} M) in 0.05 M phosphate buffer, pH **6.0** was incubated at 40°C for required periods, and the amount of released p-nitrophenol through the transglycosylation and the following hydrolysis (see Scheme 1) was determined by spectrophotometry at 400 nm after the pH value of the reaction mixture was adjusted to 9 by addition of *0.2* M sodium phosphate dibasic. The molar extinction coefficient of the p-nitrophenol was assumed to be 18000 (Raftery and Rand-Meir, 1968). The rate of the release of p-nitrophenol was calculated as the initial slope (M/min) of the line in plotting of the released amount of p-nitrophenol vs the incubation time.

Gel-filtration

After the reaction mixture **(25** ml) was adjusted to pH 4 by addition of N hydrochloric acid, p-nitrophenol released during the incubation was removed by successive extractions with ether. The pH value of the solution was again adjusted to 6. The resulting solution was applied onto a column (1.3 x *90* cm) of Bio-Gel P-2 **(100-200** mesh) and eluted by 0.05 M phosphate buffer at pH 6.0. Flow rate was 80 ml per hour.

Determination of amount of transferred acceptor

A half ml of the effluent was added to 3 ml of 0.1 N sodium hydroxide solution, and the solution was heated in boiling water bath for **30** min. Yellow color of liberated p-nitrophenol was measured as the optical density at 400 nm. The molar concentration of p-nitrophenol was calculated from the optical density using the molar extinction coefficient of 18000.

Reducing power and viscosity

The reducing power was determined by either the Somogyi method or ferricyanide method (Imoto and Yagishita, 1971). The analysis of oligosaccharides was carried out by following procedure: One ml of oligosaccharide solution was added to *2* ml of *2 N* hydrochloric acid, and heated in boiling water bath for one hr. After neutralizing the solution with 5 N sodium hydroxide solution, the reducing power was measured by ferricyanide method. The viscosity decrease due to the hydrolysis of the substrate glycol chitin was measured by the method reported in a previous paper (Hayashi ***et al.,*** 1963).

Detection of lysozyme-substrate complex

The lysozyme-substrate complex was detected by difference spectrophotometry using a solution of *0.1* % lysozyme and **0.2** % substrate solutions.

RESULTS

General features of transglycosylation

Time-course of release of p-nitrophenol

The release of p-nitrophenol showed a large initial rate for about one hour

* Molar concentration of substrate glycol chitin was calculated on the basis of residual weight of NAG.

after the initiation of the reaction as shown in Fig. 1. Thereafter, the released amount of p-nitrophenol exhibited a completely linear increase with regard to the reaction time. After 48 hr incubation, only 1.5 % of initial amount of the acceptor liberated the p-nitrophenol, i. e., lysozyme did yet not experience one-turnover at this time. The released amount of p-nitrophenol did not reach to the total amount of p-nitrophenyl moiety transferred to saccharide part, and some part of the transferred acceptor might have remained in the saccharide part without undergoing the hydrolysis.

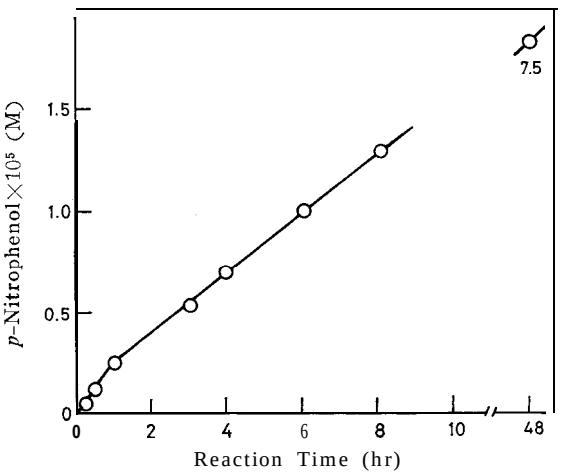


Fig. 1. Time-course of release of p-nitrophenol. Lysozyme : 2×10^{-4} M. Glycol chitin : 5×10^{-3} M. Acceptor : 5×10^{-3} M. Incubated at 40°C and pH 5.5.

pH-Dependence of release of p-nitrophenol

The maximum rates of the hydrolysis and the transglycosylation were found in nearly the same pH region, as shown in Fig. 2. Contrary to the pH-rate profile of the hydrolysis (Hayashi ***et al.,*** 1963), the transglycosylation exhibited much efficiency in a pH region from 6 to 8. When the large excess of the substrate was added to the enzyme solution, it was found that the lysozyme-substrate complex was formed fully in the same region, pH 6 to 8. Furthermore, a shoulder around pH 7 in the pH-hydrolytic activity profile was not able to be explained until nowaday. It, therefore, means that in the pH region from 6 to 8, the cleavage of β-1,4-glycosidic linkage may conduct preferentially the transglycosylation with an appropriate acceptor.

Temperature-dependence of release of p-nitrophenol

The temperature-dependence of the transglycosylation shifted toward the high temperature side with the maximum at 70°C, in comparison with that of the hydrolysis as shown in Fig. 3. The profile of the temperature-dependence of the hydrolysis exhibited a shoulder in a region from 60 to 75°C. This characteristic dependency may be thought to be reflection of specific behavior of the transglycosylation at high temperature region.

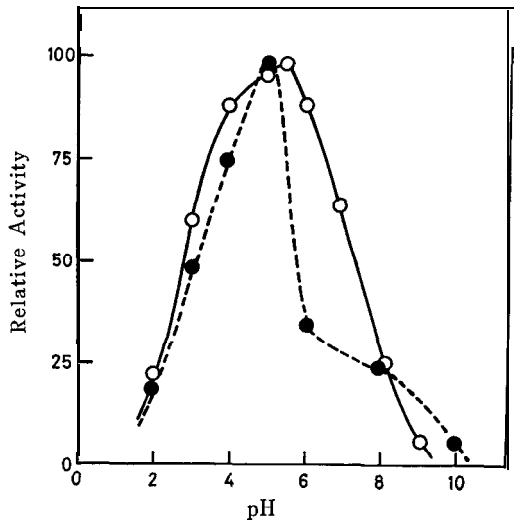


Fig. 2. pH-Dependence of release of p-nitrophenol. 0 : Release of p-nitrophenol. ●: Hydrolysis.

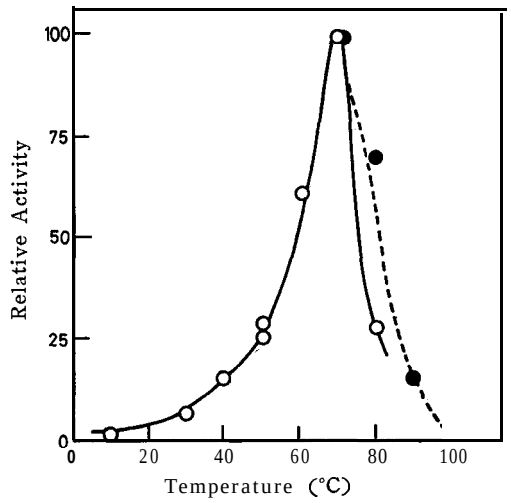


Fig. 3. Temperature-dependence of p-nitrophenol release. Final concentrations of lysozyme, glycol chitin and acceptor were 5×10^{-4} M, 0.11 % and 5×10^{-3} M, respectively. Reaction mixtures were incubated for 15 min (0) and 5 min (0).

Effects of enzyme and substrate concentrations on release of p-nitrophenol

The plottnig of the released amount of p-nitrophenol vs the concentration of the enzyme (Fig. 4-(a)) or the substrate (Fig. 4-(b)) exhibited completely straight line, similar to that observable with other enzymes. This infers that the liberation of p-nitrophenol in the experimental system is due to the process accelerated catalytically.

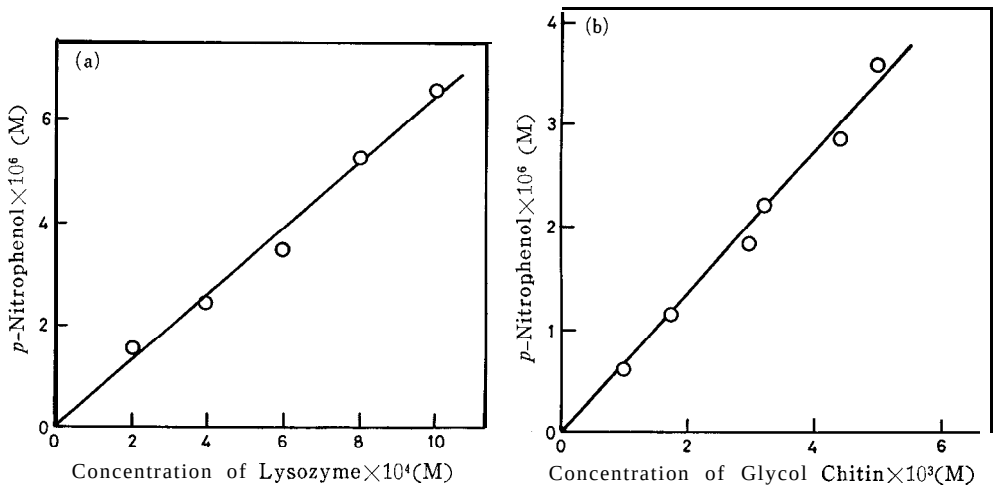


Fig. 4 (a) Effect of lysozyme concentration on release of p-nitrophenol. Concentrations of glycol chitin and acceptor were 1.66×10^{-3} M. Reaction mixture was incubated for 4 hr at 40°C and pH 5.5.
(b) Effect of substrate concentration on release of p-nitrophenol. Concentrations of lysozyme and accepotr were 3.3×10^{-4} M and 1.66×10^{-3} M respectively. Reaction mixture was incubated for 4 hr at 40°C and pH 5.5.

Effect of acceptor concentration

The effect of the acceptor concentration on the release of p-nitrophenol (Fig. 5-(a)) and the Lineweaver-Burk plot with the concentration of the acceptor (Fig. 5-(b)) indicated that the rate of the release of p-nitrophenol was of a considerably low order.

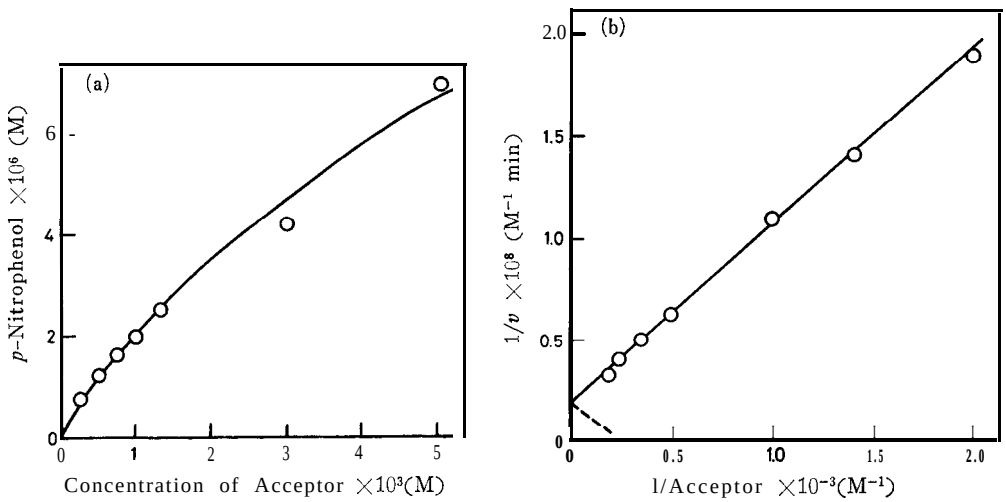


Fig. 5(a). Effect of acceptor concentration on release of p-nitrophenol. Concentrations of lysozyme and glycol chitin were 2×10^{-4}M and 3×10^{-3}M, respectively. Incubation was done under the same conditions as in Fig. 5.
(b) Lineweaver-Burk plot on acceptor. V_{max} and K_m values are estimated to be 5×10^{-8} (M, min^{-1}) and 3.85×10^{-3} (M), respectively.

Various substrates

The efficiencies of several substrates on the release of p-nitrophenol are shown in Table 1, in comparison with their relative values of digestibilities and capabilities to form the lysozyme-substrate complex. There are no clear relationships among these values. The glycol chitin which was previously and entirely digested by lysozyme, did not show any further digestbility, but it exhibited an appreciable capability as a substrate for the transglycosylation and for the following hydrolysis that causes the release of p-nitrophenol. The same tendency was observed for the other substrates. The explanation for these distinctive phenomena will be dealt later.

Table 1. Relative rates of release of p-nitrophenol and of hydrolysis. For release of p-nitrophenol, reaction mixture was incubated for 4 hr at 40°C and pH 5.5. Hydrolysis was followed by measurement of reducing power under the same conditions as above.

Substrate	Release of p-nitrophenol	Hydrolysis	Amount of ES complex
Glycol chitin	100	100	100
Glycol chitin hydrolyzed[a]	30	0	100
CM-chitin	75	—	100
CM-chitin hydrolyzed[b]	42	0	100
CM-chitin hydrolyzed[c]	40	0	100
Chitosan[d]	81	35	95
Chitosan[e]	55	10	70

a) Polymerization degree of 5. b) Low molecular weight fraction. c) High molecular weight fraction. d) 50 % deacetylation. e) 75 % deacetylation.

Modified lysozyme

In order to correlate the transglycosylation with the hydrolysis or the formation of the lysozyme-substrate complex, several modified lysozymes were subjected to the transglycosylation reaction. The extent of the modification of each preparation was conveniently represented by the remaining activity because the determination of the kinds and the numbers of modified groups were quite difficult task.

A parallel correlation was observed between the remaining activity and the ability to form the lysozyme-substrate complex. However, the transglycosylation activity did not simply correlate to the hydrolytic activity (Table 2),

Table 2. Transglycosylation activity of modified lysozyme.

Lysozyme	ES complex formation[a]	Hydrolytic activity[b]	Release of p-nitrophenol
Native lysozyme	100	100	100
WSCD-lysozyme	49.0	97.0	84.5
WR-lysozyme	57.8	60.3	6.3[c]
E-lysozyme	54.8	60.2	25.0[c]
IO-lysozyme[d]	61.0	53.0	50.0
IO-lysozyme[d]	13.0	10.7	21.0

a) Measured by difference spectrophotometry. b) Measured by viscometry. c) WR- and E-lysozymes exhibited a large release of p-nitrophenol in the absence of substrate. d) Oxidation degrees are different.

especially for the WR-lysozyme. It should be noted that WR- and E-lysozymes exhibited an abnormally high ability to release the p-nitrophenol in the absence of the substrate, and the WR-lysozyme also showed the high efficiency for the release of p-nitrophenol in a pH region from 9 to 10.

Comparison of time-course of release of p-nitrophenol and appearance of reducing power

Under the experimental conditions, the increase in the reducing power of the reaction mixture ceased after 6 hr incubation, and it was calculated at this point that lysozyme has undergone 110 turnovers (Fig. 6). However, the release of p-nitrophenol continued for a long period with a constant rate. At 12 hr incubation, lysozyme showed, however, only 0.1 turnovers in respect with the release of p-nitrophenol. From these facts, it may not be concluded that lysozyme provides much more capability for the hydrolysis than for the transglycosylation. The relative ratio of hydrolytic turnover to that of the release of *p*-nitrophenol was calculated to be 1: 8, when the molar concentrations of the water and the acceptor (5 x 10^{-3} M) were taken into consideration.

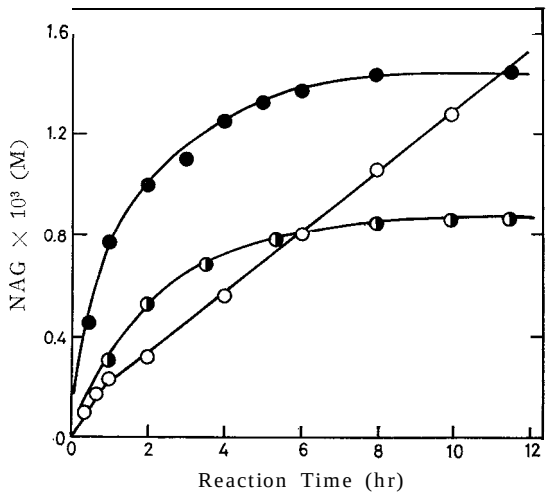


Fig. 6. Comparison of release of p-nitrophenol and appearance of reducing power. ○: Release of p-nitrophenol. ●: Reducing power (1 ml of 1.25×10^{-5} M lysozyme and 10 ml of 2.23×10^{-2} M glycol chitin). ◑: Reducing power (1 ml of 1.24×10^{-5} M lysozyme, 5 ml of 4.46×10^{-2} M glycol chitin and 5 ml of 1.32×10^{-2} M acceptor). Reaction mixture was iucubated at 40 C and pH 5.5.

The presence of the acceptor (6.61 x 10^{-3} M) in the reaction mixture caused the inhibition of the appearance of reducing power. This inhibition of the hydrolysis decreased not only the rate of appearance of reducing power but also the maximum amount of the reducing power produced finally. The presence of the acceptor at such a low concentration may not inhibit the binding of the substrate to the active site of lysozyme and the following cleavage of β-1,4-glycosidic linkage. The less amount of reducing power seen after prolonged incubation time might be explained by the consumption of the reaction inter-

mediate by an acceptor nucleophile present in the reaction mixture, resulting in the transglycosylation.

Inhibition of hydrolysis by acceptor

The inhibition of the appearance of the reducing power was studied as a function of the concentration of the acceptor (Fig. 7). The appearance of the reducing power in the control system including only lysozyme and the acceptor was the same as that in the system of the acceptor and buffer solution; there was no activity of lysozyme on liberation of p-nitrophenol from the acceptor, when the substrate was omitted from the system.

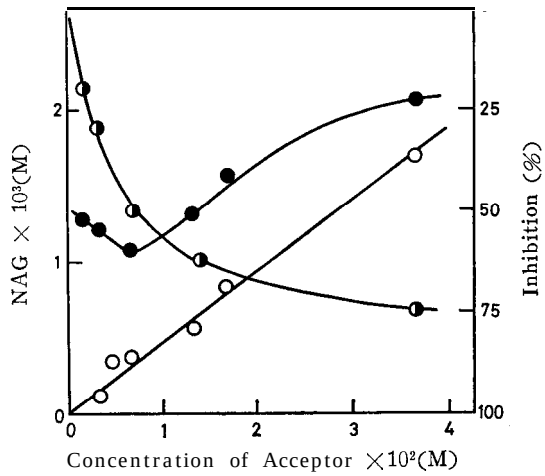


Fig. 7. Inhibitory effect of acceptor on appearance of reducing power. ○: Consisted of 1 ml of 1.24×10^{-5} M lysozyme and 10 ml of acceptor. ●: Consisted of 1 ml of 1.24×10^{-5} M lysozyme and 10 ml of 2.23×10^{-2} M glycol chitin containing acceptor. ◑: Inhibition.

Effect of additive

Release of p-nitropkenol by amino acid

The acceptor p-nitrophenyl NAG is not stable, rather being the sort of an active glycoside. It is possible that the acceptor may be hydrolyzed non-enzymatically in the reaction mixture. If this is the case, there may be a considerable confusion in the estimation of the efficiency of the transglycosylation. Table 3 shows the catalytic activities of several amino acids in the presence or the absence of the substrate glycol chitin. Amino acids have no appreciable acceleration or inhibition of the hydrolysis of the acceptor in the absence of lysozyme.

Effects of amino acids on release of p-nitropkenol by lysozyme

The effects of amino acids which were added to the reaction mixture on the release of p-nitrophenol are listed in Table 4. It is clear that no effects are observed except for the actions of cysteine and aspargine. Since cysteine exhibited an appreciable acceleration effect, sulfhydryl group is considered to be active group. In the case of aspargine, the amide is easily assumed to be cataly-

Table 3. Release of p-nitrophenol by amino acids.

Amino acid	In absence of glycol chitin[a] (10^{-5} M)	In presence of glycol chitin[a] (10^{-5} M)
None[b]	3.5	3.1
Glycine	3.2	3.2
Aspartic acid	2.5	3.5
Glutamic acid	—	3.8
Arginine	2.9	3.2
Lysine	—	3.5
Histidine	3.5	3.6
Asparagine	3.2	3.0
Tryptophan	—	3.4

a) Concentrations of amino acid, acceptor and substrate were 1.7×10^{-3} M, 5X10-3 M and 0.11 % respectively. b) Blank value. Incubated at 40°C, pH 5.5 for 48 hr.

Table 4. Effect of amino acid on release of p-nitrophenol by lysozyme.[a]

Amino acid	pH	Relative value	Amino acid	pH	Relative value
None[b]	5.5	100	Cysteine	5.7	146
Glycine	5.5	87–107[c]	Cystine	5.7	100
Alanine	5.5	88-107	Methionine	5.7	83
Valine	5.5	85-107	Aspartic acid	5.1	109
Leucine	5.5	85-111	Asparagine	5.7	147
Serine	5.5	83-106	Glutamic acid	5.2	120
Threonine	5.5	83–106	Lysine	5.7	119
Tyrosine	5.5	83-111	Arginine	5.7	116
Phenylalanine	5.5	83-102	Histidine	5.6	107
Tryptophan	5.5	80-102			

a) Sample system was lysozyme (5×10^{-4} M), glycol chitin (0.11 %), p-nitrophenyl NAG (5X10-a M) and amino acid (2×10^{-2} M). Concentrations of tryptophan, tyrosine and phenylalanine were 0.6×10^{-2} M. Reaction conditions ; at 50°C, for 4 hr. b) Blank run was carried out in the absence of amino acid. c) Data of four experiments fall in this region.

tically inactive and either a- or β-carboxyl group is thought to be active. On the other hand, nucleophilic groups such as sulfhydryl and carboxyl groups in amino acid could be assumed to be acceptor under the present experimental conditions. The mechanism by which the acceleration effects appeared may, therefore, not be simply understandable.

Effects of proteins

There seems to be a possibility that the release of p-nitrophenol in the reaction mxture may be caused in part by the non-enzymatic action of lysozyme molecule, because the acceptor is referred to be an active glycoside. In order to observe such a possibility, a few proteins were subjected to the experiment. As shown in Table 5, ovalbumin and casein exhibited an apreciable acceleration of the release of p-nitrophenol. These may arise mainly from the cysteine residues of the proteins or from some other residues such as phosphoryl group. In contrast, lysozyme alone did not show any activity for the release of p-nitrophenol from the acceptor. This is consistent with the fact that lysozyme does not contain cysteine residue. When the substrate presents in the reaction mixture, the release of p-nitrophenol by lysozyme became first obser-

Table 5. Release of g-nitrophenol by various proteins.

System[a]	Relative value of release of *p*-nitrophenol[b]	
	24 hr incubation	48 hr incubation
Lysozyme + glycol chitin + g-nitrophenyl NAG	100	100
Lysozyme+p-nitrophenyl NAG	0	0
Ovalbuminfp-nitrolphenyl NAG	46	66
Casein+*p*-nitrophenyl NAG	7	66
Gelatin+*p*-nitrophenyl NAG	8	

a) Concentrations of proteins, glycol chitin and g-nitrophenyl NAG were 0.3 %, 0.11 % and 5×10^{-3} M respectively. b) Incubated at 40°C and pH 5.5.

vable . Thus, the release of p-nitrophenol by lysozyme in the reaction mixture can be considered to be entirely the result of the enzymatic catalysis.

Effects of sugars

The effects of sugars (final concentration: 2 M) added to the reaction mixture on the release of p-nitrophenol are shown in Table 6. All sugars except for sucrose and L-arabinose inhibited the release of p-nitrophenol of the transglycoylation to various extents. The acting points of the sugars as inhibitors are assumed to be (1) competitive binding to the active site, (2) deccreasing the activity of the catalytic group, (3) competitive binding at the subsites E and F (binding sites for acceptor) and (4) competive reaction as another acceptor with the reaction intermediate. The inhibition mechanism of individual sugars, therefore, cannot be concluded simply from the experimental data. D-Glucose exhibited the largest inhibition among used sugars except for NAG. Galactose was a poor acceptor because of its -OH configuration at the C, position. Then the small inhibition of galactose might arise from the competive binding at the subsite E. D-Glucose and D-galactose have same configuration, except for the configuration at the C_4 position. The excess inhibition of D-glucose than D-galactose may be due to the competitive reaction as an acceptor to the reaction of p-nitrophenyl NAG. NAG at a considerably low concentration showed a pro-

Table 6. Effect of saccharides on release of p-nitrophenol by lysozyme.

Saccharide[a]	Relative value
None	100
Sucose	108
Lactose	69.2
Maltose	83.0
D-Glucose	24.6
D-Mannose	43.0
D-Galactose	60.0
D-Fructose	72.3
L-Arabinose	105
Raffinose	78.5
NAG	6.0[b], 16.0[c]

a) Saccharide was added to the system of lysozyme, substrate and acceptor and the mixture was incubated for 4 hr at 40°C and pH 5.5. Concentration of saccharide was 2.0 M except for NAG. b) Concentration was 0.5M. c) Concentration was 0.057 M.

nounced inhibition. This seems to arise from its good capability as an acceptor, competing with the acceptor p-nitrophenyl NAG in the transglycosylation.

Effect of NAG

The effect of NAG added to the reaction mixture in place of the substrate is shown in Fig. 8, together with the result from the reaction system containing glycol chitin as the substrate. Since NAG is not hydrolyzable substance, the release of p-nitrophenol must be derived by another unique mechanism. NAG forms the complex with lysozyme with the equilibrium constant of 15-20 M^{-1} (Chipman *et al.*, 1967). In the presence of 0.5 M NAG, only 5 % of lysozyme is calculated to be in free form and 95 % is in complex form in which NAG binds at the subsite C (Rupley *et al.*, 1966). If the binding of acceptor p-nitrophenyl NAG at the subsites D and E were cooperatively enhanced by the NAG bound at the subsite C, the release of p-nitrophenol in the absence of the substrate would be rationally explained. This may be called cooperative binding and cooperative hydrolysis of the acceptor p-nitrophenyl NAG. The fraction of released amount of p-nitrophenol by such hydrolysis, however, cannot be quantified in the present time.

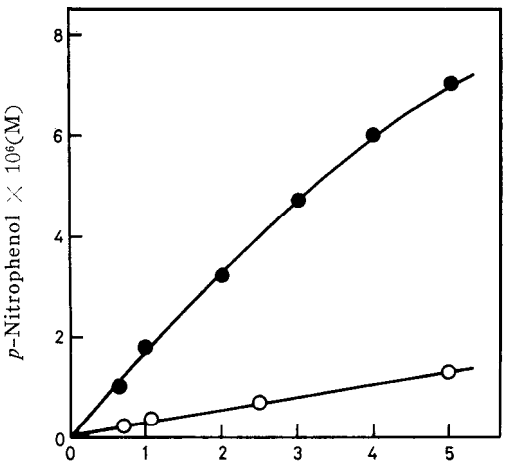


Fig. 8. Release of p-nitrpphenol. ●: Lysozme (2×10^{-4}M) + p-nitrophenyl NAG (5×10^{-3}M) + glycol chitin. ○: Lysozyme (2×10^{-4}M) fp-nitrophenyl NAG (5×10^{-3} M) + NAG. Reactions were carried out for 4 hr at 40°C and pH 5.5.

Effects of alcohols

The inhibitory effects of ethanol on the release of p-nitrophenol and the hydrolysis (by viscometry) of glycol chitin are shown in Fig. 9-(a). In general, the effect of many inhibitors tends to be overestimated in viscometry than in other assay methods. Since there is parallelism between both inhibitions, the effects of ethanol seem to be produced by simple change in the environment of the cleavage reaction of the glycosidic linkage, although it is known that ethanol also could be an acceptor of the transglycosylation (Rupley *et al.*, 1968; Ikeda and Hamaguchi, 1970). The inhibitory effects of various alcohols are shown in

Fig. 9-(b) as a function of incubation time. Apparent acceleration of various alcohols would be connected to their capabilities as acceptor in the transglycosylation.

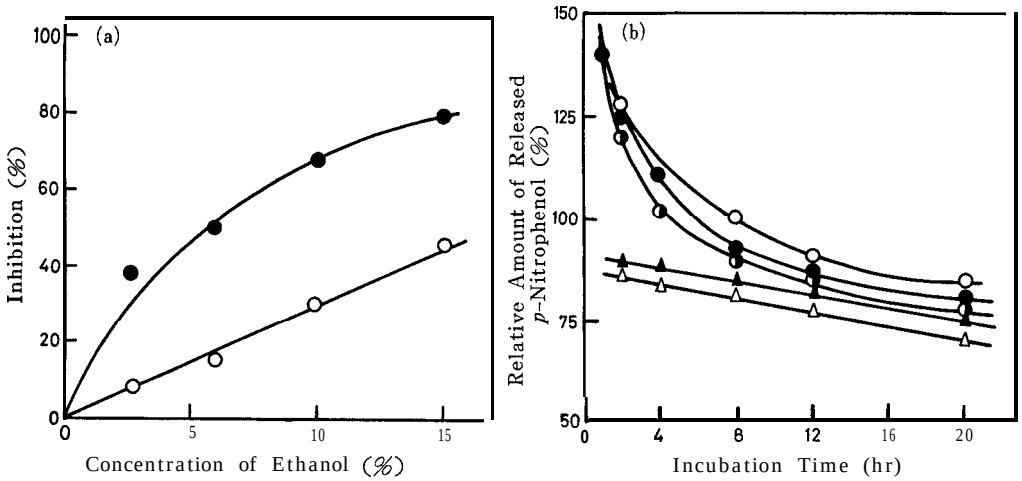


Fig. 9. (a) Inhibition by ethanol. 0 : Release of p-nitrophenol (at 24 hr incubation) ●: Hydrolysis measured by viscometry (at 3 min incubation).
(b) Effects of various alcohols on release of p-nltrophenol as a function of incubation time. 0 : Butanol. ●: n-Propanol. ◑: Isopropanol. ▲: Methanol. △: Ethanol.

Effect of sodium chloride

The inhibitory effect of sodium chloride on the release of p-nitrophenol was nearly identical to the effect on the hydrolysis of glycol chitin measured by reducing power method or viscometry (Fig. **10).** The hydrolysis (viscometry) in the reaction mixture not containing the acceptor was profoundly inhibited by sodium chloride, as had been reported in a previous paper (Imoto ***et al.,*** 1969). The reason why the presence of the acceptor p-nitrophenyl NAG lowered the inhibitory effect of sodium chloride on the hydrolysis measured by viscometry, cannot be solved at the present time.

Effect of urea

It has been well known that urea inhibits reversibly the hydrolytic activity of lysozyme. A similar effect of the urea solution was observed for the release of p-nitrophenol (Fig. 11). The difference in the degrees of inhibition for the hydrolysis and for the release of p-nitrophenol may be explained in a way similar to the case of ethanol inhibition.

Analysis of transglycosylation products

Gel-filtration of reaction product

The pattern of gel-fiitration of the reaction mixture incubated for 2 hr is shown in Fig. **12.** The sacchrides were separated into four fractions, and *p*-nitrophenol was liberated from three fractions. The fraction I composed of mainly the unreacted acceptor and a trace of NAG. The fraction II may be composed of (NAG),, (NAG), and the p-nitrophenyl glycoside of (NAG),. Frac-

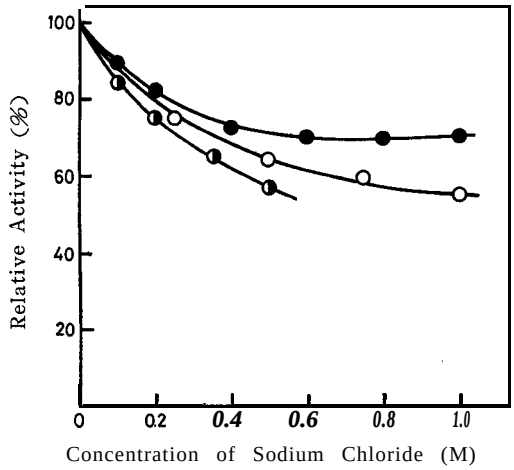
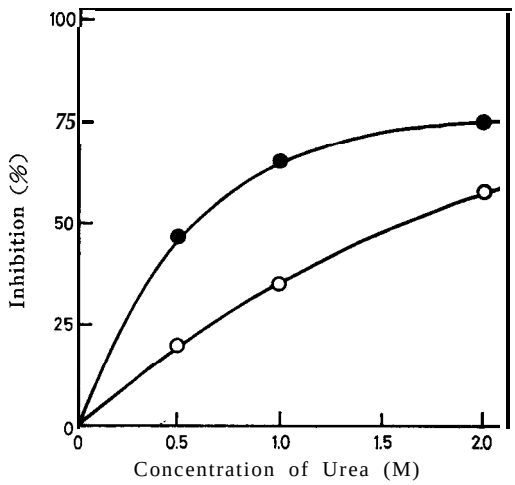


Fig. 10. Inhibition by sodium chloride. ●: Release of p-nitrophenol ; lysozyme (2 $\times 10^{-5}$ M) + glycol chitin (0.11 %) + p-nitrophenyl NAG (5×10^{-3} M), incubated for 4 hr at 40°C and pH 5.5. 0 : Reducing power ; lysozyme (1.24 X 10^{-5} M) + glycol chitin (0.5 %) + p-nitrophenyl NAG (7 $\times10^{-3}$ M), incubated for 4 hr at 40°C and pH 5.5. ◑: Viscosity ; lysozyme (2.73 $\times10^{-7}$ M) + glycol chitin (0.08 %) + *p*-nitrophenyl NAG (1.75×10^{-3} M), incubated for 3 min at 30°C and pH 5.5.



Fig, 11. Inhibitory effect of urea on release of p-nitrophenol. 0 : Lysozyme (2 $\times$ 10^{-4} M) + glycol chitin (0.11%) + p-nitrophenyl NAG (5 $\times10^{-3}$ M), incubated for 24 hr at 40°C and pH 5.5. ●: Inhibition for hydrolytic activity measured by viscometry.

tion III did not contain the p-nitrophenyl moiety. Fraction IV consisted of oligosaccharides larger than (NAG),. p-Nitrophenyl moiety was quite selectively accumulated to the fractions II and IV.

The reaction mixture incubated for 48 hr exhibited the gel-filtration pattern shown in Fig. 13. The pattern is very similar to that shown in Fig. 12, except

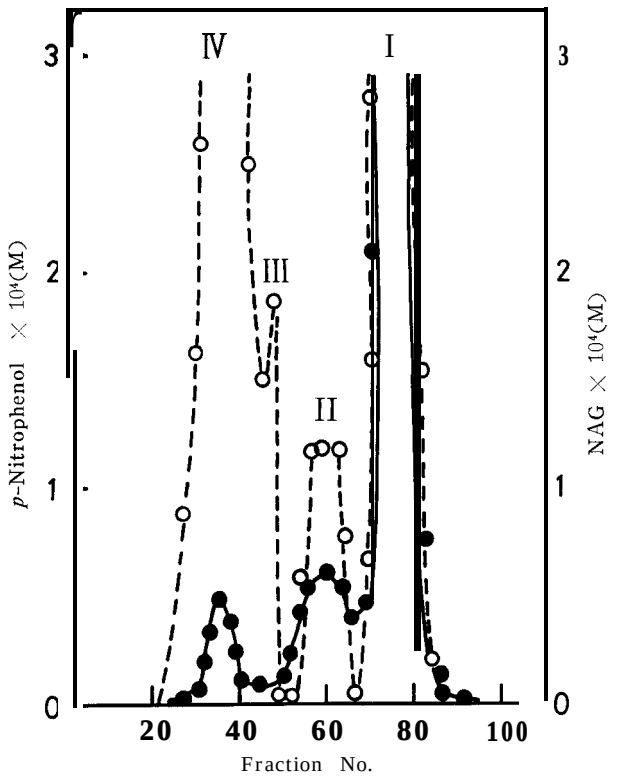


Fig. 12. Gel-filtration on Bio-Gel P-2 of reaction mixture incubated for 2 hr. 0 : Reducing power. ●: p-Nitrophenol.

that the distribution of p-nitrophenyl moiety is much more complicated and the peaks of oligosaccharides shifted toward more low molecular side. The gel-filtration pattern (not shown in figure) of the reaction mixture incubated for 16 hr was almost the same as that shown in Fig. 13.

Time-course of transglycosylation

The time-course of transglycosylation with p-nitrophenyl NAG as the acceptor is shown in Fig. 14, and Table 7. In contrast to the smooth increase in the released amount of p-nitrophenol, the accumulation of p-nitrophenyl moiety in the fragmented substrate (oligosaccharides) was quite characteristic; the *p*-nitrophenyl moiety was rapidly transferred to the fragmented substrate in the initial stages, at least, within 2 hr incubation, and thereafter the accumulated amount was decreased very gradually.

The appearance of the reducing power during the incubation was also remarkable in the initial stages as shown in Fig. 6. Thus, as far as the initial stages of incubation were considered, it can be concluded that the increase in the reducing power and the accumulation of the p-nitrophenyl moiety are roughly in prallel. Upon prolonged incubation, there was no more increase in the reducing power. It is clear that the continuous release of p-nitrophenol with a constant

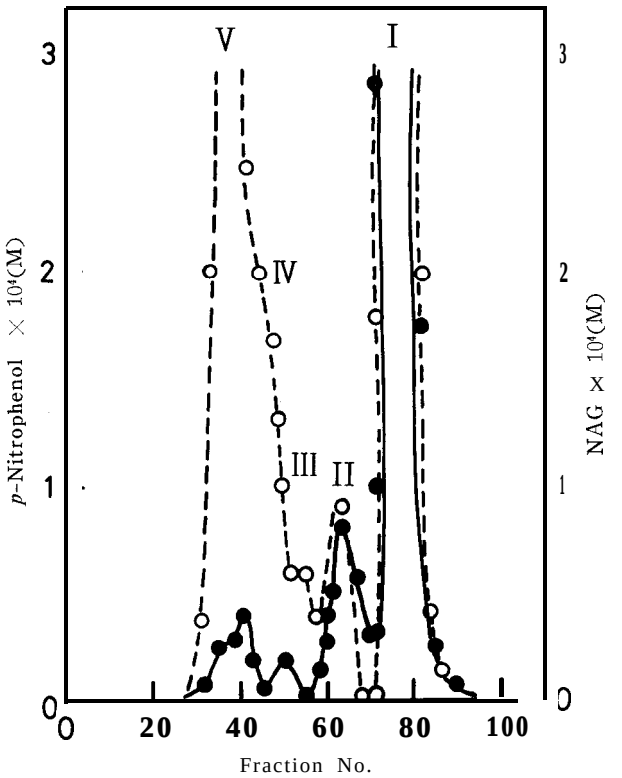


Fig. 13. Gel-filtration on Bio-Gel P-Z of reaction mixture incubated for 48 hr. ○ : Reducing power. ● : p-Nitrophenol.

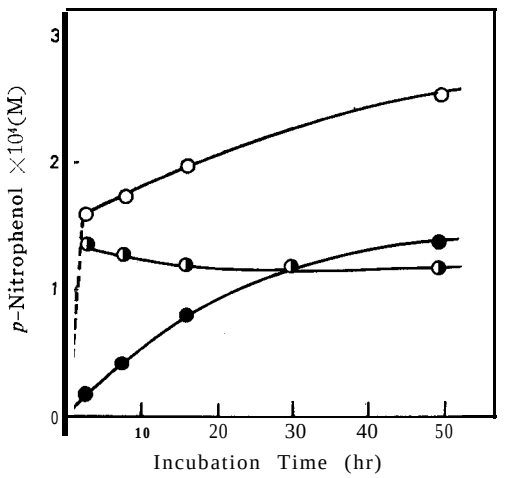


Fig. 14. Time-course of transglycosylation. ● : Release of p-nitrophenol. 0 : Total acceptor transferred into oligosaccharides. ◑ : Accumulated acceptor.

Table 7. Transfer of acceptor.

Incubation time (hr)	Transferred acceptor(10^{-4}M)	Released p-nitrophenol (10^{-4}M)	Accumulated acceptor (10^{-4} M)
2			
6	1.73 1.60	0.16 0.29	1.42
15			1.44
30	2.24 1.92	0.70 1.10	1.22
			1.24
48	2.52	1.35	1.20

rate was not derived from the consumption of p-nitrophenyl moiety accumulated in the initial stages of the incubation. The near constancy of the accumulated p-nitrophenyl moiety in a wide time-range may infer that the transglycosylation of the acceptor to the oligosaccharide fragments and the hydrolysis of *p*-nitrophenyl moiety in oligosaccharides was well balanced; that is, the released amount of p-nitrophenol. at a prolonged incubation time was proportional quantitatively to the extent of the transglycosylation of the acceptor. If the release of p-nitrophenol is caused by successive processes, the bond-cleavage of β-1,4-glycosidic linkage, the transfer of the acceptor and the following cleavage of p-nitrophenyl glycosidic linkage, it is clear that the intermediate formed by catalytic cleavage of β-1,4-glycosidic linkage only reacts with acceptor, but not with the water molecule at a prolonged incubation time.

Under the experimental conditions, the oligosaccharides produced by lysozyme catalyzed hydrolysis of the substrate are also the acceptors. The real transglycosylation, therefore, is expected to take place more than estimated from the accumulated amount of the acceptor p-nitrophenyl NAG or released amount of p-nitrophenol.

Oligosaccharide substrate

Release of p-nitrophenol with various substrates

Figure 15 shows the time-courses of the release of p-nitrophenol with various substrates. The concentrations of all substrates were fixed at 0.4 %. The time-courses of the release of p-nitrophenol with glycol chitin, (NAG), and (NAG), coincided surprisingly with each other under the experimental conditions, showing the non-dependency on the polymerization degree of the substrates. The time-course of (NAG), exhibited a concave profile, and after 10 hr incubation, the rate of the release of p-nitrophenol approached to those with the substrates above $(NAG)_5$. Disaccharide (NAG), and NAG showed almost no activity for the release of p-nitrophenol, when they were compared to the other substrates.

Release of p-nitrophenol with (NAG),

The time-course of the release of p-nitrophenol with $(NAG)_5$ at various concentrations is shown in Fig. 16-(a). The rate of the release was calculated from the released amount of p-nitrophenol at 2 hr incubation and it was plotted in Fig. 16-(b) as a function of the concentration of $(NAG)_5$. The plotting exhibited a typical profile of the saturation curve. The minimum saturation concentration was 1 x 10^{-3} M (0.103 %), and V_{max} and K_m were found to be 6 x 10^{-7} M min^{-1} and 2.5 x 10^{-4} M, respectively. From the Lineweaver-Burk plot (not shown in figure), V_{max} and K_m were calculated to be 8.2×10^{-7} M min^{-1} and 2.2 x 10^{-4} M,

respectively.

Release of p-nitrophenol with $(NAG)_4$

The rate of the release of p-nitrophenol with (NAG), as the substrate is

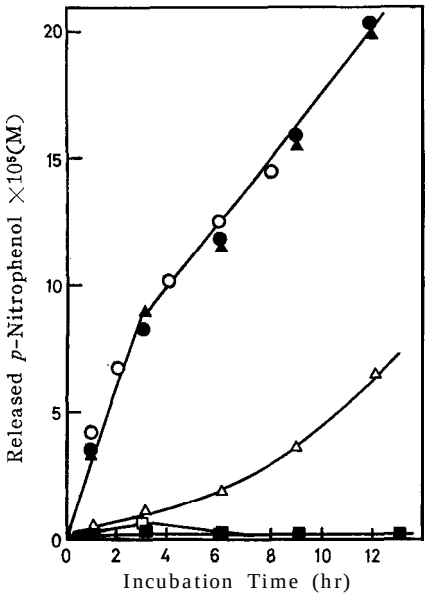


Fig. 15. Time-course of release of p-nitrophenol. ■ : NAG. □ : $(NAG)_2$. A: $(NAG)_4$. ▲ : $(NAG)_5$. ● : $(NAG)_6$. 0 : Glycol chitin.

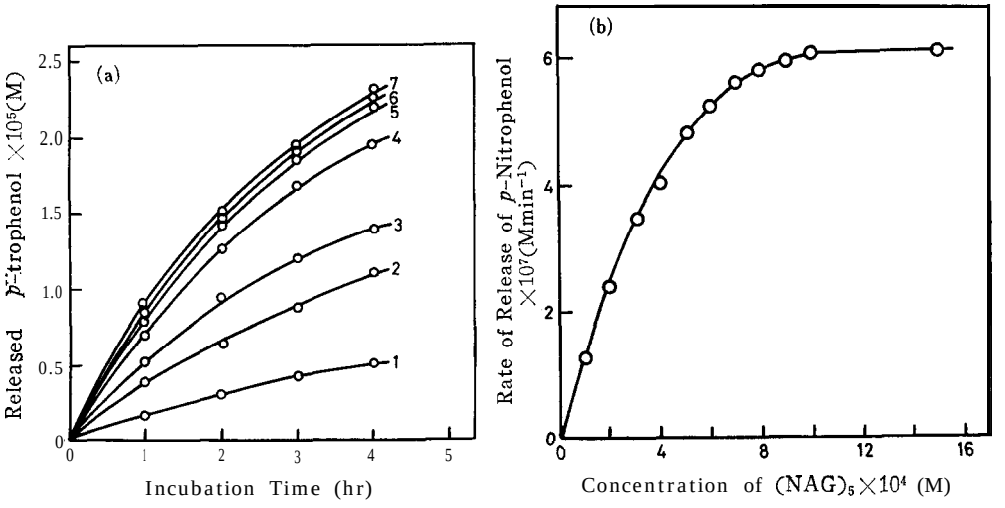


Fig. 16. (a) Release of p-nitrophenol with pentasaccharide of NAG as substrate. Concentrations of $(NAG)_5$ were, 1 : 1×10^{-4}M, 2 : 2×10^{-4}M, 3 : 3×10^{-4}M, 4 : 5×10^{-4}M, 5 : 7.5×10^{-4}M, 6 : 1×10^{-3}M, 7 : 2×10^{-3}M.
(b) Effect of concentration of pentasaccharide of NAG on release of p-nitrophenol.

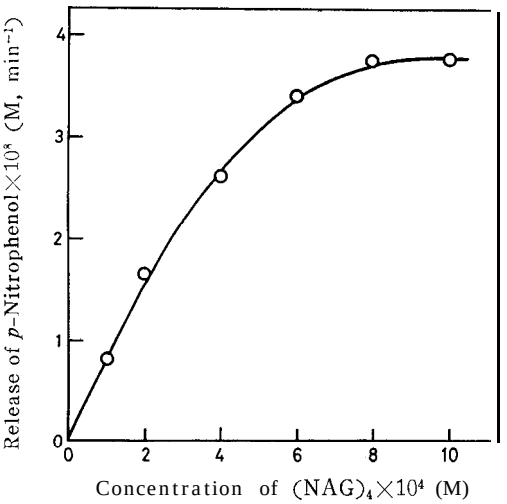


Fig. 17. Effect of concentration of tetrasaccharide of NAG on release of p-nitrophenol.

plotted in Fig. 17 as a function of the substrate concentration. The rate-con. centration profile shows the shape similar to that for the (NAG),. The kinetic parameters for some substrates are listed in Table 8. Although the V_{max} of (NAG), is about 1/20 of that of the (NAG),, K_m and minimum saturation concentration were nearly the same for both substrates.

Table 8. Kinetic parameters.

Substrate	V_{max}	K_m	Saturationc conc. (M)
$(NAG)_4$	3.6×10^{-8}	2.2x10-4	8×10^{-4}
(NAG),	6.0×10^{-7}	2.6×10^{-4}	8×10^{-4}
	(1.51×10^{-6})[a]	(9.1×10^{-5})[a]	
Glycol chitin	1.2x10-6	1.64×10^{-2} [b]	
		3.5x10-3 [c]	

a) Values in bracket represent ones for hydrolysis. b) Calculated by residual weight of NAG. c) Calculated by (NAG), units.

Rate of hydrolysis of various substrates

The change in reducing power of various substrates incubated with lysozyme are shown in Fig. 18 as a function of incubation time. The concentration of all substrates was fixed at 0.01 %. During the incubation, it was observed that NAG, (NAG), and (NAG), exhibited a slight loss in reducing power. In inital stage of the incubation (1.5 hr), there was an appreciable increase in the reducing power of (NAG),, while the increase in the reducing power of glycol chitin ceased within 6 hr incubation. The initial rate in the increase in the reducing power of glycol chitin was 6 times larger than that of the (NAG),. The clear difference in the rate of the hydrolysis and the transglycosylation will be discussed later in more detail.

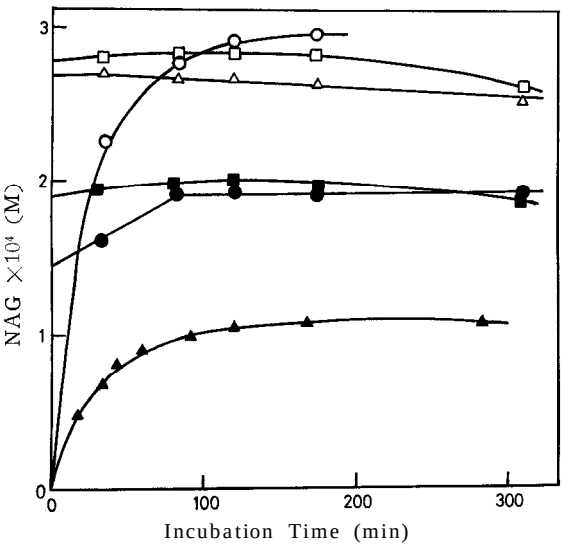


Fig. 18. Hydrolyses of various substrates. □ : NAG (4.3×10^{-4} M, 0.01 %). A : (NAG), (2.2×10^{-4} M, 0.01 %). ■ : $(NAG)_4$ 1.1X10-4 M, 0.01 %). ● : $(NAG)_5$ (8.6×10^{-5} M, 0.01 %). ▲ : Glycol chitin (0.05 %). 0 : Glycol chitin (0.1 %).

Hydorlysis of (NAG) $_5$

In order to make a comparison with the release of p-nitrophenol, the hydrolysis of the $(NAG)_5$ was followed in detail. Fig. 19 shows the time-dependence of the new formation of reducing power at various concentrations of $(NAG)_5$. In Fig. 20-(a), the plotting of rate of the hydrolysis of (NAG), calculated from the value at 40 min incubation is shown as a function of the (NAG), concentra-

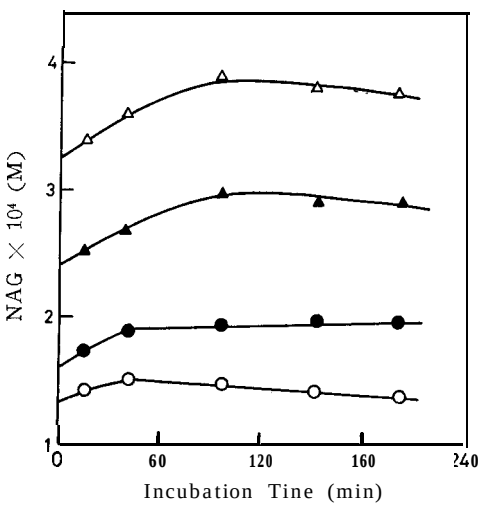


Fig. 19. Time-course of hydrolysis of pentasaccharide of NAG. Concentrations of $(NAG)_5$ o : 4.6×10^{-5} M. ● : 6.1×10^{-5} M. A : 9.15×10^{-5} M. △ : 12.1×10^{-5} M.

tion. Fig. 20-(b) shows the Lineweaver-Burk plot made by the data shown in Fig. 20-(a). The values of V_{max} and K_m were found to be 1.51 x 10^{-6} M min^{-1} and 9.1 x 10^{-5} M, respectively.

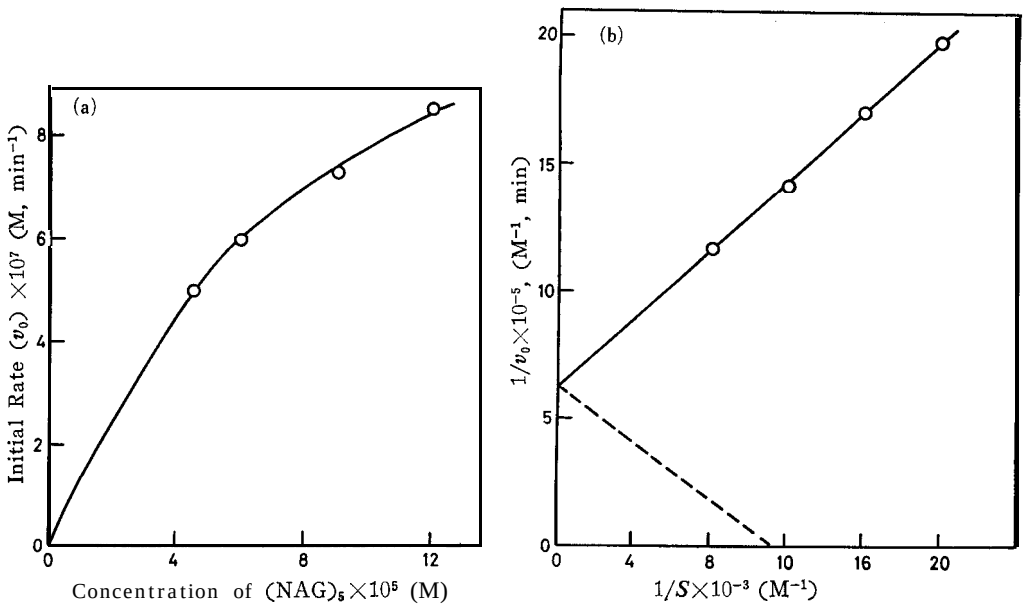


Fig. *20.* (a) Concentration-dependence of hydrolysis of pentasaccharide of NAG. (b). Lineweaver-Burk plot.

Acceptor ability of various sugars

Effect of enzyme concentration on release of p-nitrophenol

Lysozyme at varied concentration was incubated with 5 x 10^{-3} M p-nitrophenyl NAG and 5 x 10^{-4} M (NAG),. The plotting of the rate of the release of *p*-nitrophenol vs the enzyme concentration is shown in Fig. 21. It was found that the lysozyme concentration of 5 x 10^{-4} M may be most suitable for the follow ing experiments.

Time-course of release of p-nitrophenol

Typical example of the time-course of the release of p-nitrophenol in the presence or the absence of the various sugars is shown in Fig. 22. Because of linearity observable till 2 hr after the initiation of the reaction, the rate v of the release of p-nitrophenol was determined as the initial slope (M min^{-1}) of the line in the time-course.

Lineweaver-Burk plot

The effects of D-glucose and NAG are shown in Fig. *23* as a function of their concentrations. It should be noted that the concentration of NAG was about half of that of D-glucose. The plottings of l/v *vs* l/A based upon the data shown in Fig 23 are shown in Fig. *24,* where *A* represents the concentration of the acceptor p-nitrophenyl NAG. The plots exhibit the straight lines and the

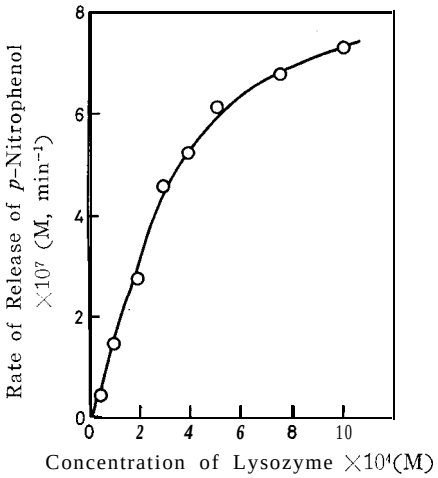


Fig. 21. Effect of lysozyme concentration. Varied concentration of lysozyme was incubated with (NAG), (5×10^{-4} M) and p-nitrophenyl NAG (5×10^{-3} M) at 40°C and pH 5.5.

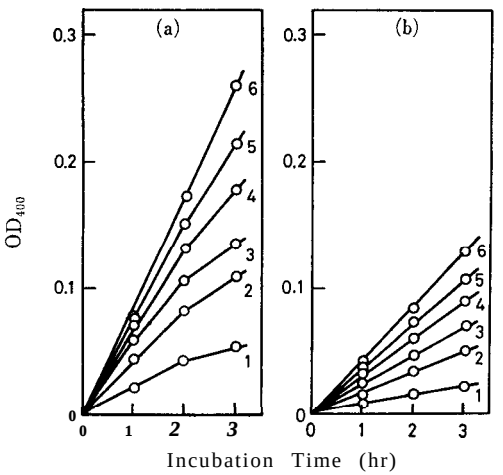


Fig. 22. Time-course of release of p-nitrophenol. Varied concentration of *p*-nitrophenyl NAG was incubated with lysozyme (5×10^{-4}M) and (NAG), (5×10^{-4} M) at 40°C and pH 5.5. (a) : Control. (b) : Containing 0.05 M NAG. Concentrations of *p*-nitropheny NAG were 1 : 1×10^{-3} M, 2 : 2×10^{-3} M, 3 : 3×10^{-3} M, 4 : 4×10^{-3} M, 5 : 5×10^{-3} M, 6 : 7.5×10^{-3} M.

line for control and those for systems containing the inhibitors meet on the same point on l/v axis. This indicates that the transglycosylation of p-nitrophenyl NAG was inhibited competitively by D-glucose and NAG according to the following equation :

$$\frac{1}{v} = \frac{K_p}{V_{max}A} + \frac{1}{V_{max}}$$

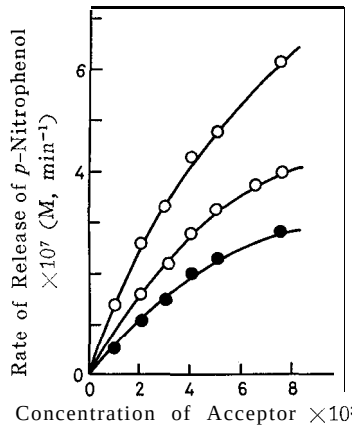


Fig. 23. Effect of acceptor concentration. Various concentrations of g-nitrophenyl NAG were incubated with lysozyme and $(NAG)_5$ in the absence (0) and presence (◑) of 0.4 M D-glucose or in the presence of 0.05 M NAG (0). Experimental conditions were the same as those in Fig. 22.

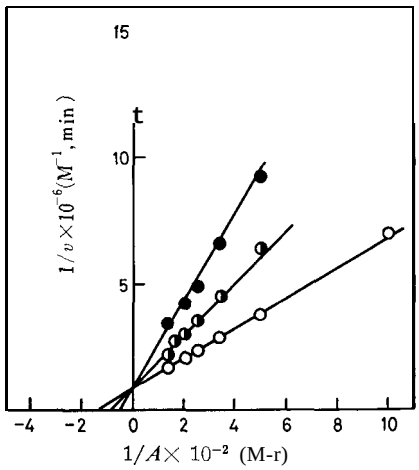


Fig. 24. Lineweaver-Burk plots for inhibitory effects of D-glucose and NAG. Composition of reaction mixture was the same as that in Fig. 22. 0 : Control. ◑ : Containing 0.4 M D-glucose. ● : Containing 0.05 M NAG.

$$K_p = K_m\left(1 + \frac{I}{K_i}\right)$$

where Z represents the sugar (inhibitor) concentration, K_p is Michaelis constant in the presence of sugar, K_i the inhibition constant of sugar.

The V_{max} and K_m were obtained from the intercepts on the vertical axis and on the $1/A$ axis, and found to be 1.1×10^{-6} M min^{-1} and 6.6×10^{-3} M, respectively. The K_i value of the sugars was calculated form the intercept K_p on the l/A axis and found to be 0.026 M for NAG and 0.509 M for D-glucose.

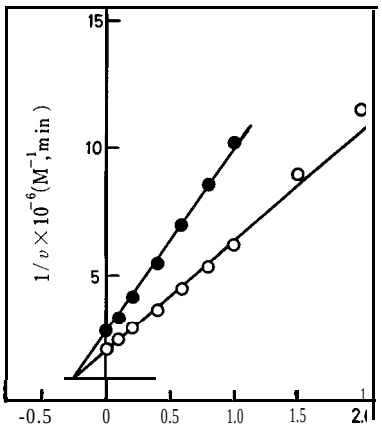


Fig. 25. Dixon plot of inhibitory effect of NAG. Composition of reaction mixture was the same as that in Fig. 22 except that concentration of NAG was varied. Concentrations of p-nitrophenyl NAG ○ : 3×10^{-3} M. ● : 5×10^{-3} M.

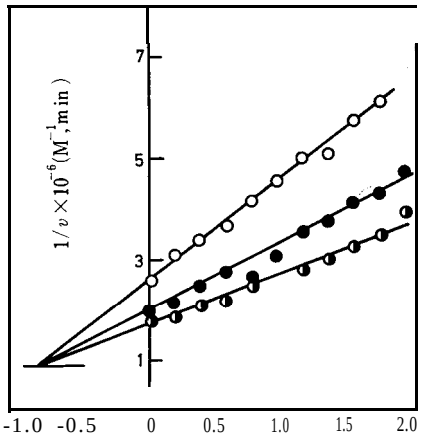


Fig. 26. Dixon plot of inhibitory effect of D-xylose. Concentrations of p-nitrophenyl NAG ○ : 3×10^{-3} M, ● : 5×10^{-3} M, ◑ : 6.5×10^{-3} M.

Dixon plot

To make sure that sugars used in the present experiment behave as competitive inhibitor for the release of p-nitrophenol or the transglycosylation and that the value of K_i obtained from Lieweaver-Burk plot is of reasonable, the plotting of l/v vs sugar concentration was analyzed (Dixon and Webb, 1964). The plots for NAG and for D-glucose as examples are shown in Figs. ***25*** and ***26.*** The ***K,*** value for NAG obtained from the intercept on Z-axis in Fig. ***25*** is in good agreement with that obtained from Fig. ***24.*** The type of inhibition and inhibition constants obtained for all sugar inhibitors are summarized in Table 9.

Table 9. Inhibition of lysozyme catalyzed transglycosylation of p-nitrophenyl NAG by sugars. Concentrations of lysozyme and (NAG), were 5×10^{-4} M. Reaction was carried out at 40°C and pH 5.5. C : Competitive inhibition. N : Noncompetitive inhibition.

Sugar	Type of inhibition	K_i(M)	Acceptor ability	$-\Delta G^0$ (Cal/mole)
NAG	C	0.026	20	2270
Methyl-a-NAG	C	0.09	5.6	1500
Methyl- ß-NAG	C	0.018	25	2500
Glucosamine HCl	C or N	0.30	1.7	750
D-Glucose	C	0.509	1.0	410
D-Mannose	C	0.776	0.65	158
2-Deoxy-D-glucose	C	0.50	1.0	431
D-Xylose	C or N	0.95	0.56	29
D-Galactose	C or N	2.52	0.2	--575
L-Arabinose	C or N	2.35	0.22	-532
Maltose	C	2.40	0.21	-545
Lactose	C	2.36	0.22	-535
2-Benzamido-2-deoxy-D-glucose	C	0.033	16	2120
D-Fructose	C	8.25	0.063	-1330

Free energy change

Table 9 also includes the standard free energy change in apparent value, $-\Delta G^0$, accompanied by the binding of sugar inhibitors to the subsites E (and F) of lysozyme, which was calculated by the following equation:

$$-\Delta G^0 = RT\ln\left(\frac{1}{K_i}\right)$$

where R and T are the gas constant and the absolute temperature. Most of the sugars used behaved as the competitive inhibitor for the transfer of p-nitrophenyl NAG and the release of p-nitrophenol. D-Xylose, D-galactose and L-arabinose showed more complicated types of the inhibition.

DISCUSSION

The release of p-nitrophenol originally included in the acceptor molecule through the transglycosylation reaction in the presence of the substrate may be schematically represented by Scheme 1. The released amount of p-nitrophenol does not represent the total amount but only parts of the transferred acceptor to oligosaccharide fragments. However, it is possible to assume that the released amount of p-nitrophenol could be an index of the extent of the transglycosylation reaction.

One of the characteristics of lysozyme catalyzed reaction has been thought to be a relatively high efficiency of the transglycosylation. When the mode of the transglycosylation is investigated in detail and compared with that of the hydrolysis, much information about the mechanism of lysozyme catalysis could be accumulated. The present experimental data show that the release of *p*-nitrophenol or transglycosylation continued with a constant rate for a long period, whereas the increase in the reducing power due to the hydrolysis of glycol chitin

ceased after 6 hr incubation. This distinctive features of the transglycosylation reaction will further help to elucidate the mechanism of lysozyme catalysis.

According to the above scheme on the release of p-nitrophenol, it is absolutely necessary that the β-1,4-glucosaminide linkage must once be cleaved before such a releasing reaction occurs. On the other hand, it was observed that the substrate in the reaction mixture incubated for more than 6 hr showed no more increase in the reducing power and degraded to oligosaccharides with average polymerization degree of bout 5. Thus, the ratio of the rate of hydrolysis of the substrate to that of the transglycosylation seems to depend upon the polymerization degree of the substrate. When polymer substrate had been degraded to oligosaccharides, the ratio may decrease to a very small value. In such a case, the C, carbonium ion formed by cleavage of glycosidic bond may react selectively with nucleophiles (acceptor) present in the reaction mixture, but scarcely with the water molecule. Consequently, in the late priod of lysozyme catalyzed reaction, only transglycosylation took place, and this seems to be arisen from the increase in oligosaccharide species in the reaction mixture, which were able to behave as acceptor of the transglycosylation reaction.

The low efficiency of the hydrolysis or low rate of attack of the water molecule to C, carbonium ion may connect with fine mechanism of lysozyme catalysis or with the structure of the water molecules surrounding the substrate molecule.

It is most probable that the hydrolysis and the transglycosylation have the same C_1 carbonium ion as a common intermediate. The bifurcation occurs at the consumption of C, carbonium ion. The additive affecting the formation of C_1 carbonium ion would alter the both rates of the hydrolysis and the transglycosylation. When additive affects the reaction step following the C_1 carbonium ion intermediate, the change in the rate of either reaction would be observable. The experimental results showed that hydrolysis was inhibited in parallel with transglycosylation by nearly all the additives used. This fact means that the additives affected the reaction step of C_1 carbonium ion formation, but not steps after that. This is very peculiar findings, and reason why the hydrolysis or the transglycosylation was not able to be inhibited individually, should be solved by succeeding experiments, since this may profoundly relate to the mechanism of lysozyme catalysis.

As stated before, the rate of release of p-nitrophenol does not necessarily mean the real rate of the transglycosylation. It was attempted, therefore, to correlate the time-course of the release of p-nitrophenol with that of the whole transglycosylation. As a rseult, it was found that, except for the initial stages of the incubation, the released amount of p-nitrophenol was exactly proportional to the extent of the transglycosylation. The release of p-nitrophenol may consist of two separate steps, the transfer of the aceptor into the reaction intermediate to form p-nitrophenyl glycoside of fragmented substrate and the following hydrolysis of p-nitrophenyl moiety located in the end of transferred product. In the initial stages of the reaction, the rate of the transfer of acceptor exceeds that of the release of p-nitrophenol. Consequently, a large part of p-nitrophenyl moiety became to be accumulated in the fargmented substrate. With proceeding of the reaction, both rates became comparable and finally the latter exceeded

very slightly the former. Thus, the accumulated amount of p-nitrophenyl moiety has been kept nearly constant in the incubation times more than 2 hr.

From the findings described above, two important points can be emphasized : (1) The cease of the increase in the reducing power in the reaction mixture does not mean the cease of lysozyme action. In a prolonged incubation time over 8 hr, the enzyme still acts on the glycosidic linkages, breaking the C_1-O bond to form the reaction intermediate C_1 carbonium ion, which reacts selectively with the acceptor generating the new glycosidic linkage. *(2)* In the reaction mixture incubated for at least 8 hr, the substrate has been degraded to oligosaccharides with an average polymerization degree of about 5. It is, therefore, easily expected that when oligosaccharides are used as the substrate, lysozyme catalyze only the transglycosylation but not the hydrolysis. This appears to be the most distinguishing characteristic of lysozyme catalysis.

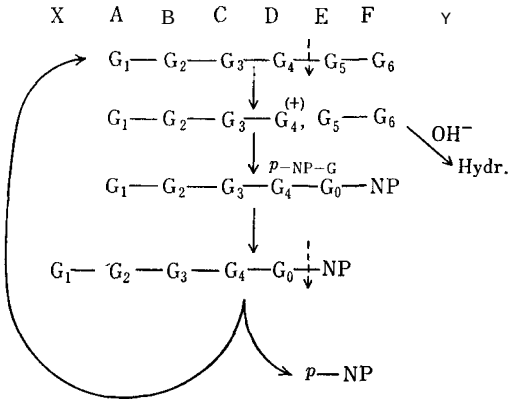
To ascertain above expectation, the hydrolytic and transglycosylation processes were analyzed using oligosaccharides of NAG, especially the behavior of the (NAG), being studied in detail with comparing to that of polymer substrate glycol chitin. The rates of the release of p-nitrophenol were the same for the (NAG),, (NAG), and glycol chitin, when their concentrations were fixed at the same value in percent units. This may explain the experimental result that the rate of the release of p-nitrophenol with glycol chitin as the substrate was constant independently of the incubation time. Thus, it was obvious that capabilities of these substrates to release the p-nitrophenol, based upon the molar concentration, was reversely dependent on the molecular weight of the substrate.

The K_m values obtained from the release of p-nitrophenol were quite different from the reciprocal value of equilibrium constant measured on the lysozyme-substrate complex formation (Chipman et al., 1967; Rupley et *al.*, 1966 ; Ikeda and Hamaguchi, 1970). This may be reasonable, because the release of p-nitrophenol involves several other steps than a simple binding of the substrate in the active site. The association constant of lysozyme-(NAG), complex formation was found to be of order of $10^5\ M^{-1}$ (Dahlquist et *al.*, 1966), and the reciprocal value of K_m of the release of p-nitrophenol with the substrate $(NAG)_5$ was calculated to be $4 \times 10^3\ M^{-1}$. In the transglycosylation followed by the release of p-nitrophenol, the K_m value of the (NAG), and (NAG), were nearly the same, although V_{max} was clearly different. Because of the complexity of the transglycosylation, this finding could not be explained at the present step.

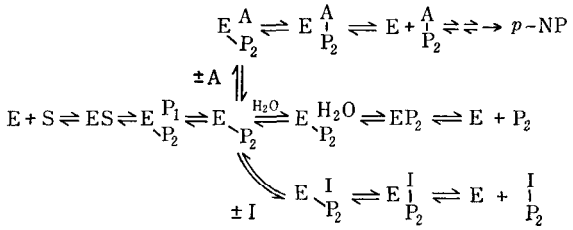
Contrary to the release of p-nitrophenol through the transglycosylation, the appearance of the reducing power from various substrates exhibited quite different features. At 0.01 % substrate concentration, only the $(NAG)_5$ and glycol chitin were hydrolyzed by lysozyme. However, the hydrolysis of the $(NAG)_5$ ceased wihin 1.5 hr incubation. The kinetic parameters of the hydrolysis of the $(NAG)_5$ were $V_{max} = 1.51 \times 10^{-6}$ M min^{-1}, $K_m = 9.1 \times 10^{-5}$ M. The V_{max} of the hydrolysis was twice larger than that of the transglycosylation and K_m was about half of that of the transglycosylation. The distinct difference between the hydrolysis and the transglycosylation of oligosaccharide was the time-course of the reactions, which completely resembled those observed when glycol chitin was used as the substrate. Thus, it was confirmed again that when the oligosaccharides were used as the substrate, lysozyme catalyzed only the transglycosyla-

tion, but not the hyrolysis.

Many saccharides (sugars) structurally resembled to the sugar moiety of acceptor p-nitrophenyl NAG may bind at the subsites E and F, and react competitivey with the reaction intermediate. Actually, the release of p-nitrophenol through the transglycosylation was repressed by the presence of sugars (acceptor analogs). The modes of inhibition by most of the sugars used on the transglycosylation were categorized to be competitive one. The kinetic scheme of the transglycosylation was assumed to consist of successive steps as shown in Scheme 2; the enzyme-substrate complex formation, cleavage of glycosidic linkage, attack of the acceptor to the reaction intermediate and finally the release of agricon. As mentioned above, the inhibition by sugars may consist of two different effects ; one is the competitive binding and the other the competitive reaction to the intermediate to generate a new glycosidic linkage. The meaning of K_i or $-\Delta G^0$ value may therefore be a much more complicated one than that of a typical competitive inhibition widely observable in general enzymatic reaction. However, the authors simply assumed that the K_i or- ΔG^0 value covers only the events in the binding process, but not those in the competitive reaction with



Scheme 1. Release of p-nitrophenol through transglycosylation. X-Y : Binding sites on lysozyme molecule. G_1-G_6: Hexasaccharide of NAG. p-NP : p-Nitrophenol. p-NP-G_0 : Acceptor p-nitrophenyl NAG.



Scheme 2. Kinetic scheme for hydrolysis and transglycosylation. E : Enzyme. S : Substrate. A : Acceptor. E-P_2 : Lysozyme-intermediate complex. I : Sugar added (acceptor analog, inhibitor), P_1,P_2 : Reaction products.

the intermediate.

The value of $-\Delta G^0$ for NAG was larger by 1.86 kcal/mole than that for D-glucose. This may be attributable to the contribution of the 2-acetamido group of NAG to the binding at the binding subsites. It was found that 2-benzamido-2-deoxy-D-glucopyranose has slightly smaller value of $-\Delta G^0$ than that of NAG. This may arise from the steric effect of a bulky benzoyl residue. The differences in values of $-\Delta G^0$ among NAG, glucosamine hydrochloride, D-glucose and 2-deoxy-D-glucose suggest that the acetyl group at the C_2 position interacts with the binding sites strongly. This fact coincides with the Phillips' model (Blake *et al.*, 1967), which shows a specific hydrogen bonding between carbonyl oxygen of 2-acetamido group of NAG bound at the subsite E and the side chain amide group of Asn 44 of the enzyme. The value of $-\Delta G^0$ for methyl β-NAG was larger by 0.23 kcal/mole than that of NAG, whereas methyl α-NAG smaller by 0.77 kcal/mole. This indicates that β-anomer is more accessible to the binding site. The ratio of K_i value of D-glucose to that of any other acceptor analog (sugar) was defined as "acceptor ability". Maltose showed the ability of 0.21, while cellobiose showed about 6. The P-configuration of C_1-OH at the binding subsite E may be important factor for the binding of the saccharides.

The difference in $-\Delta G^0$ value between D-glucose and D-mannose was about 0.25 kcal/mole, which was presumed to be attributable to the difference in C_2-OH configuration. The $-\Delta G^0$ value for 2-deoxy-D-glucose was however the same as that for D-glucose. There seems to be no interaction between C_2-OH group and the binding site E. From the difference in $-\Delta G^0$ value between D-glucose and D-galactose (0.55 kcal/mole) and between D-xylose and L-arabinose (1.0 kcal/mole), it was speculated that there is a large contribution of C_4-OH configuration to the binding of the acceptor analog. Such an effect of C,-OH configuration may interrelate to the fact that newly formed 1,4-glycosidic linkages through the transglycosylation have only β-anomeric structure and that D-galactose and L-arabinose gave β-1,2-glycosidic linkage and D-xylose gave β-1,2- and β-1,4-linkages through the transglycosylation. There was a fairly large difference in $-\Delta G^0$ value between D-glucose and D-xylose (0.44 kcal/mole), in spite of that there was no difference between D-galactose and L-arabinose. This seems to support the proposal of Sharon and Pollock (1970), that there was the interaction of C_6-CH_2OH at binding site E with methyl group of Val 109 of the enzyme.

Thus, the present experimental results can offer only discrete information for understanding of the mechanism of lysozyme action. For the explanation of high efficiency of the transglycosylation, more detailed experiments should be carried out in the near future.

REFERENCES

Blake, C. C. F., L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips and V. R. Sarma 1967 Crystallographic studies of the activity of hen egg-white lysozyme. *Proc. Roy. Soc.*, B167 : 378-388

Chipman, D. M. 1971 A Kinetic Analysis of the Reaction of Lysozyme with Oligosaccharides from Bacterial Cell Walls. ***Biochemistry, 10 : 1714-1722***

Chipman, D. M., V. Grisaro and N. Sharon 1967 The Binding of Oligosaccharides Containing N-Acetylglucosamine and N-Acetylmuramic Acid to Lysozyme. *J. Biol.Chem., 242: 4388-4394*

Dahlquist, F. W., L. Tao and M. A. Raftery 1966 On the Binding of Chitin Oligosacharides to Lysozyme. *Proc Natl. Acad. Sci., 56: 26-30*

Dixon, M. and E. C. Webb 1964 *Enzymes*. Academic Press, New York

Fraenkel-Conrat H. 1950 The Essential Groups of Lysozyme, with Particular Reference to Its Reaction with Iodine. *Arch. Biochem. Biophys., 27* : 109-124

Hartdegen, F. J. and J. A. Rupley 1967 The Oxidation by Iodine of Tryptophan 108 in Lysozyme. *J. Am. Chem. Soc., 89: 1743-1745*

Hayashi, K., T. Imoto and M. Funatsu 1963 The Enzyme-substrate Complex in a Muramidase Catalyzed Reaction. *J. Biochem., 54: 381-387*

Hayashi, K., N. Yamasaki and M. Funatsu 1964 Muramidase Catalyzed Hydrolysis of Glycol Chitin. *Agr. Biol. Chem., 28: 517-523*

Hayashi, K., N. Fujimoto, M. Kugimiya and M. Funatsu 1969 The Enzyme-substrate Complex of Lysozyme with Chitin Derivatives. *J. Biochem., 65: 401-405*

Ikeda, K. and K. Hamaguchi 1970 Interation of Alcohols with Lysozyme. *J. Biochem., 68: 785-794*

Imoto, T., Y. Doi, K. Hayashi and M. Funatsu 1969 Characterization of Enzyme-substrate Complex of Lysozyme. *J. Biochem., 65: 667-671*

Imoto, T. and K. Yagishita 1971 A Simple Activity Measurement of Lysozyme. *Agr. Biol. Chem., 35* : *1154-1156*

Konstas, S., I. Photaki and L. Zervas 1959 Überführung von D-Glucosamin in Oxazolon- und Oxaozlinderivate. *Ber. 92* : 1288-1293

Kravchenko, N. A. 1967 Lysozyme as a transferase. *Proc. Roy. Soc.,* B167 : 429-430

Leaback, D. H. and B. Weissmann 1963 p-Nitrophenyl N-acetyl-β-D-glucosamine *In* "Biochemical Preparation" ed. by G. B. Brown, 10 : 118-121

Pollock, J. J., D. M. Chipman and N. Sharon 1967 The Active Site of Lyszyme : Some Properties of Subsites E and F. *Biochem. Biophys. Res. Comm., 28: 779-784*

Pollock, J. J. and N. Sharon 1970 Studies on the Acceptor Specificity of the Lysozyme-Catalyzed Transglycolylation Reaction. *Biochemistry, 9* : 3913-3925

Raftery, M. A. and T. Rand-Meir 1968 On Distinguishing between Possible Mechanistic Pathway during Lysozyme-Catalyzed Cleavage of Glycosidic Bonds. *Biochemistry, 7* : 3281-3289

Rupley, J. A. 1964 The Hydrolysis of Chitin by Concentrated Hydrochloric Acid and the Preparation of Low-molecular-weight Substrate for Lysozyme. *Biochim. Biophys. Acta,* **83** : 245–255

Rupley, J. A. 1967 The binding and cleavage by lysozyme of N-acetylucosamine oligosaccharide. *Proc. Roy. Soc.,* B167: 416-428

Rupley, J. A., L. Butler, M. Gerring, F. J. Hartdegen and R. Pecoraro 1966 Studies on the Enzymatic Activity of Lysozyme. *Proc. Natl. Acad. Sci., 56: 1088-1095*

Rupley, J. A. and V. Gates 1967 Studies on the Enzymatic Activity of Lyszoyme. *Proc. Nail. Acad. Sci., 57: 496-510*

Rupley, J. A., V. Gates and R. Bilbery 1968 Lysozyme Catalysis, Evidence for a Carbonium Ion Intermediate and Participation of Glutamic Acid 35. *J. Am, Chem. Soc.,* **90** : 5633-5634

Zilliken, F., C. S. Rese, G. A. Brown and G. György 1955 Preparation of Alkyl N-Acetyl-α- and -β-D-glucosaminides and their Microbiological Activity for *L. bifidus. Arch. Biochem. Biophys., 54* : 329-397