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学　位　論　文

Studies on the Incorporation Mechanism of Aminosugar into Bacterial Cellulose

（バクテリアセルロースへのアミノ糖の導入機構に関する研究）

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STUDIES ON THE INCORPORATION MECHANISM OF AMINOSUGAR INTO BACTERIAL CELLULOSE

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A DOCTORAL DISSERTATION

DIVISION OF BIOLOGICAL SCIENCE,
GRADUATE SCHOOL OF SCIENCE,
HOKKAIDO UNIVERSITY

CONTENTS

ABSTRACT

A novel mucopolysaccharide, bacterial cellulose (BC) containing *N*-acetylglucosamine (GlcNAc) residues in main chain through β-1,4 glucosidic linkage, was produced by the bacterium *Acetobacter xylinum*. Incorporation of GlcNAc residues into BC has been achieved by the successive transfers of the strain to the culture medium containing GlcNAc instead of Glc. However, few investigations have been conducted to determine metabolic pathways of GlcNAc so far. The fine mechanism of incorporation from exogenous GlcNAc of the culture medium has not become evident. The purpose of this study is to clarify the incorporation mechanism of GlcNAc by *A. xylinum*. Thus culture conditions of *A. xylinum* and its uptake of carbon sources were investigated in order to elucidate the flow of GlcNAc in the biosynthetic pathway of BC. Further, several carbon sources were employed to examine the specificity of substrate for cellulose production.

A. xylinum ATCC 10245 was used in this investigation. The adaptation of *A. xylinum* to GlcNAc was carried out by repetitive subculturing to Schramm-Hestrin (SH) liquid medium containing only GlcNAc. Then cultures were transferred to SH mixed medium containing Glc and GlcNAc and incubated at 28°C statically for the production of BC pellicles. Since sugar consumption by the bacteria is essential to uptake of carbon sources for cellulose production, sugar concentration in the culture medium and variation of sugar composition were observed time dependently during incubation. It was shown that Glc was consumed preferentially in SH mixed medium containing both Glc and GlcNAc. GlcNAc was utilized as a minor carbon source for cellulose production when all Glc disappeared from the culture medium. The amount of incorporated GlcNAc residues into BC increased with increase of the ratio of GlcNAc concentration in the culture medium. Although the sugar component in the culture medium became only GlcNAc due to exhaustion of Glc, production of pellicles continued and major constituent residue of BC was found to be Glc. These results indicate that GlcNAc which taken up by the bacteria, was converted into Glc by the equilibrium function of enzymatic systems including

deacylase and aminotransferase. Resulted Glc was provided as a carbon source to the general biosynthetic pathway.

Other aminosugars, glucosamine (GlcN), galactosamine (GalN) and mannosamine (ManN), were applied to the cultures of *A. xylinum* to examine enzymatic stereoregulations in metabolic pathway of aminosugars. GlcN and GalN were taken up by *A. xylinum* to incorporate GlcNAc residues into BC, whereas ManN was not achieved to incorporate. It is proposed that the equilibrium among three aminosugars, GlcNAc, GlcN and Glc, is established in the flow of hexose by the participation of GlcN. The GlcNAc incorporation mechanism by the addition of GalN might be proposed as epimerization at a first step to prepare GlcN, and then deamination occurred to produce Glc as a carbon source for cellulose production. As the stereospecific deamination and amination processes are suggested, an aminotransferase and an epimerase are assumed to be present among other significant enzymes in the metabolic pathway of *A. xylinum*.

An aminotransferase which catalyzes amination and deamination reactions rises to metabolic pathway, thus ammonium ion seems to takes part in the equilibrium. The application of several ammonium salts were found to be effective on the incorporation of aminosugar residues into BC even in the absence of aminosugars, when the cultures of *A. xylinum* were incubated under rotatory and aerated conditions. As little incorporation was observed in the case of static culture, aeration was assumed to be an essential factor to the aminosugar incorporation in the presence of ammonium salts. Ammonium chloride seemed to be the best additive to enhance aminosugar incorporation, because the highest value of GlcNAc content obtained by the addition of it. It was proposed that GlcN which arose from enzymatic equilibrium of Glc and ammonium ion, was converted into GlcNAc to be provided to the biosynthetic pathway of cellulose, since incorporated aminosugar residue was estimated to be GlcNAc.

Attention was focused on sugar phosphate which is an intermediate of biosynthetic pathway of BC. Two pathways of GlcNAc for biosynthesis of BC containing GlcNAc residues were proposed; (1) GlcNAc was metabolized through general pathway after the conversion into Glc, (2) GlcNAc as it is was led to sugar phosphates (GlcNAc-6-P, GlcNAc-

1-P) and sugar nucleotide (UDP-GlcNAc), and became the substrate of cellulose synthase. Thus sugar phosphates are important as intermediates for the biosynthetic pathway of BC. As GlcNAc-6-phosphate enhanced the incorporation of GlcNAc residues into BC in the culture of *A. xylinum*, it was proposed that pathway of GlcNAc via GlcNAc phosphate was present in biosynthetic pathway.

Since chitin is a biodegradable homopolysaccharide consisted of GlcNAc residues, phosphorylated chitin is expected to be suitable carbon as a source to the incorporation of GlcNAc residues into BC and enhancement of their content. The influence of phosphoryl chitin on the biosynthesis of BC was investigated by the addition of phosphorylated chitin derivatives to the culture medium of *A. xylinum*. Phosphorylation of chitin could be achieved by using of tosylated chitin as a synthetic intermediate, while it was very hard to prepare phosphorylated derivative from intact chitin so far. Incorporation of GlcNAc was accelerated by the addition of phosphoryl chitins to SH GlcNAc medium. Biodegradable phosphoryl chitin seemed to be hydrolyzed to monomeric sugar during incubation and its hydrolysates were utilized as a source of hexose phosphate by the bacteria.

The results obtained from the present investigation indicate that the conversion of hexoses and its equilibrium functions which are established by enzymatic systems rises to metabolic pathway of *A. xylinum*. It became evident that glucose analogues such as GlcNAc, GlcN are metabolized by proposed enzymatic systems with recognition of stereo-specificity to produce β-1,4-glucan. As the incorporation of GlcNAc residues into BC was achieved only by the alternation of the medium components, such a flow of hexoses is inevitable for the bacteria to adapt to a new environment.

CHAPTER I

GENERAL INTRODUCTION

Cellulose is the most abundant carbohydrate in nature, which is a linear polysaccharide consisted of D-glucopyranose through β-1,4 glycosidic linkage. It forms the principal constituent of cell walls in higher plants, forming the main structural element. It occurs in an almost pure form (98%) in cotton fibers and to a lesser extent in flax (80%), jute (60-70%) and wood (40-50%) and has also been found as a constituent of some algae and as a product of bacterial synthesis[1].

Among the bacteria, the genus *Acetobacter* produces abundant quantities of cellulose, which is called bacterial cellulose(BC). In particular, the gram negative *Acetobacter xylinum* has received much attention for the study of cellulose biosynthesis. Unlike the algae and higher plants, the cellulose of *A. xylinum* is not produced as a cell wall component, but as an extracellular pellicle of essentially pure cellulose. Thus many studies have addressed the physical and biological aspects of BC produced by *A. xylinum* as a model for cellulose biogenesis.

Various physical properties of BC were investigated, especially, structural analyses of cellulose relevant to biosynthesis, the mode of polymerization and the formation of fibrils and crystalline structure. Cellulose displays a diverse range of conformations and crystalline packing arrangements, as well as fiber structure, it crystallizes in four different forms, the cellulose I, II, III and IV polymorphs[2]. Crystallographic analyses clarified that all native forms of cellulose including BC exist in the cellulose I form (glucan chains are aligned parallel), although a form cellulose I is thermodynamically less stable than cellulose II (an antiparallel orientation). X-ray analysis showed that the membranes dried with a flat surface parallel to a glass plate provided a typical selective uniplanar orientation of the (101) crystallographic plane[3]. Cross-polarization/magic-angle spinning (CP/MAS) ^{13}C-NMR spectroscopy has also proved to be useful in characterizing crystallinity of BC[4]. The

average degree of polymerization(d.p.) were determined by viscosity measurement, d.p. of BC was estimated to be 2,000-3,700[5] or 5700[6].

A. xylinum grows in the nutrient medium and produces cellulosic pellicle at the surface of medium by using glucose(Glc) as a carbon source generally. Synthesis of cellulose from added Glc to the culture medium was demonstrated by using of radiolabeled Glc, it was clear that the major carbon source was the exogenous Glc in cellulose production[7]. The range of utilizable substrates for cellulose production demonstrated under the conditions including hexoses, hexanoates, three and four carbon substrates[8-10], and it depended on the cell history and on the conditions of the assay[11]. It suggested that an adaptive effect of carbohydrate composition of the growth medium contributed to use of substrates except glucose.

Much effort has gone into the study to elucidate the biosynthetic route of BC in *A. xylinum*. Pathway of carbon metabolism was shown in Scheme 1-1. Essentially, the presence of four enzymatic steps has been suggested in the pathway of cellulose synthesis from Glc to cellulose as follows; (1) phosphorylation of Glc by glucokinase[12], (2) the transphosphorylation of glucose-6-phosphate (Glc-6-P) to glucose-1-phosphate (Glc-1-P) by phosphoglucomutase[13], (3) the synthesis of uridine-5'-diphosphoglucose (UDP-Glc) by pyrophosphorylase[14] and (4) the cellulose synthase reaction to polymerize Glc units[15,16]. Cellulose synthesis starts in *A. xylinum* from Glc-6-P synthesized through two alternative pathways[17]. One pathway is direct phosphorylation of exogenous Glc. The other is an indirect one via pentose cycle and gluconeogenesis pathway, which is induced from oxalacetate via pyruvate in *A. xylinum*. The immediate sugar nucleotide precursor of cellulose synthesis is UDP-glucose[18]. Further in higher plant system, it is proved that UDP-Glc is a precursor to secondary wall cellulose in the cotton fiber by the observation of the carbon flow *in vivo* from Glc to end products [19]. It is clear that cellulose polymerization is a membrane associated event, cellulose synthase (UDP-glucose:1,4-β-D-glucan 4-β-D-glucosyltransferase, E.C. 2.4.1.12) is located in cytoplasmic membrane[20]. Solubilization of cellulose synthase has been achieved by treatment of membranes from *A. xylinum* with digitonin[15]. And synthesis of cellulosic fibrils from UDP-Glc *in vitro* was

allowed by solubilization of synthase[21]. Enhancement in the *in vitro* synthesis of cellulose has been accomplished by use of cellulose synthase in the presence of guanosin 5'-(γ-thio)-tri-phosphate, a protein factor and polyethylene glycol-4000[22]. In the genetic engineering, gene manipulation was attempted to improve *Acetobacter* strains for the productivity of BC by means of recombinant DNA technique[23]. Analysis of DNA sequence of *Acetobacter* cellulose-synthesizing operon characterized the gene coded for a polypeptide, which was identified as the cellulose synthase[24].

Recently, it was reported that the incorporation of *N*-acetylglucosamine (GlcNAc) residues into BC has been achieved by the application of new preculturing process and the modification of the culture medium of *A. xylinum*[25]. Thus novel polysaccharide, heteropolysaccharide consisted of Glc and GlcNAc residues, was produced in the medium containing GlcNAc as a carbon source by the use of *A. xylinum*, which was previously adapted to GlcNAc (Scheme 1-2). One of the most important process of that is adaptation of the bacterial strains to GlcNAc, it was carried out by the successive transfer to GlcNAc as a liquid medium or by incubation on a solid medium containing GlcNAc. The amount of incorporated GlcNAc residues into BC was up to 4 mol% detected by amino acid analysis of acid hydrolysates. Since resulted BC showed susceptibilities for not only cellulase but also chitinolytic enzymes such as lysozyme and chitinase, it proved that GlcNAc residue was incorporated through β-1,4 glycosidic linkage in BC chain with random distribution[26]. In addition, the higher plane orientation and Young's modulus than those of normal BC were observed by X-ray analysis and the measurement of tensile strength, respectively[27]. It was demonstrated that incorporation of a few mol% of GlcNAc residues into BC afforded several properties unique to this novel mucopolysaccharide.

Few investigations have been conducted to determine metabolic pathways of GlcNAc and the fine mechanism of incorporation so far. The pathway of GlcNAc from exogenous GlcNAc of the culture medium to incorporated GlcNAc into BC has not become evident. The purpose of this study is to clarify the incorporation mechanism of GlcNAc.

Firstly, cultures of *A. xylinum*, which adapted to GlcNAc as a liquid medium, were performed in SH medium containing only GlcNAc as a carbon source or mixed with Glc.

Time courses of sugar concentration and variation of sugar composition were observed during incubation, because information concerning uptake and consumption of sugars including GlcNAc by the bacteria is essential to determine carbon sources for cellulose production. It become evident that the relationships between sugar component and incorporated GlcNAc content, yield of pellicles in Chapter II. An enzymatic equilibrium among sugars was assumed in the biosynthetic pathway prior to cellulose production.

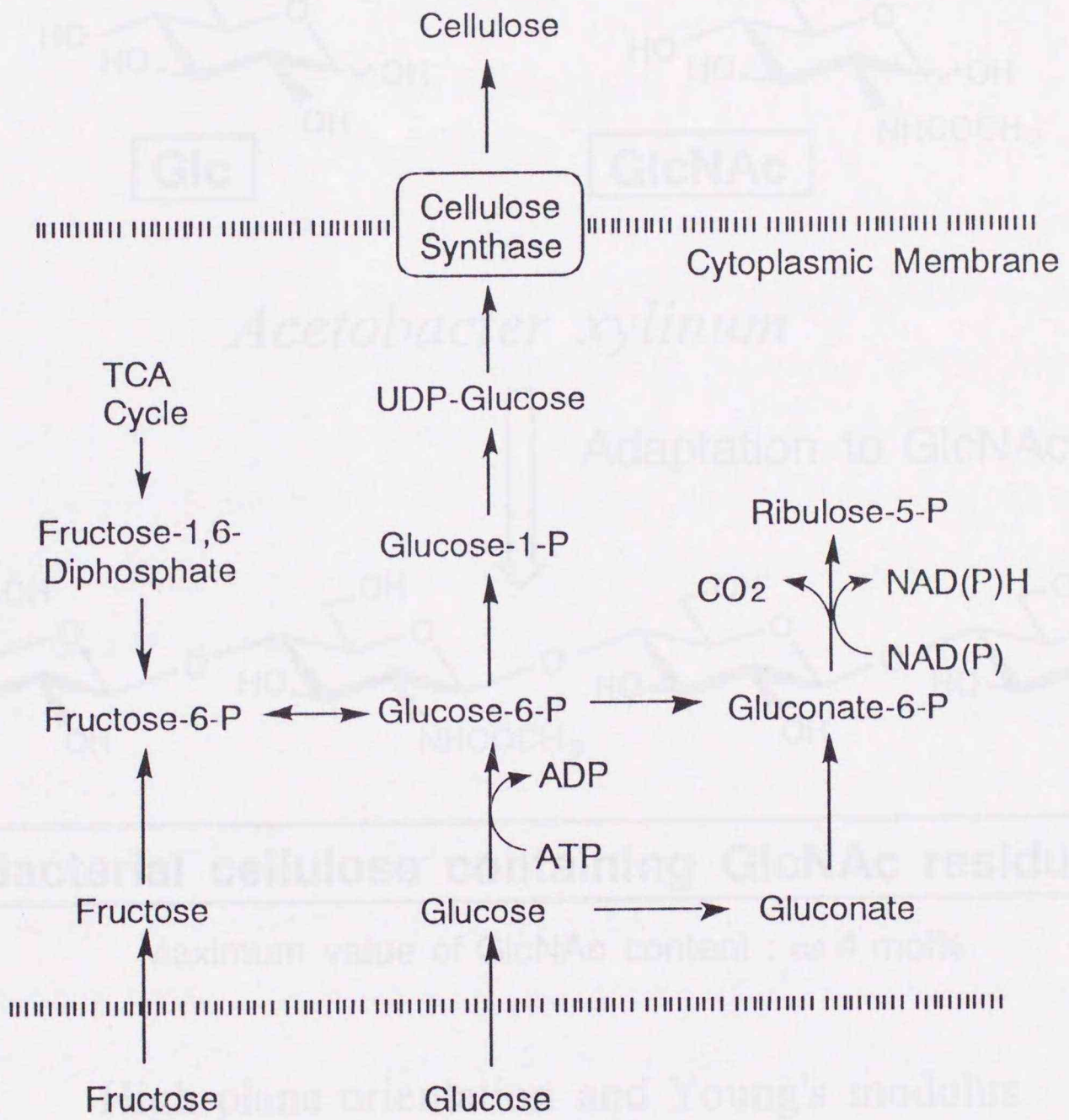
In Chapter III, other aminosugars except GlcNAc, glucosamine (GlcN), galactosamine (GalN) and mannosamine (ManN), were applied to the cultures of *A. xylinum*. GlcN is a deacetylated form of GlcNAc, GalN and ManN are epimer of GlcN. The processes that sugar is subject to deamination or acetylation were estimated by the observation of the relationships between sugar component and incorporated aminosugar into pellicles. Enzymatic stereoregulations in metabolic pathway of aminosugar were also examined by the application of sugar sources having different stereostructures.

It is presumed that *A. xylinum* should convert aminosugars into Glc for cellulose production, since BC was produced even in the medium containing only GlcNAc or GlcN. It seemed that enzymatic systems catalyzed such a conversion and the equilibrium between aminosugars and Glc were assumed in the bacteria. An aminotransferase which catalyzes amination and deamination reactions rises to metabolic pathway, thus ammonium ion seems to takes part in the equilibrium. In this Chapter IV, the cultures of *A. xylinum* were performed in the presence of ammonium salt, it is expected that the equilibrium was shifted to the side of aminosugars due to increase of concentration of ammonium ion. And incorporation of aminosugar residues into BC was investigated in the culture medium consisted of several ammonium salts and Glc instead of aminosugars.

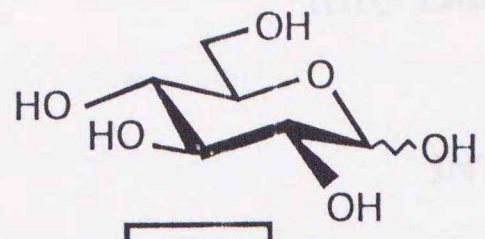
Attention was focused on sugar phosphate which is an intermediate of biosynthetic pathway of BC, in practice, GlcNAc phosphate and chitin phosphate were applied to the cultures of *A. xylinum* as medium components. GlcNAc phosphate seems to be available sugar source to examine direct incorporation pathway of GlcNAc into BC. It is anticipated that phosphorylated derivative of chitin becomes a utilizable sugar source, because chitin is a glucan consisted of GlcNAc residues through β-1,4 glycosidic linkage, further constant

supply of sugar source to the culture medium during incubation may be achieved by the addition of phosphorylated polysaccharide, while commercial sugar phosphates are hardly obtained owing to its instability. In Chapter V, a few phosphorylated chitin derivatives were prepared and the influence of phosphoryl chitin on the cultures of *A. xylinum* was examined, since it is expected that added phosphoryl chitin is hydrolyzed by glycosidases of the bacteria in incubation stage. On biosynthesis of cellulose, phosphorylated chitin derivatives were added to the culture medium at various concentration, the amount of GlcNAc residues into BC was examined. Molecular weight and its distribution of phosphoryl chitins of medium component were determined by GPC analysis in order to observe degradation of additives during incubation.

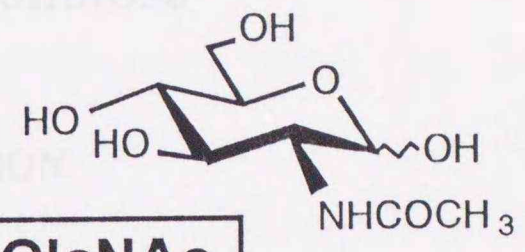
Chemically modified chitin derivatives such as deacetylated, carboxymethylated, sulfated chitin have already been applied in the biological field due to its biodegradability[28-33]. In general, when chitin derivatives are applied to biological materials including bacteria, it is essential to prepare with controlling degree of substitution, introduction site and so on. However chemical modification of chitin is very difficult due to rigid crystalline structure formed by intra- and intermolecular hydrogen bonds[34]. Thus in the case of phosphorylation, the product which regulated degree of substitution and its site was hardly obtained by reported methods without depolymerization[35-38]. In this experiment, tosylated chitin is prepared as a synthetic intermediate for phosphorylation. When substitution reaction to hydroxyl groups of chitin is carried out, alkali-chitin is such an important precursor that preparation method of that determines the properties of the final product. Therefore, in this tosylation of chitin, alkali-chitins were prepared by two different procedures, that is, in heterogeneous and homogeneous systems. The relationships between reaction conditions and degree of phosphorylation, substitution site and molecular weight were investigated, furthermore, susceptibilities of chitin phosphates for glycosidases such as lysozyme and chitinase were also examined as one of indications of biodegradability.



Scheme 1-1. Pathway of carbon metabolism in *A. xylinum*.



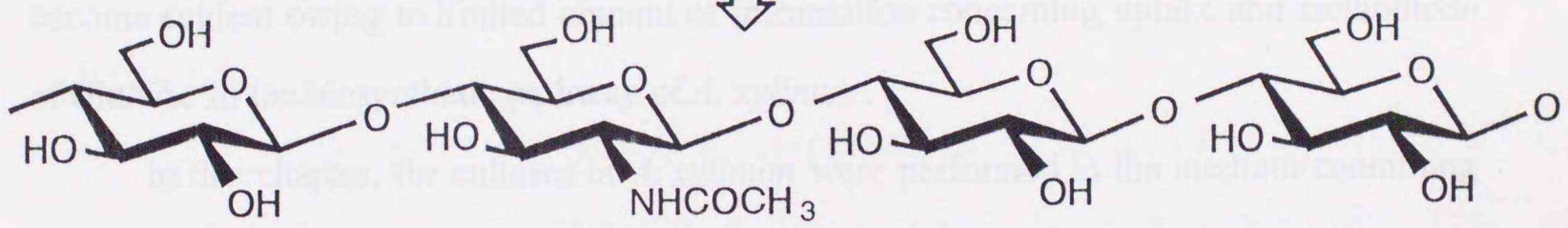
Glc



GlcNAc

Acetobacter xylinum

Adaptation to GlcNAc



Bacterial cellulose containing GlcNAc residues

Maximum value of GlcNAc content : *ca* 4 mol%

High plane orientation and Young's modulus
Susceptibilities for cellulase, lysozyme and chitinase

Scheme 1-2. Preparation of bacterial cellulose containing *N*-acetylglucosamine residues.

CHAPTER II

Incorporation Mechanism of *N*-Acetylglucosamine Residues into Bacterial Cellulose

INTRODUCTION

Acetobacter xylinum produces BC on the surface of the liquid medium including Glc as a carbon source generally. The incorporation of GlcNAc residues into BC was achieved by the successive transfer to GlcNAc as liquid medium (containing GlcNAc as a carbon source, instead of Glc) under the culture of *A. xylinum* strains, which were adapted to GlcNAc. However, the details of the incorporation mechanism of GlcNAc residues have not become evident owing to limited amount of information concerning uptake and metabolism of GlcNAc in the biosynthetic pathway of *A. xylinum*.

In this chapter, the cultures of *A. xylinum* were performed in the medium containing GlcNAc as a single sugar source (SH GlcNAc medium) or in mixed medium which was a combination of Glc and GlcNAc (SH mixed medium) to observe sugar consumption during cultivation. And the relationship between time course of sugar composition in the culture medium and the amount of incorporated GlcNAc residues into BC was investigated in order to elucidate the mechanism of bacterial metabolism. Productivity of BC pellicles was also examined in connection with sugar composition of the medium.

EXPERIMENTAL

Bacterial strain and culture conditions

The bacterial strain used in this investigation was *Acetobacter xylinum* ATCC 10245. All cultures were incubated statically at 28°C in Schramm - Hestrin (SH) medium[7] containing GlcNAc instead of Glc as listed in Table 2-1. When combinations of Glc and GlcNAc (SH mixed medium) were applied, the weight ratio of Glc and GlcNAc was set to 7:3.

Adaptation of *A. xylinum* to GlcNAc

The strain of *A. xylinum* has been maintained in SH Glc medium by repetitive subculturing. A 0.5 ml aliquot of 3 days' culture was transferred to 15 ml of SH GlcNAc medium. This culture was transferred to SH GlcNAc medium every 3 days of culture repeatedly.

The numbers of transfers were set to more than 5 times, because the incorporation of GlcNAc residues into pellicle reached a maximum value in the case of five transfers to liquid GlcNAc medium when cultures were incubated in SH mixed medium containing Glc and GlcNAc[25].

Production of pellicles

A 0.5 ml aliquot of the cultures in SH GlcNAc medium was inoculated into 15 ml of SH medium containing 2.0% of GlcNAc or 0.6% of GlcNAc and 1.4% of Glc and incubated statically at 28°C for 7 days. In the case of long period incubation more than a week, a 15 ml aliquot of culture was inoculated into 200 ml of the culture medium and incubated.

Purification of pellicles

The pellicles produced at the surface of the culture medium were harvested and boiled in 2% (w/v) sodium dodecyl sulfate (SDS) aqueous solution for 3 hours and in 4% (w/v) aqueous NaOH solution for 1.5 hours, successively. The pellicles were rinsed with

deionized water extensively until neutral. Resulted pellicles were dried on stainless steel plates at 60° C or lyophilized.

Estimation of incorporated aminosugar into pellicle

Dried pellicles were swollen in 85% phosphoric acid as described in the text[39] and lyophilized after extensive rinsing with deionized water. The samples were hydrolyzed in 2N HCl at 100°C for 12 h under reduced pressure. After the hydrolysis, excess acid was removed by evaporation to dryness under reduced pressure over NaOH. The residue was applied to amino acid analysis to estimate the amount of incorporated aminosugar content referring that of GlcNAc as standard.

Sugar composition of culture medium

The portions of culture medium were taken out during incubation and filtrated through cellulose acetate membrane of 0.45 μm (Millipore) to remove bacteria. The filtrates were diluted with deionized water to appropriate concentration and deproteinized by addition of deproteination reagent[40]. After removal of denatured protein by centrifugation, concentrations of sugar in the culture medium were measured. The amount of sugar components were estimated by the modification of Schales' method[41] for total amount of sugar, glucose-oxidase peroxidase system[42] for Glc and Morgan-Elson (Reissig) method[43] for GlcNAc.

Table 2-1. Composition of medium components [a]

Components of medium	Concentration (w/v%)
Sugar [b]	2.0
Bacto peptone [c]	0.5
Yeast extract [c]	0.5
Disodium hydrogenphosphate	0.27
Citric acid	0.115

[a] The initial pH value is 6.0

[b] Concentrations of sugar in SH mixed medium are 1.4% of Glc and 0.6% of aminosugar

[c] Difco Laboratories

RESULTS

Sugar consumption

Time courses of sugar composition in the mixed medium consisted of Glc and GlcNAc are shown in Figure 2-1. The arrows show the repeated addition of fresh SH mixed or SH GlcNAc medium after harvesting pellicles produced on the surface of the culture medium. In the initial stage of incubation, Glc concentration decreased immediately prior to that of GlcNAc. Reduction of GlcNAc concentration was barely found in the mixed medium after addition of fresh medium. So long as Glc existed in the culture medium, GlcNAc was little consumed by *A. xylinum*. The preference of Glc consumption was found in the case of mixed medium containing Glc and GlcNAc. However, concentration of GlcNAc began to decrease gradually when all Glc was disappeared from the culture medium. Thus, production of pellicles was continued though sugar component was only GlcNAc. It suggests that GlcNAc is taken up by *A. xylinum* as a carbon source.

When one batch incubation was carried out without addition of fresh medium, sugar consumption was observed as shown in Figure 2-2, Glc concentration was diminished from the beginning of incubation. After all Glc was consumed, total amount of sugar increased slightly, whereas that of GlcNAc was hardly changed. It seems that the bacteria transformed medium component into reducing sugar or deamination GlcNAc.

Incorporation of GlcNAc residues into BC

Time course of Glc fraction in SH mixed medium and the amount of incorporated GlcNAc residues into pellicle are shown in Figure 2-3. The incorporated GlcNAc content became higher when sugar component was only GlcNAc after consumption of all Glc from the culture medium. A linear relationship was found between incorporated GlcNAc content and the ratio of GlcNAc concentration in mixed medium as shown in Figure 2-4. It seems that uptake and incorporation of GlcNAc were accelerated by increase of the ratio of GlcNAc concentration in the culture medium. Thus GlcNAc which was taken up by *A. xylinum,* seems to be utilized directly for the biosynthesis of BC.

However, as a major constituent residue of resulted BC was Glc even when sugar component became only GlcNAc caused by exhaustion of all Glc, GlcNAc which was taken up by the bacteria, seems to be converted into Glc to utilize as a carbon source. It was suggested that produced polysaccharide was constructed mainly from Glc which arose from catalytic conversion of GlcNAc by some enzymatic system in the metabolic cycle of bacteria.

Production of pellicles

The relationship between the change of Glc fraction in SH mixed medium and the yield of pellicles is shown in Figure 2-5. The yields of pellicles were nearly constant while Glc was present in the culture medium, because most of Glc was consumed as a carbon source for the cellulose production. Subsequently, the yields of pellicles were extremely depressed after the disappearance of Glc from the culture medium. But production of pellicles was continued even in GlcNAc medium, it suggests that *A. xylinum* could consume GlcNAc for cellulose production. It is evident that resulted pellicles are shown to be exclusively 1,4-β-D-glucan, because the products are insoluble in hot aqueous alkaline solution similarly to reported previously[15,20,22].

Figure 2-6 shows the relationship between the amount of incorporated GlcNAc residues and the yields of pellicles. The GlcNAc content in pellicle starts to increase at the time when the yield reduces sharply. While productivity of BC became lower probably due to exhaustion of Glc from the culture medium, BC of high GlcNAc content could be obtained. The harvested quantities of BC were quite small in GlcNAc alone medium, but production of pellicles was continued by the bacteria and incorporated GlcNAc content became higher. The cellulose production seemed to be depressed by the increase of GlcNAc ratio in the culture medium.

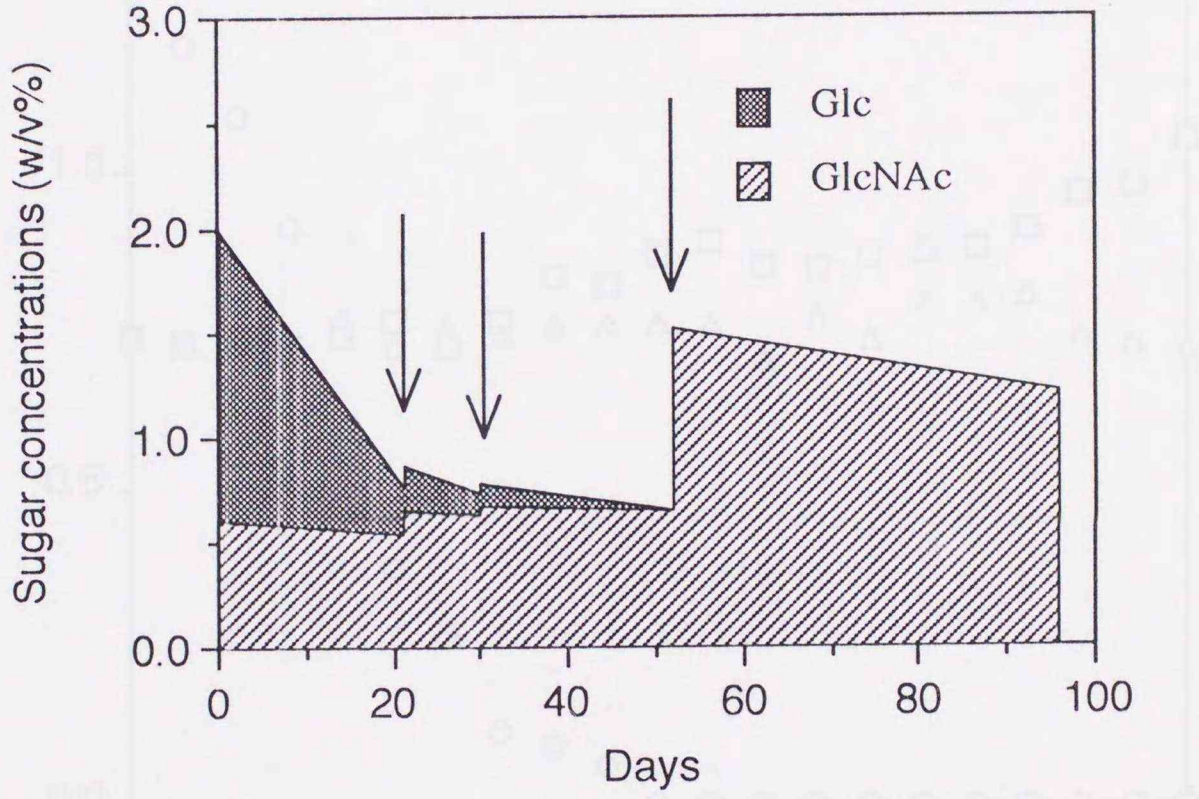


Figure 2-1. Time course of Glc and GlcNAc concentrations in SH mixed medium. The arrows show addition of fresh SH mixed or SH GlcNAc medium.

▩ : Glc, ▨ : GlcNAc

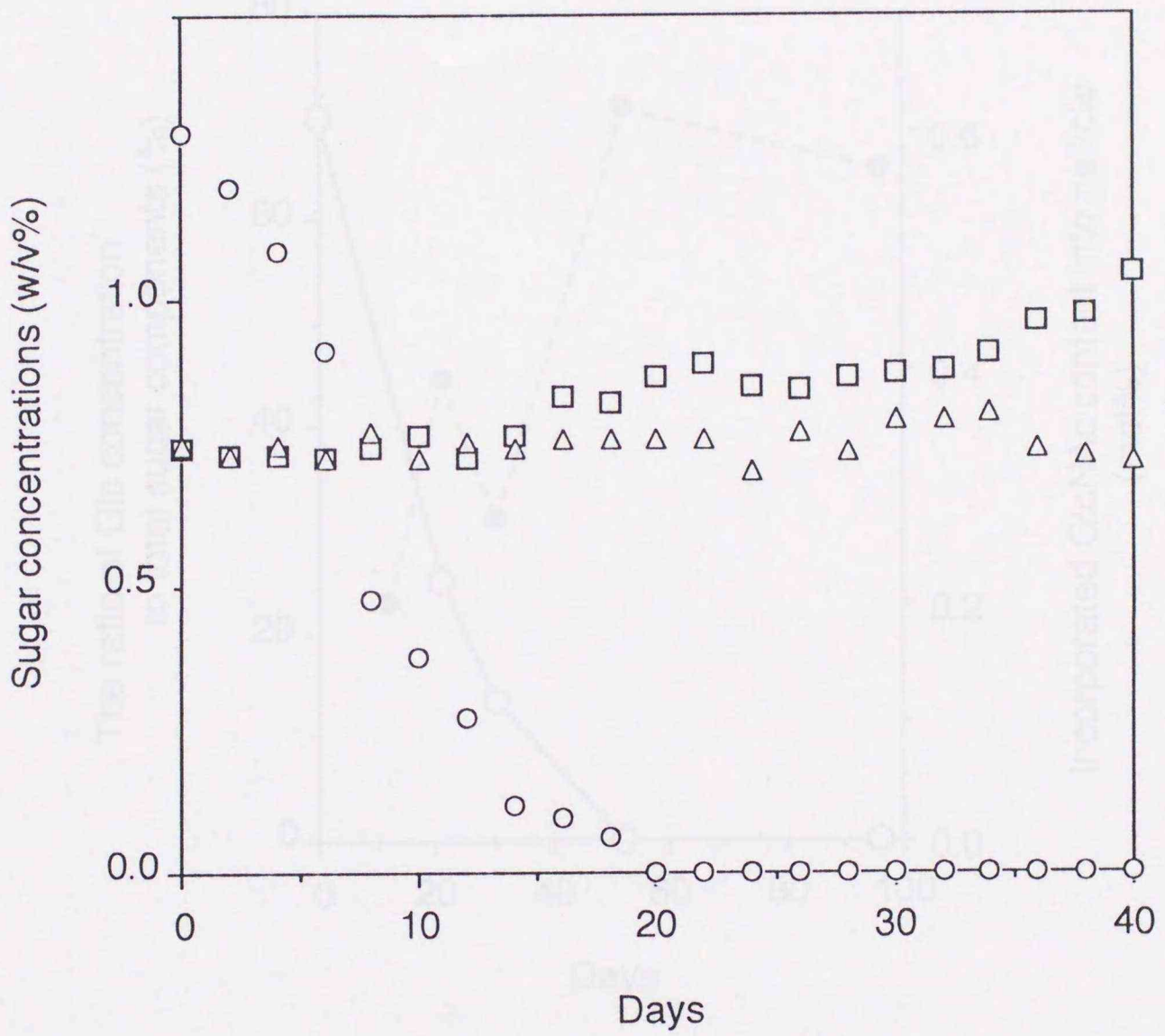


Figure 2-2. Time course of sugar concentrations in SH mixed medium containing 1.4% of Glc and 0.6% of GlcNAc : (O) Glc; (Δ) GlcNAc; (□) Total amount of reducing sugar.

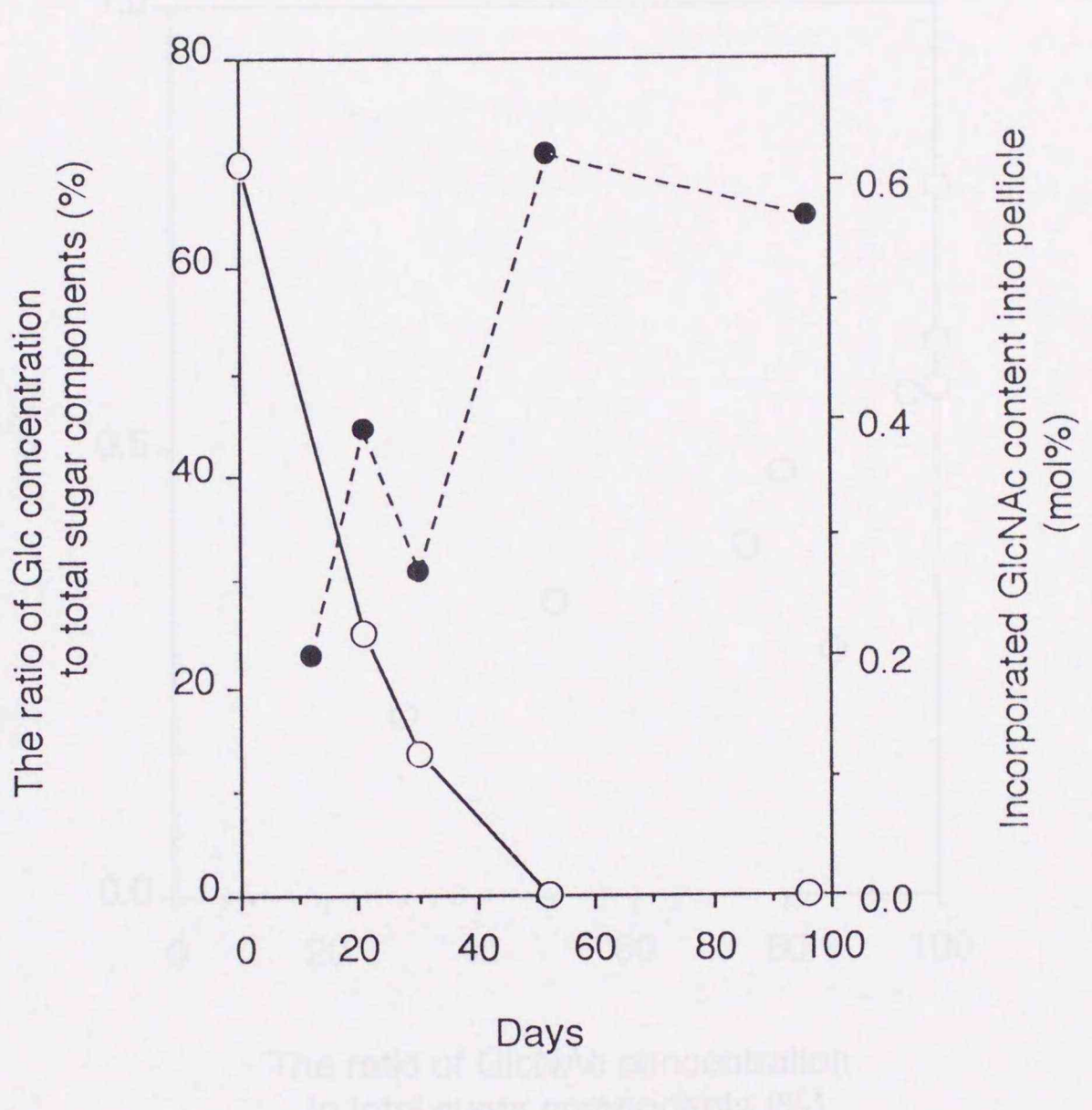


Figure 2-3. The relationship between the ratio of Glc concentration to total sugar components in SH mixed medium (O) and incorporated GlcNAc content into pellicle (●).

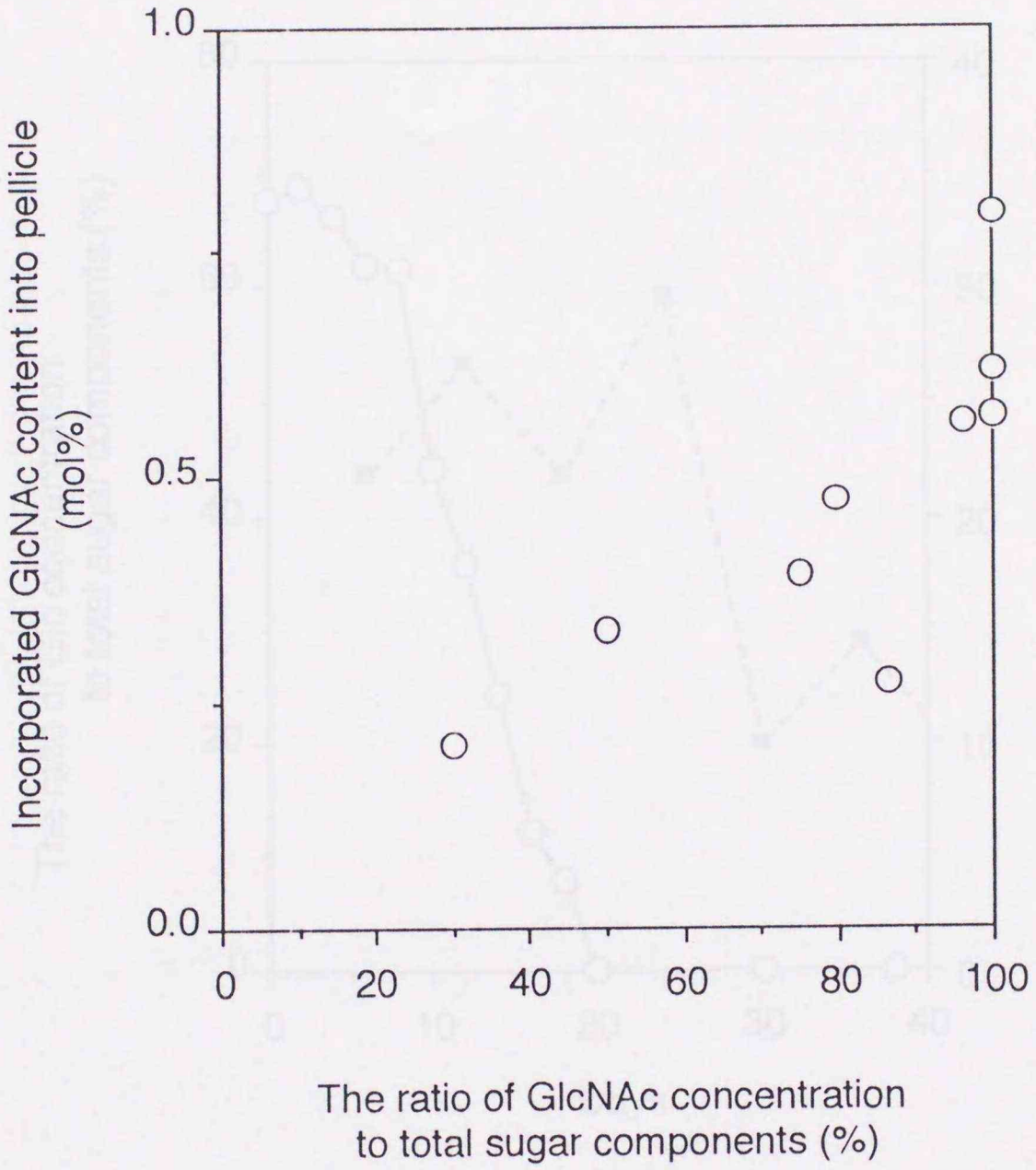


Figure 2-4. The relationship between the ratio of GlcNAc concentration to total sugar components in SH mixed medium and incorporated GlcNAc content into pellicle.

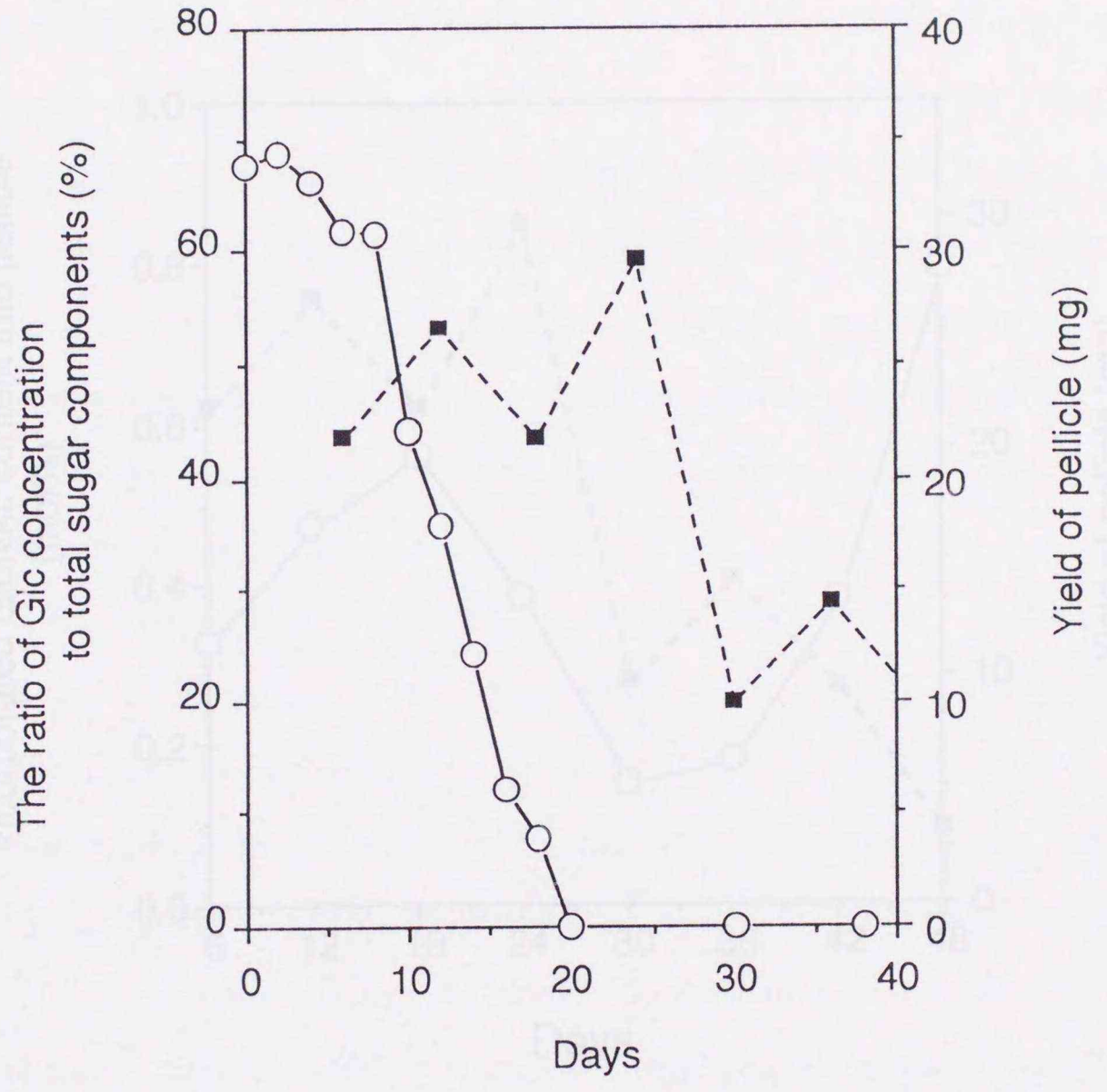


Figure 2-5. The relationship between the ratio of Glc concentration to total sugar component in SH mixed medium (O) and the yield of pellicles (■). Initial volume of the medium was 200ml. Pellicles were harvested at intervals of 6 days.

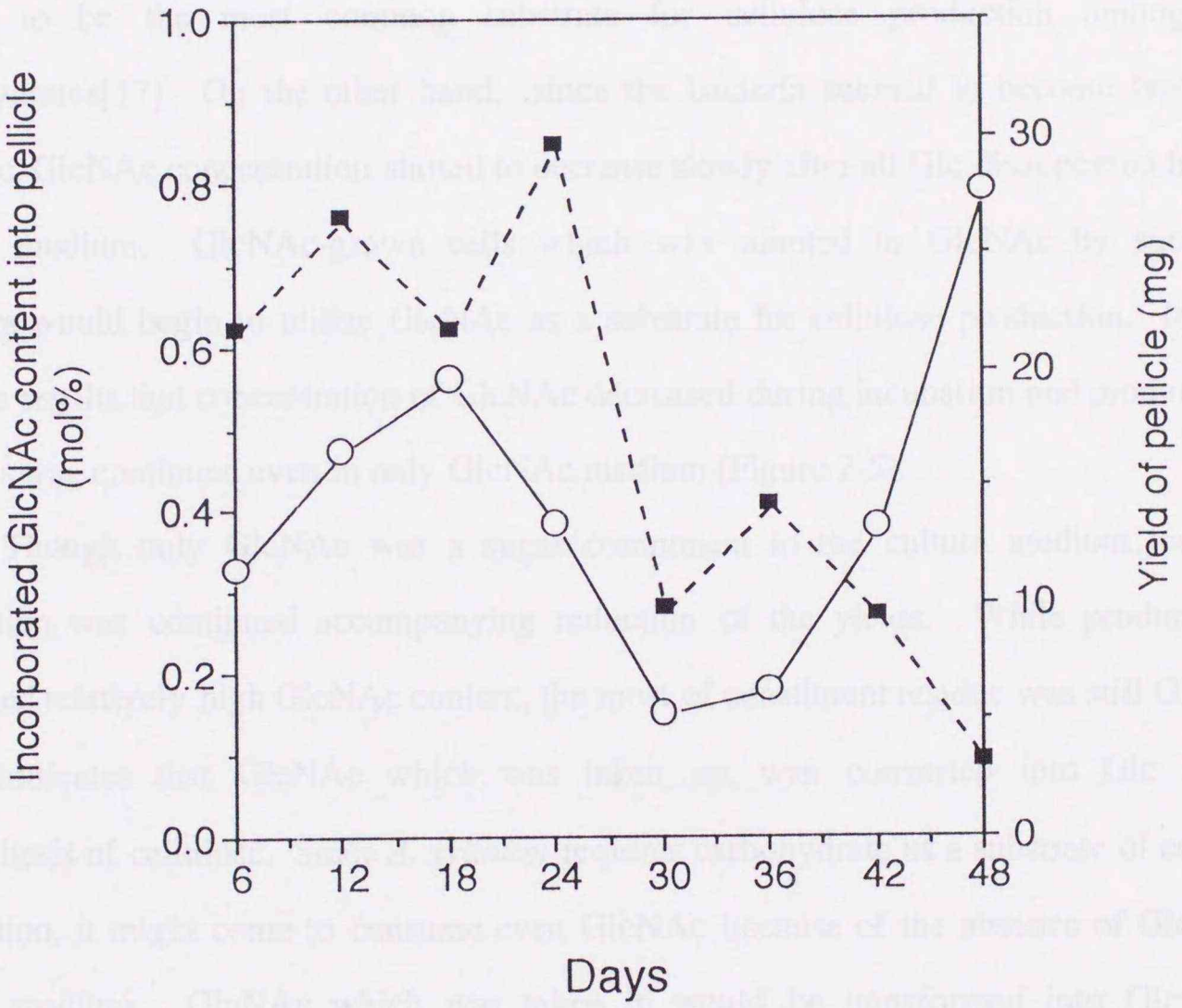


Figure 2-6. The relationship between the amount of incorporated GlcNAc into pellicle (O) and the yield of pellicle (■).

DISCUSSION

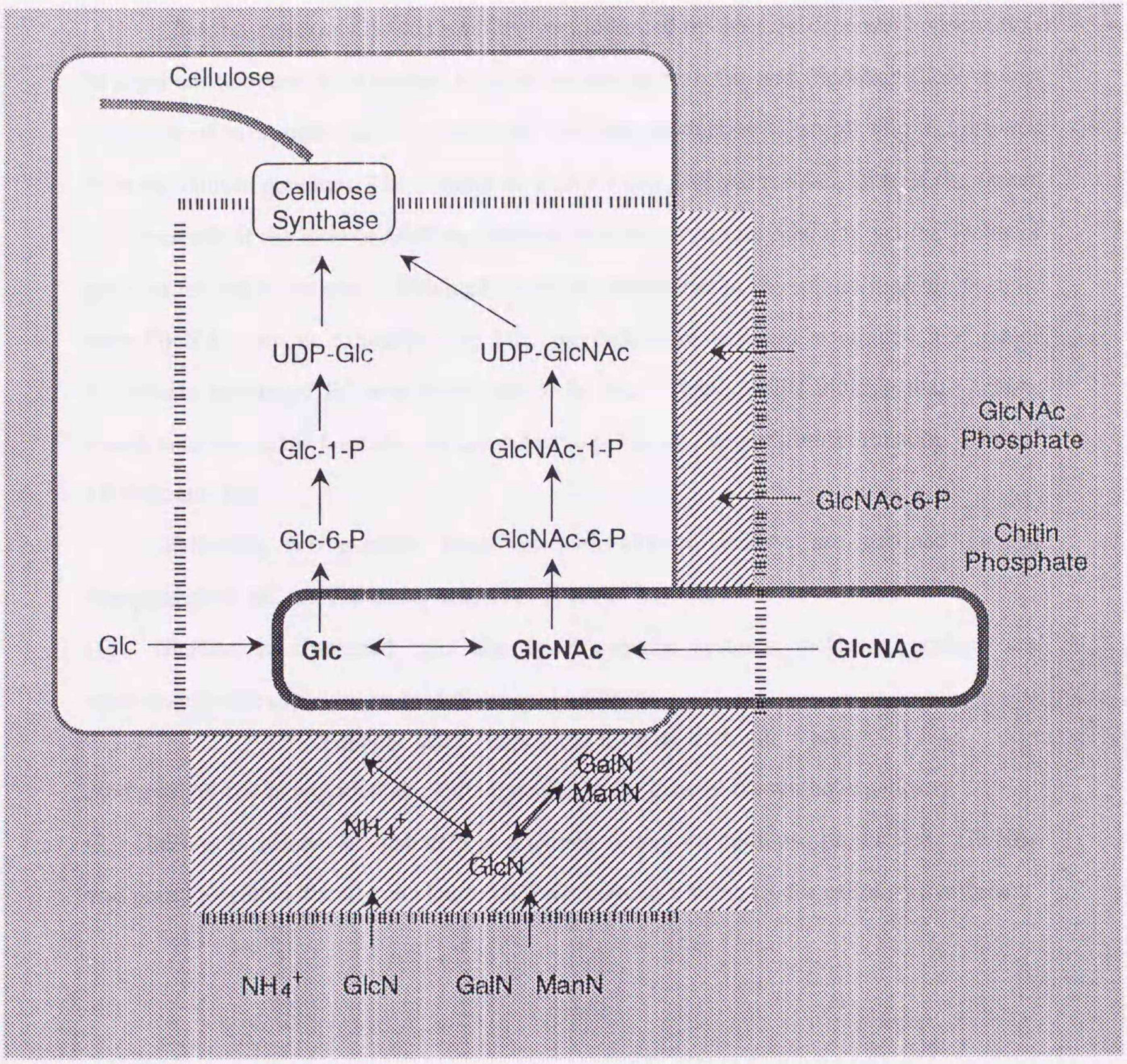
Observation of sugar consumption during incubation revealed that Glc was consumed prior to GlcNAc in SH mixed medium containing both sugar sources (Figure 2-1, 2-2). Since constant yields of pellicles were observed so long as Glc was present in the culture medium, most of Glc was utilized by the bacteria for cellulose production. Glc is known to be the most common substrate for cellulose production among other carbohydrates[17]. On the other hand, since the bacteria seemed to become familiar to GlcNAc, GlcNAc concentration started to decrease slowly after all Glc disappeared from the culture medium. GlcNAc-grown cells which was adapted to GlcNAc by successive transfers would begin to utilize GlcNAc as a substrate for cellulose production. It agrees with the results that concentration of GlcNAc decreased during incubation and production of pellicles was continued even in only GlcNAc medium (Figure 2-5).

Though only GlcNAc was a sugar component in the culture medium, cellulose production was continued accompanying reduction of the yields. While produced BC contained relatively high GlcNAc content, the most of constituent residue was still Glc. The result indicates that GlcNAc which was taken up, was converted into Glc for the biosynthesis of cellulose. Since *A. xylinum* requires carbohydrate as a substrate of cellulose production, it might come to consume even GlcNAc because of the absence of Glc in the culture medium. GlcNAc which was taken in would be transformed into Glc in the metabolic cycle of bacteria, because GlcNAc is unfamiliar hexose to biosynthetic pathway. So it is suggested that such enzymatic systems which catalyze hexose conversion are present in *A. xylinum*, and they seems to be induced and activated by an adaptation process of preculturing.

The amount of incorporated GlcNAc residues into pellicle increased with the increase of GlcNAc ratio in the mixed medium, and GlcNAc content reached the highest value when sugar component became only GlcNAc (Figure 2-4). GlcNAc consumption was found concurrently in the GlcNAc alone medium during incubation (Figure 2-1). The incorporation seemed to be accelerated when uptake of GlcNAc increased due to a rise of

GlcNAc concentration comparing to that of Glc in the culture medium. Thus the formation of GlcNAc phosphate would arise directly to BC production by phosphorylation of exogenous GlcNAc due to its high concentration in the medium and adaptive effect, consequently the incorporation into BC was promoted.

Two possible metabolic pathways of GlcNAc should be present in *A. xylinum*. The flow of hexoses are shown in Scheme 2-1. One is the conversion of GlcNAc into Glc for cellulose production after the bacteria took up it (area enclosed by thick line). Deacetamidation or a combination of deacetylation and deamination reactions were proposed to involve in the pathway in order to transform GlcNAc into Glc, then resulted Glc was provided as a carbon source to the general biosynthetic pathway from Glc to cellulose. It presumes that some enzymatic systems which catalyze the conversion between GlcNAc and Glc exist in *A. xylinum*. Enzymes such as acylase and aminotransferase seem also to be activated by adaptation to GlcNAc by means of repetitive subculturing because of the demand of Glc for cellulose production. The other is utilization of GlcNAc after direct phosphorylation. GlcNAc was phosphorylated directly by glucokinase and consequently led to UDP-GlcNAc as a substrate for cellulose synthase. The immediate utilization of exogenous GlcNAc seems to be caused by relatively high concentration of GlcNAc in the culture medium.



Scheme 2-1. The flow of GlcNAc in the metabolic pathway of *A. xylinum*.

CONCLUSION

The preference of Glc consumption was indicated by the time dependent observation of sugar consumption in SH mixed medium containing both Glc and GlcNAc. GlcNAc was also utilized as a minor carbon source for cellulose production when all Glc disappeared from the culture medium. The amount of incorporated GlcNAc residues into BC increased with increase of the ratio of GlcNAc concentration in the culture medium, yet the yields of pellicles tended to decrease. Although the sugar component in the culture medium became only GlcNAc due to exhaustion of Glc, production of pellicles continued and major constituent residue of BC was found still to be Glc. These results indicate that GlcNAc which taken up in the bacteria was converted into Glc by the equilibrium function between GlcNAc and Glc.

Following two possible metabolic pathways of GlcNAc are assumed for the biosynthesis of BC and the incorporation of GlcNAc residue.

(1) GlcNAc is converted into Glc by enzymatic systems including acylase and aminotransferase as shown in the following equilibrium.

$$\text{GlcNAc} \rightleftarrows \text{GlcN} + \text{CH}_3\text{COOH} \rightleftarrows \text{Glc} + \text{NH}_4^+$$

And resulted Glc is provided as a carbon source to the general biosynthetic pathway.

(2) GlcNAc is utilized immediately as a carbon source for cellulose production. GlcNAc was phosphorylated directly and led to UDP-GlcNAc as a substrate for cellulose synthase.

CHAPTER III

Stereostructural Participation of Aminosugars in the Incorporation Mechanism

INTRODUCTION

As the existence of enzymatic equilibrium between GlcNAc and Glc was proposed in the metabolic pathways of *A. xylinum* as described in Chapter II, it is assumed that enzyme catalyze deacetamidation or a combination of deacetylation and deamination. Glucosamine(GlcN), a deacetylated form of GlcNAc, is expected to play an intermediate role on the conversion of GlcNAc into Glc. Thus, in this chapter, GlcN was employed to the culture medium of *A. xylinum* to examine participation of GlcN in the equilibrium state as intermediate between GlcNAc and Glc.

Moreover, the stereospecificity of carbon source for the metabolism of the bacteria was investigated to clarify the incorporation mechanism. Thus three hexosamines, GlcN, galactosamine(GalN) and mannosamine(ManN) were employed as carbon sources together with Glc (Chemical structures are shown in Figure 3-1). The cultures of *A. xylinum* were performed in the medium containing aminosugars as a single sugar source or in the mixed medium containing Glc and each aminosugar. The relationship between time course of sugar composition in the culture medium and the amount of incorporated GlcNAc residues into BC were investigated in order to elucidate the stereospecific utilization of carbon sources by *A. xylinum*.

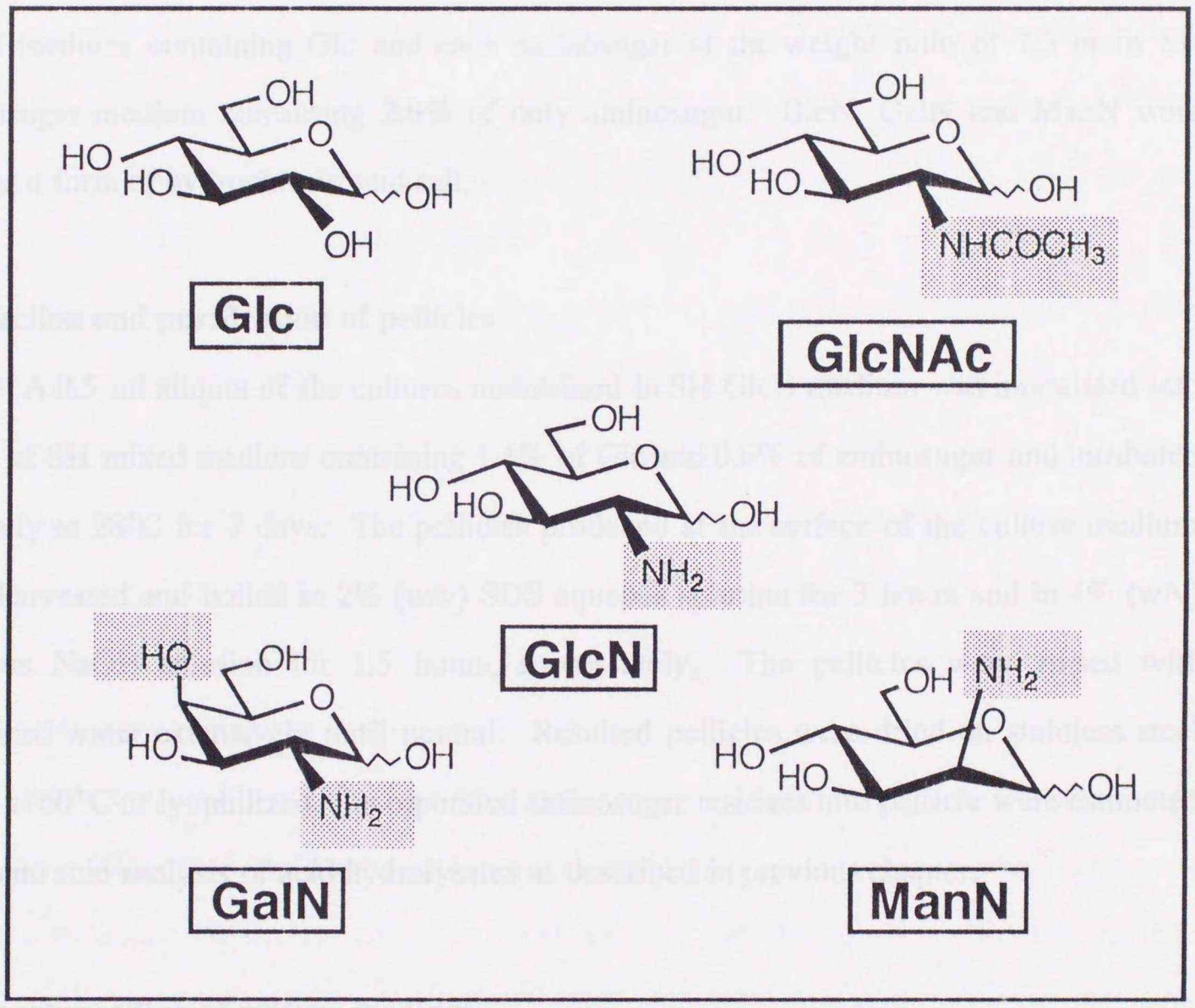


Figure 3-1. Chemical structures of aminosugars.

EXPERIMENTAL

Bacterial strain and culture conditions

The bacterial strain was *Acetobacter xylinum* ATCC 10245 which was adapted previously to GlcN by repetitive subculturing. All cultures were incubated statically at 28° C in SH medium containing Glc and each aminosugar at the weight ratio of 7:3 or in SH aminosugar medium containing 2.0% of only aminosugar. GlcN, GalN and ManN were used as a form of hydrochloric acid salt.

Production and purification of pellicles

A 0.5 ml aliquot of the cultures maintained in SH GlcN medium was inoculated into 15 ml of SH mixed medium containing 1.4% of Glc and 0.6% of aminosugar and incubated statically at 28°C for 7 days. The pellicles produced at the surface of the culture medium were harvested and boiled in 2% (w/v) SDS aqueous solution for 3 hours and in 4% (w/v) aqueous NaOH solution for 1.5 hours, successively. The pellicles were rinsed with deionized water extensively until neutral. Resulted pellicles were dried on stainless steel plates at 60° C or lyophilized. Incorporated aminosugar residues into pellicle were estimated by amino acid analysis of acid hydrolysates as described in previous chapter.

Sugar composition of culture medium

Sugar concentration was measured by the modification of Schales' method for the amount of total sugar and glucose-oxidase peroxidase system for Glc in the similar manner as described in previous chapter.

IR spectra

IR spectra of pellicles were taken with HORIBA fourier transform infrared spectrometer FT-210 at a resolution of 4 cm^{-1}.

RESULTS

Sugar consumption

Time course of sugar consumption was observed during incubation in SH mixed medium as shown in Figure 3-2. Glc was consumed prior to any kind of aminosugars at the early stage of incubation. Concentrations of GlcN and GalN started to decrease a little before the exhaustion of Glc similarly to GlcNAc mixed medium, whereas that of ManN was hardly changed. These results indicate that GlcN and GalN could be taken up and utilized as carbon sources by the bacteria.

Time courses of aminosugar consumption in the medium consisted of only aminosugar are shown in Figure 3-3. The cultures were transferred to the same medium components up to 4 times at the intervals of a week. ManN was not consumed except first transfer while both GlcN and GalN were consumed from the early stage of transfers. It seems that ManN was scarcely accepted by the bacteria as a carbon source.

Incorporation of aminosugar residues into BC

The amount of incorporated aminosugar residues into pellicle was found to increase gradually in SH mixed medium according to the numbers of transfers as shown in Figure 3-4. But little incorporation was observed by ManN, it seems that ManN is almost insensitive to the adaptation by repetitive subculturing. GlcN and GalN increased the incorporation of aminosugar effectively with increase in the numbers of transfers. In addition, since only GlcN was detected by amino acid analysis of acid hydrolysate, GalN which taken up by the bacteria might be converted into GlcN by epimerization. IR spectrum of harvested pellicle produced in the medium containing GlcN is shown in Figure 3-5. The aminosugar residue incorporated into BC was presumed to be only GlcNAc, because amide I and II bands which derived from acetamide group of GlcNAc residue were observed. Thus GlcN seems to be acetylated followed by incorporation.

Although carbon source was only aminosugar, the production of pellicles and a little incorporation of GlcNAc residues were observed in SH GlcN medium similar to the case of

GlcNAc alone medium. As a major constituent residue of produced BC was Glc even when SH GlcN medium was applied, the presence of enzymatic systems such an aminotransferase were also suggested in the metabolic pathway of *A. xylinum*. GlcN was assumed to be transformed into Glc by the deamination, and was utilized as a carbon source for cellulose production. As ManN is an epimer of GlcN at C-2 position, axial amino group of ManN seemed to be hardly deaminated and converted into hydroxyl group due to the stereospecific recognition of enzymes.

Production of pellicles

Yields of pellicles produced in SH mixed medium consisted of Glc and each aminosugar at the weight ratio of 7:3 are shown in Figure 3-6. A similar pellicle yield were shown in all cultures of every transferring numbers, because Glc is a major carbon source for cellulose production. The average yields of pellicles produced in 15 ml of SH medium for a week are shown in Figure 3-7, The average values were about 2/3 of the yield harvested in SH Glc medium. Their values corresponded to the initial concentration of Glc fed in the culture medium.

Yields of pellicles produced in SH GlcN medium were quite low comparing with those in the medium containing Glc. Little pellicle was harvested in the medium containing only GalN or ManN.

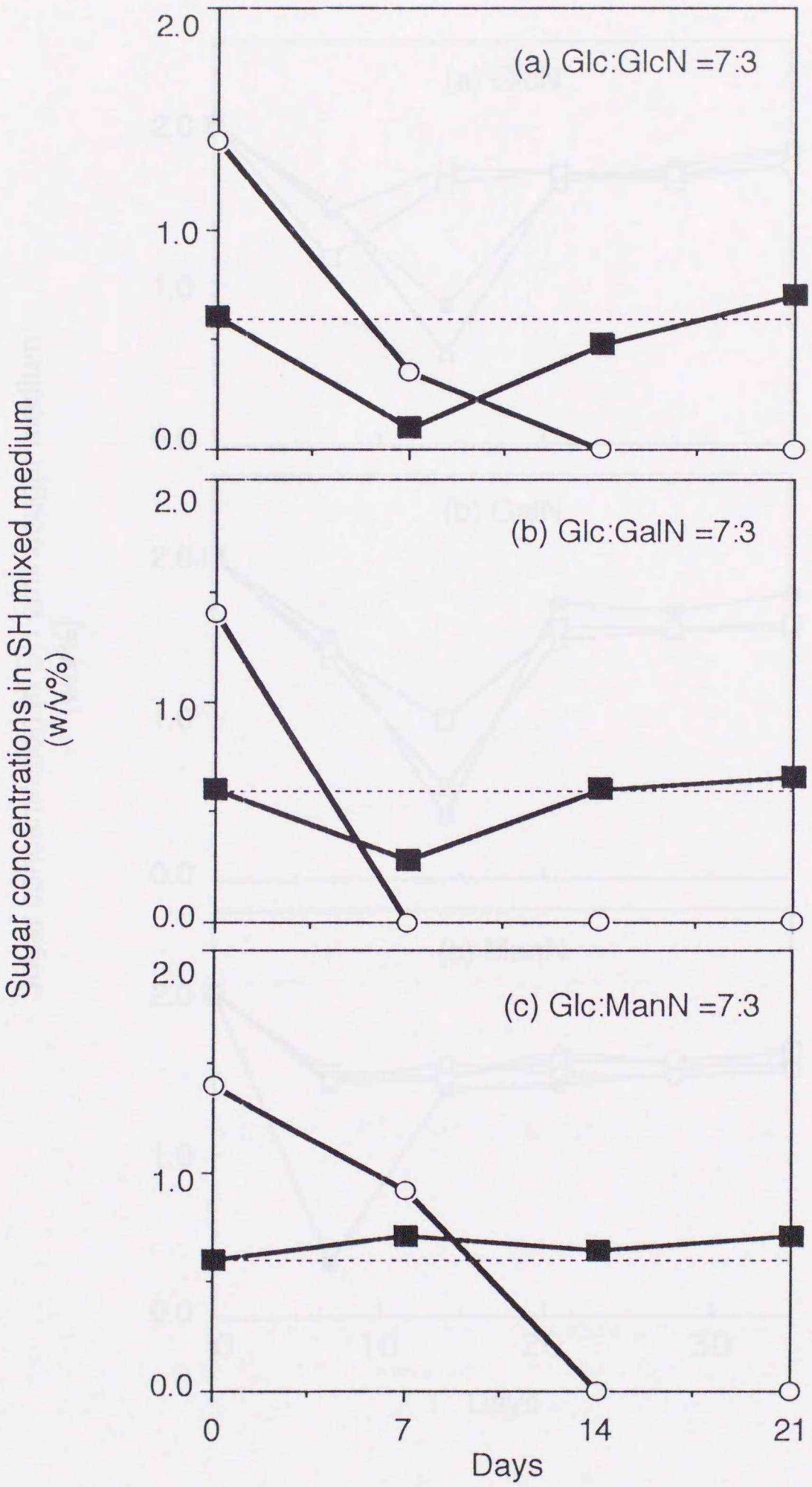


Figure 3-2. Time course of sugar concentrations in SH mixed medium containing 1.4% of Glc and 0.6% of aminosugar: (a) GlcN; (b) GalN; (c) ManN. The broken line shows initial concentration of aminosugar.

(O) : Concentration of Glc

(■) : Concentration of aminosugar

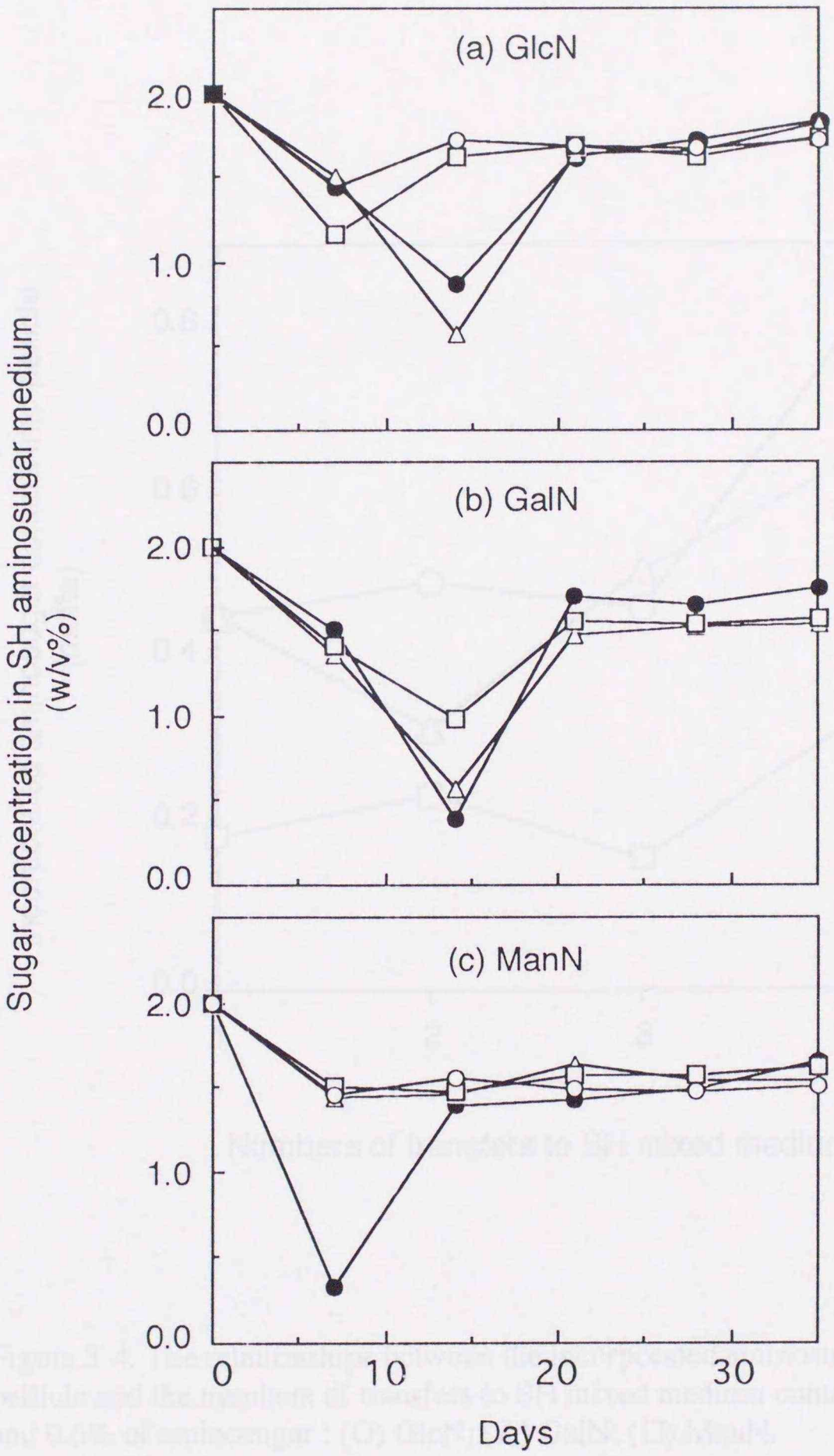


Figure 3-3. Time course of aminosugar concentration in SH aminosugar medium containing 2% of aminosugar: (a) GlcN; (b) GalN; (c) ManN. The numbers of transfers: (●) 1; (△) 2; (□) 3; (O) 4 times.

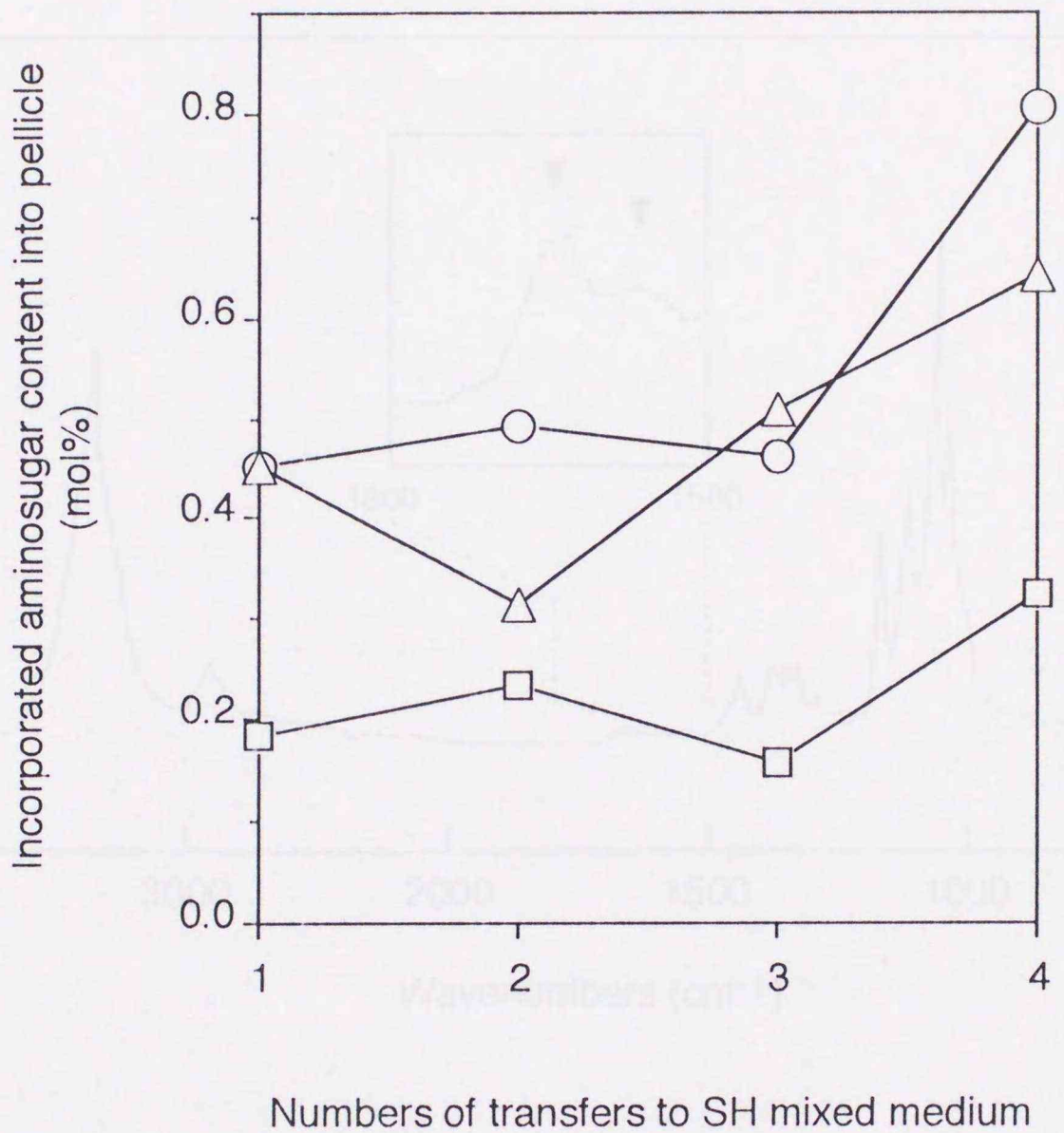


Figure 3-4. The relationships between the incorporated aminosugar content into pellicle and the numbers of transfers to SH mixed medium containing 1.4% of Glc and 0.6% of aminosugar : (O) GlcN; (Δ) GalN; (□) ManN.

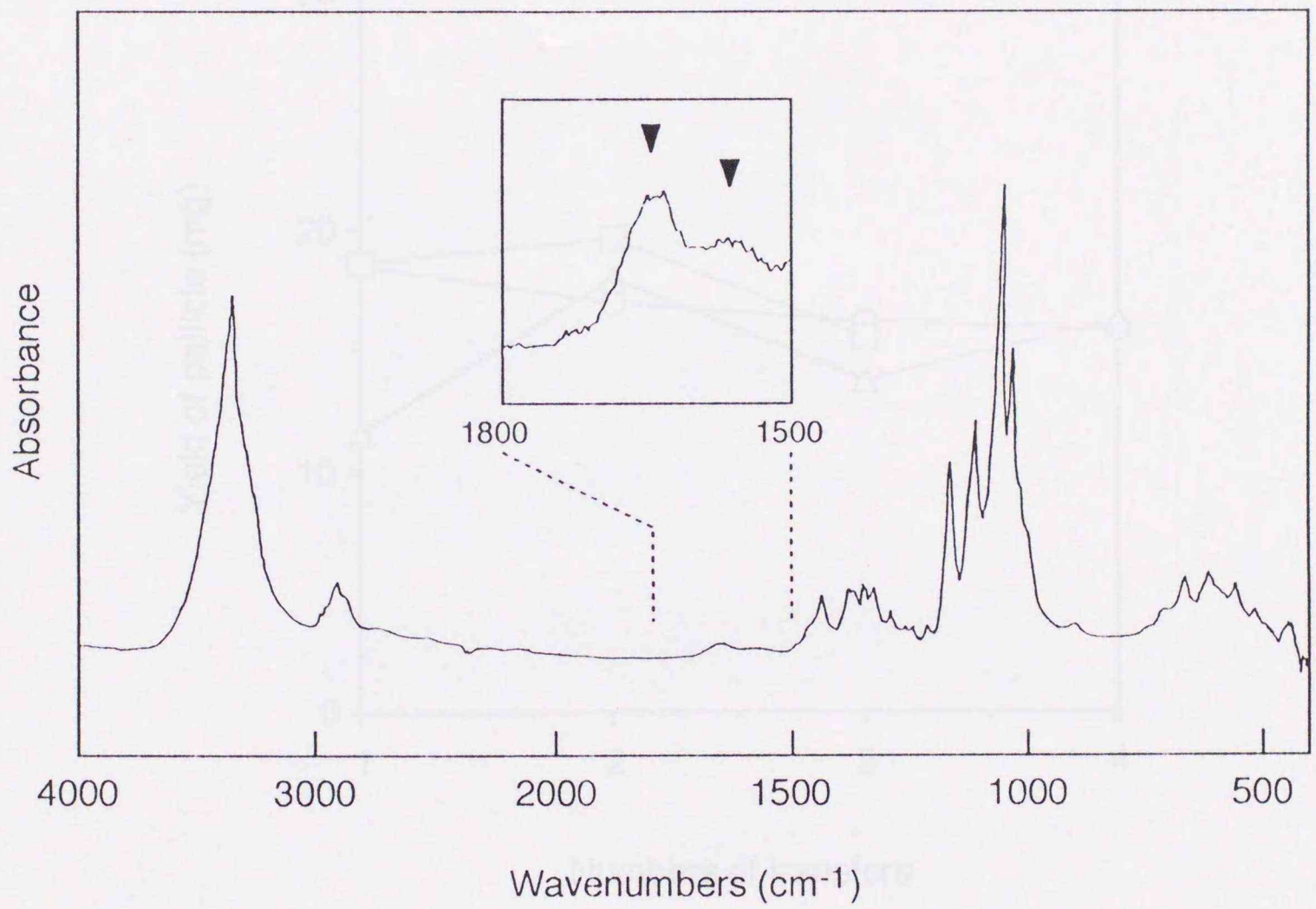


Figure 3-5. IR spectrum of pellicle produced in SH mixed medium containing Glc and GlcN at the weight ratio of 7:3.

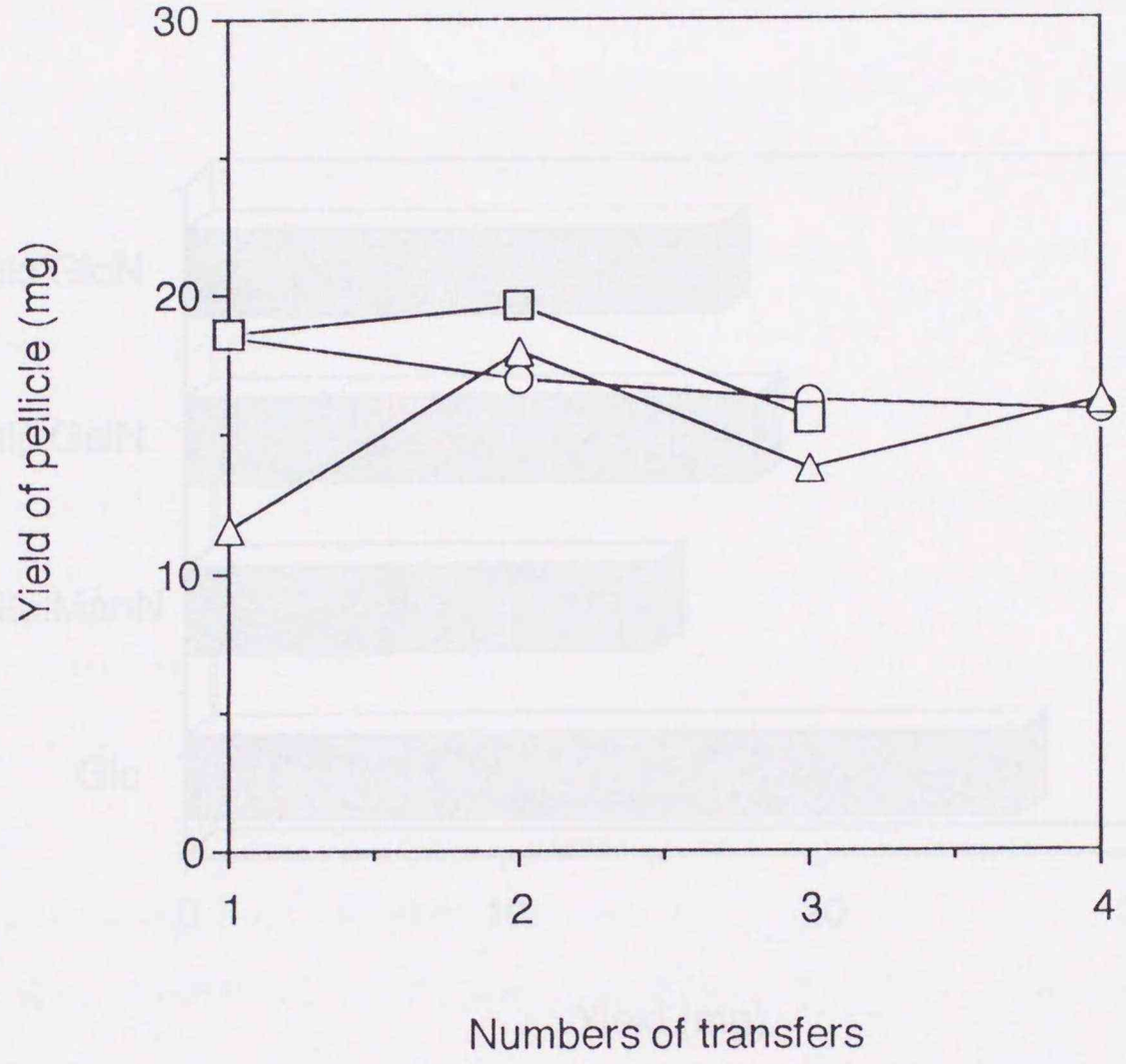


Figure 3-6. The relationship between yield of pellicle and the numbers of transfers to SH mixed medium containing 1.4% of Glc and 0.6% of aminosugar: (O)GlcN; (△) GalN; (□) ManN. Cultures were incubated statically at 28°C for a week.

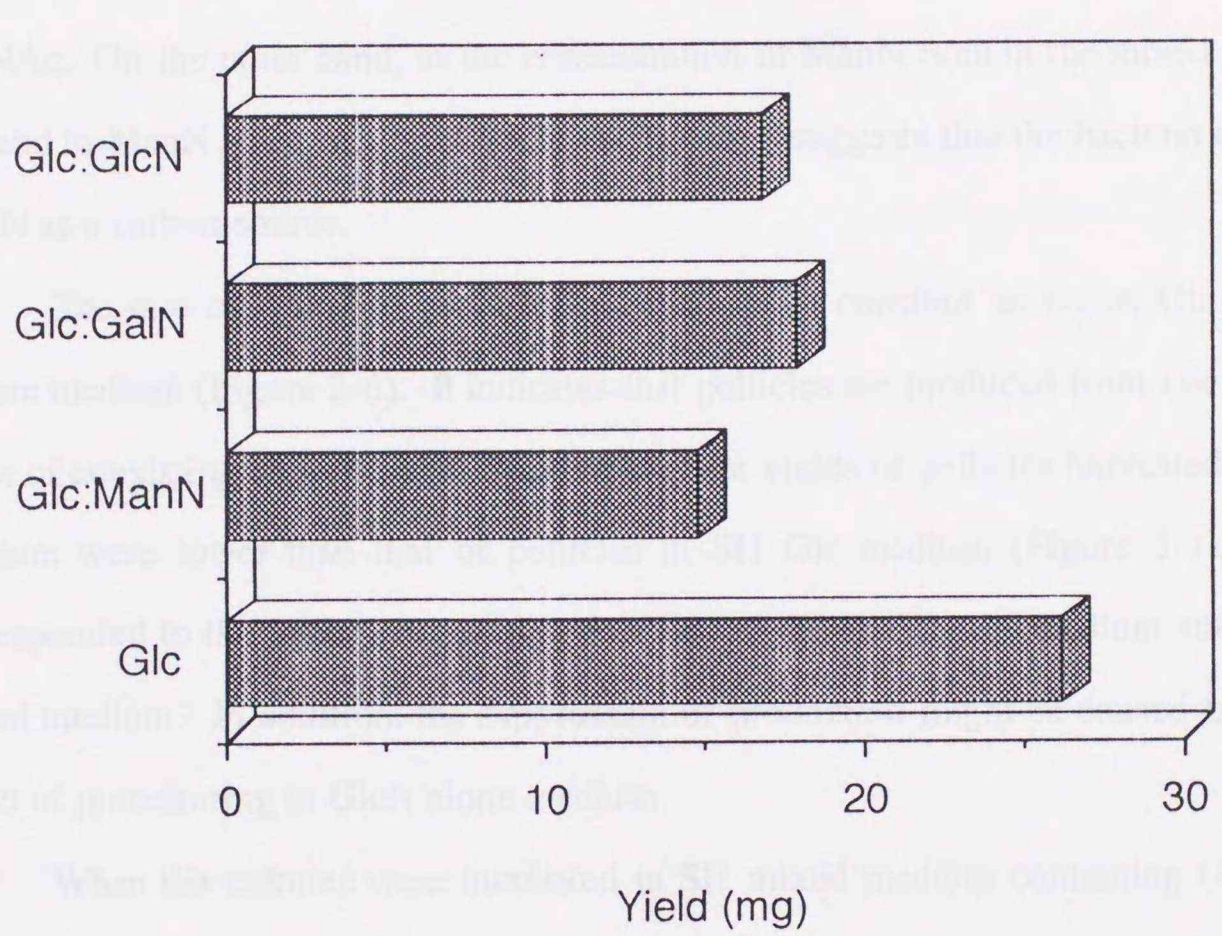


Figure 3-7. Average yield of pellicles produced in SH Glc medium and SH mixed medium containing Glc and aminosugar at the weight ratio of 7:3. Cultures were incubated statically at 28°C for a week. Volume of medium was 15ml.

DISCUSSION

From the observation of sugar consumption in SH mixed medium during incubation, it was evident that Glc was consumed prior to any aminosugars (Figure 3-2). And GlcN and GalN seem to be taken up by the bacteria as a minor carbon source, when adaptation to aminosugar was carried out by successive transfer to GlcN medium in these cultures. The tendency of preferential Glc consumption in SH mixed medium was the same as the case of GlcNAc. On the other hand, as the concentration of ManN both in the mixed medium with Glc and in ManN alone medium hardly decreased, it suggests that the bacteria did not accept ManN as a carbon source.

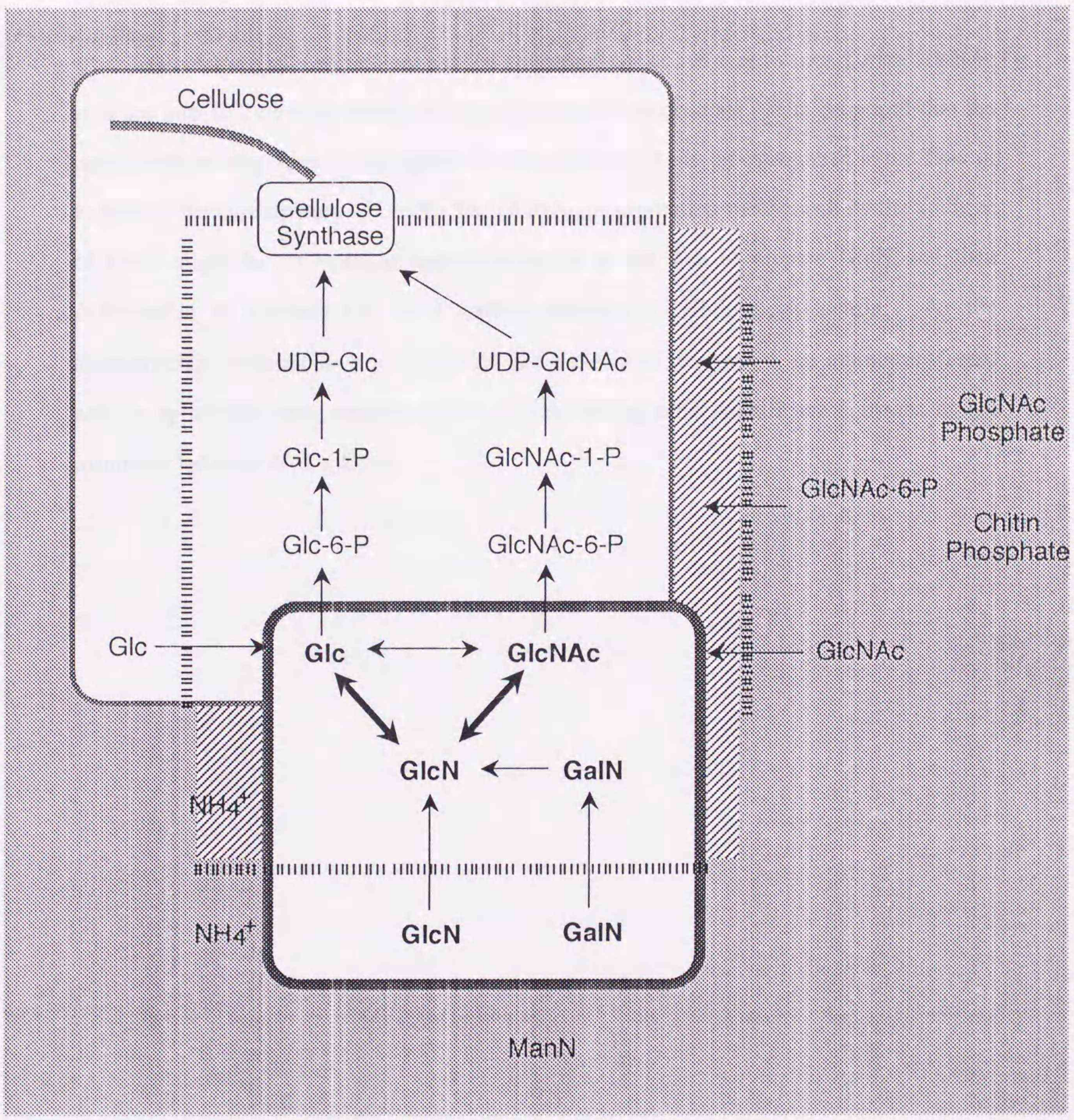
The rate of cellulose production seems to be constant as far as Glc exists in the culture medium (Figure 3-6). It indicates that pellicles are produced from Glc regardless of kinds of coexisting aminosugars. But the average yields of pellicles harvested in SH mixed medium were lower than that of pellicles in SH Glc medium (Figure 3-7), their values corresponded to the initial concentration of Glc, 2.0% in SH Glc medium and 1.4% in SH mixed medium. In addition, the suppression of production might be caused by an adaptive effect of preculturing in GlcN alone medium.

When the cultures were incubated in SH mixed medium containing GlcN or GalN, only GlcN was detected by amino acid analysis of acid hydrolysates and the adsorption derived from acetamide group was observed in IR spectrum of pellicle (Figure 3-5). Thus incorporated aminosugar residues into BC were estimated to be GlcNAc, though GlcNAc was absent in the culture medium. Moreover, the amount of incorporated GlcNAc residues increased according to the numbers of transfers (Figure 3-4). It is suggested that GlcN and GalN are taken up as carbon sources by the bacteria, especially GalN might be utilized after a conversion into GlcN by epimerization, and consequently incorporated into BC. BC is polymerized by cellulose synthase via a direct substitution mechanism, in which the phosphoester-activating group at the anomeric carbon of UDP-sugar is displaced by the C-4 hydroxyl group of another glucosyl residue with inverting the α configuration to form a β-glucosidic bond[17]. Thus it was inevitable that GalN was epimerized when it was

incorporated into BC chain, because configuration of hydroxyl group at C-4 position is involved in the biosynthesis of β-1,4 glucan. On the other hand, aminosugar content was insensitive to the transfer numbers in the case of SH mixed medium of ManN. The results obtained in the study of application of GalN and ManN suggest that not 2-epimerase but 4-epimerase is involved in enzymatic systems related to the conversion of aminosugars.

Though a sugar component of the culture medium was only GlcN (SH GlcN medium), consumption of GlcN was observed (Figure 3-3) and production of pellicle was continued. In addition, the amount of incorporated GlcNAc residues were quite small and most of constituent sugar of produced BC were Glc residues. These results indicate that GlcN was led to GlcNAc as mentioned above or transformed into Glc for cellulose production in the metabolic pathways of *A. xylinum*, and GlcN would take part in the equilibrium between GlcNAc and Glc as a metabolic intermediate of hexose.

Proposed flow of hexose is displayed in Scheme 3-1. GlcN which is taken up by the bacteria is converted into Glc for cellulose production or into GlcNAc for the incorporation pathway. GlcN seems to play an intermediate role and take part in the equilibrium between GlcNAc and Glc. It is proposed that the equilibrium among three hexoses, GlcNAc, GlcN and Glc, was established by the application of GlcN to the culture medium and induced by adaptation. It suggests that enzymatic systems which catalyze such conversion reactions exist in *A. xylinum*. Enzymes including deacylase and aminotransferase seem to be activated by the repetitive subculturing of bacteria. The hypothesis to assume the existence of aminotransferases in the bacterial metabolism might be assisted by the fact that the rate of aminosugar consumption and polysaccharide production were relatively slow comparing with that of the cultures in SH Glc medium. GalN is transformed into GlcN by epimerase owing to different configuration of C-4 hydroxyl group. ManN is hardly metabolized due to axial amino group at C-2 position, aminotransferase proposed did not recognize ManN as a substrate. Configuration of amino group of hexose might be related to incorporation of a carbon source and enzymatic recognition.



Scheme 3-1. The flow of aminosugars in the metabolic pathway of *A. xylinum*.

CONCLUSION

GlcN and GalN were shown to be consumed by *A. xylinum* to incorporate GlcNAc residues into BC whereas ManN was not achieved to incorporate. It is proposed that the equilibrium among three aminosugars, GlcNAc, GlcN and Glc, is established in the flow of hexose by the participation of GlcN. The GlcNAc incorporation mechanism by the addition of GalN might be proposed as epimerization at a first step to prepare GlcN and then deamination to produce Glc as a carbon source for cellulose production. As the stereospecific deamination and amination processes were suggested, an aminotransferase and an epimerase were assumed to be present among other significant enzymes in the metabolic pathway of *A. xylinum*.

CHAPTER IV

Contribution of Ammonium Ion to the Incorporation of Aminosugar Residues into Bacterial Cellulose

INTRODUCTION

Incorporation of GlcNAc residues into BC main chain has been achieved by the application of several aminosugars to the culture of *A. xylinum* as described in former chapters. The strain *A. xylinum* should be adapted to aminosugars prior to incorporate them by repetitive subcultures. Production of pellicles on the surface of the medium and a little amount of GlcNAc residues incorporated into BC were found even in the culture medium consisted of only GlcNAc or GlcN. Although a carbon source in the medium was only aminosugar, Glc was a major constituent residue of resulted BC. This result may indicate that an aminosugar is transformed into Glc to utilize for cellulose production. It is proposed that the equilibrium might exist between GlcNAc and Glc which catalyzed by enzymatic system such an aminotransferase. The ammonium ion concentration would be increased gradually by the deamination of GlcNAc, hydroxyl groups are transformed into acetamide groups by the enzyme through equilibrium shift due to increment of ammonium ion concentration. Thus the related enzyme reaction may proceed reversibly to provide GlcNAc again. Then various ammonium salts were employed to the Glc medium to examine the possibility of aminosugar incorporation into BC. As *A. xylinum* is strictly aerobic bacteria, sufficient air supply is expected to activate growth of bacteria. In this study, the incubation system was also investigated to improve the incorporation of aminosugar, in which an aeration incubation system was applied to the culture of *A. xylinum*. The hypothesis would be confirmed by the GlcNAc incorporation under the strong influence of artificial ammonium ion.

EXPERIMENTAL

Bacterial strain and culture conditions

A. xylinum ATCC 10245 which was maintained in SH Glc medium was used. SH Glc medium containing 0.2(w/v)% of various ammonium salts was applied : ammonium sulfate, $(NH_4)_2SO_4$; ammonium chloride, NH_4Cl; ammonium phosphate, $(NH_4)_2HPO_4$; ammonium citrate, $(NH_4)_2HC_6H_5O_7$.

Glucose-6-phosphate(Glc-6-P) was applied to the culture of *A. xylinum* instead of Glc. (Glucose 6-phosphate monosodium salt, SIGMA Lot. 12H3805) The cultures were incubated in SH medium containing 2% of Glc-6-P and 0.2% of ammonium chloride for a week statically or under rotatory - aerated conditions.

Rotatory culture apparatus was employed in these cultivation system as illustrated in Figure 4-1. In this apparatus, aeration was performed by bubbling air sterilized through cotton wool from the bottom of tilted culture tank and an axle fixed stainless steel meshes rotates to stir the culture medium.

Production of cellulosic fibrils

A 15 ml aliquot of 3 days' culture in SH Glc medium was inoculated into 2,500 ml of the culture medium containing ammonium salt, and stirred gently at 6r.p.m. with aeration at a flow rate of 2 liter/min at 28° C for a week. Fibrils produced on the meshes were collected by filtration. Purification procedures were the same as those of pellicles.

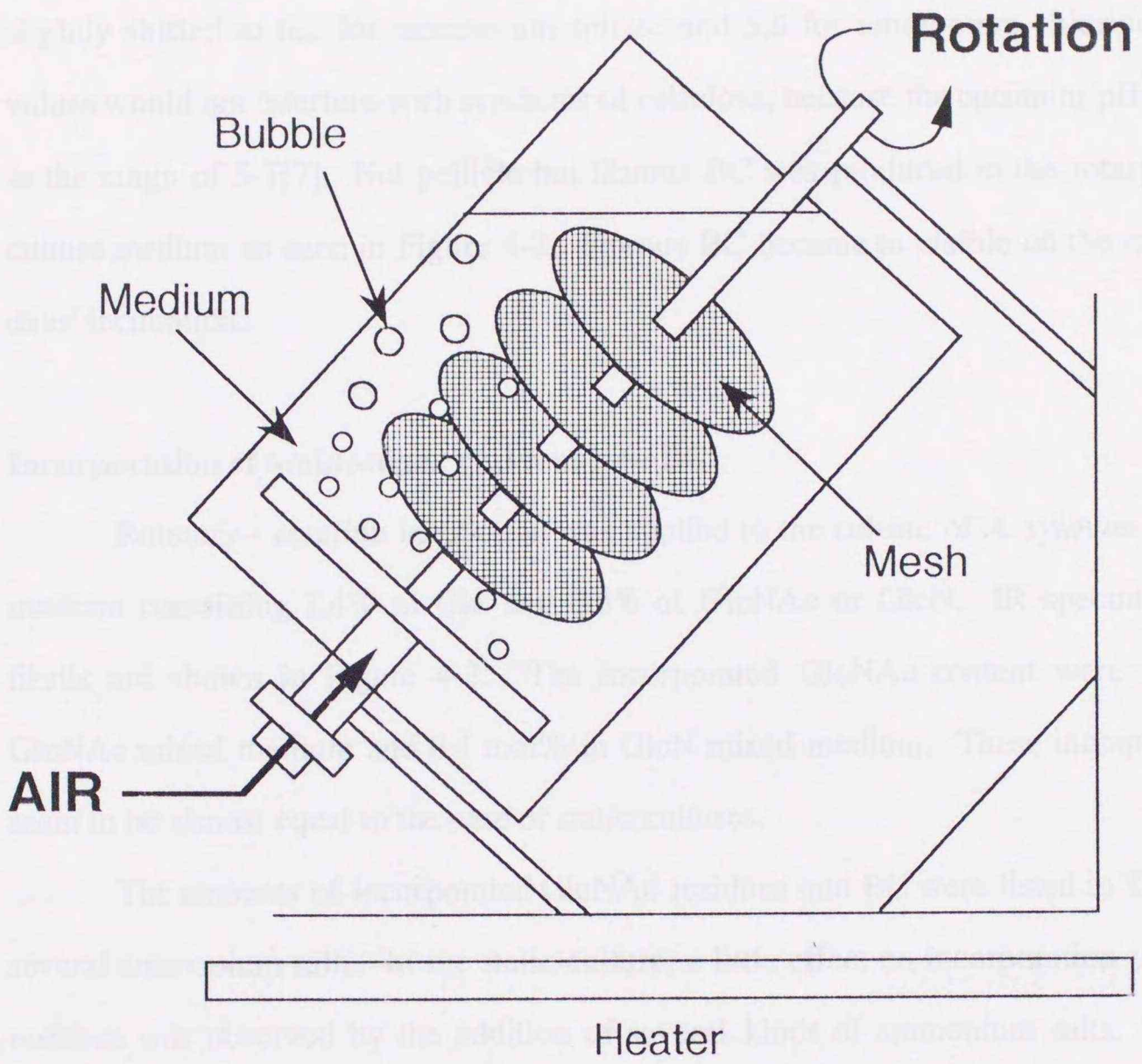


Figure 4-1. Outline of rotatory culture apparatus.
(Volume of medium is 2500ml.)

RESULTS

Production of BC

The initial pH value of the medium containing 0.2% (w/v) ammonium salts was slightly shifted to 6.2 for ammonium sulfate and 5.8 for ammonium chloride. These pH values would not interfere with synthesis of cellulose, because the optimum pH was reported in the range of 5-7[7]. Not pellicle but fibrous BC was produced in the rotary and aerated culture medium as seen in Figure 4-2. Fibrous BC became to visible on the meshes after 3 days' incubation.

Incorporation of aminosugar residues into BC

Rotatory - aeration incubation was applied to the culture of *A. xylinum* in SH mixed medium containing 1.4% of Glc and 0.6% of GlcNAc or GlcN. IR spectra of produced fibrils are shown in Figure 4-3. The incorporated GlcNAc content were 0.3 mol% in GlcNAc mixed medium and 0.4 mol% in GlcN mixed medium. These incorporated values seem to be almost equal to the case of static cultures.

The amounts of incorporated GlcNAc residues into BC were listed in Table 4-1 with several ammonium salts. In the static culture, a little effect on incorporation of aminosugar residues was observed by the addition of several kinds of ammonium salts. On the other hand, significant incorporation was observed by rotatory - aerated culture in the presence of ammonium salts. Ammonium chloride seems to be the best additive to enhance GlcNAc incorporation and its amount of incorporated residues was remarkably higher than that of pellicle produced in static culture of SH GlcNAc medium. Since static incubation was not effective as much as the incubation with aeration, aeration seems to be an important factor to utilize ammonium ion which was present in the culture medium. In addition, when stirred culture was incubated without aeration in SH Glc medium containing 0.2% of ammonium chloride, incorporation of GlcNAc residues into harvested fibril was hardly observed. Thus it suggests that the aeration is an essential factor to affect the incorporation of aminosugar residues into BC.

IR spectra of BC produced in rotatory - aerated culture with ammonium salts were shown in Figure 4-4. As the adsorption bands of amide I and II were observed around 1650 and 1550 cm^{-1}, incorporated aminosugar residues into resulted BC were assumed to be *N*-acetylated form.

The dependence of concentration of ammonium chloride was examined in rotatory - aerated cultures. The relationship between incorporated aminosugar content and the concentration of ammonium chloride added to the culture medium is shown in Figure 4-5. GlcNAc residues were hardly incorporated at the concentrations of 0.1 and 0.4%, whereas GlcNAc content reached its maximum value around 0.2-0.3%. It seemed that 0.2% of ammonium salt was the optimum concentration for the aminosugar incorporation. The concentration of ammonium ion was so low that reversible reaction of aminotransferase was not performed at the concentration of 0.1%. Since the yield of BC fell extremely at the concentration of 0.4%, the growth of bacteria seemed to be suppressed.

The amounts of incorporated GlcNAc residues into BC produced in SH medium containing Glc-6-P and 0.2% of ammonium chloride were listed in Table 4-2. In the static culture, little effect on incorporation of aminosugar residues was observed. On the other hand, significant incorporation was observed by rotatory - aerated culture in the presence of Glc-6-P and ammonium chloride, but its value was lower than that of Glc and ammonium salt.

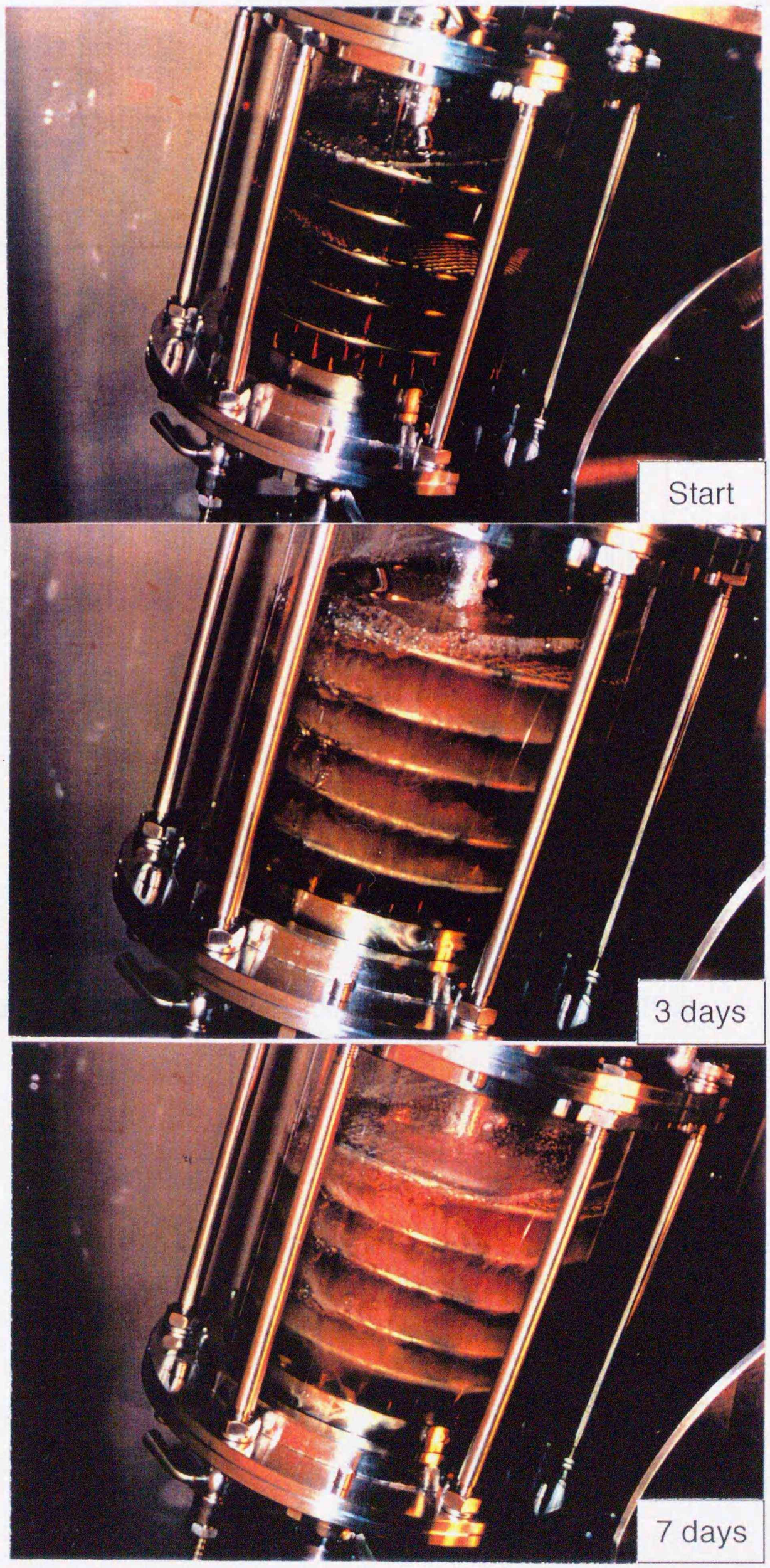


Figure 4–2. Photographs of rotatory aerated culture of *A. xylinum*. The culture medium was SH Glc medium containing 0.2% of ammonium sulfate.

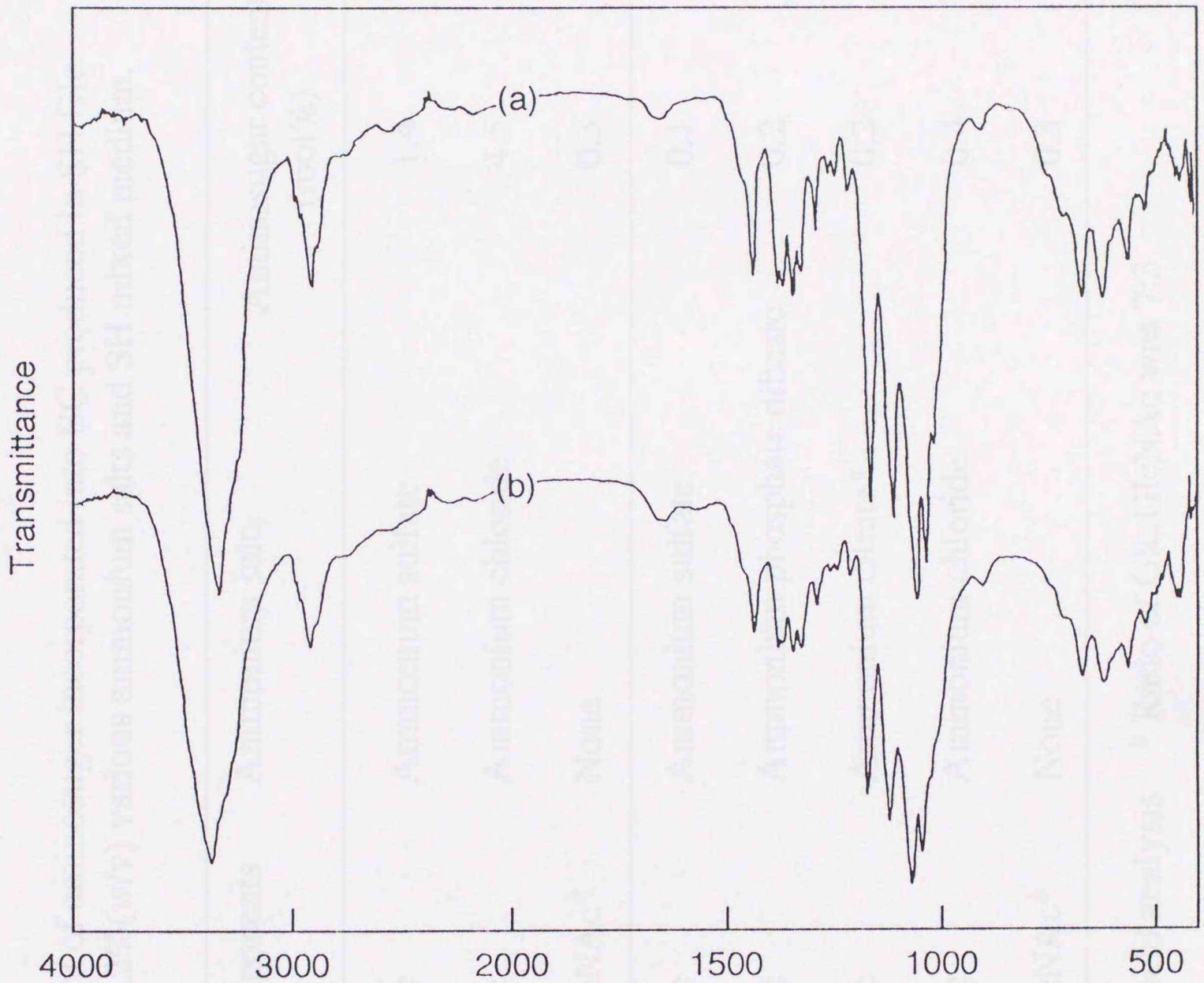


Figure 4-3. IR spectra of BC fibrils produced in SH mixed medium containing (a) Glc and GlcNAc, (b) Glc and GlcN at the weight ratio of 7:3 under rotatory culture with aeration.

Table 4-1. The amount of aminosuger incorporated into BC produced in SH Glc medium containing 0.2%(w/v) various ammonium salts and SH mixed medium.

Incubation	Sugar components	Ammonium salts	Aminosugar content [a] (mol%)
Rotatory	Glc	Ammonium sulfate	1.4
	Glc	Ammonium chloride	4.5
	Glc:GlcNAc [b]	None	0.3
Static	Glc	Ammonium sulfate	0.1
	Glc	Ammonium phosphate dibasic	0.2
	Glc	Ammonium citrate [c]	0.2
	Glc	Ammonium chloride	0.4
	Glc:GlcNAc [b]	None	0.3

[a] Determined by amino acid analysis [b] Ratio of Glc:GlcNAc was 7:3

[c] $(NH_4)_2HC_6H_5O_7$

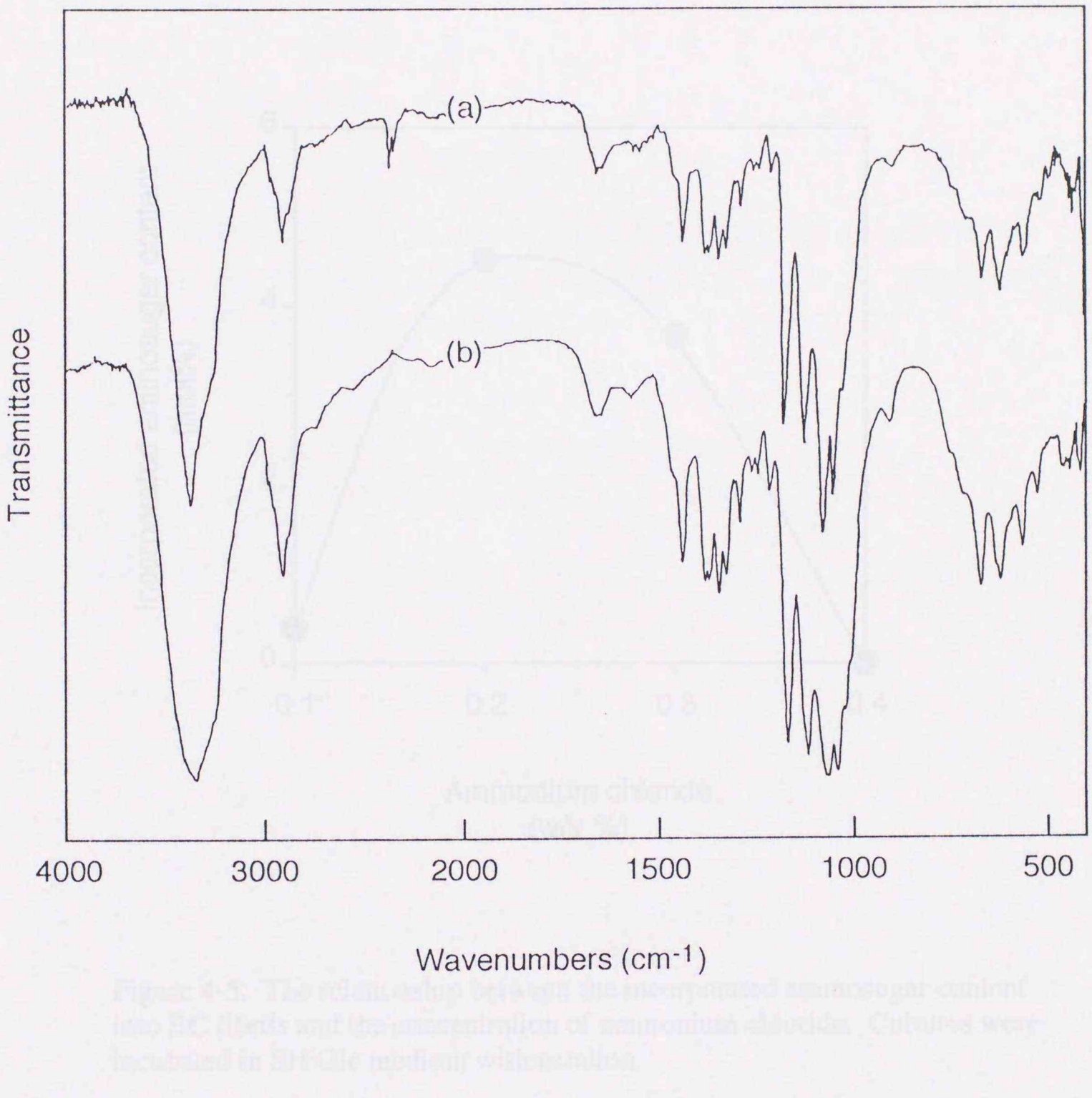


Figure 4-4. IR spectra of BC fibrils produced in SH Glc medium containing (a) ammonium sulfate and (b) ammonium chloride under rotatory culture with aeration.

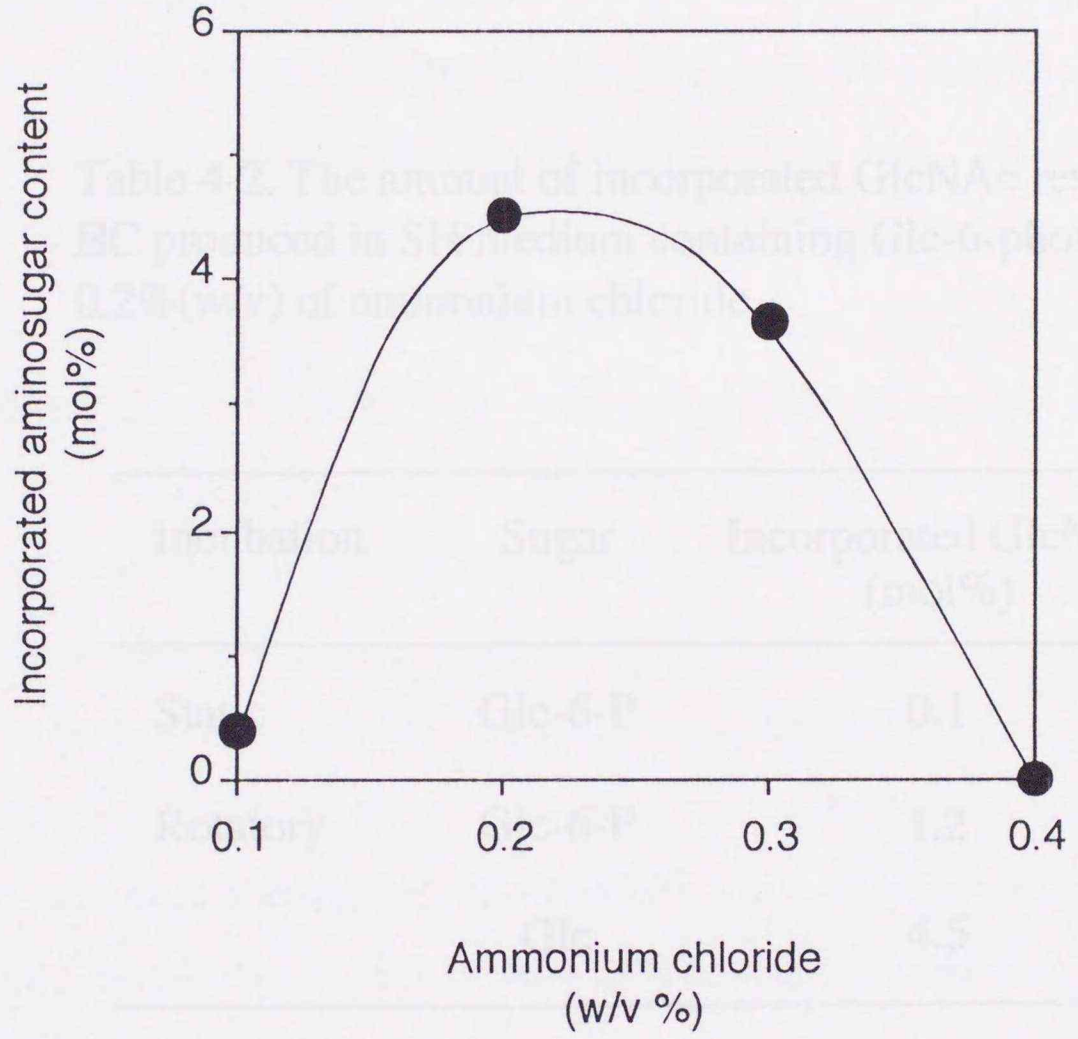


Figure 4-5. The relationship between the incorporated aminosugar content into BC fibrils and the concentration of ammonium chloride. Cultures were incubated in SH Glc medium with aeration.

Table 4-2. The amount of incorporated GlcNAc residues into BC produced in SH medium containing Glc-6-phosphate and 0.2%(w/v) of ammonium chloride.

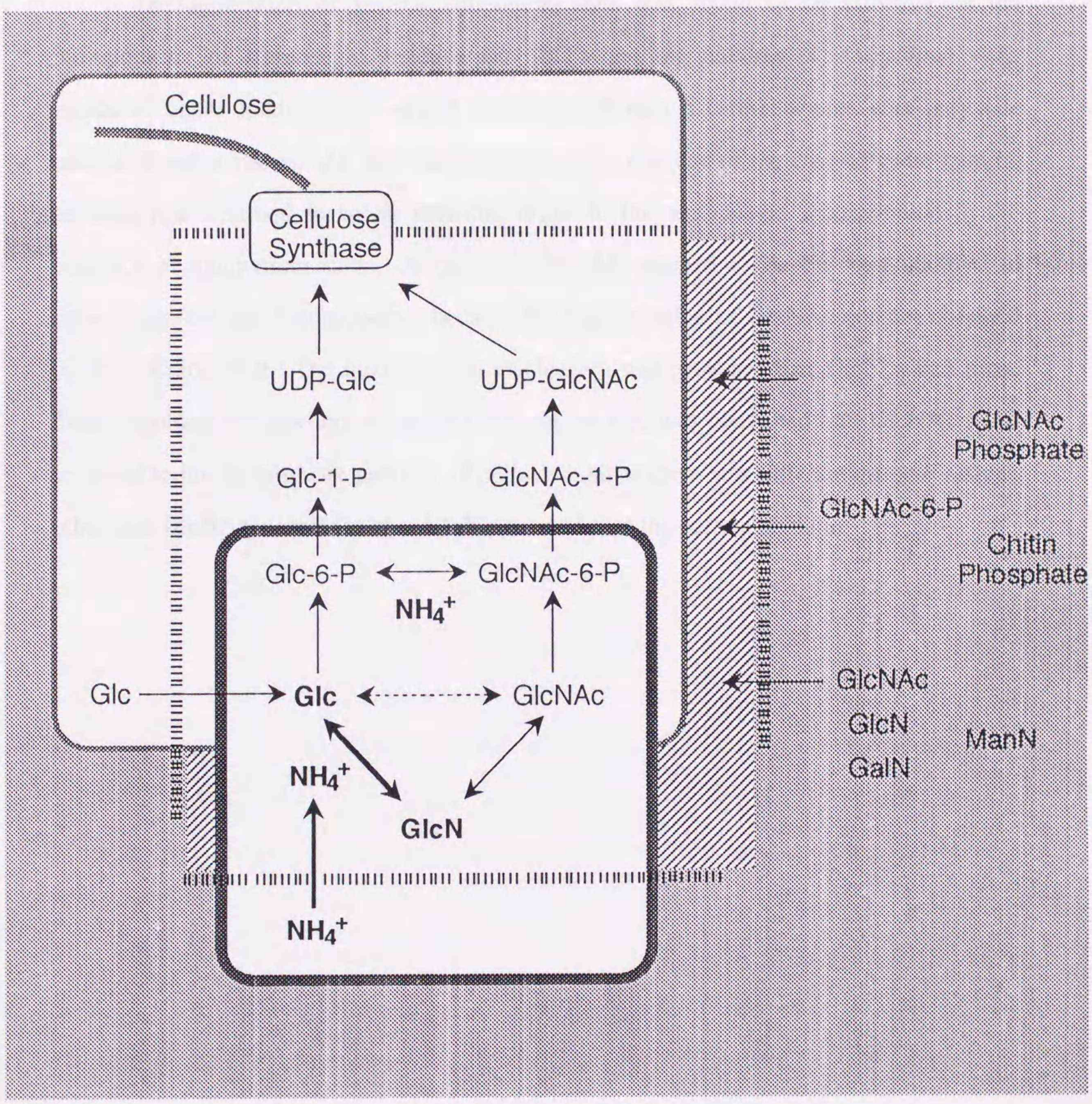
Incubation	Sugar	Incorporated GlcNAc (mol%)
Static	Glc-6-P	0.1
Rotatory	Glc-6-P	1.2
	Glc	4.5

DISCUSSION

Formation of BC fibril was observed in rotatory culture (Figure 4-2). It has been reported that highly orientated BC was produced under stirred culture accompanying decrease of yields of BC[44], formation of fibril seems to be inevitable under stirred culture.

Incorporation of aminosugar residues into BC was achieved by the addition of ammonium salts to SH Glc medium when cultures were incubated rotatory with aeration (Table 4-1). Further, incorporated aminosugar was found to be *N*-acetylated form by IR analysis of resulted fibril (Figure 4-4). The obtained values of GlcNAc content are higher than those of pellicles produced in SH GlcNAc medium and SH mixed medium with aminosugars. Especially, ammonium chloride seems to be the best salt among other additives, because the highest value of incorporated GlcNAc residues was shown when it added to the medium. On the other hand, little effect of ammonium salt on incorporation was observed under static conditions. It seems that regulated aeration is essential to incorporate GlcNAc residues in the presence of ammonium salts.

These results suggest that an ammonium ion participated in the conversion of Glc into GlcN as an equilibrium species of enzymatic systems which catalyze such a conversion would exist in the metabolism of *A. xylinum*. The equilibrium between aminosugars and Glc with ammonium ion was proposed as shown in Scheme 4-1. Exogenous Glc was transformed into GlcN with reversible reaction of aminotransferase which caused by rather high concentration of ammonium ion in the bacteria body. Resulted GlcN was provided to the incorporation pathway after acetylation similar to the case of exogenous aminosugars. It is suggested that the addition of ammonium salts to the culture medium makes equilibrium shift to the side of GlcN. Moreover, its equilibrium would shift after phosphorylation of Glc, since the incorporation was observed by the application of Glc-6-P and ammonium chloride (Table 4-2). But such an incorporation was observed only in rotatory - aerated culture. As agitation of the culture medium was very gentle, it did not seem to disturb cellulose production.



Scheme 4-1. The equilibrium system among Glc and aminosugars with ammonium ion.

CONCLUSION

The application of several ammonium salts was found to be effective on the incorporation of aminosugar residues into BC when the cultures of *A. xylinum* were incubated under rotatory and aerated conditions, though a carbon source was only Glc instead of aminosugars. As little incorporation was observed in the case of static culture, aeration was assumed to be an essential factor to the aminosugar incorporation in the presence of ammonium salts. Ammonium chloride seemed to be the best additive to enhance aminosugar incorporation, because the highest value of GlcNAc content obtained by the addition of it. The incorporation mechanism was proposed that GlcN which arose from enzymatic equilibrium of Glc and ammonium ion, was converted into GlcNAc to be provided to the biosynthetic pathway of cellulose, since there was little aminosugar residue other than GlcNAc even if GalN or GlcN were added to the culture medium.

CHAPTER V

Participation of Phosphorylated Chitin Derivatives in Aminosugar Incorporation

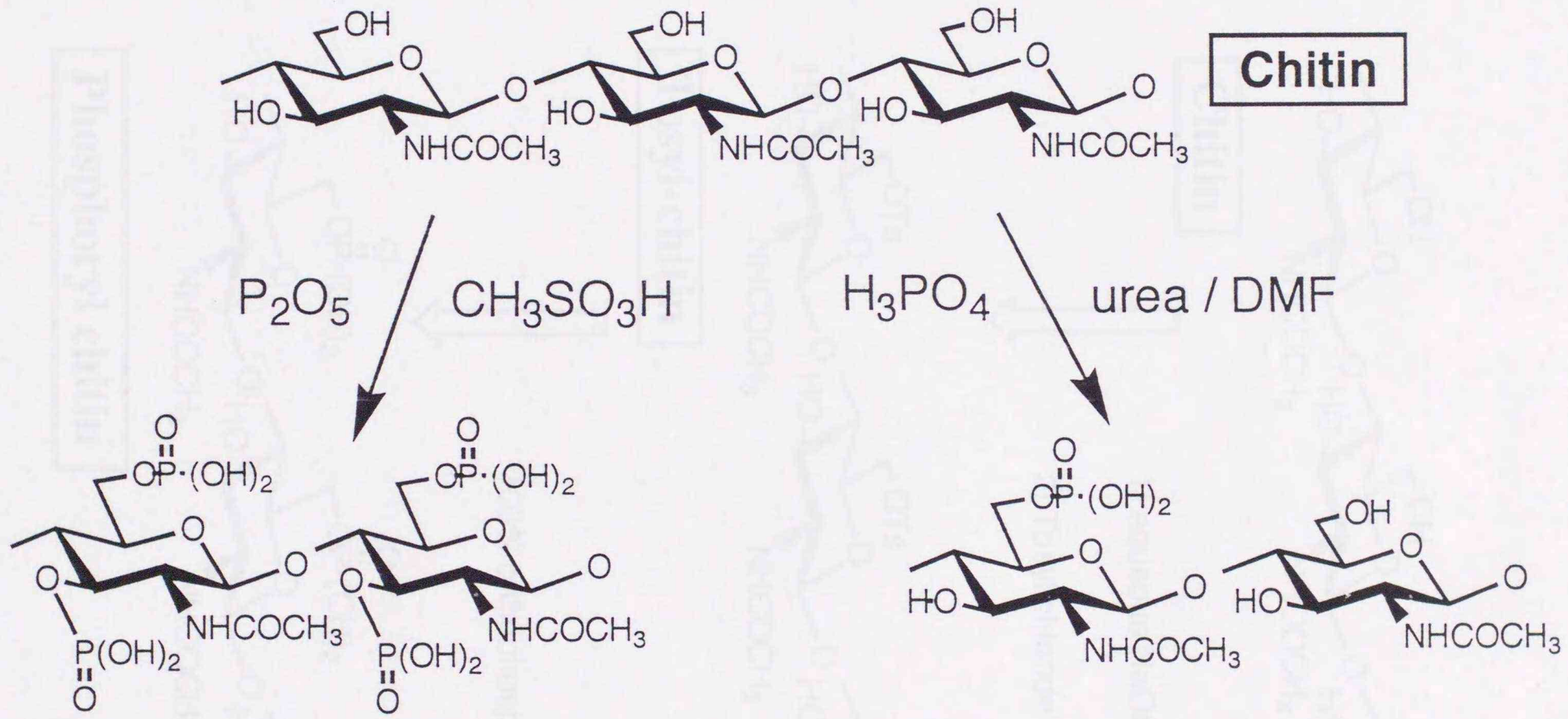
INTRODUCTION

On the time dependent observation of sugar consumption in SH mixed medium, it was clear that *A. xylinum* started to take up GlcNAc after exhaustion of Glc. Moreover, BC production continued even when medium component became only GlcNAc, and the major constituent sugar of produced BC was still Glc residues. Most of GlcNAc which were taken up by the bacteria seems to be incorporated into BC after conversion of GlcNAc into Glc, since GlcNAc content of resulted BC was maintained in low level even in SH GlcNAc medium. It was suggested that the enzyme which catalyzes such a sugar conversion, exists in *A. xylinum*. Two pathways of GlcNAc were assumed for biosynthesis of BC incorporated GlcNAc residues; (1) GlcNAc was metabolized through general pathway after the conversion into Glc, (2) GlcNAc as it is was led to sugar phosphates (GlcNAc-6-P, GlcNAc-1-P) and sugar nucleotide (UDP-GlcNAc), and became the substrate of cellulose synthase. Thus the sugar phosphates would be important as intermediates for the biosynthetic pathway of BC.

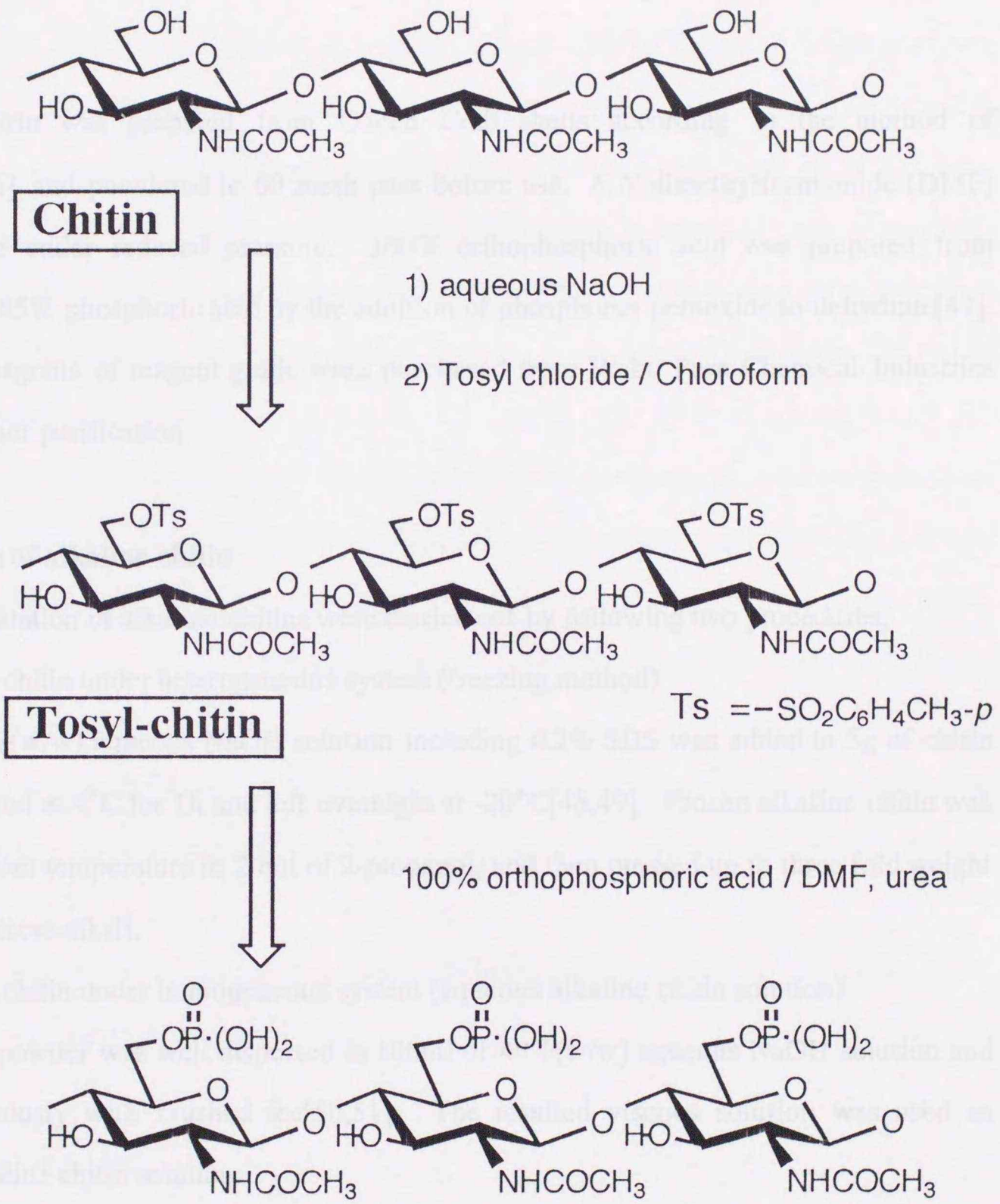
Since chitin is a biodegradable homopolysaccharide consisted of GlcNAc residues, phosphorylated chitin is expected to be suitable substrate to examine the incorporation mechanism of GlcNAc residues into BC and to enhance its content. In this chapter, the influence of phosphoryl chitin on the biosynthesis of BC was investigated by the addition of several phosphorylated chitin derivatives or GlcNAc-6-phosphate to the culture medium of *A. xylinum* .

In order to investigate the influence of chitin derivatives on biological system of bacteria such as *A. xylinum*, it is essential to prepare chitin derivatives under the regulated conditions for the site specific substitution. The chemical modification of chitin is fairly

difficult, because chitin forms rigid crystalline structure due to intra- and intermolecular hydrogen bonds[34]. Therefore, in the case of phosphorylation, the product which regulated degree of substitution and its site was hardly obtained without depolymerization by reported methods[35-38] (Scheme 5-1). In this study, tosylated chitin (Ts-chitin) is prepared as a synthetic intermediate for site specific phosphorylation as depicted in Scheme 5-2, because improvement of reactivity is expected by the introduction of Ts group as a leaving group to chitin molecule. In addition, site specific substitution is also anticipated, as bulky Ts group is introduced into only primary hydroxyl group[45]. When substitution reaction will be carried out to hydroxyl groups of chitin, alkaline chitin is such an important precursor that preparation method of that determines the properties of the final product. Therefore, in this tosylation of chitin, alkaline chitins were prepared by two different procedures, that is, in heterogeneous and homogeneous systems. And the relationships between reaction conditions and degree of phosphorylation, substitution site and molecular weight were investigated, furthermore, susceptibilities of chitin phosphates for glycosidases such as lysozyme and chitinase were also examined as one of indications of biodegradability.



Scheme 5-1. Phosphorylation of chitin.



Scheme 5-2. Synthetic route of phosphoryl chitin through tosyl chitin.

V-1. Preparation of Phosphoryl Chitins and Its Properties

EXPERIMENTAL

Materials

α-chitin was prepared from Queen Crab shells according to the method of Hackman[46], and powdered to 60 mesh pass before use. *N,N*-dimethylformamide (DMF) was distilled under reduced pressure. 100% orthophosphoric acid was prepared from commercial 85% phosphoric acid by the addition of phosphorus pentoxide to dehydrate[47]. The other reagents of reagent grade were purchased from Wako Pure Chemical Industries without further purification.

Preparation of alkaline chitin

Preparation of alkaline chitins were carried out by following two procedures.

(1) Alkaline chitin under heterogeneous system (freezing method)

20ml of 40%(w/w) aqueous NaOH solution including 0.2% SDS was added to 5g of chitin powder, stirred at 4°C for 1h and left overnight at -20°C[48,49]. Frozen alkaline chitin was thawed at room temperature in 20ml of 2-propanol, and then pressed up to three fold weight to remove excess alkali.

(2) Alkaline chitin under homogeneous system (aqueous alkaline chitin solution)

5g of chitin powder was well dispersed in 100ml of 40%(w/w) aqueous NaOH solution and stirred vigorously with crushed ice[50,51]. The resulted viscous solution was used as aqueous alkaline chitin solution.

Tosylation

90g of tosyl chloride (20 mol. equiv. to GlcNAc residue)-chloroform(150ml) solution was added to each alkaline chitin prepared in heterogeneous and homogeneous systems, and stirred mechanically at 0°C for 1h and for another 3h at room temperature. The product was collected by filtration, successively washed with deionized water until

neutral, followed by rinsed with methanol and ether, and dried *in vacuo*. Yield, 5.5g from heterogeneous system (Ts.fz-chitin), 8.5g from homogeneous system (Ts.aq-chitin).

Phosphorylation of Ts-chitin

2.0g of Ts.fz-chitin (degree of tosylation was 0.38)-DMF(50ml) suspension was added to the reaction bath which consisted of urea (120g) and DMF (80ml). 7.5g of 100% orthophosphoric acid (10 mol. equiv. to GlcNAc residue) was added dropwise to the above mixture and stirred at 150°C for 4h. After cooling to room temperature, the mixture was suspended in 400ml deionized water, neutralized with 1N HCl, and separated into water soluble and insoluble part by centrifugation. Insoluble part was suspended in 2% NaOH aqueous solution, stirred at room temperature for 24h in order to form sodium salt. This water insoluble product was washed with deionized water, methanol and acetone, and dried *in vacuo*. Yield, 0.8g (water insoluble P-Ts.fz-chitin). Soluble part was initially dialyzed against deionized water to remove urea, salt, *etc.*, further dialyzed against deionized water after adjusting pH with diluted aqueous NaOH solution and lyophilized. Yield, 1.2g (water soluble P-Ts.fz-chitin).

In the phosphorylation of Ts.aq-chitin, purification was carried out in the similar manner to water soluble part of P-Ts.fz-chitin, since all phosphorylated product became water soluble (P-Ts.aq-chitin).

Phosphorylation in phosphorus pentoxide-methanesulfonic acid system was carried out similar to reported procedures[37,38].

IR and NMR spectra

IR spectra of chitin derivatives were took by KBr pellet. ^{13}C-NMR spectra of water soluble chitin phosphates were obtained by using of Bruker NMR spectrometer ASX-300 in D_2O at 50°C. The assignment of NMR signals of phosphorylated derivatives was referred to those of carboxymethylated chitin[48,49] and sulfated chitosan[52].

X-ray diffraction

X-ray diffractograms were obtained by the reflection method with Rigaku automatic diffractometer RAD-II SR using Cu Kα radiation (1.541Å).

Degree of substitution

The degrees of tosylation and phosphorylation were estimated from elemental analysis value of sulfur and phosphorus, respectively. Phosphorus content of the products was estimated by molybdenum blue method[53].

Estimation of molecular weight

Average molecular weights of chitin phosphates were estimated by GPC method using pullulan as standard. HPLC analysis was performed on Asahipak GFA-30F column and eluted at a flow rate of 0.5 ml/min with a mobile phase of water at 50°C. Effluent was detected by using a Shimadzu JC-6A with refractive-index detector.

Enzyme assay

Egg-white lysozyme (EC 3.2.1.17, 50,000units/mg, Seikagaku Kogyo) and chitinase (EC 3.2.1.14, 0.045units/mg, Wako Pure Chemical) were used without further purification.

Phosphoryl chitins were dissolved or suspended in 0.1M acetate buffer (pH4.5) at the concentration of 1.5mg/ml. 1ml of enzyme solution (0.15mg/ml) was added to 2ml of above substrate solution, and incubated at 37°C with shaking. Enzyme was inactivated by the addition of 1ml of deproteination reagent[40]. After removal of denatured protein and insoluble substrate by centrifugation, the amount of reducing ends in 1.5ml of the supernatant were measured by the modified Schales' method[41].

RESULTS

Phosphorylation of chitin through Ts-chitin

In the tosylation reaction of two alkaline chitins which prepared in heterogeneous and homogeneous systems, degrees of tosylation were 0.38 and 0.81, respectively. IR spectra of Ts-chitin are shown in Figure 5-1. Introduction of Ts group was confirmed by the peaks at 1600cm^{-1} (SO, st.), 1175cm^{-1} (S=O, st.), 810, 650, 545cm^{-1} (S-O-C, st.). X-ray diffractograms show that Ts.aq-chitin became completely amorphous, while Ts.fz-chitin slightly held crystalline structure as seen in Figure 5-2. Ts.aq-chitin was soluble in DMF owing to high substitution of Ts group, whereas Ts.fz-chitin was not. Ts.fz-chitin swelled only in dimethylsulfoxide.

The resulted P-Ts.fz-chitin was the mixture of water soluble and insoluble parts, and the degrees of phosphorylation were 0.71 for water soluble fraction and 0.13 for insoluble one, respectively. Since phosphorylation reaction in DMF was proceeded in heterogeneous system, two derivatives with different solubilities might be obtained. However, in the case of direct phosphorylation of chitin, the product was poorly substituted derivative (degree of phosphorylation was 0.16) and insoluble in water. In this way, even lower tosylated derivative which was prepared in heterogeneous system, was available to intermediate for phosphorylation. In the phosphorylation of Ts.aq-chitin, the degree of phosphorylation was 0.58, and whole product was soluble in water. IR spectra of phosphoryl chitins are shown in Figure 5-3. The peaks derived from phosphoric ester appeared at 1230cm^{-1} (P=O, st.) and 920cm^{-1} (P-O, st.) with disappearance of the peaks of Ts group. It suggests that the residual Ts group was not remained at all on phosphoryl chitins prepared from Ts-chitin, and it was also proved by the result that no sulfur content derived form Ts group was detected by elemental analysis of the products. The relationships between degree of phosphorylation and reaction conditions are shown in Figure 5-4. The degree of phosphorylation increased with increase of the amount of orthophosphoric acid (2-10 mol. equiv. to GlcNAc residue) and reaction time (1-4h). Thus it suggested that any degree of phosphorylation could be obtained by the reaction conditions. Phosphorylation of chitin in phosphorus pentoxide-

methanesulfonic acid system gave extremely high substituted derivative (P-MSA-chitin) and its degree of phosphorylation was 1.90.

The average molecular weight of phosphorylated derivatives estimated by GPC method using pullulan as standard are listed in Table 5-1. As the molecular weight of P-MSA-chitin was $2.8x10^4$, remarkable depolymerization seemed to be occurred due to acidic solvent used, though its phosphorylation was carried out below 0°C. In contrast with P-MSA-chitin, molecular weight of P-Ts.fz-chitin and P-Ts.aq-chitin were relatively high value in the range of $7.6x10^4$ to $9.8x10^4$. It shows that phosphorylation through Ts-chitin is effective synthetic route without depolymerization.

^{13}C-NMR spectra of water soluble phosphoryl chitins are shown in Figure 5-5, 5-6 and their chemical shifts are listed in Table 5-2. According to ^{13}C-NMR spectrum of P-MSA-chitin, the downfield displacement of C-3 and C-6 were observed due to the substitution of phosphoric esters (Figure 5-5). The signals of C-6 and C-6' which was shifted to low magnetic field by the substitution were observed in spectrum of P-Ts.aq-chitin, on the other hand, both C-6' and C-3' were appeared in that of P-Ts.fz-chitin (Figure 5-6). These results indicate that the substitution site of phosphoric ester of P-Ts.aq-chitin was only C-6 site on GlcNAc residue and that of P-Ts.fz-chitin was both on C-6 and C-3 sites. Thus, phosphoric ester seems to be introduced site-selectively to Ts.aq-chitin, but not to Ts.fz-chitin.

Susceptibilities of phosphoryl chitins for glycosidases

The time course of lysozymic hydrolysis of phosphoryl chitins are shown in Figure 5-7. Not only water soluble part of P-Ts.fz-chitin but also insoluble part seems to be hydrolyzed, since the amount of reducing ends increased time dependently. Contrary to this, the amount of reducing ends of P-Ts.aq-chitin was hardly changed in this experimental period. It suggests that susceptibility of P-Ts.aq-chitin for lysozyme was not shown in spite of water solubility. The results of chitinase hydrolysis are similar to the case of lysozyme as seem in Figure 5-8. The reducing ends of water soluble P-Ts.fz-chitin increased time dependently, whereas those of P-Ts.aq-chitin did not. These results might indicate that even

P-Ts.fz-chitin which is 3,6-*O*-phosphorylated derivative is susceptible for glycosidases such as lysozyme and chitinase, however, P-Ts.aq-chitin which is 6-*O*-phosphorylated derivative shows little susceptibility. It is assumed that susceptibilities of phosphoryl chitin derivatives relate to preparation methods of alkaline chitin, which is a precursor of Ts-chitin.

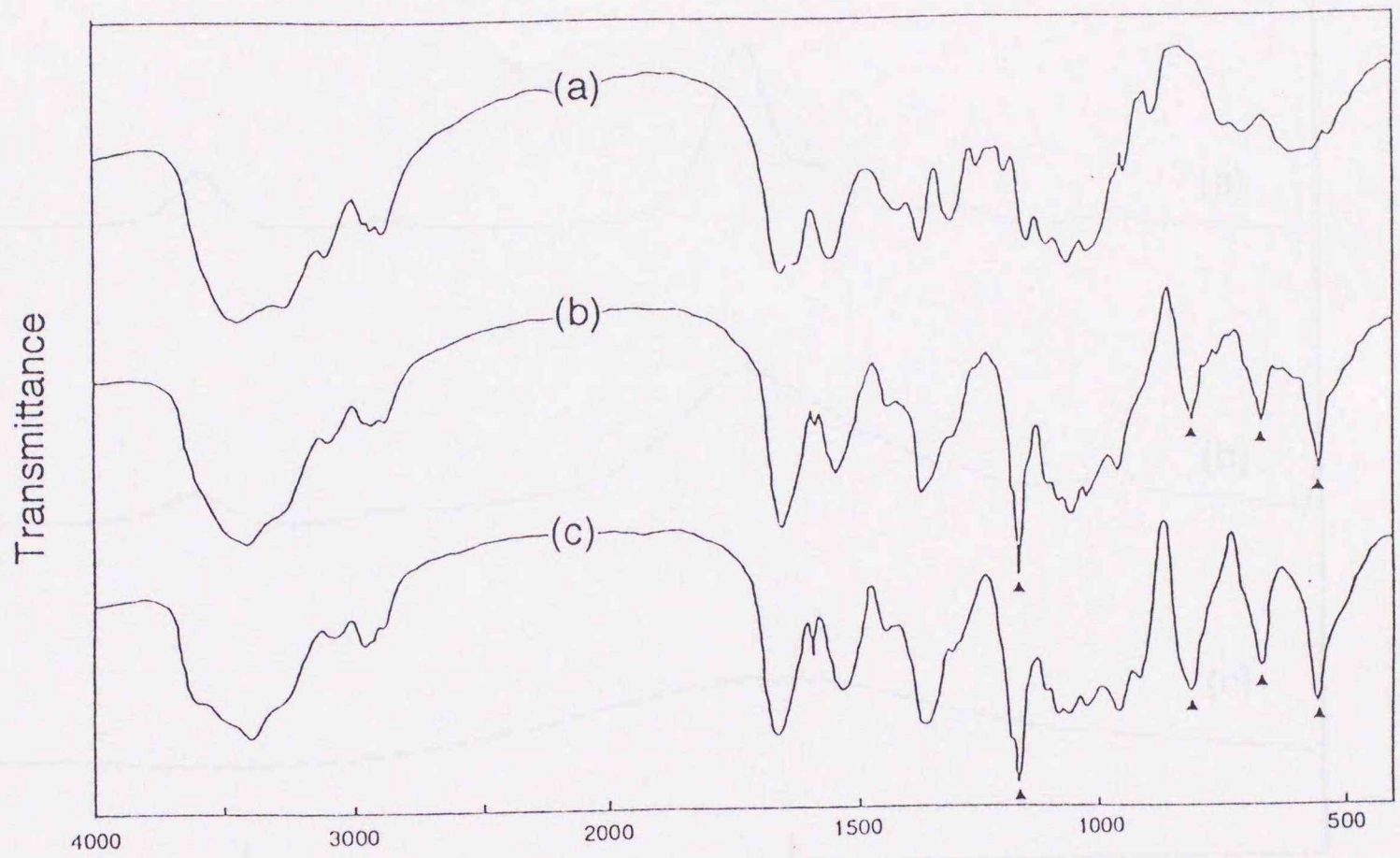


Figure 5-1. IR spectra of (a) α-chitin, (b) Ts.fz-chitin, D.S. 0.34 for tosyl group and (c) Ts.aq-chitin, D.S. 0.98 for tosyl group.

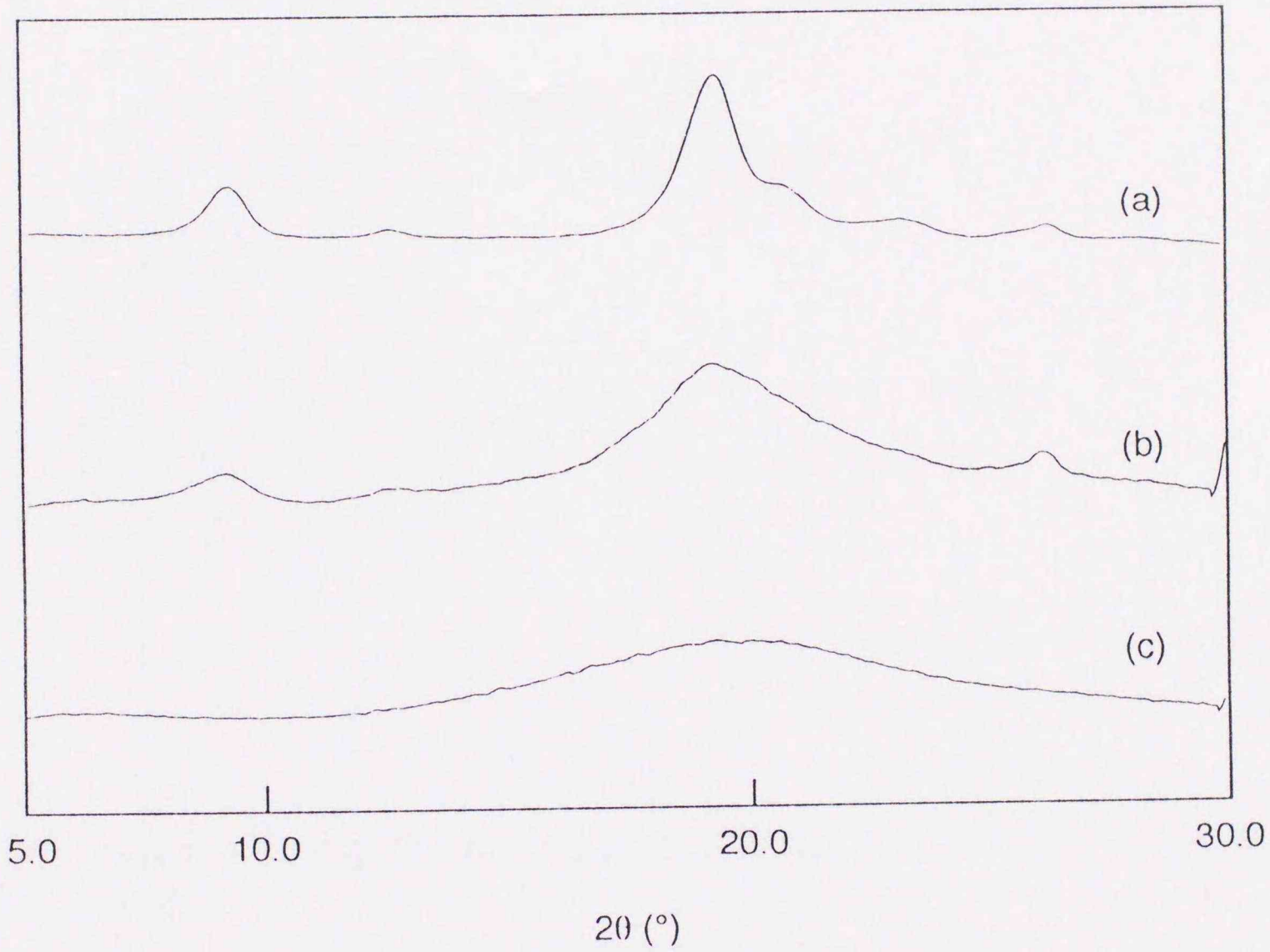


Figure 5-2. X-ray diffractograms of (a) α-chitin, (b) Ts.fz-chitin and (c) Ts.aq-chitin.

(a)
(b)
(c)
(d)
Wavenumbers (cm⁻¹)

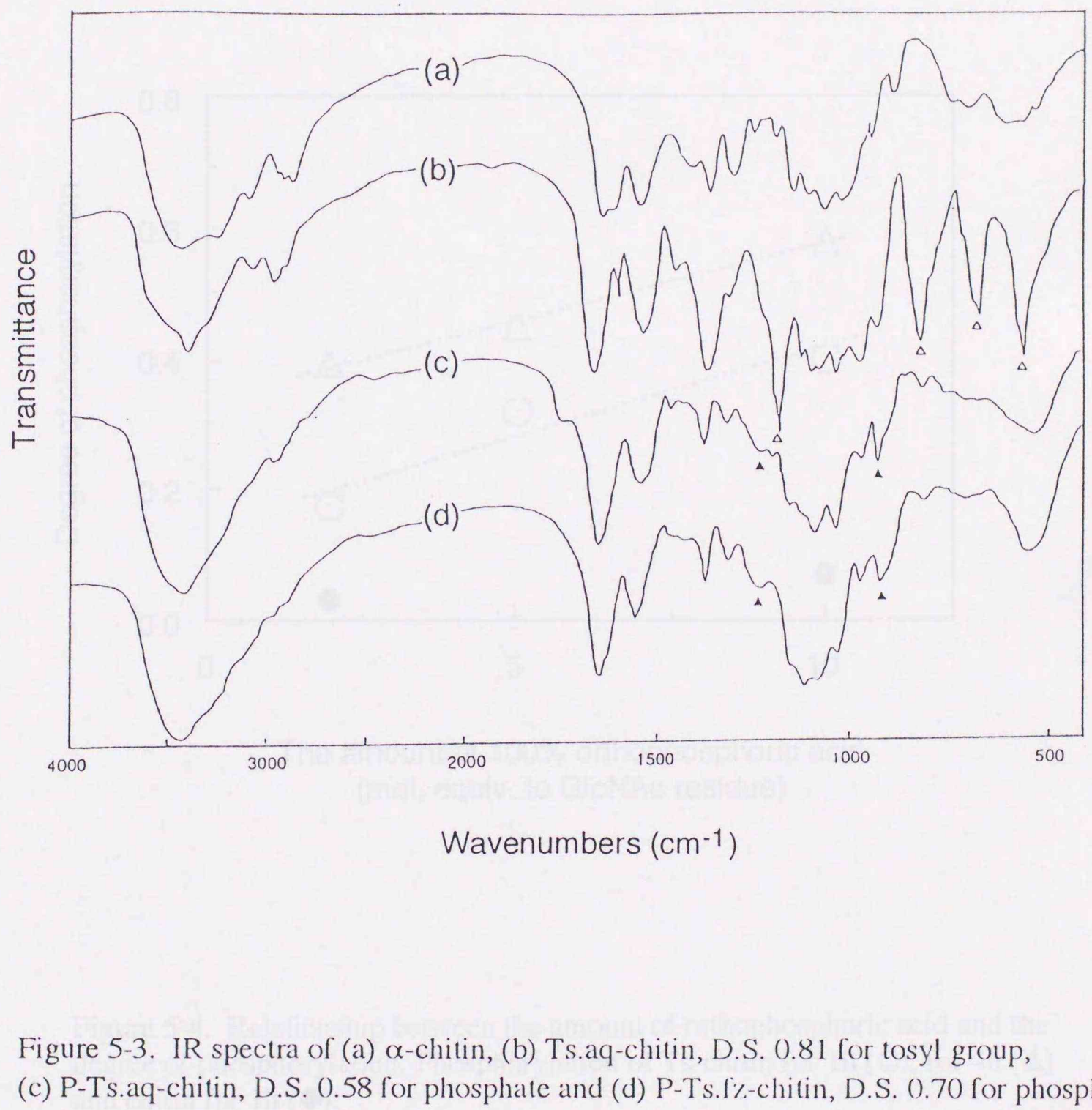


Figure 5-3. IR spectra of (a) α-chitin, (b) Ts.aq-chitin, D.S. 0.81 for tosyl group, (c) P-Ts.aq-chitin, D.S. 0.58 for phosphate and (d) P-Ts.fz-chitin, D.S. 0.70 for phosphate.

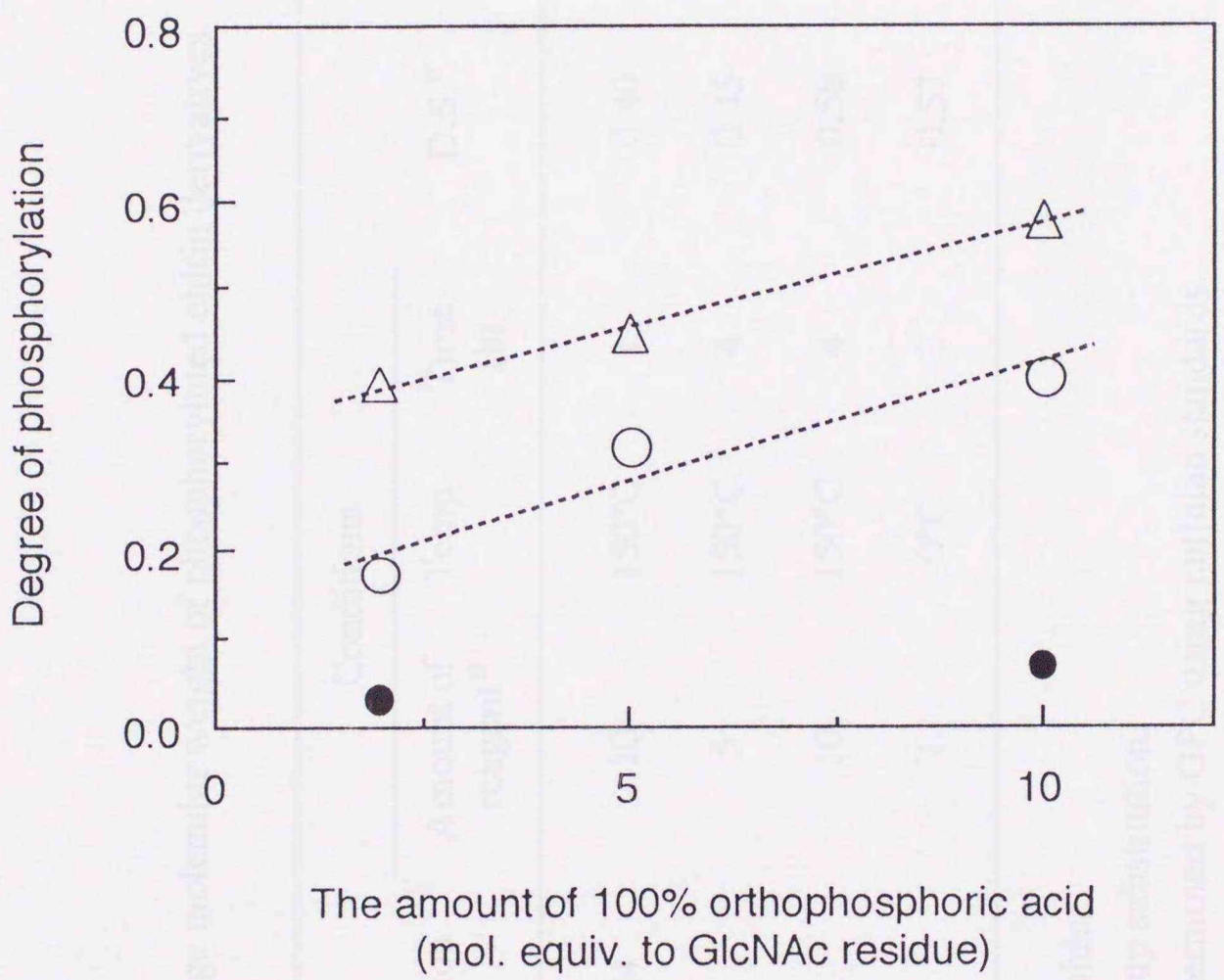


Figure 5-4. Relationship between the amount of orthophosphoric acid and the degree of phosphorylation. Phosphorylation of Ts-chitin for 1h (○), for 4h (△) and chitin for 1h (●).

Table 5-1. Average molecular weight of phosphorylated chitin derivatives.

Starting material	Phosphorylation reagent	Conditions: Amount of reagent[a]	Temp.	Time (h)	D.S.[b]	$\overline{Mw}$[c] ($\times 10^{-4}$)
Ts-chitin	100% H_3PO_4	10	150°C	1	0.40	9.8
		5	150°C	4	0.45	7.6
		10	150°C	4	0.58	8.1
Chitin	P_2O_5	1	0°C	3	0.57	2.8

[a] mol. equiv. to GlcNAc residue.
[b] Degree of phosphoryl group substitution.
[c] Molecular weight was determined by GPC using pullulan standards.

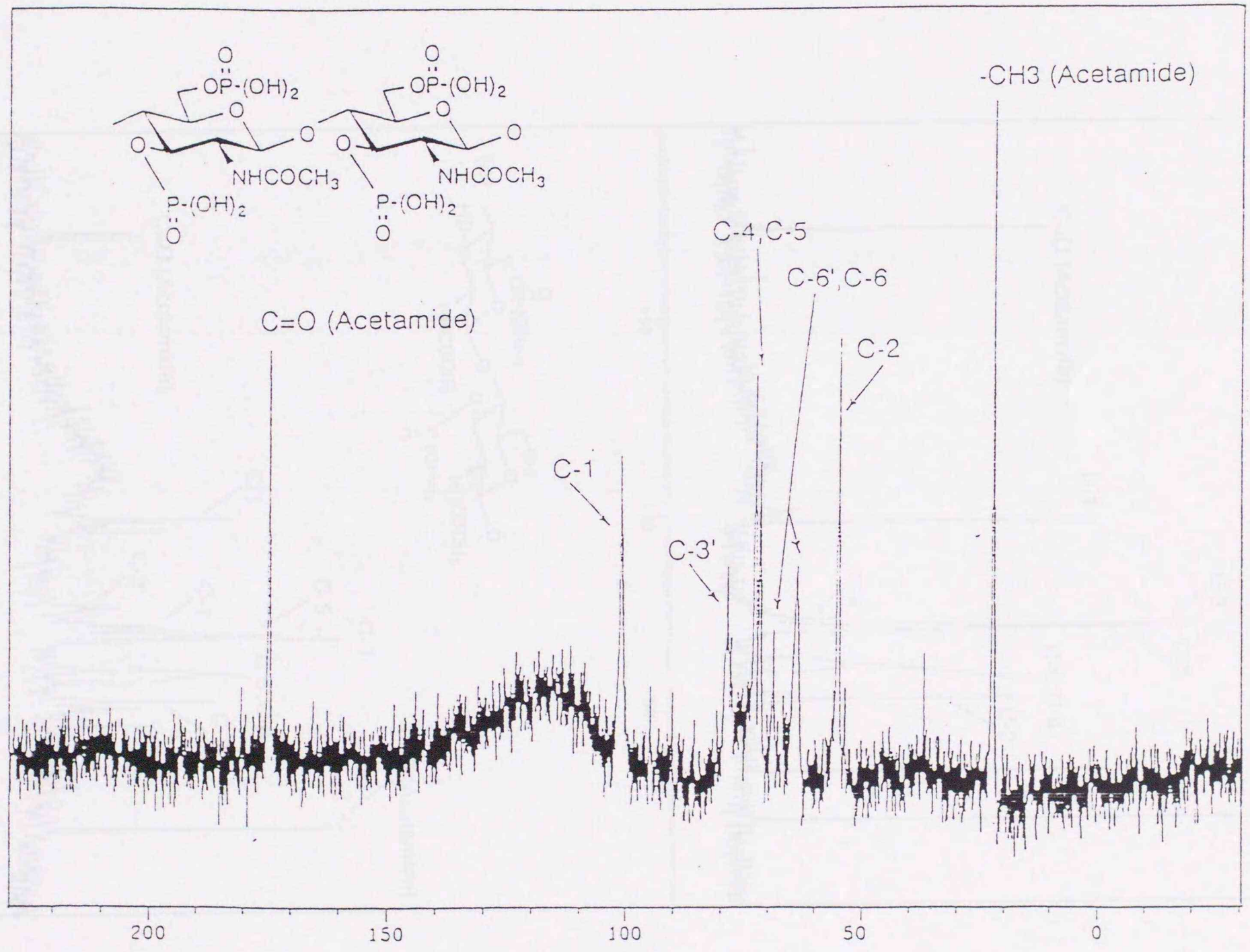


Figure 5-5. ^{13}C-NMR spectrum of P-MSA-chitin (D.S. 1.90) in D_2O at 50°C.

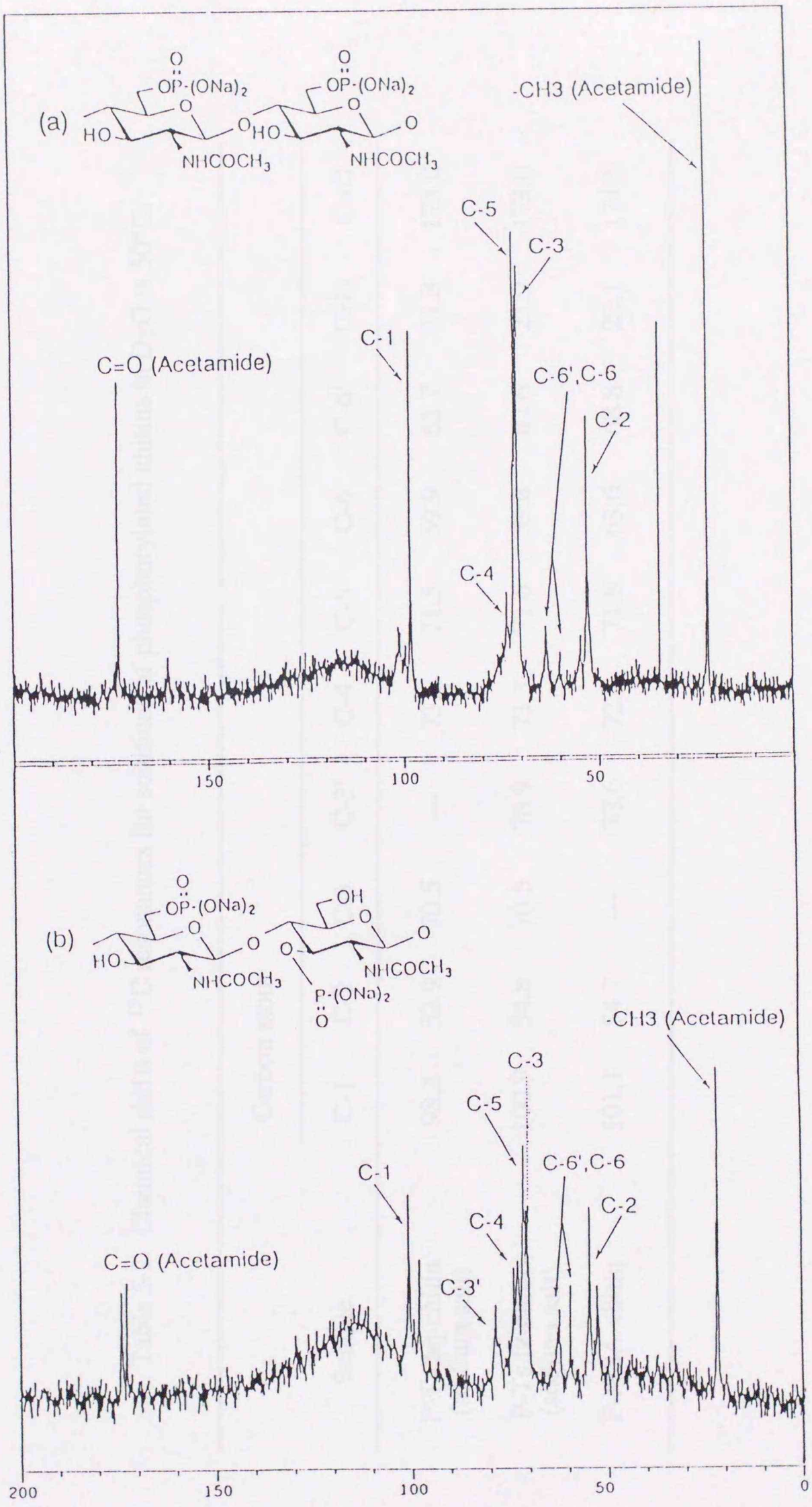


Figure 5-6. ^{13}C-NMR spectra of (a) P-Ts.aq-chitin (D.S. 0.58, sodium salt) and (b) P-Ts.fz-chitin, (D.S. 0.70, sodium salt) in D_2O at 50°C.

Table 5-2. Chemical shifts of ^{13}C resonances for solutions of phosphorylated chitins in D_2O at 50°C.

	Carbon atom									
Sample	C-1	C-2	C-3	C-3'	C-4	C-5	C-6	C-6'	CH_3	C=O
P-Ts.aq-chitin (sodium salt)	98.2	52.9	70.5	---	73.6	71.5	59.9	63.7	21.8	173.0
P-Ts.fz-chitin (sodium salt)	100.9	54.8	70.5	78.9	73.3	71.6	59.8	62.6	21.9	173.0
P-MSA-chitin	101.1	54.7	---	78.6	72.8	71.8	63.6	68.8	22.1	174.5

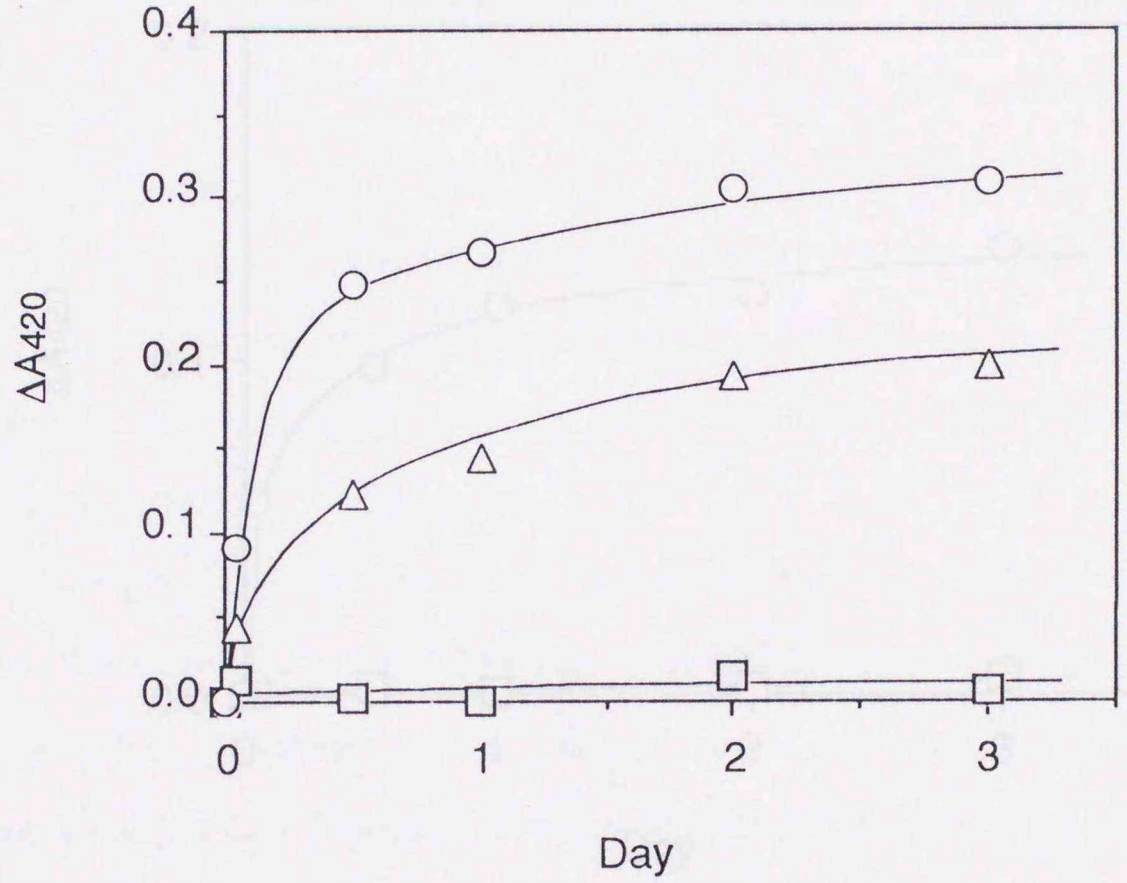


Figure 5-7. Time course of lysozymic hydrolysis of P-Ts.fz-chitin, water soluble(O),water insoluble(Δ) and P-Ts.aq-chitin(□) in 0.1M acetate buffer (pH4.5) at 37°C.

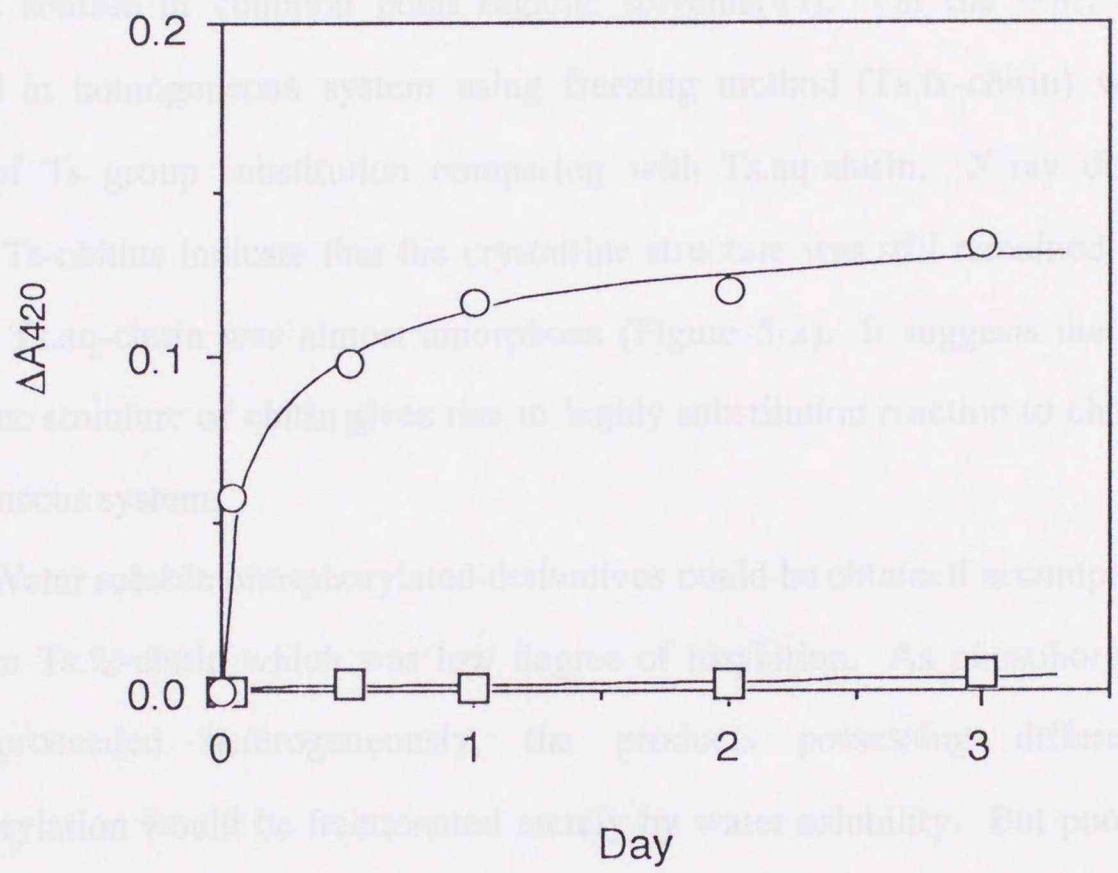


Figure 5-8. Time course of chitinase hydrolysis of water soluble P-Ts.fz-chitin (○) and P-Ts.aq-chitin(□) in 0.05M acetate buffer (pH5.2) at 37°C.

DISCUSSION

There is a difference between two systems for preparation of alkaline chitin, homogeneous system makes chitin dissolve completely, whereas heterogeneous system makes it be gel. Thus high reactivity is expected in homogeneous system, practically, highly tosylated derivatives (Ts.aq-chitin) has been obtained. This agrees with the result that Ts-chitin is soluble in common polar organic solvents[45]. On the other hand, Ts-chitin prepared in homogeneous system using freezing method (Ts.fz-chitin) was not so high degree of Ts group substitution comparing with Ts.aq-chitin. X-ray diffractograms of resulted Ts-chitins indicate that the crystalline structure was still remained in Ts.fz-chitin , whereas Ts.aq-chitin was almost amorphous (Figure 5-2). It suggests that the collapse of crystalline structure of chitin gives rise to highly substitution reaction to chitin molecules in homogeneous system.

Water soluble phosphorylated derivatives could be obtained accompanying insoluble part from Ts.fz-chitin which was low degree of tosylation. As phosphorylation of Ts.fz-chitin proceeded heterogeneously, the products possessing different degree of phosphorylation would be fractionated merely by water solubility. But poor reactivity was shown by direct phosphorylation with orthophosphoric acid to chitin probably owing to its rigid crystalline structure. It suggests that the reactivity and water solubility of the products are improved by tosylation step even in heterogeneous system. The regulation of degree of phosphorylation was achieved by the choosing of reaction conditions of Ts.aq-chitin (Figure 5-4). In addition, as no residual Ts group was detected in the final products, Ts group seems to be excellent leaving group for chitin under these conditions.

In former method, that is phosphorus pentoxide and methanesulfonic acid system[37,38], water soluble derivative of high degree of phosphorylation could be prepared, however, remarkable depolymerization happened owing to acidic catalyst and solvent. On the other hand, the average molecular weight of phosphorylated derivatives through Ts-chitin were relatively high value (Table 5-1). Molecular weight of water soluble 6-*O*-carboxymethyl(CM)-chitin, which was prepared from frozen alkaline chitin with

monochloroacetic acid, was estimated to 6-10x10^4 by viscometric measurement[54,55]. Since molecular weight of P-Ts.aq-chitin and water soluble P-Ts.fz-chitin were similar values to that of CM-chitin, applied phosphorylation route through Ts-chitin seems to be available to avoid depolymerization.

Substitution site of phosphoryl group on GlcNAc residue became evident from the observation of ^{13}C-NMR spectra (Figure 5-6). Phosphorylation of Ts.fz-chitin was not site specific substitution, because phosphoryl group was introduced to C-6 and C-3 sites of GlcNAc residue. It suggests that phosphorylation proceeds into not only Ts group of C-6 but also hydroxyl group of C-3, because the degree of phosphorylation of P-Ts.fz-chitin exceeds the degree of tosylation of Ts.fz-chitin. As substitution site of P-Ts.aq-chitin was found only at C-6 of GlcNAc residue, phosphorylation of Ts.aq-chitin progressed site-selectively. Site specific phosphorylation was achieved due to high substitution of Ts group into chitin.

The difference between P-Ts.fz-chitin and P-Ts.aq-chitin was observed in enzymatic susceptibilities. P-Ts.fz-chitin was susceptible for both lysozyme and chitinase, whereas P-Ts.aq-chitin was not, though they were water soluble derivative (Figure 5-7, 5-8). In general, it is known that there is close relationships between susceptibilities for lysozyme and substitution site of chitin derivatives. In the case of CM-chitin, susceptibility was extended when C-6 hydroxyl group was substituted, and it depressed extremely when further substitution was progressed to C-3 site[56]. But it is suggested that susceptibilities of phosphorylated chitin derivatives for lysozyme did not relate to substitution site of phosphoric ester on GlcNAc residue, because P-Ts.fz-chitins substituted at C-6 and C-3 sites were highly hydrolyzed by lysozyme. Since only procedures of tosylation were different between P-Ts.fz-chitin and P-Ts.aq-chitin, one was in heterogeneous system and the other was in homogeneous system, it is clearly concluded that the preparation method of alkaline chitin is closely correlated with susceptibilities for enzymes.

V-2. Influence of Phosphoryl Chitins on the Incorporation of Aminosugar Residues into Bacterial Cellulose

EXPERIMENTAL

Culture conditions

A. xylinum was precultured to adapt to GlcNAc by successive transfer to SH GlcNAc medium. 0.5ml of aliquots of the 3 days' culture were inoculated to 15ml of SH mixed medium containing Glc and GlcNAc-6-phosphate at the weight ratio of 7:3 or 3:7 and incubated statically at 28°C for a week. (GlcNAc-6-phosphate disodium salt, SIGMA Lot. 52H7817) Water soluble phosphorylated chitin derivatives were employed to SH GlcNAc medium at the range of concentrations from 0.1 to 1.0(w/v)%. Purification of pellicles and estimation of incorporated aminosugar content into them were the same as former chapters.

Analysis of culture medium

HPLC analysis of the culture medium was performed to observe the state of chitin phosphate during incubation. The portions of culture medium were taken out time dependently and filtrated through cellulose acetate membrane of 0.45 μm (Millipore) to remove bacteria. Their solutions were loaded on connected two columns (Asahipak GFA-30F) and eluted at a flow rate of 0.5 ml/min with a mobile phase of water at 50°C. Effluent was analyzed by using a Shimadzu JC-6A with refractive-index detector.

Acetylation of BC pellicle

Acetylation of pellicle was carried out in acetic anhydride and sulfuric acid system as follows. 50mg of pellicle was suspended in 8ml of glacial acetic acid. 0.9ml of acetic anhydride (10mol equiv. to hydroxyl group) and a catalytic amount of sulfuric acid were added to above suspension, and stirred at 20°C for 6h. The reaction mixture was poured into 100ml of ice-cold water and washed with deionized water extensively by filtration. Finally the product was lyophilized (Yield 79mg).

Acid hydrolysis of phosphoryl chitin

Phosphorylated chitin (P-MSA-chitin) was hydrolyzed with hydrochloric acid. Briefly, 1.4 g of P-MSA-chitin was dissolved in 180ml of 1N aqueous HCl and stirred at 40°C for 24h. The solution was concentrated and dialyzed against deionized water using cellulose tube which fractionated molecular weight of about 12,000. Its outer solution was collected and concentrated, successively dialyzed by using of dialyzing tube which was molecular weight 3,500 cutoff (Spectrapor, 3787-H45). The inner solution of 3,500 cutoff membrane was collected by lyophilization as a medium molecular weight fraction (AH-1, Yield 0.08g). The outer solution of 3,500 cutoff was concentrated and precipitated with methanol. The precipitate was dried under reduced pressure (AH-2, Yield 0.86g). Molecular weight of each fraction was estimated by GPC method.

RESULTS

Influence of GlcNAc phosphate on cultures

Monomeric sugar phosphate was applied to the culture in SH mixed medium instead of GlcNAc. The amount of incorporated GlcNAc into pellicle produced in the mixed medium containing GlcNAc-6-P was higher than that of GlcNAc and increased with increase the ratio of GlcNAc-6-P to Glc as listed in Table 5-3. IR spectra of resulted pellicles are shown in Figure 5-9. Enhancement of incorporated GlcNAc residues was confirmed because of the appearance of amide I and II adsorption bands. These results indicate that GlcNAc phosphate was taken up and utilized by the bacteria as an intermediate of biosynthetic pathway.

Influence of phosphoryl chitins on cultures

The pH value of the culture medium was hardly changed by the addition of phosphorylated derivatives. As shown in Figure 5-10, the amount of incorporated GlcNAc residues into pellicle produced in SH GlcNAc medium containing phosphoryl chitins increased with increase the concentration of additives. It shows that incorporation of GlcNAc residues into pellicle was promoted by the addition of phosphoryl chitins. And GlcNAc contents of pellicle produced by the application of P-Ts.fz-chitin which was hydrolyzed by lysozyme were higher than that of P-Ts.aq-chitin which was poorly biodegradable. IR spectra of pellicles produced in SH GlcNAc medium containing P-Ts.fz-chitin are shown in Figure 5-11. The peaks derived from acetamide group were observed clearly and their intensities depend on the concentration of additive. It is suggested that the biodegradability of phosphoryl chitin was significant in the process of incorporation of aminosugar. But GlcNAc content of pellicle produced with P-MSA-chitin did not depend on the concentrations of P-MSA-chitin, and further, no pellicle was harvested in medium containing 1.0% of P-MSA-chitin. P-MSA-chitin seems to interfere with synthesizing cellulose.

The GlcNAc content of pellicles which was produced in SH Glc medium containing phosphoryl chitins are shown in Table 5-4. The incorporated GlcNAc content became a significant amount in the medium containing P-Ts.fz-chitin comparing with other phosphorylated derivatives. As incorporation of GlcNAc was observed even in SH Glc medium without GlcNAc, added P-Ts.fz-chitin seems to be used as a substrate for cellulose production following to chitinolytic hydrolysis.

Influence of hydrolysates of phosphoryl chitins

P-MSA-chitin which was not effective on GlcNAc incorporation in SH GlcNAc medium, was hydrolyzed under acidic condition and fractionated into two parts (AH-1 and AH-2) by dialyzing. Its oligomeric hydrolysates were added to the culture medium and incubated similarly. Molecular weight of hydrolysates were estimated to about 4,000 for AH-1 and 300-400 for AH-2. The fraction of AH-2 corresponded to mono- and disaccharides. The incorporated GlcNAc content was hardly changed even in both SH GlcNAc and SH Glc medium as seen in Table 5-5. Since this P-MSA-chitin was highly substituted derivative, phosphoric esters of hydrolysates still remained even after acidic hydrolysis (Substitution at C-3 and C-6 sites was confirmed by ^{13}C-NMR spectrum of the fraction AH-1). It suggests that sugar diphosphate is not catalyzed by transferase or isomerase in the metabolic pathway of bacteria.

GPC analysis of the culture medium

The average molecular weights of phosphoryl chitins in the culture medium were measured time dependently by GPC analysis as shown in Figure 5-12, 13, 14. Molecular weight of P-Ts.fz-chitin was slightly decreased in SH GlcNAc medium. The peak of polymer was broadened in SH Glc medium, its molecular weight distribution was 2.74 at the beginning of incubation and it changed to 4.46 after 2 weeks (Figure 5-12). The result indicates that P-Ts.fz-chitin was degraded during incubation. In the case of P-MSA-chitin, a similar tendency to P-Ts.fz-chitin was observed as seen in Figure 5-14. On the other hand, the molecular weight of P-Ts.aq-chitin and its distribution was hardly changed in both SH

Glc and SH GlcNAc medium (Figure 5-13). Biodegradable phosphoryl chitins seem to be hydrolyzed by glycosidases, degraded into oligomer and/or monomer and consequently taken up by the bacteria as a carbon source. It is proposed that their oligomeric and/or monomeric phosphoryl chitins were supposed to be utilized as intermediates in synthetic pathway and led to substrates for cellulose synthase of *A. xylinum*.

Acetylation of pellicle

Acetylation of pellicle was carried out in acetic anhydride and sulfuric acid system which was general method to prepare cellulose acetate[57]. Starting material was a pellicle which was produced in SH mixed medium containing 0.5% of P-Ts.fz-chitin. Acetylated product was readily dissolved in acetone and aqueous acetone, and insoluble part was not found in the solution.

Treatment of pellicle with saturated $CaCl_2$ methanol solution

As saturated calcium chloride dihydrate methanol solution is known to be good solvent system for chitin[58-60], pellicles which produced in the medium containing phosphoryl chitin were treated with this solvent to extract contaminated chitin molecule. However, the GlcNAc content in pellicles never changed even after extraction. This result might indicate that there is no contaminated chitin in the BC pellicle. This means that added phosphoryl chitin was not converted to chitin by the removal of phosphoric ester during cultivation of *A. xylinum*. But, no phosphorus content was detected by the elemental analysis of harvested pellicle.

Table 5-3. The amount of incorporated GlcNAc residues into pellicles produced in SH mixed medium containing Glc and GlcNAc-6-phosphate.

Sugar components	Ratio (wt)	Incorporated GlcNAc (mol%)
Glc : GlcNAc	7 : 3	0.3
	3 : 7	1.2
Glc :GlcNAc-6-P	7 : 3	1.3
	3 : 7	3.4

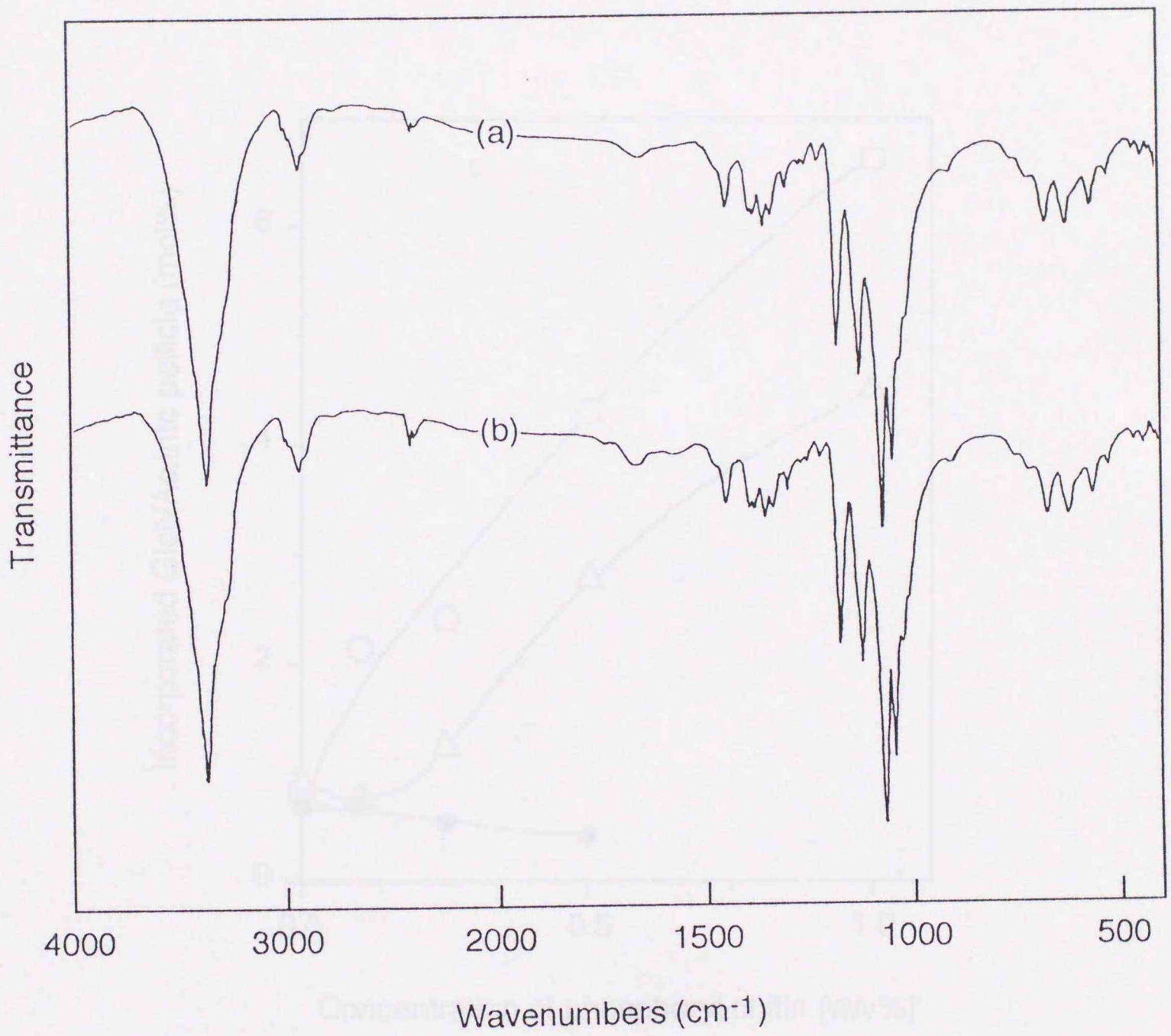


Figure 5-9. IR spectra of pellicles produced in SH mixed medium containing Glc and GlcNAc-6-phosphate at the weight ration of (a) 7:3 and (b) 3:7.

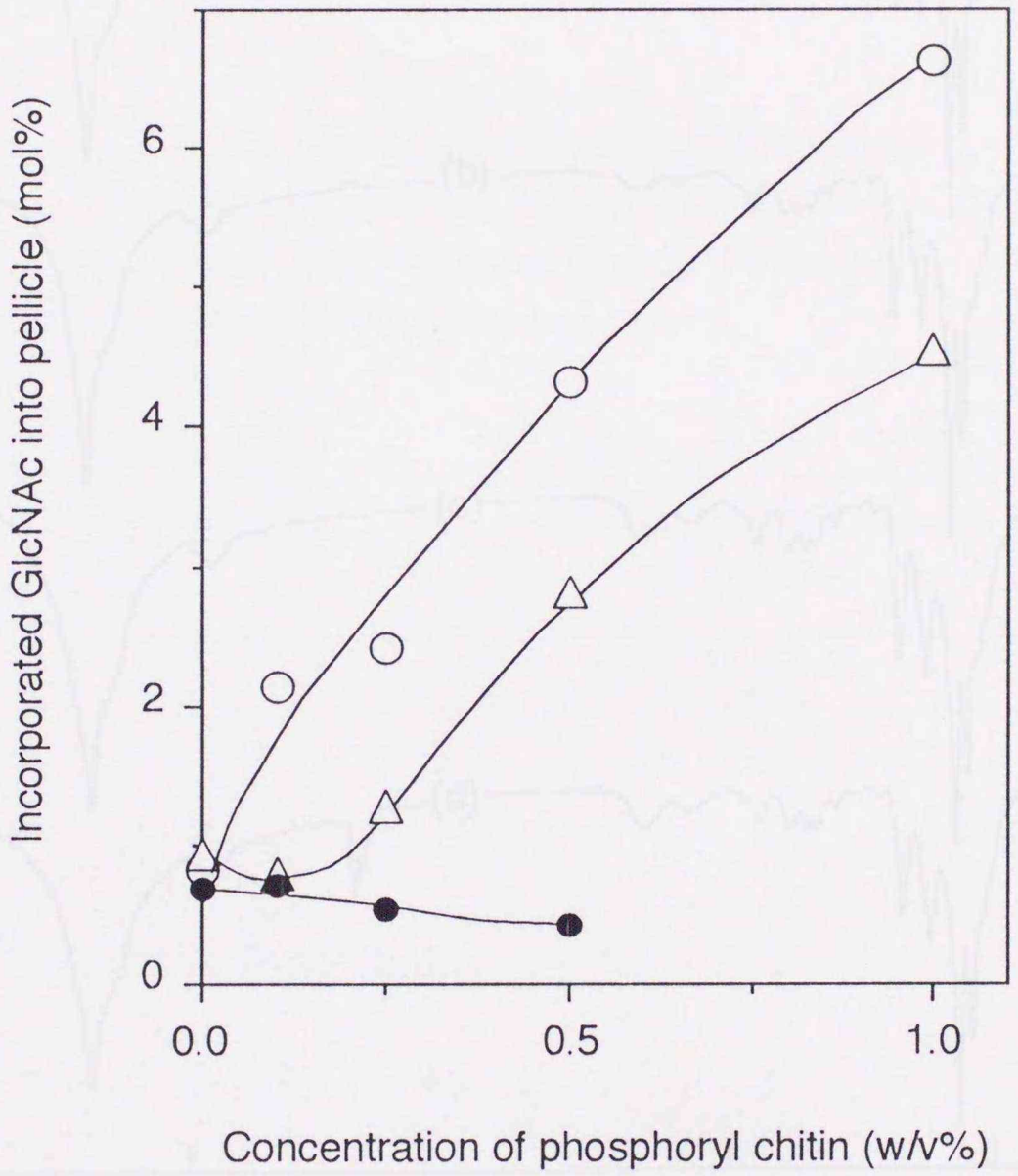


Figure 5-10. The relationship between the amount of incorporated GlcNAc residues into pellicle and concentration of water soluble P-Ts.fz-chitin(O), P-Ts.aq-chitin(Δ) and P-MSA-chitin(●) in SH GlcNAc medium.

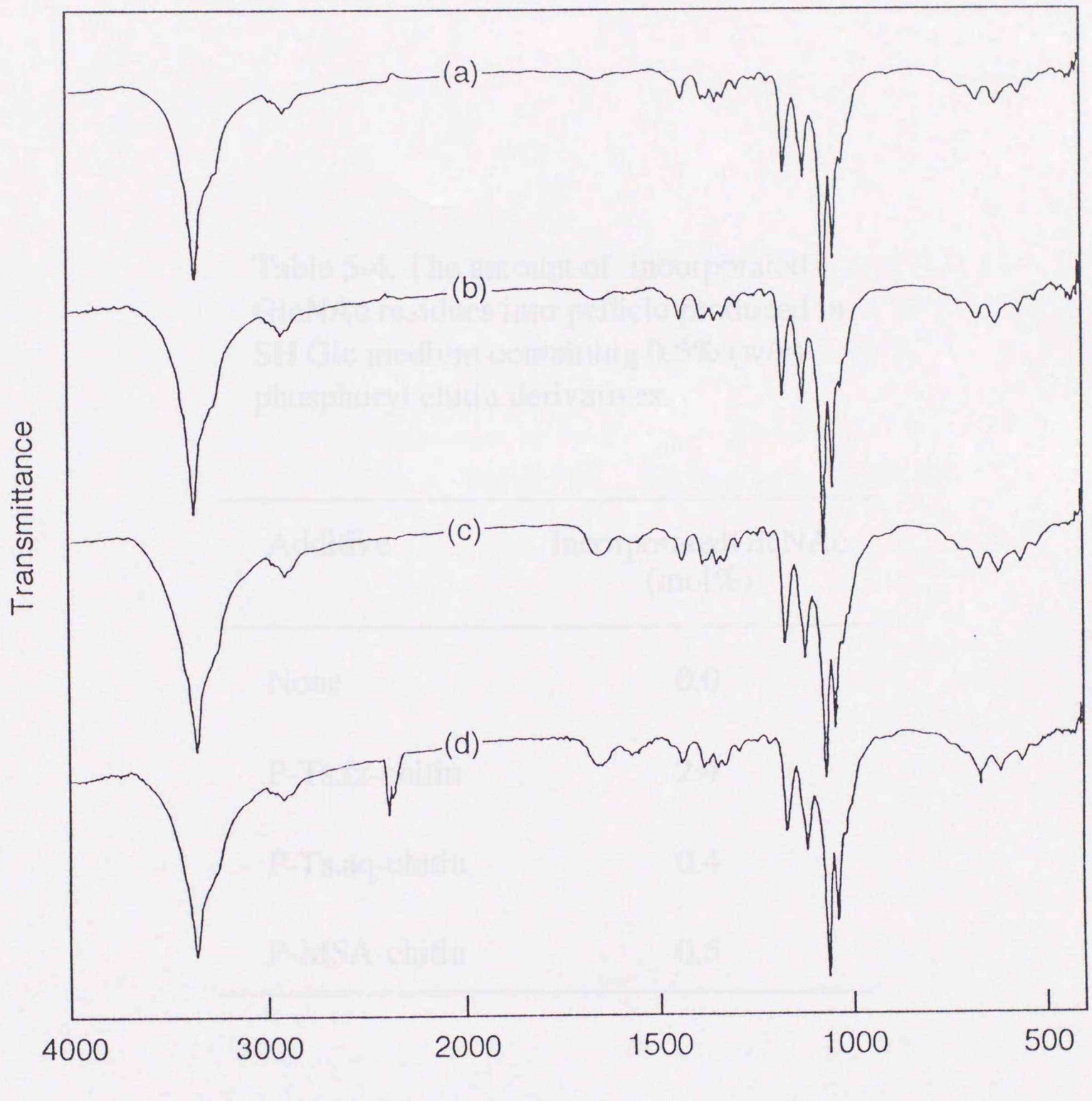


Figure 5-11. IR spectra of pellicles produced in SH GlcNAc medium containing P-Ts.fz-chitin at the concentration of (a) 0.1%, (b) 0.25%, (c) 0.5% and (d) 1.0%.

Table 5-4. The amount of incorporated GlcNAc residues into pellicle produced in SH Glc medium containing 0.5% (w/v) phosphoryl chitin derivatives.

Additive	IncorporatedGlcNAc (mol%)
None	0.0
P-Ts.fz-chitin	2.4
P-Ts.aq-chitin	0.4
P-MSA-chitin	0.5

Table 5-5. The amount of incorporated GlcNAc residues into pellicle produced in the culture medium containing 0.5% (w/v) of acid hydrolysates of phosphoryl chitin.

Culture medium	Additive[a]	Incorporated GlcNAc (mol%)
SH GlcNAc	None	0.8
	AH-1	0.2
	AH-2	0.2
SH Glc	None	0.0
	AH-2	0.7

[a] Molecular weight of hydrolysates was 4,000 for AH-1 and 300-400 for AH-2 estimated by GPC method.

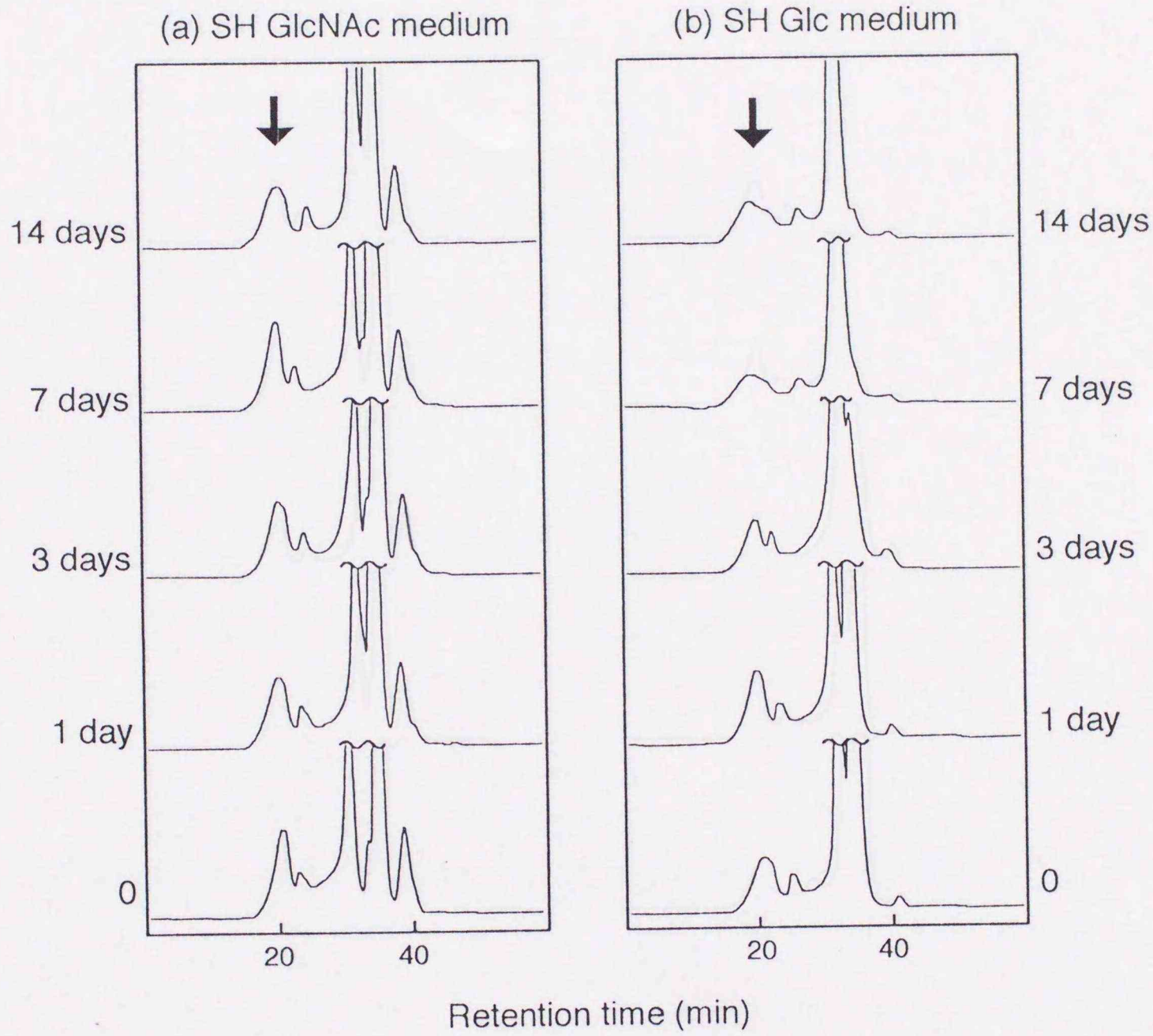


Figure 5-12. GPC profiles of (a) SH GlcNAc medium and (b) SH Glc medium containing 0.5% of P-Ts.fz-chitin. The arrow indicates the peak of P-Ts.fz-chitin eluted.

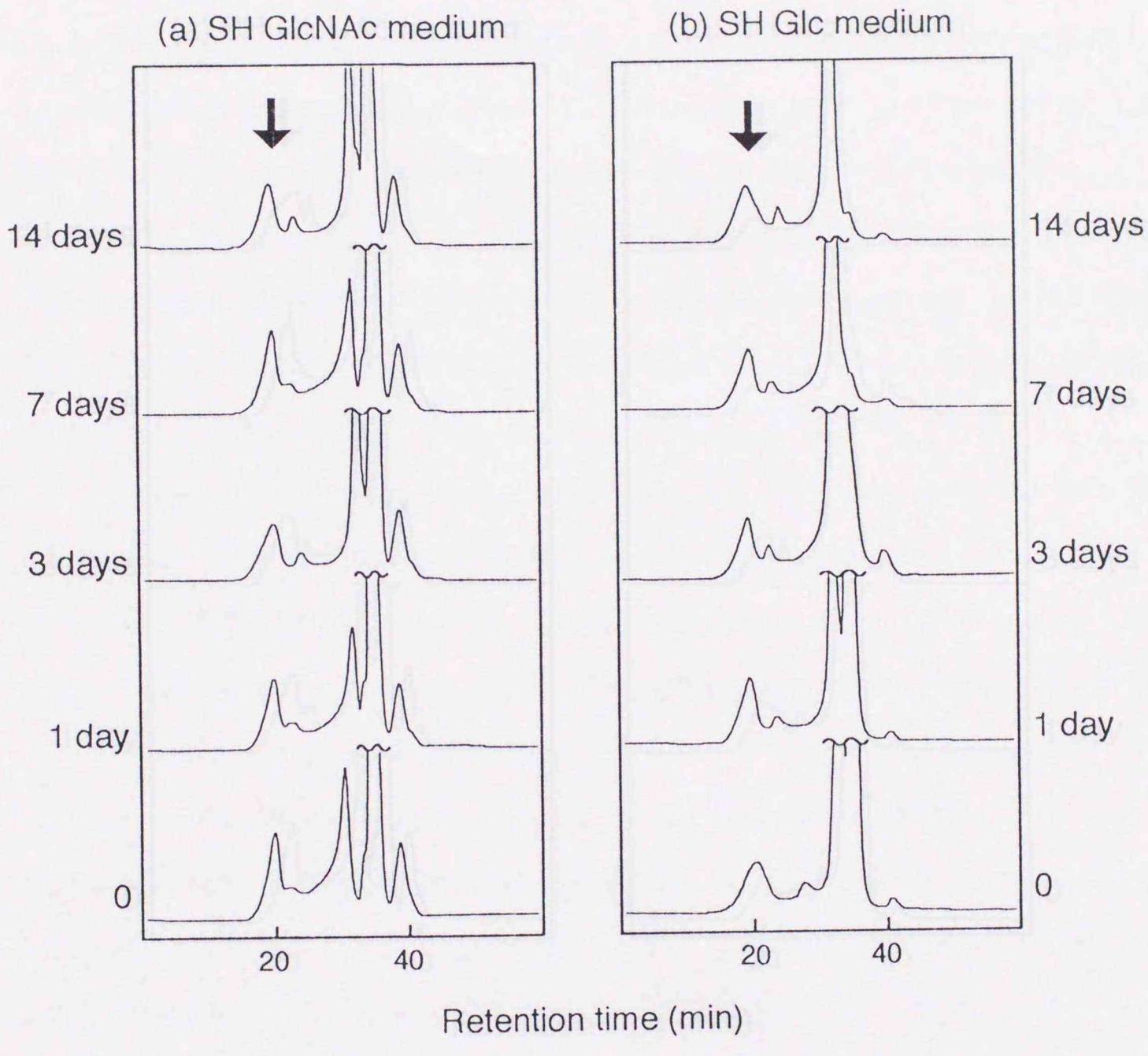


Figure 5-13. GPC profiles of (a) SH GlcNAc medium and (b) SH Glc medium containing 0.5% of P-Ts.aq-chitin. The arrow indicates the peak of P-Ts.aq-chitin eluted.

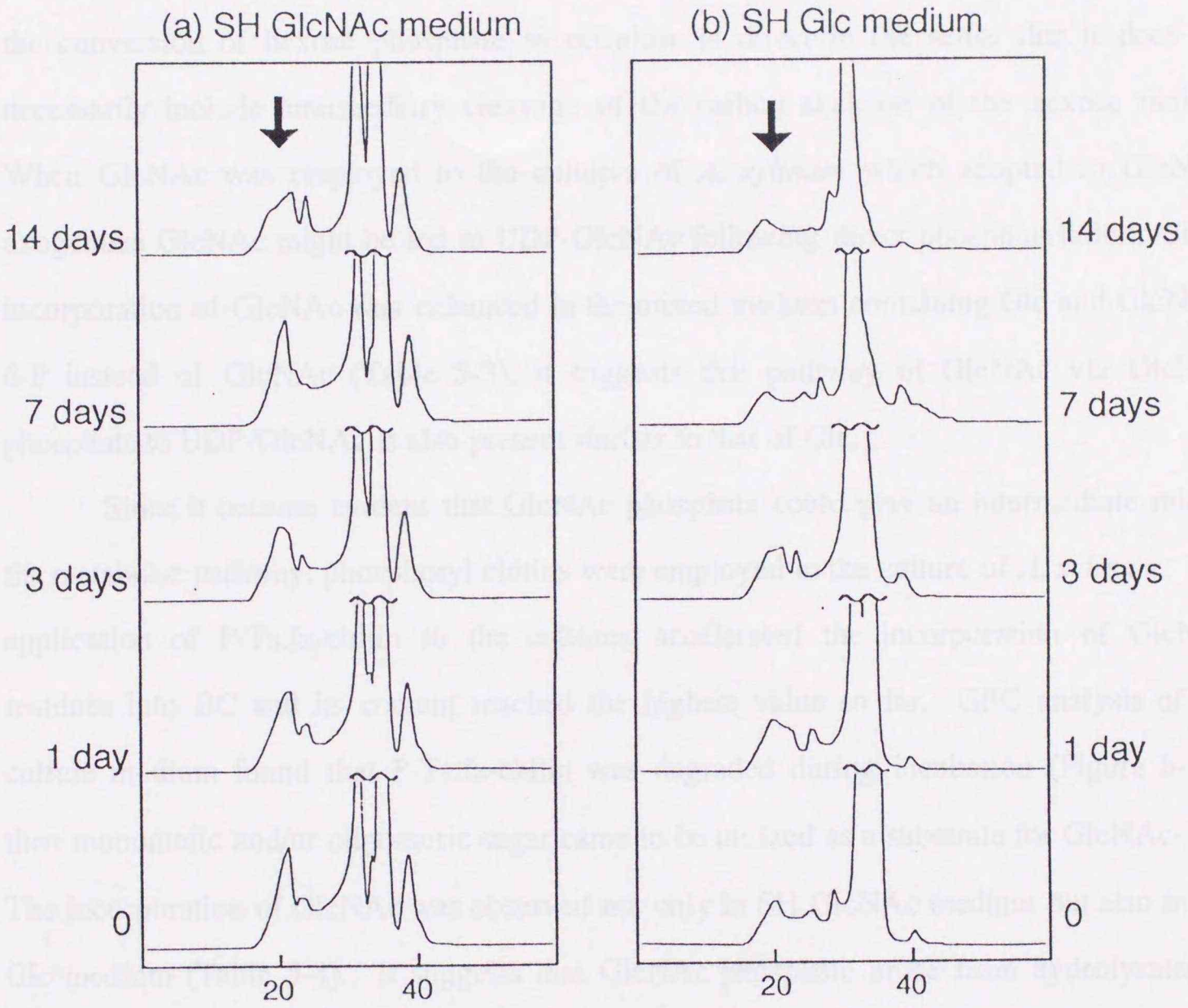


Figure 5-14. GPC profiles of (a) SH GlcNAc medium and (b) SH Glc medium containing 0.5% of P-MSA-chitin. The arrow indicates the peak of P-MSA-chitin eluted.

DISCUSSION

Hexose phosphate is a common intermediate in cellulose synthesis of *A. xylinum*[9,61], and it arises directly by phosphorylation of exogenous hexose[12]. Further, the conversion of hexose phosphate to cellulose is direct in the sense that it does not necessarily include intermediary cleavage of the carbon skeleton of the hexose moiety. When GlcNAc was employed to the cultures of *A. xylinum* which adapted to GlcNAc, exogenous GlcNAc might be led to UDP-GlcNAc following direct phosphorylation. Since incorporation of GlcNAc was enhanced in the mixed medium containing Glc and GlcNAc-6-P instead of GlcNAc (Table 5-3), it suggests that pathway of GlcNAc via GlcNAc phosphate to UDP-GlcNAc is also present similar to that of Glc.

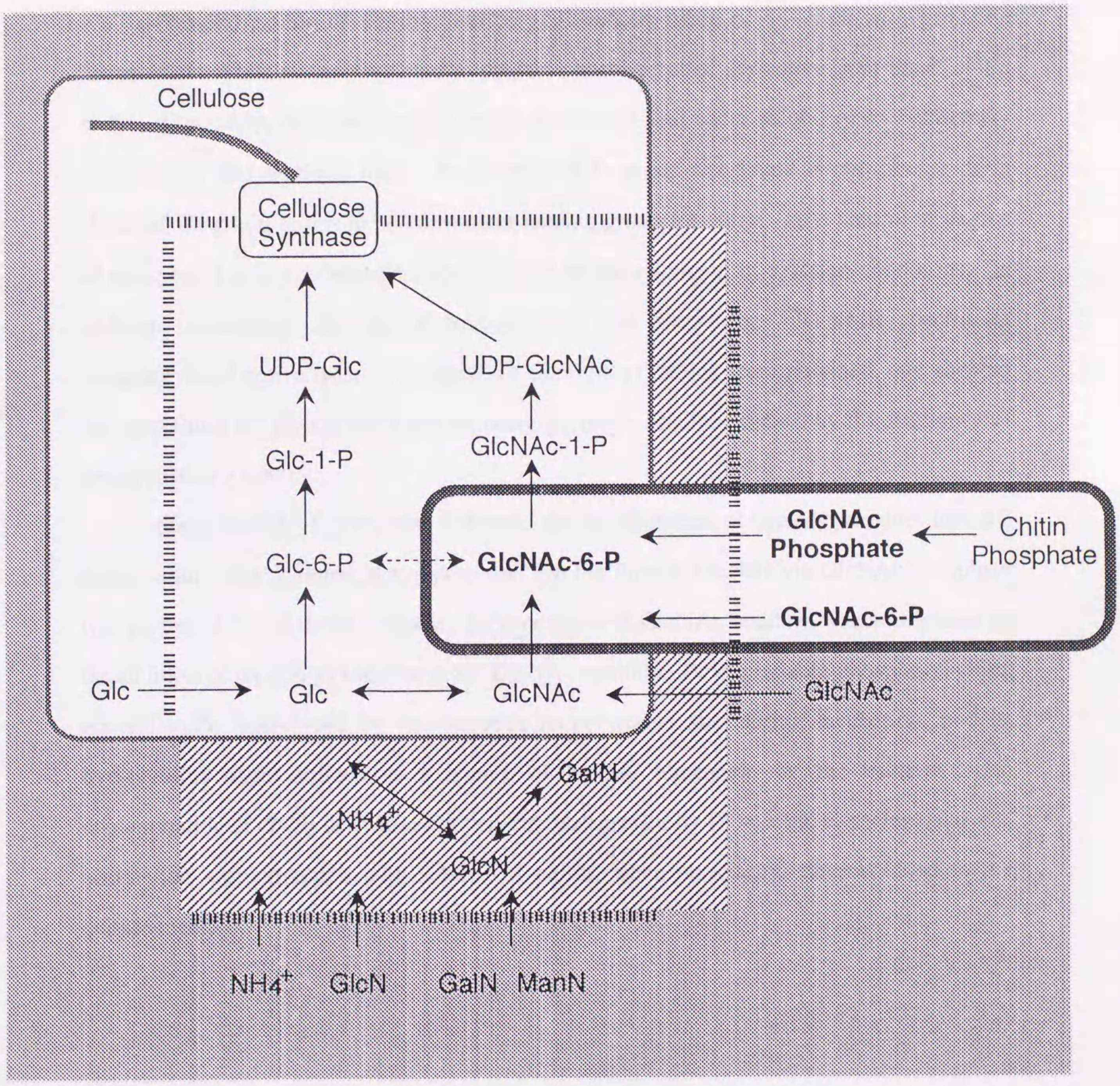
Since it became evident that GlcNAc phosphate could play an intermediate role in the metabolic pathway, phosphoryl chitins were employed to the culture of *A. xylinum*. The application of P-Ts.fz-chitin to the cultures accelerated the incorporation of GlcNAc residues into BC and its content reached the highest value so far. GPC analysis of the culture medium found that P-Ts.fz-chitin was degraded during incubation (Figure 5-12), then monomeric and/or oligomeric sugar came to be utilized as a substrate for GlcNAc-1-P. The incorporation of GlcNAc was observed not only in SH GlcNAc medium but also in SH Glc medium (Table 5-4). It suggests that GlcNAc phosphate arose from hydrolysates of phosphoryl chitin which degraded by some glycosidases as a carbon source.

The flow of GlcNAc phosphate was proposed as illustrated in Scheme 5-3. Initially phosphoryl chitin is degraded to permeable size, then added GlcNAc-6-P or hydrolyzed GlcNAc phosphate are taken up and isomerized to GlcNAc-1-P by phosphoglucomutase. And it is led to UDP-GlcNAc as a substrate of cellulose synthase, finally GlcNAc residue was incorporated into BC.

P-MSA-chitin which was highly phosphorylated derivatives was not effective on the incorporation, it seems that 3,6-*O*-substituted phosphoric esters inhibit isomerization to GlcNAc-1-P or synthesis of UDP-GlcNAc, even if it was hydrolyzed to monomeric sugar. Practically, little incorporation of GlcNAc was observed by the addition of hydrolysates of

P-MSA-chitin both in SH GlcNAc and SH Glc medium (Table 5-5). It suggests that sugar diphosphate is not catalyzed by enzymes such as transferase and isomerase in metabolic pathway.

No adsorption of phosphoric ester was observed in IR spectra of the pellicles produced in the medium containing phosphoryl chitins and also no phosphorus content was detected by elemental analysis. Moreover, when their pellicles were acetylated, all products were dissolved in acetone or aqueous acetone solution. Thus, chitin molecule would not contaminated in the pellicle, because acetylated chitin is not soluble in acetone and water at all[62]. Even though phosphoryl chitin was converted to chitin by removal of phosphoric esters, it should be extracted with saturated $CaCl_2 \cdot 2H_2O$ methanol solution which is good solvent of chitin. However, the GlcNAc content of pellicle did not change by the treatment with its solvent. These results indicate that phosphoryl chitin did not contaminate and were not miscible with BC pellicles produced in the medium containing them.



Scheme 5-3. The flow of GlcNAc phosphate in the metabolic pathway of *A. xylinum*.

CONCLUSION

Phosphorylation of chitin could be achieved by using of Ts-chitin as a synthetic intermediate, while it was very hard to prepare phosphorylated derivative from intact chitin so far. Especially, depolymerization could be avoided and water solubility was extremely improved by this synthetic route. As no residual Ts group was found in phosphoryl chitin obtained, Ts group seems to be convenient leaving group for chitin. And control of degree of phosphoryl group substitution was allowed by the choosing of reaction conditions. In addition, substituted site on GlcNAc residue was shown by ^{13}C-NMR spectra of phosphorylated derivatives. Two kinds of phosphoryl chitins were obtained, one showed susceptibilities for glycosidases and the other did not, it might be caused by the difference of preparation procedures.

Since GlcNAc-6-phosphate enhanced the incorporation of GlcNAc residues into BC in the culture of *A. xylinum*, it was proposed that the flow of GlcNAc via GlcNAc phosphate was present in biosynthetic pathway. Incorporation of GlcNAc residues was accelerated by the addition of phosphoryl chitins to SH GlcNAc medium. Biodegradable phosphoryl chitin seemed to be hydrolyzed by the enzymes to permeable size during incubation, and its hydrolysates were utilized as a source of hexose phosphate by the bacteria. No contamination of chitin derivatives in pellicles was proven by IR spectra, elemental analysis, acetylation and extraction with solvent of chitin, when phosphoryl chitins added to the culture medium.

CHAPTER IV

CONCLUDING REMARKS

The incorporation mechanism of aminosugar into BC were clearly shown by this study, as follows;

1. The time dependent observation of sugar consumption indicated that Glc was consumed by *A. xylinum* preferentially as a major carbon source for cellulose production in the mixed medium containing both Glc and aminosugar. GlcNAc was also utilized as a minor carbon source after exhaustion of Glc in the culture medium.

2. Although the sugar component in the culture medium became only GlcNAc, production of pellicles continued and major constituent residue of BC was found to be Glc. GlcNAc which was taken up by *A. xylinum*, was converted into Glc by the equilibrium function of enzymatic systems including deacylase and aminotransferase. Resulted Glc was provided as a carbon source to the general biosynthetic pathway.

3. GlcN was taken up by *A. xylinum* and incorporated aminosugar residue was found to be GlcNAc. It is assumed that the equilibrium among three aminosugars, GlcNAc, GlcN and Glc, is established in the flow of hexose by the participation of GlcN.

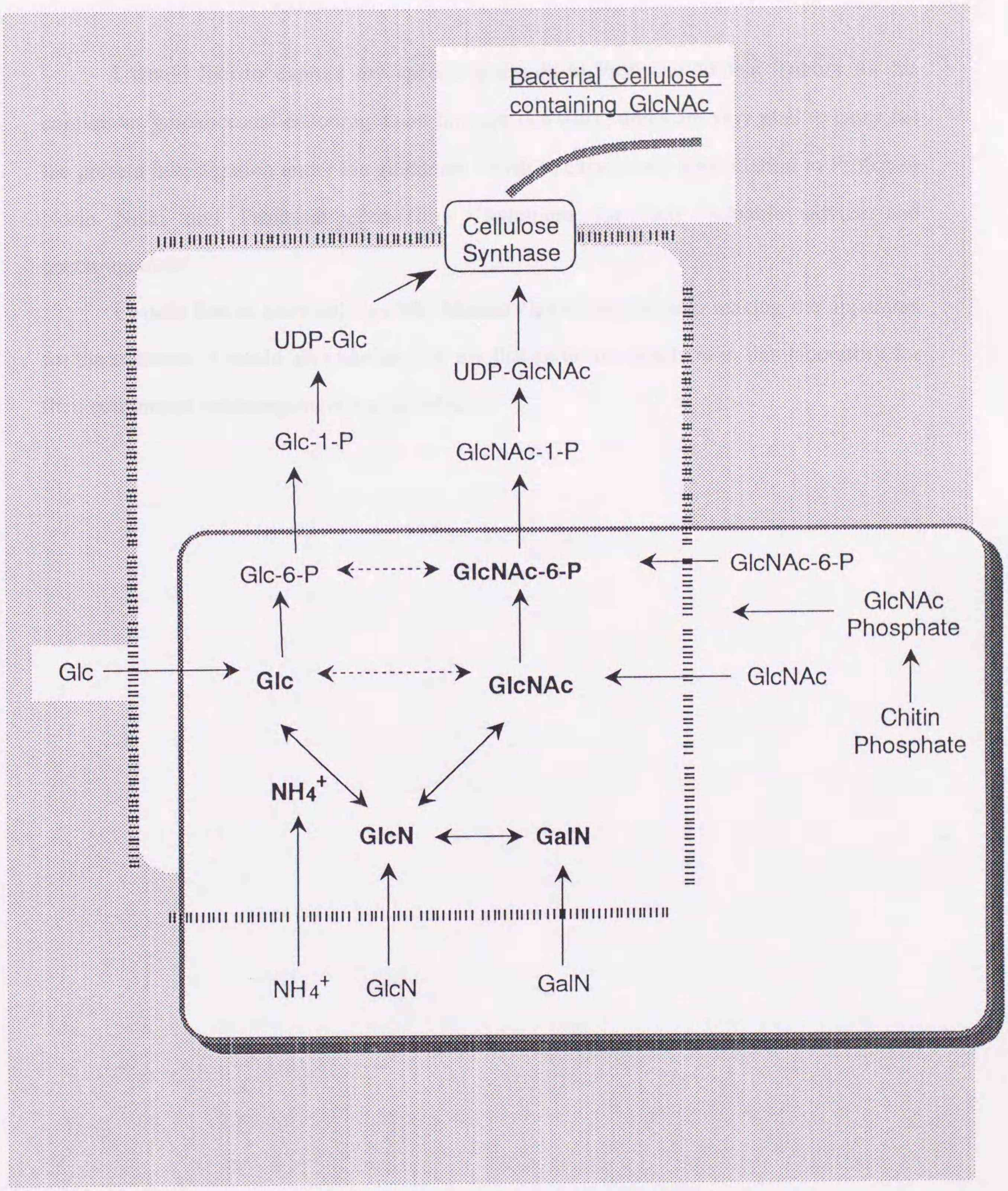
4. GlcN and GalN were taken up by *A. xylinum* to incorporate GlcNAc residues into BC whereas ManN was not accepted to incorporate. GalN might be subjected to epimerization to convert into GlcN, because GalN was not detected in acid hydrolysates of produced BC. As the stereospecific deamination and amination processes were suggested, aminotransferase and epimerase were also assumed to be present among other significant enzymes in the metabolic pathway of *A. xylinum*.

5. The application of several ammonium salts was effective on the incorporation of aminosugar residues into BC, when the cultures of *A. xylinum* were incubated under rotatory and aerated conditions. As little incorporation was observed in the case of static culture, aeration was assumed to be an essential factor to the aminosugar incorporation in the presence of ammonium salts. Ammonium chloride seemed to be the best additive to enhance aminosugar incorporation. The incorporation mechanism was proposed that GlcN which arose from enzymatic equilibrium of Glc and ammonium ion, was converted into GlcNAc similar to the case of exogenous aminosugars, since incorporated aminosugar residue was estimated to be GlcNAc residue by amino acid analysis and IR spectrum.

6. Since GlcNAc-6-phosphate enhanced the incorporation of GlcNAc residues into BC in the culture of *A. xylinum*, it was proposed that pathway of GlcNAc via GlcNAc phosphate seemed to be present in biosynthetic pathway. GlcNAc phosphate seems to play a intermediate role of the incorporation pathway.

7. Incorporation of GlcNAc was accelerated by the addition of phosphoryl chitins to SH GlcNAc medium. Phosphoryl chitin seems to be hydrolyzed to permeable size during incubation and its hydrolysates were utilized as a source of hexose phosphate by *A. xylinum*.

The results obtained from the present investigations indicate that the conversion of hexoses and its equilibrium functions which are established by enzymatic systems rise to metabolic pathway of *A. xylinum* as illustrated in Scheme 6-1. It became evident that glucose analogues such as GlcNAc, GlcN are metabolized by proposed enzymatic systems with recognition of stereospecificity to produce β-1,4-glucan. As the incorporation of GlcNAc residues into BC was achieved only by the alternation of the medium components, such a flow of hexoses is inevitable for the bacteria to adapt to a new environment.



Scheme 6-1. Proposed metabolic pathway of hexoses in *A. xylinum*.

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REFERENCES

1 Kennedy, J.F., White, C.A. Ed., *Bioactive Carbohydrates In Chemistry, Biochemistry and Biology,* Ellis Horwood, Chichester (1983).

2 Delmer, D.P. *Adv. Carbohydr. Chem. Biochem.*, **41**, 105-153 (1983).

3 Takai, M., Tsuta, Y., Hayashi, J. and Watanabe, S. *Polym. J.,* 7, 157-164 (1975).

4 Horii, F., Yamamoto, H., Kitamaru, R., Tanahashi, M. and Takayoshi, H. *Macromolecules*, **20**, 2949-2951 (1987).

5 Marx-Figini, M. and Pion, B.G. *Biochim. Biophys. Acta*, **338**, 382-393 (1974).

6 Takai, M., Tsuta, Y. and Watanabe, S. *Polym. J.*, 7, 137-146 (1975).

7 Hestrin, S. and Schramm, M. *Biochem. J.*, **58**, 345-352 (1954).

8 Benziman, M. and Burger-Rachamimov, H. *J. Bacteriol.*, **84**, 625-630 (1962).

9 Weinhouse, H. and Benziman, M. *Biochem. J.*, **138**, 537-542 (1974).

10 Weinhouse, H. and Benziman, M. *J. Bacteriol.*, **127**, 747-754 (1976).

11 Schramm, M., Gromet, Z. and Hestrin, S. *Biochem. J.*, **67**, 669-679 (1957).

12 Benziman, M. and Rivetz, B. *J. Bacteriol.*, **111**, 325-333 (1972).

13 Gromet, Z., Schramm, M. and Hestrin, S. *Biochem. J.*, **67**, 679-689 (1957)

14 Valla, S., Coucheron, D.H., Fjærvik, E., Kjosbakken, J., Weinhouse, H., Ross, P., Amikam, D. and Benziman, M. *Mol. Gen. Genet.,* **217**, 26-30 (1989).

15 Aloni, Y., Cohen, R., Benziman, M. and Delmer, D. *J. Biol. Chem.*, **258**, 4419-4423 (1983).

16 Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D. Weinberger-Ohara, P., Mayer, R., Braun, S., de Vroom, E.,van der Marel, G.A., van Boom, J.H. and Benziman, M. *Nature*, **325**, 279-281 (1987).

17 Ross, P., Mayer, R. and Benziman, M. *Microbiol. Rev.*, **55**, 35-58 (1991).

18 Swissa, M., Aloni, Y., Weinhouse, H. and Benizman, M *J. Bacteriol.*, **143**, 1142-1150 (1980).

19 Carpita, N. C. and Delmer, D. P. *J. Biol. Chem.*, **256**, 308-315 (1981).

20 Bureau, T.E. and Brown, R.M., Jr. *Proc. Natl. Acad. Sci. USA*, **84**, 6985-6989 (1987).

21 Lin, F.C., Brown, R.M., Jr., Cooper, J.B. and Delmer, D.P. *Science*, **230**, 822-825 (1985).

22 Aloni, Y. Delmer, D.P. and Benziman, M. *Proc. Natl. Acad. Sci. USA*, **79**, 6448-6452 (1982).

23 Fujiwara, M., Maruyama, M., Takai, M. and Hayashi, J. p.17-22. In *CELLULOSICS: Chemical, Biological and Material Aspects* , Kennedy, J.F., Phillips, G.O. and Williams, P.A. Ed., Ellis Horwood, Chichester (1993).

24 Saxena, I.M., Kudlicka, K., Okuda, K. and Brown, R.M., Jr. *J. Bacteriol.*, **176**, 5735-5752 (1994).

25 Ogawa, R., and Tokura, S. *Carbohydr. Polym.*, **19**, 171-178 (1992).

26 Ogawa, R., Miura, Y., Tokura, S. and Koriyama, T. *Int. J. Biol. Macromol.*, **14**, 343-347 (1992).

27 Ogawa, R., Sato, M, Miura, Y. Fujiwara, M., Takai, M. and Tokura, S. p.35-40. In *CELLULOSICS: Chemical, Biological and Material Aspects*, Kennedy, J.F., Phillips, G.O. and Williams, P.A. Ed., Ellis Horwood, Chichester (1993).

28 Tokura, S. and Azuma, I. *Chitin Derivatives in Life Science*, Sapporo, JAPAN (1991)

29 Tokura, S. Miura, Y. Kaneda, Y. and Uraki, Y. *Polymeric Delively System*, **25**, 351-361 (1993).

30 Tokura, S., Kaneda, Y. Miura, Y. and Uraki, Y. *Carbohydr. Polym.*, **19**, 185-190 (1992).

31 Murate, J., Saiki, I., Makabe, T., Tsuta, Y., Tokura, S. and Azuma, I. *Cancer Res.*, **51**, 22-26 (1991).

32 Nishimura, K., Nishimura, S.-I., Nishi, N., Saiki, I., Tokura, S. and Azuma, I. *Vaccine*, **2**, 93-99 (1984).

33 Nishimura, K., Nishimura, S.-I., Nishi, N., Numata, F., Tone, Y.,Tokura, S. and Azuma, I. *Vaccine*, **3**, 379-384 (1985).

34 Muzzarelli, R.A.A. *CHITIN*, Pergamon Press, Oxford (1977).

35 Sakaguchi, T, Horikoshi, T. and Nakajima, A. *Nippon Nogeikagaku Kaishi*, **53**, 149-156 (1979).

36 Sakaguchi, T., Horikoshi, T. and Nakajima, A. *Agric. Biol. Chem.*, **45**, 2191-2195 (1981).

37 Nishi, N., Nishimura, S.-I, Ebina, A, Tsutsumi, A. and Tokura, S. *Int. J. Biol. Macromol.,* **6**, 53-54 (1984).

38 Nishi, N., Maekita, Y., Nishimura, S.-I., Hasegawa, O. and Tokura, S. *Int. J. Biol. Macromol.*, **9**, 109-114 (1987).

39 Reese, E.T. and Mandels, M. *Methods in Carbohydrate Chemistry*, Vol. 3. Academic Press, New York, p. 141 (1963).

40 Merrill, A. T. *J. Biol. Chem.*, **60**, 257-266 (1924).

41 Imoto, T. and Yagishita, K. *Agr. Biol. Chem.*, **35**, 1154-1156 (1971).

42 Ebell, L. *Phytochemistry*, **8**, 25-36 (1969).

43 Reissig, J.L., Strominger, J.L. and Leloir, L.F. *J. Biol. Chem.*, **217**, 959 (1955).

44 Ogawa, R., Ohtsuki, M., Miura, Y. and Tokura, S.,*Sen-i Gakkaishi*, **48**, 434-436 (1992).

45 Kurita, K., Yoshino, H., Yokota, K., Ando, M., Inoue, S., Ishii, S. and Nishimura, S.-I. *Macromolecules*, **25**,3786-3790 (1992).

46 Hackman, R.H. *Aust. J. Biol. Sci.*, **7**, 168-178 (1954).

47 Katsuura, K. and Mizuno, H. *Sen-i Gakkaishi*, **22**, 510-514 (1966).

48 Tokura, S., Yoshida, J., Nishi, N. and Hiraoki, T. *Polym. J.*, **14**, 527-536 (1982).

49 Tokura, S., Nishi, N., Tsutsumi, A. and Somorin, O. *Polym. J.*, **15**, 485-489 (1983).

50 Senju, R. and Okimasu, S. *Nippon Nogei Kagaku Kaishi,* **23**, 432-437 (1950).

51 Sannan, T., Kurita, K. and Iwakura, Y. *Makromol. Chem.*, **176**, 1191-1195 (1975).

52 Hirano, S., Hasegawa, M. and Kinugawa, J. *Int J. Biol. Macromol.*, **13**, 316-317, (1991).

53 Nakamura, M. *Nogei Kagakushi,* **24**, 197 (1951).

54 Inoue, Y., Kaneko, M. and Tokura, S. *Rep. Proc. Polym. Jpn.*, **25**, 759-760 (1982).

55 Tokura, S, Baba, S., Uraki, Y., Miura, Y., Nishi, N. and Hasegawa, O. *Carbohydr. Polym.*, **13**, 273-281 (1990).

56 Nishimura, S.-I., Nishi, N. and Tokura, S. *Carbohydr. Res.*, **146**, 251-258 (1986).

57 Fordyce, C.R. *Adv. Carbohydr. Chem. Biochem.*, **1**, (1945).

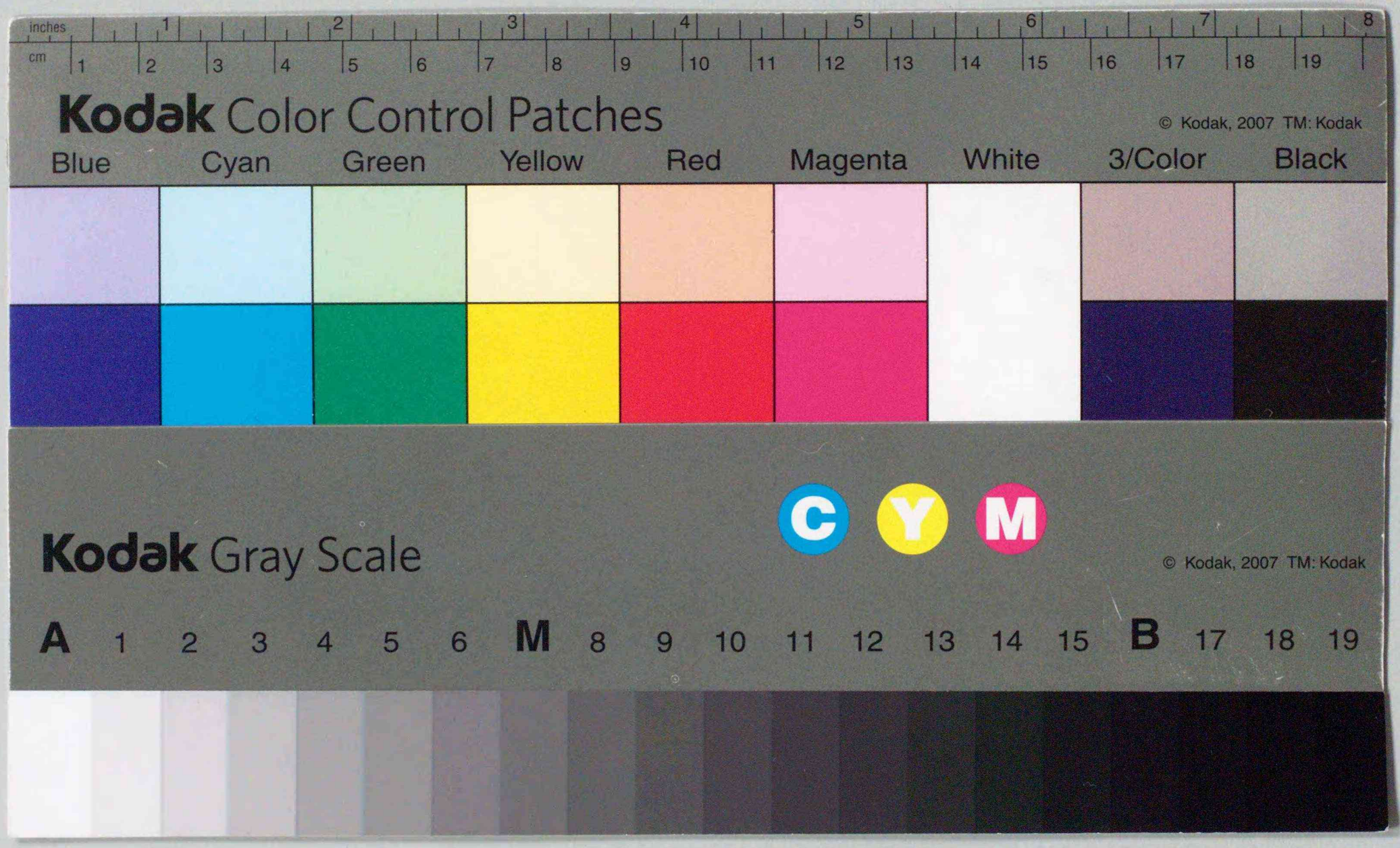
58 Tokura, S., Shirai, A., Kaneko, H., Itoyama, K. and Takahashi, K. *Proceeding of the International Seminar on Alginate, Chitin, and Chitosan*, Royal Norwegian Embassy, Tokyo, Japan (1993).

59 Tokura, S., Shirai, A., Kaneko, H., Itoyama, K., Ren, L.-D. and Sekiguchi, H. *Carbohydr. Polym.* (1994) in submission.

60 Shirai, A., Takahashi, K., Rujiravanit, R., Nishi, N. and Tokura, S. *Proceeding of Asia-Pacific Chitin and Chitosan Symposium,* Bangi, Malaysia (1994).

61 Schramm, M., Gromet, Z. and Hestrin, S. *Nature*, **179**, 28-29 (1957).

62 Nishi, N., Noguchi, J., Tokura, S. and Shiota, H. *Polym. J.*, **11**, 27-32 (1979).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