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CHEMICAL SYNTHESIS OF NATIVE-TYPE CELLULOSE AND ITS ANALOGUES VIA ENZYMATIC POLYMERIZATION

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ABSTRACT

The first successful chemical synthesis of cellulose was achieved by a polycondensation of β-cellobiosyl fluoride monomer catalyzed with cellulase, an extracellular hydrolytic enzyme of cellulose, in a mixed solvent of acetonitrile and acetate buffer. The product, *synthetic cellulose*, was the crystalline allomorph cellulose II, a thermodynamically more stable form. More detailed examinations of the polymerization conditions led to the formation of the native cellulose I, a metastable allomorph, for the first time. The key to the success was to use partially purified cellulase and an appropriate mixed solvent of acetonitrile/buffer. The formation of the two allomorphs of cellulose implies that the polarity of the glucan chain ordering can be controlled in a test tube. Based on these findings, a new concept "choroselectivity," meaning spacial control in ordering the macromolecular chain, has been proposed. Cellulose analogues, 6-*O*-methylated cellulose and xylan, have been synthesized regio- and stereoselectively by using the enzymatic polymerization technique.

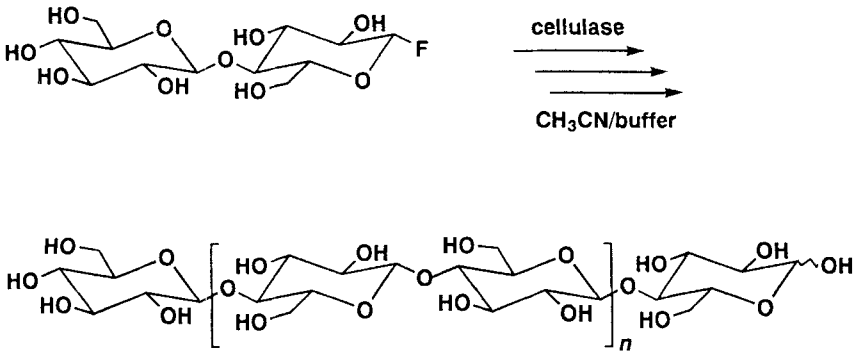
INTRODUCTION

Cellulose is produced photochemically on earth and is one of the most important naturally occurring polymeric materials [1]. In macromolecular science, cellulose has been a symbolic substance because several significant and fundamental concepts of polymers were born through the investigation of cellulose at the early stage of polymer science in the 1920s [2]. In-vitro synthesis of cellulose, therefore, has been a challenging dream for synthetic chemists for more than a half century. Since its first trial in 1941, many attempts have been made, which include a selective dehydration polycondensation of a 2,3,6-tri-protected glucose [3] and a ring-opening polymerization of bicyclic acetal compounds [4, 5]. However, steroregular polysaccharides having the desired structure were not obtained due to the lack of regio- and stereoselectivity.

ENZYMATIC POLYMERIZATION OF β-CELLOBIOSYL FLUORIDE

The first in-vitro synthesis of cellulose via a nonbiosynthetic pathway was achieved in 1991 by an enzymatic polymerization of β-cellobiosyl fluoride as substrate monomer for cellulase, an extracellular hydrolytic enzyme of cellulose, in an acetonitrile/buffer mixed solvent [6] (Scheme 1). The enzyme promotes transglycosylation of the cellobiosyl moiety toward the 4′-hydroxy group of another cellobiosyl fluoride eliminating hydrogen fluoride. The best ratio for acetonitrile and buffer concerning the yield of "synthetic cellulose" so far has been 5:1.

X-ray as well as ^{13}C-NMR analyses showed that its crystal structure is of type II, a thermodynamically more stable form. The formation of the stereoregular β(1→4) linkage is explained by the formation of a glycosyl-enzyme intermediate or a glycosyl oxocarbenium ion at an active site of cellulase with the elimination of fluoride anion [7]. This reactive intermediate is then attacked by the 4′-hydroxy group of another monomer or propagating polymer which locates in a subsite of the enzyme, leading to the stereoselective formation of the β(1→4) linkage. Conse-



SCHEME 1.

quently, the stereochemistry of the product is retention of configuration via "double inversion" concerning the anomeric carbon atom of the β-cellobiosyl fluoride [8].

In this enzymatic polymerization the choice of a leaving group on the anomeric center of cellobiose monomer was found to be very important. Various β-cellobiose derivatives (β-cellobiosyl fluoride, methyl β-cellobioside, allyl β-cellobioside, trifluoroethyl β-cellobioside, methyl β-thiocellobioside, phenyl β-cellobiosyl sulfoxide, and 1-*O*-acetyl β-cellobiose) have been employed for the enzymatic polymerization using cellulase as catalyst [9]. Among these activated cellobiose substrates, β-cellobiosyl fluoride gave the best result with respect to the yield of synthetic cellulose.

We also prepared β-cellotriosyl fluoride and β-cellotetraosyl fluoride as novel substrate monomers, and investigated their hydrolysis and polymerization behavior in the presence of cellulase. These substrates were found to be rapidly hydrolyzed in aqueous solution, indicating that they can be recognized as a substrate by the cellulase. A polymerization reaction was performed in an aqueous organic solvent (acetonitrile:buffer = 3:1) giving rise to cellooligomers and cellulose [10].

FIRST VISUALIZATION OF SYNTHETIC CELLULOSE

The visualization of cellulose microfibrils starting from the smallest building block of cellobiose derivatives is of great interest in basic science in connection with the mechanism of cellulose biosynthesis in nature. One of the most powerful tools for direct visualization of the cellulose-forming process is transmission electron microscopy (TEM). To date the majority of the microscopic studies of cellulose morphology and biosynthesis have been conducted from a biological viewpoint. The microfibrillar structure of cellulose was observed in vivo in association with organized particles known as terminal complexes [11]. In-vitro cellulose synthesis via a biosynthetic pathway was accomplished by an isolated enzyme system from a bacterium [12] and cotton [13]. These approaches have always been based on the fact that the biosynthetic polymerization process utilizes an activated glucose monomer of uridine diphosphate glucose (UDP-glucose) and cellulose synthase as catalyst. So far, no attempt for the direct observation of the cellulose-forming process via a nonbiosynthetic pathway has been made by means of TEM, where neither an activated saccharide monomer nor a polymerization catalyst has its origin in the cellulose biosynthesis. According to the enzymatic polymerization of β-cellobiosyl fluoride, it is possible to form an elongated polymer chain of cellulose molecules in solution, enabling visualization of cellulose formation and the subsequent crystallization process.

The novel system of cellulose synthesis has been followed for the first time by TEM [14, 15]. The enzymatic polymerization was found to be a rapid process; cellulose formation was detected as early as 30 seconds after initiation. A micellar phase separation occurred at the initial stage of the reaction. Irregular aggregates of cellulose were formed at the boundary of the micellar particles, suggesting that the interface is the site of polymerization (indicated by arrowheads in Fig. 1a). The electron diffraction pattern of the product showed the typical pattern of the crystal structure of cellulose II, a thermodynamically more stable allomorph [16] (Fig. 1b).

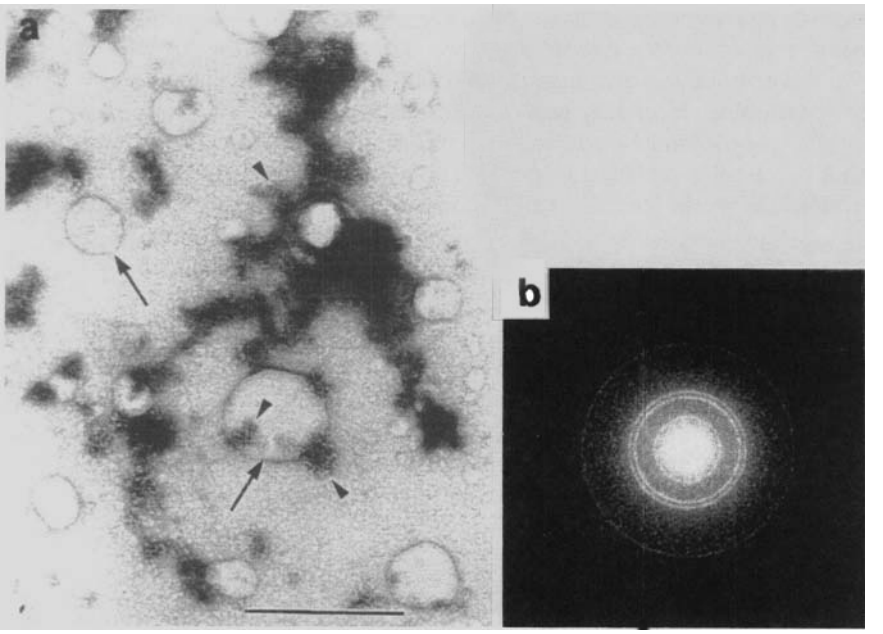


FIG. 1. (a) Electron micrograph of synthetic cellulose II. Note the product aggregated adhering to the circular traces of micelle-like phase separation (scale bar is 500 nm). (b) Electron diffraction pattern of synthetic cellulose II. The rings correspond to spacing of 7.19, 4.41, 4.03, and 2.20 Å, corresponding to $(1\bar{1}0)$, (110), (200), and (310), respectively, from inside to outside.

The enzyme used in this study was a mixture of many cellulolytic and noncellulolytic enzyme components.

FIRST IN-VITRO SYNTHESIS OF NATIVE-TYPE CELLULOSE I VIA ENZYMATIC POLYMERIZATION

No in-vitro or abiogenic process has been reported that produces cellulose I, either by recrystallization or by polymerization. However, the challenge for the formation of synthetic cellulose I, a metastable allomorph, has been successfully accomplished by means of a partially purified cellulase-catalyzed polymerization of β-cellobiosyl fluoride in an optimized acetonitrile/acetate buffer system [17].

At the early stage for this challenge, we used an acetonitrile/buffer ratio of 5:1. We attempted to purify the crude enzyme and to find the cellulose component(s) responsible for the polymerization. During this work we observed by TEM that the $\beta(1\rightarrow4)$ glucan products obtained from a partially purified preparation occasionally contained fibrillar materials dispersed among the type II cellulose. These elongated fibrils were very similar to the morphology of extended fibrils of cellulose I formed in vivo, and they gave a similar reaction to a specific cellulose probe, colloidal gold-CBH I [18]. To overcome the difficulty of obtaining more sufficient fibrillar materials, we experimented with a different ratio of acetonitrile/buffer. We

then found that the most effective production of the fibrillar materials was realized with an acetonitrile/buffer ratio of 2:1. In addition to a greater abundance of fibrillar materials, larger bundle-like aggregates were readily synthesized (Fig. 2a). This allowed an electron diffraction (ED) analysis of the fibrils which clearly indicated a cellulose I diffraction pattern (Fig. 2b) [17].

The use of the organic solvent serves two functions: 1) it creates micelles which promote critical aggregation of the catalytic sites, and 2) it favors a shift of the equilibrium reaction toward chain extension rather than hydrolytic cleavage. Sufficient purification to obtain the active enzyme component for cellulose synthesis was necessary to achieve a critical density of catalytic subunits to promote extended parallel glucan chain formation (Fig. 3a). On the other hand, only thermodynamically stable antiparallel chains were formed when the crude enzyme mixture was used where each active enzyme must be located apart due to other nonactive protein components (Fig. 3b). The present findings may provide clues for a further understanding of in-vivo biosynthetic mechanisms leading to native cellulose I formation.

CHOROSELECTIVITY

It is well accepted that selectivity in polymer synthesis can be classified in the following three categories: chemoselectivity, regioselectivity, and stereoselectivity. All of these terms have been defined for selectivity concerning covalent bond formation between two reaction centers. In order to achieve precise architecture of macromolecules of well-defined structure, all of these selectivites must be controlled. As mentioned above, two allomorphs of cellulose, cellulose II with antiparallel glucan chains and cellulose I with parallel glucan chains, can be selectively synthesized in vitro using the enzymatic polymerization of β-cellobiosyl fluoride monomer, and this selectivity can be controlled by changing the degree of purification of the

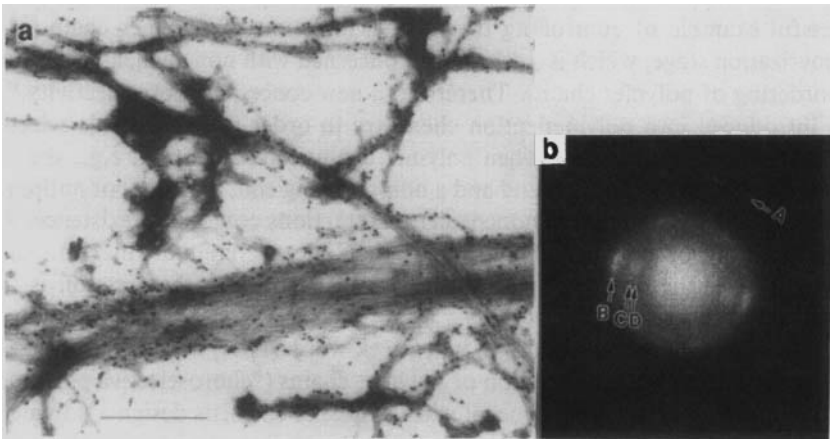


FIG. 2. (a) Electron micrograph of synthetic cellulose I synthesized by the partially purified cellulase with an acetonitrile/buffer ratio of 2:1 (scale bar is 250 nm). (b) Electron diffraction pattern of synthetic cellulose I. A = 2.6 Å, B = 4.0 Å, C = 5.4 Å, D = 6.0 Å.

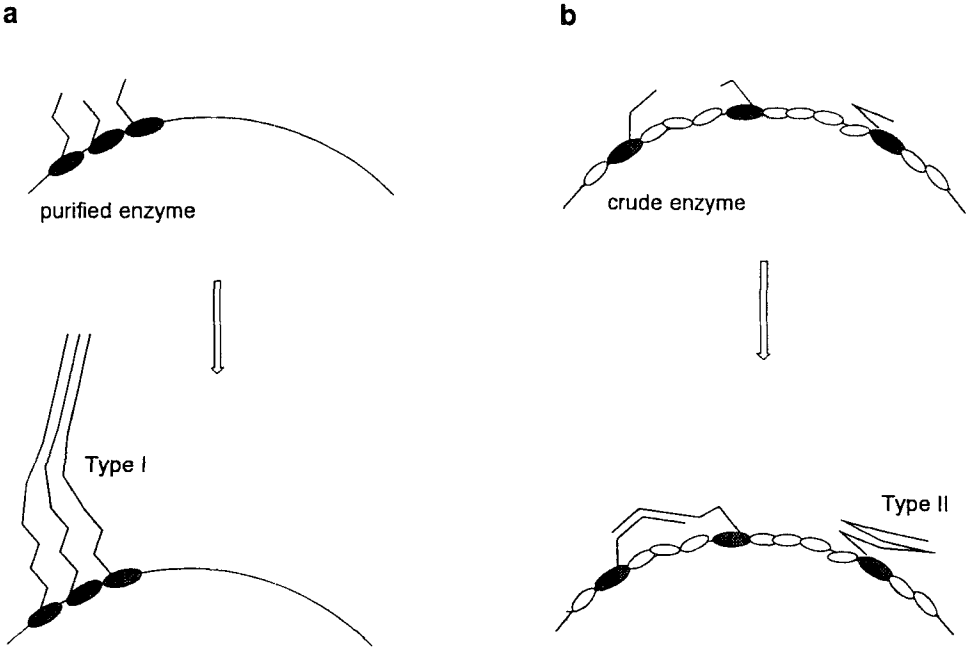
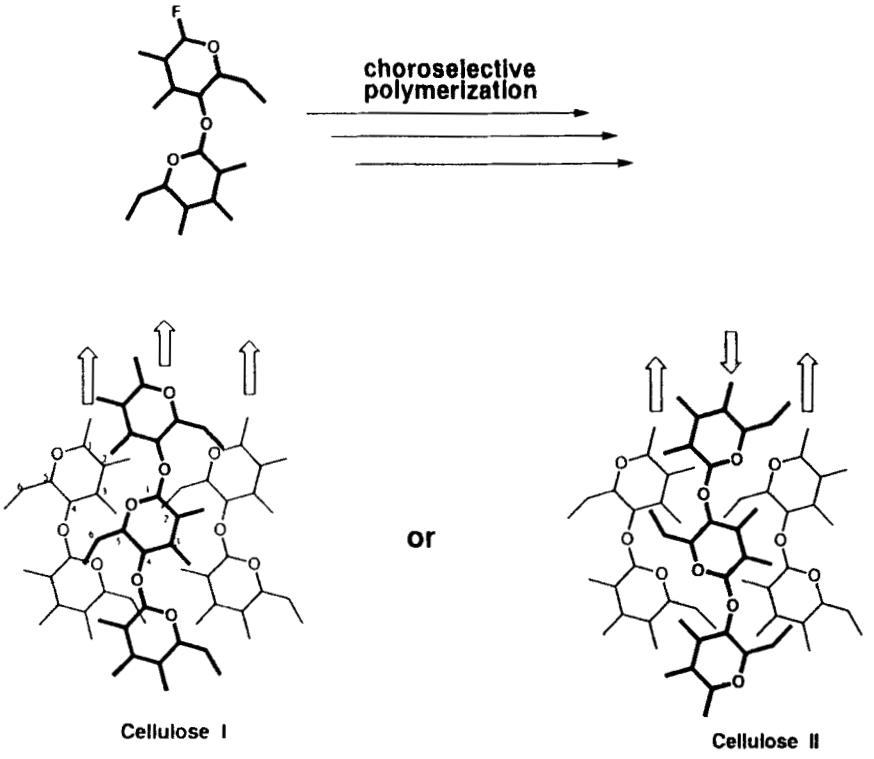


FIG. 3. Possible models. (a) Formation of synthetic cellulose I with extended parallel glucan chains by the purified enzyme. (b) Formation of synthetic cellulose II with antiparallel glucan chains by the crude enzyme mixture.

enzyme catalyst and the polymerization conditions. These results indicate that the relative intermolecular direction of glucan chains has successfully been controlled in the propagating process of polymerization. This is, to our best knowledge, the first successful example of controlling the relative direction of polymer chains at the polymerization stage, which is a selectivity concerned with noncovalent, intermolecular ordering of polymer chains. Therefore, a new concept, "choroselectivity," has been introduced into polymerization chemistry in order to express this selectivity [19–22] (Scheme 2). Namely, when polymer chains have direction, e.g., the cellulose molecules have a reducing end and a non-reducing end, a parallel or antiparallel directional relationship due to noncovalent interactions comes into existence. And, if the assembly of polymer chains shows that one preferential direction of the allomorph over the other is formed during polymerization, the reaction is to be defined as "choroselective."

The term "choro" has its origin in a Greek word χώρος, meaning "space" [22]. Such control of the spacial direction of polymer chains ("choroselective polymerization") would be an important concept when synthetic chemists design a polymerization reaction which considers not only the repeating unit structure, sequence, stereochemistry, molecular weight and its distribution, end-group structures, etc., but also the other chemical and physical properties of the resulting polymers.

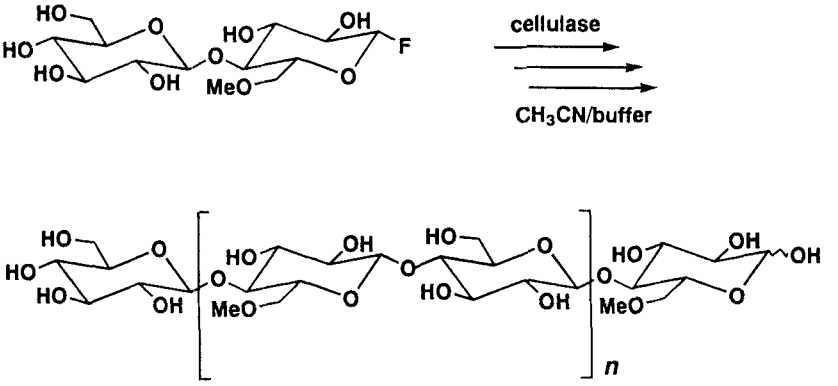


SCHEME 2.

APPLICATIONS TO SYNTHESIS OF MODIFIED CELLULOSE AND XYLAN

Synthetic strategy for constructing a functionalized cellulose has so far been based on the modification of a hydroxy group in natural cellulose by a chemical reaction. Actually, the derivatization of cellulose has been studied extensively with the aim of developing high performance cellulosic materials. Very recently we developed a new method for the synthesis of modified cellulose by using enzymatic polymerization. New cellobiosyl fluoride derivatives 6-*O*-methyl, 6′-*O*-methyl, and 6,6′-di-*O*-methyl β-cellobiosyl fluorides were prepared as monomers for enzymatic polymerization, aimed at the preparation of regioselectively methylated cellulose derivatives [23]. A 6-*O*-methylated cellulose derivative with an alternatingly methyl group has been obtained by the enzymatic polycondensation reaction of 6-*O*-methyl β-cellobiosyl fluoride monomer (Scheme 3).

Xylan, a xylose polymer having a β(1→4) glycosidic linkage in the main chain, is one of the most important components of hemicellulose in plant cell walls. Natural xylan normally contains 4-*O*-methylglucuronic acid or L-arabinose as a minor unit in the side chain. The synthesis of poly- and oligosaccharides consisting of only the xylose unit is therefore of great interest from the viewpoint of material science. In this framework, the first in-vitro synthesis of xylan by enzymatic poly-



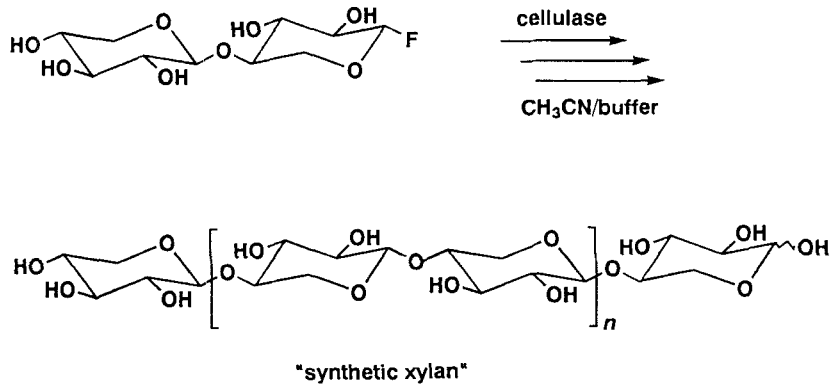
SCHEME 3.

merization using β-xylobiosyl fluoride as a substrate for cellulase has been achieved (Scheme 4) [24].

The enzymatic polymerization of β-xylobiosyl fluoride proceeded smoothly at 30°C to produce white fine powders which are insoluble in any solvent. The CP MAS solid ^{13}C NMR of the product showed the typical signals for a xylan-type saccharide structure. These results indicate that the polycondensation process is under perfect control of regio- and stereochemistry. X-ray diffraction also supported the exclusive formation of synthetic xylan.

CONCLUSION

Cellulose has been synthesized in a test tube for the first time by an enzymatic polymerization of β-cellobiosyl fluoride monomer. The formation of native cellulose I in vitro has been realized by using this novel enzymatic methodology, which



SCHEME 4.

brought about a new concept of "choroselectivity" in polymer chemistry. This new methodology for polysaccharide synthesis is a promising tool for the preparation of highly designed polysaccharide derivatives, and it continues to broaden the scope of macromolecular architecture by a biocatalyst for future use.

ACKNOWLEDGMENTS

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[15] On this occasion of Professor Rånby's issue, one of the present authors (S.K.) wishes to take the opportunity to describe here very briefly how the cooperative work between two independent research groups of S.K. at Tohoku University and of Professor R. M. Brown Jr. at University of Texas at Austin started. This is because the cooperation of the two groups, having

very much different research backgrounds, has been so fruitful and Professor Rånby actually catalyzed the contact of the two groups. In May 1989, S.K. visited Sweden and gave a talk on the preliminary results of the enzymatic polymerization for chemical synthesis of cellulose at the Royal Institute of Technology. After the talk Professor Rånby suggested S.K. contact Professor Brown whose main research area is biosynthesis of cellulose. Then S.K. wrote to Professor Brown, who invited S.K. to Austin on the occasion of S.K.'s attendance at the American Chemical Society meeting in Atlanta in April 1991. This was the first meeting of the two research groups. Both sides thoroughly discussed about in-vivo and in-vitro cellulose syntheses as well as important unsolved problems, and agreed to start cooperative work with exchanging researchers. S.K. was then funded from Monbusho, Japanese Government, for two years, 1992 and 1993, to carry out the international cooperation. Professor Rånby paid attention to the progress of the cooperation. We greatly thank him for his stimulating interest to this cooperation. The group at Tohoku University expresses thanks to Professor Brown, Mr. J. H. Lee (University of Texas), Dr. S. Kuga (University of Tokyo), and Dr. K. Okuda (Kochi University) for their enthusiastic cooperative work.

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[22] At the symposium in Stockholm, S.K. presented this paper on April 20, 1995. S.K. used a new term, "allos-selectivity," to mean the spacial direction (three-dimensional direction) control, in which "allos" was from the Greek αλλος [19-21]. After the presentation, Professor N. Hadjichristidis, University of Athens, commented that the terminology was not well suited and he suggested to S.K. the term "choroselectivity" from the greek word χῶρος (choros) meaning "space." We thank him for his very valuable suggestion.

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