ISSN 0366-5119

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БИОЛОГИЧЕСКИЙ ЖУРНАЛ АРМЕНИИ

BIOLOGICAL JOURNAL of ARMENIA

Published from 1948 in Armenian. Russian and English.

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The present issue is published within the frame of the Project A-326 of the International Science and Technology Center.

Издание номера осуществлено в рамках Проекта А-326 Международного Научно-технического Центра.

ISSN 0366-5119.

PREFACE

Increasing interest for microbial production of cyclodextrins reflects the general trend in the use of biocatalysis/biotransformation having evident advantages to fermentation and traditional technologies.

The history of study of cyclodextrins exceeds 100 years but this field is still developing intensively covering more fundamental and applied issues. Along with enlargement of the areas of practical application of cyclodextrins comprising pharmaceutics, cosmetics, food and chemical industries there is a dramatic increase in R&D for obtaining of new cyclodextrins and their derivatives. Outstanding achievements in gene cloning and overexpression of cyclomaltodextrin glucanotransferases genes provide a solid background for high economic production of cyclodextrins and their expansion into the world market.

Having specific and unique activity for formation of cyclic inclusion complexes, cyclodextrins may catalyze chemical reactions and are excellent models of enzymes. In this respect the catalysis occurring after such complexing represents an efficient tool for study of "host-guest" relationships. Cyclodextrins nowadays are more available and cheap compounds used for complexing purposes with a broad perspectives of practical application in many fields of science and industry.

From microbiological point of view cyclodextrins represent the products of microbial biosynthesis closely related to the cell differentiation, particularly to the sporulation. On the other hand, summarizing the literature and our experimental data we can conclude on the existence of the species specificity in production of certain types of these substances. So, the strategy in the search of new cyclodextrins has to be based on the screening of new species of their producers what attracts a keen interests of microbiologists.

The Institute of Microbiology of the National Academy of Sciences of Armenia is engaged in the study of cyclodextrins biosynthesis for 20 years. In the beginning the research efforts aimed to establish biosynthetic activity of cyclodextrins production by different species of aerobic spore-forming bacilli, especially their extremophilic forms. Later the essential progress in this field was associated with the active involvement of the research team headed by Dr. V. Abelian. Many microbial species including extremophilic forms of bacilli and actinomycetes have been isolated, studied and applied for the production of cyclodextrins. The action patterns of cyclomaltodextrin glucanotransferases has been elucidate and new branched, substituted as well as polymeric cyclodextrins have been synthesized and tested in the solutions treatment, enantiomer resolution of optically active compounds and for stabilization of different substances and drugs.

Due to substantial support of the International Science and Technology Center within the Project A-326 the researches mentioned have been developed in many spheres of production and use of cyclodextrins with large international co-operation.

On behalf of our personnel and my own behalf I wish to express sincere thanks to all authors participated in this issue.

Dr. Evrik Afrikian
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Member of the National Academy of Sciences of Armenia

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Հայաստանի Գիտությունների Ազգ ակադ Միացեալիա, Հայաստանի կենսաբանական Հանդես
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National Academy of Sciences of Armenia, Biological Journal of Armenia

Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

LARGE CYCLODEXTRINS

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The existence of large cyclodextrins, cyclic α-D-(1—>4) glucans with a degree of polymerisation higher than eight, has been proved during the past decade. A number of 4-α-glucanotransferases have been shown to be able to produce large cyclodextrins consisting of up to several hundred glycosyl units, from both amylose and amylopectin. Large cyclodextrins with degree of polymerisation up to 31 have been isolated to purity by use of elaborate purification schemes, enabling studies of their structural and complex forming properties. The solid state structures of the large cyclodextrins with a degree of polymerisation 10, 14 and 26, respectively, have revealed interesting new structural features of this family of molecules. This review summarises the studies of the large cyclodextrins, a varied and highly interesting group of molecules.

За прошедшее десятилетие доказано существование крупномолекулярных циклодекстринов — циклических α—D—(1—>4) глюканов со степенью полимеризации выше, чем восемь. Показано, что ряд 4—α—глюканотрансфераз способен продуцировать крупномолекулярные циклодекстрины, состоящие из нескольких сотен гликозильных единиц как из амилозы, так и амилопектина. Высокомолекулярные циклодекстрины со степенью полимеризации до 31 выделены в чистом виде с использованием детально разработанных схем очистки, которые позволяют изучить их структурные и комплексообразующие свойства. Структуры твердого состояния крупномолекулярных циклодекстринов со степенью полимеризации 10, 14 и 26 соответственно выявляют новые интересные структурные особенности молекул этого семейства. Данный обзор обобщает исследования о крупномолекулярных циклодекстринах — разнообразной и весьма интересной группе молекул.

Խոշորմոլեկուլային ցիկլոդեքստրինների ութից ավելի պոլիմերիզացիայի աստիճանով ցիկլիկ α—D—(1—>4) գլյուկանների, գոյությունը ապացուցվել է անցած տասնամյակի ընթացքում. Ցույց է տրվել, որ մի շարք 4- α—գլյուկանոտրանսֆերազներ կարող են առաջացնել խոշորմոլեկուլային ցիկլոդեքստրիններ կազմված ինչպես ամիլոզի, այնպես էլ ամիլոպեկտինի մինչև մի քանի հարյուր գլիկոզիլային միավորներից. Խոշորմոլեկուլային ցիկլոդեքստրինները մինչև 31 պոլիմերիզացիայի աստիճանով անջատվել են մաքուր վիճակում, օգտագործելով մանրակրկիտ մշակված մաքրման սխեմաներ, որոնք թույլ են տալիս ուսումնասիրել նրանց կառուցվածքային և համալիրագոյացման առանձնահատկությունները. Խոշորմոլեկուլային ցիկլոդեքստրինների համապատասխանաբար 10, 14 և 26 պոլիմերիզացիայի աստիճանով պինդ կառուցվածքները բացահայտել են մոլեկուլների այս ընտանիքի նոր հետաքրքիր կառուցվածքային հատկանիշները. Այս ակնարկը ամփոփում է խոշորմոլեկուլային ցիկլոդեքստրինների բազմազան և խիստ հետաքրքիր մոլեկուլների խմբի, վերաբերյալ ուսումնասիրությունները.

Introduction

In 1891, Villier discovered a crystalline material, which he named "cellulosine", from *Bacillus amylobacter* digest of potato starch [1]. This discovery is regarded as the first published record of the compounds later to be known as cyclodextrins. Years later Schardinger found two crystalline polysaccharides during his investigations of food spoilage, which he called "crystalline dextrin α" and "crystalline dextrin β" [2]. After the development of a relatively simple method for the purification of "α-dextrin" and "β-dextrin" and a new fraction "γ-dextrin", by Freudenberg and Jacobi in 1935, it became possible to carry out thorough studies on the chemical composition of these molecules [3]. A year later, this lead Freudenberg and co-workers to propose a cyclic structure for these molecules, which was confirmed in a series of studies published in the late thirties [4,5]. At present date it is well known that cyclodextrins are annular molecules, comprised of α-D-(1→4)-linked glycosyl units of varying numbers. The most common and commercially successful cyclodextrins are the α-, β-, and γ-cyclodextrins which consists of 6, 7, and 8 glucose molecules, respectively [6-8]. These molecules have a high ability to complex a wide range of, especially hydrophobic, molecules due to a hydrophobic cavity and a hydrophilic outer surface. The inclusion complexes thus formed, also known as guest-host complexes, can have highly altered properties compared to that of the guest molecule alone; including altered solubility, stability, reactivity, volatility and bioavailability. These properties are currently used in numerous applications in the pharmaceutical, agro-chemical, food and chemical industries [6-10]. Furthermore, cyclodextrins and their derivatives have become the molecules of choice in the area of analytical chemistry for the separation of structural, positional, and stereo isomers [11-14]. In addition to the stabilising effect of cyclodextrins, protecting guest molecules against degradation, they possess an "enzyme-like" catalytic property to accelerate chemical reactions, including hydrolysis of certain compounds. Therefore cyclodextrins have achieved considerable attention as enzyme models [6,15-17]. As a result of both scientific and commercial interests, cyclodextrins are frequently used as objects for the study of molecular interactions and they are one of the most studied class of molecules within the field of supramolecular chemistry [18].

The first indications of the existence of cyclodextrins comprising more than 8 glycosyl units was published in 1948 by Freudenberg and Cramer [19]. A decade later these findings were substantiated by French and co-workers, who reported the isolation and partial characterisation of large cyclodextrins with 9, 10, 11 and 12 glycosyl units in the macrocycle [5,20,21]. The reports on the large cyclodextrins by French and co-workers has for many years been regarded as dubious, since they were not able to experimentally distinguish the large cyclodextrins from branched cyclodextrins [20,21]. As late as 1988, Szejtli expressed his doubts, in his monograph "Cyclodextrin Technology", to whether cyclodextrins larger than γ-cyclodextrins exists [6].

Only during the past decade, the existence of the large cyclodextrins has been fully proved [22-28]. At present cyclodextrins containing up to 31 glycosyl units has been purified and characterised [28] and the existence of even larger cyclodextrins with degrees of polymerisation up to several hundreds of glycosyl units have been proved [28-38]. An increasing availability of large cyclodextrins either as pure substance or as mixtures, at least on the laboratory scale, has facilitated an increasing number of studies of their properties, particularly with regard to inclusion complex formation [23,36,39-47]. In this review an overview of the nomenclature, production, purification, structures and properties of the varied group of molecules known as the large cyclodextrins will be presented.

Nomenclature

"Cellulosine" was the first name used for cyclodextrins, which were obtained as crystalline cellulose-like products from bacterial starch digests [1]. Later, Schardinger isolated two non-reducing crystalline compounds, dextrins A and B, which was renamed "crystallized dextrin α" and "crystallized dextrin β"[2]. γ-dextrin was introduced in 1935 by Freudenberg and Jacobi [3]. As a result of the pioneering work of Schardinger, the cyclodextrins have often been denoted "Schardinger dextrins" in the older literature.

The term "cyclodextrin" has for many years served as a general name for the cyclic α-D-(1→4)-linked D-glucose oligosaccharides consisting of 6, 7 and 8 glycosyl units, well known as the α-, β- and γ-cyclodextrins, respectively. However, as emphasised by Lichtenthaler and co-workers [48,49] the term cyclodextrin only specifies the nature of the saccharide (dextrose was an early synonym for glucose), and does not contain information on the nature of the intersaccharidic linkages. The semisystematic names, such as cyclomaltohexaose for the cyclodextrin consisting of 6 α-(1→4)-linked glycosyl units, have been used almost consistently as descriptor for the small cyclodextrins along with the Greek letter prefix version. The use of the semisystematic names for cyclodextrins was recommended by the Joint Commision on Biochemical Nomenclature in 1996 *"by citing the prefix cycio, followed by the terms indicating the type of intersaccharidic linkages (e.g. "malto" for α-(1→4)linked glucose units), the number of units (e.g. "hexa" for "six") and the termination "-ose"* [50]. A systematic nomenclature was proposed where cyclic oligosaccharides composed of a single type of residue could be named *"by giving the systematic name of the glycosyl residue, preceded by the linkage type in parentheses, preceded in turn by "cycio-" with a multiplicative suffix (i.e. "cyclohexakis-" etc.)"* (e.g. cyclohexakis-(1→4)-α-D-glycosyl for α-cyclodextrin). A similar systematic nomenclature (e.g. cyclo-α(1→4)-glucohexaoside for α-cyclodextrin) has been proposed by Lichtenthaler and co-workers [48,49].

Large cyclodextrins have, as a natural continuation of the generic names of the α-, β-, and γ-cyclodextrins, been given Greek letters as prefix by French and co-workers [5,20,21] a tradition that has been continued recently [22-27]. Until now cyclodextrin with from 6 to 21 glycosyl units have been described with a Greek letter prefix in the literature (table 1). However, the Greek alphabet is finite and will not be able to accommodate the growing number of large cyclodextrins described. The last cyclodextrin to be able to benefit from a Greek letter prefix, will be ω-CD (cyclomaltononacosaose, CD_{29}). Moreover, although researchers are familiar with the generic names for the small cyclodextrins, α-, β- and γ-cyclodextrin, the use of the Greek letter prefix for the large cyclodextrins is confusing and non-descriptive of the size of the macrocycle. Large cyclodextrins have often been designated "cycloamylose" (abbreviated CAn, where n designated the number of glucose molecules in the macrocycle). However, this is a non-systematic name whose use has been discouraged [50]. The designation "large-ring cyclodextrin" has often been used to distinguish the large cyclodextrins from large derivatives of α-, β- and γ-cyclodextrin.

Throughout this review the generic names will be used for α-, β- and γ-cyclodextrin, whereas the semisystematic names, which includes the number of glycosyl units in the macrocycle, will be used for the large cyclodextrins (abbreviated, CDn, where n designates the number of glycosyi units); (table 1). α-, β- and γ-cyclodextrins will collectively be referred to as the small cyclodextrins.

Production and purification of large cyclodextrins

The large amount of literature on the production of the cyclodextrins, does not consider the production of cyclodextrins larger than γ-cyclodextrin. One reason is that their analysis is very difficult, giving that they comprise a group of fairly similar molecules and that they occur in mixtures with linear oligosaccharides. A number of chromatographic methods, based on gel-filtration or reverse-phase separation principles, have been employed for the analysis of the large cyclodextrins after enzymatic removal of linear oligosaccharides. However, they are normally only capable of separating a narrow range of cyclodextrins. The separation of α-, β-, γ-cyclodextrin and CD_9 by use of capillary electrophoresis has been published [52], however, this technique will not be able to separate the vast range of large cyclodextrins. The most appropriate method for the analysis of large cyclodextrins has so far proved to be high performance anion exchange chromatography (HPAEC) combined with the highly sensitive pulsed amperometric detection (PAD) [28-38]. By use of HPAEC-PAD it has been possible to resolve large cyclodextrins containing up to more than 60 glucose molecules.

As indicated by the trace amounts of large cyclodextrins found in reaction mixtures of cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) and starch by French and co-workers [5,20,21] and more recently from commercial cyclodextrin powder [22-27,53] CGTases are capable of producing cyclodextrins containing more than 8 glycosyl units in the macrocycle. The first conclusive report on the production of large cyclodextrins by this enzyme was published in 1997 by Terada and co-workers [32]. Previously, it had been a common belief that CGTases were exo-acting enzymes, only capable of producing α-, β-, and γ-cyclodextrin and thus only these cyclodextrins had been analysed to account for the cyclisation activities of the CGTases [6]. However, Terada and co-workers showed that the CGTases indeed were endo-acting enzymes, which initially produce a wide range of cyclodextrins from CD_9 to at least CD_{60}, together with the conventional α-, β-, and γ-cyclodextrin. Using synthetic amylose as substrate, large cyclodextrins were preferentially produced by CGTase from *Bacillus* A2-5a in the initial stages of the reaction [28,32,38]. Prolonged incubation lead to a gradual conversion into smaller cyclodextrins, with β-cyclodextrin as the major final product. Similar time-course of product formation was seen using *Bacillus macerans* and *Bacillus stearothermophilus* CGTase, except that α-cyclodextrin was the major final product [38]. The initial production of CD_9 by CGTases from 12 different bacterial strains has been demonstrated, substantiating that the production of cyclodextrins larger than γ-cyclodextrin is a common feature of CGTases [54]. Trace amounts of large cyclodextrins, with γ-cyclodextrin being the smallest, have even been discovered to be produced initially by both exo- and endo-acting amylases [55]. However, the amylolytic enzymes subsequently degraded these cyclodextrins due to their high hydrolytic activity.

Similar to the CGTases, other 4-α-glucanotransferases have been found to be very effective in producing large cyclodextrins [29-37]. Amylomaltase (E.C. 2.4.1.25) from *E. coli* and *Thermus aquaticus*, 4-α-glucanotransferase from *Pyrococcus kodakaraensis* KOD1 and potato D-enzyme (E.C. 2.4.1.25) produced CD_{17}, CD_{22}, CD_{16} and CD_{17}, as the smallest cyclodextrins, respectively. On synthetic amylose, potato D-enzyme and *Thermus aquaticus* amylomaltase produced a wide range of large cyclodextrins, which during incubation gradually were reduced in size. The final yield of large cyclodextrins from these enzymes were >95% and 84%, respectively. The average molecular weight of the cyclodextrins produced by potato D-enzyme was 15,000, corresponding to an average degree of

polymerisation of 92. In contrast, probably due to a small hydrolytic activity, the large cyclodextrins produced initially by the *E. coli* amylomaltase and 4-α-glucanotransferase from *Pyrococcus kodakaraensis* KOD1, were degraded after prolonged incubation.

Degradation of waxy corn amylopectin by potato D-enzyme yielded two fractions, separable by gel filtration with an average molecular weight of 30,000 and 3,000, respectively [31,33,36]. Fraction II appeared earlier than fraction I, and contained large cyclodextrins with only α-D-1→4 or both α-D-1→4 and α-D-1→6 glucosidic bonds (63.1 % and 16.8 %, respectively). This fraction was believed to derive from transglycosylation reactions on the outer chains of the amylopectin. The large cyclodextrins (containing only α-D-1→4 glucosidic bonds) were larger in size than what could be expected from the chain length of the outer chains of amylopectin, showing that potato D-enzyme was able to produce longer side chains prior to the cyclisation by inter-chain transglycosylation reactions. As a result of the initial production of large cyclodextrins the side-chains of the amylopectin were shortened. Subsequently, so-called cyclic cluster dextrins were produced by intra-cluster cyclisation reactions. These molecules were found in fraction I, together with a large portion of noncyclic glucan. The combination of both α-D-1→4 and α-D-1→6 glucosidic bonds found in large cyclodextrins produced by the action of potato D-enzyme on amylopectin, have also been found from the action of *Bacillus stearothermophilus* branching enzyme (E.C. 2.4.1.18) on amylose and amylopectin [56,57].

In the first report on the isolation of large cyclodextrins by Pulley and French (1961), high temperature cellulose column chromatography was used to isolate CD_9 to CD_{12} prepared from glycogen by use of *Bacillus macerans* CGTase [20]. Later highly pure preparations of CD_9 to CD_{21} have been prepared by Ueda and coworkers from commercial cyclodextrin powders, Dexypearl K-50 (Ensuiko Sugar Refining Co., Yokohama, Japan) and Celdex SG-30 (Nihon Shokuhin Kako Co., Tokyo, Japan) [22-27,53]. These isolation procedures included enzymatic treatment of the cyclodextrin powders with glucoamylase and pullulanase, followed by incubation with yeast, to remove linear oligosaccharides. Subsequently, α-, β-, and γ-cyclodextrin, were removed by precipitation with an organic compound. The large cyclodextrins were then isolated to purity by use of several chromatographic methods. Up to three different chromatographic steps were needed to isolate these molecules. Similarly, Koizumi and co-workers reported the purification of 23 large cyclodextrins ranging from CD_9 to CD_{31} by use of repeating reverse-phase chromatography [28]. Mixtures of large cyclodextrins can easily be obtained by use of gel filtration chromatography [28] or by a combination of exo-acting amylases, pullulanase and yeast, similar to the initial purification steps performed by Ueda and co-workers [22-27,53].

As evident from the published reports on the isolation of large cyclodextrins, a number of tedious chromatografic separation steps are required to obtain these compounds. In contrast, the small cyclodextrins α-, β-, and γ-cyclodextrin), are easily obtained through the enzymatic reaction of CGTase on starch, followed by precipitation of the individual cyclodextrin using an organic compound, often referred to as a selective complexant [58]. This procedure ensures a relatively cheap production of large quantities of these cyclodextrins, a major reason for their commercial success. In order to provide pure, large cyclodextrins at a reasonable cost, production schemes similar to those used for the small cyclodextrins have to be elaborated. However, so far, no selective complexants for the large cyclodextrins have been found. Akasaka and co-workers showed that CD_9 could be precipitated by use of macrocyclic compounds. γ-cyclodextrin and to some extent β-cyclodextrin also formed precipitates with these compounds [47]. Whether or not, these organic compounds would enable enhanced production of CD_9 in a production setup, still needs to be clarified.

Table 1. Nomenclature and some properties of cyclodextrins

Glycosyl units	Semisystematic name	Generic name[a]	Abbreviation	Molecular weight	Aqueous[d,e] solubility [g per 100 ml]	Surface tention[d,e] (mN/ m)	Specific[c] rotation $[\alpha]^{25}_D$	Half life of[c,f] ring opening (h)
6	cyclomaltohexaose	α-cyclodextrin	α-CD	972.9[b]	14.5	72	147.8	33
7	cyclomaltoheptaose	β-cyclodextrin	β-CD	1135.0[b]	1.85	73	161.1	29
8	cyclomaltooctaose	γ-cyclodextrin	γ-CD	1297.2[b]	23.2	73	175.9	15
9	cyclomaltononaose	δ-cyclodextrin	CD_9	1459.3[b]	8.19	73	187.5	4.2
10	cyclomaltodecaose	ω-cyclodextrin	CD_{10}	1621.4[b]	2.82	72	204.9	3.2
11	cyclomaltoundecaose	η-cyclodextrin	CD_{11}	1783.6[b]	>150	72	200.8	3.4
12	cyclomaltododecaose	θ-cyclodextrin	CD_{12}	1945.7[b]	>150	72	197.3	3.7
13	cyclomaltotridecaose	ι-cyclodextrin	CD_{13}	2107.9[b]	>150	72	198.1	3.7
14	cyclomaltotetradecaose	κ-cyclodextrin	CD_{14}	2270.0[b]	2.30	73	199.7±1.0	3.6
15	cyclomaltopentadecaose	λ-cyclodextrin	CD_{15}	2432.2[b]	>120	73	203.9±0.4	2.9
16	cyclomaltohexadecaose	μ-cyclodextrin	CD_{16}	2594.3[b]	>120	73	204.2±0.7	2.5
17	cyclomaltoheptadecaose	ν-cyclodextrin	CD_{17}	2756.4[b]	>120	72	201.0±0.6	2.5
18	cyclomaltooctadecaose	ξ-cyclodextrin	CD_{18}	2918.6[b]	-	-	-	-
19	cyclomaltononadecaose	ο-cyclodextrin	CD_{19}	3080.7[b]	-	-	-	-
20	cyclomaltoeicosaose	π-cyclodextrin	CD_{20}	3242.9[b]	-	-	-	-
21	cyclomaltoheneicosaose	-	CD_{21}	3405.0[b]	-	-	-	-
22	cyclomaltodoicosaose	-	CD_{22}	3567.2[c]	-	-	-	-
23	cyclomaltotricosaose	-	CD_{23}	3729.3[c]	-	-	-	-
24	cyclomaltotetracosaose	-	CD_{24}	3891.4[c]	-	-	-	-
25	cyclomaltopentacosaose	-	CD_{25}	4053.6[c]	-	-	-	-
26	cyclomaltohexacosaose	-	CD_{26}	4215.7[c]	-	-	-	-
27	cyclomaltoheptacosaose	-	CD_{27}	4377.9[c]	-	-	-	-
28	cyclomaltooctacosaose	-	CD_{28}	4540.0[c]	-	-	-	-
29	cyclomaltononacosaose	-	CD_{29}	4702.2[c]	-	-	-	-
30	cyclomaltotriacontaose	-	CD_{30}	4864.3[c]	-	-	-	-
31	cyclomaltohentriacontaose	-	CD_{31}	5026.5[c]	-	-	-	-
n	-	-	CD_n	n·162.14	-	-	-	-

[a][22-27], [b]calculated from the molecular formula and confirmed by mass spectroscopy [22-28], [c] calculated from the molecular formula and confirmed by mass spectroscopy [28], [d] observed at 25°C, [e] [25,51], [f] In 1M HCl at 50°C.

With respect to the biological role of cyclodextrin production by CGTase, it has been argued that the extracellular production of α- and β-cyclodextrin by various bacterial strains constitutes a unique uptake pathway for starch degradation products [59]. In *Klebsiella oxytoca* strain M5al only α- and β-cyclodextrin can be transported across the bacterial membranes [60-61]. As a consequence of this, the large cyclodextrins, including γ-cyclodextrin, cannot be utilised directly by this organism without extracellular rearrangement to linear oligosaccharides or α- and β-cyclodextrin. Furthermore, the enzyme responsible for the intracellular degradation of the cyclodextrins (cyclodextrinase, E.C. 3.2.1.54) displayed a low affinity towards γ-cyclodextrin and starch, compared to α- and β-cyclodextrin. It should be kept in mind that α- and β-cyclodextrin are not readily degradable by other amylolytic enzymes, e.g. α-amylases. The preference for α- and β-cyclodextrin as products/substrates for CGTase/cyclodextrinase producing bacteria can thus be viewed as means to reserve the energy contained in the starch [36,59]. Microorganisms lacking this specific cyclodextrin uptake pathway will only be able to utilise a minimum of the energy stored in α- and β-cyclodextrin. The large cyclodextrins do not represent a reserved pool of substrates, since their susceptibility to enzymatic degradation can be compared to that of starch. An extracellular production of large cyclodextrins will not give a CGTase producing microorganism an advantage over non-CGTase producing microorganisms. Furthermore, the specific environments in which the large cyclodextrin producing enzymes have to function, may hinder the formation of large cyclodextrins. The ability of large cyclodextrin producing enzymes, such as amylomaltase and potato D-enzyme to produce larger cyclodextrins with a very high degree of polymerisation *in vitro* from amylose, may be hampered *in vivo* by the presence of a large number of small acceptor molecules (e.g. glucose, sucrose and small maltooligosaccharides). The presence of a high amount of acceptor molecules will favour the inter-chain transglycosylation reactions of these enzymes and thus prevent the formation of cyclodextrins. The biological role of these enzymes is so far not clear [36]. The *in vitro* production of large cyclodextrins may not represent the *in vivo/in situ* production of cyclodextrins at all, nor, the dominant enzymatic activity of the large cyclodextrin producing enzymes *in vivo*.

Structures of large cyclodextrins

The solid state structures of CD_9 [22], CD_{10} [62-64] CD_{14} [62,63,65] and CD_{26} [66] have been reported. The detailed structural features of these solid state structures have been reviewed previously by Saenger and co-workers [67] and will only briefly be treated in this review.

In contrast to the annular shape displayed by the small cyclodextrins, the crystal structure of CD_9 displays a distorted elliptic boat-like shape, resulting in an elongated slit-like cavity [22]. CD_{10} and CD_{14} also display distorted structures, containing a ~180° rotation of two diametrically opposed glucosidic bonds (figure 1) [62-65,67]. The bonds between the glucose molecules at the two band-flipped sites are oriented in *trans* conformation, while the remaining glucosidic bonds retain the normal *cis* conformation. A similar double band-flip motif was found in the crystal structure of CD_{26} [66,67]. This molecule consisted of two parallel left-handed, single helices of almost two turns, connected by two band flipped glucosidic bonds. The structure of each helix resembled V-amylose with six glycosyl units pr. repeating unit.The band-flip motif has only been observed in these cyclodextrin crystal structures and has been suggested to occur to relieve steric strain [62,63,65]. Alternatively, it may be argued that the band-flips constitute an allowed structure, due to higher

conformational freedom of the macrocycle and thus not necessarily a strain induced conformation. Albeit, the band-flip motif reveals a new previously unknown structural feature of α-D-1→4 glucans, so far, no evidence, besides the crystal structures, has been obtained to support its existence in solution. By ^{13}C-NMR only one sharp signal pr. carbon atom has been obtained for the range of cyclodextrins from α-cyclodextrin to CD_{31} [23-28]. This indicates that the glycosyi units are identical on the NMR timescale. Increasing the number of glycosyl units in the macrocycle mainly affect the ^{13}Cl and ^{13}C4 signals (figure 2). For α-, β-, and γ-cyclodextrin, the ^{13}Cl and ^{13}C4 signals, occur at ~102 ppm and ~82 ppm, respectively. The resonances gradually shifts upward from CD_9 (^{13}Cl at 100.9 ppm and ^{13}C4 at 79.2 ppm) to CD_{10} and CD_{11} (^{13}Cl at 99.7 ppm and 99.8 ppm, respectively, and ^{13}C4 at 78.0 ppm and 78.3 ppm, respectively).

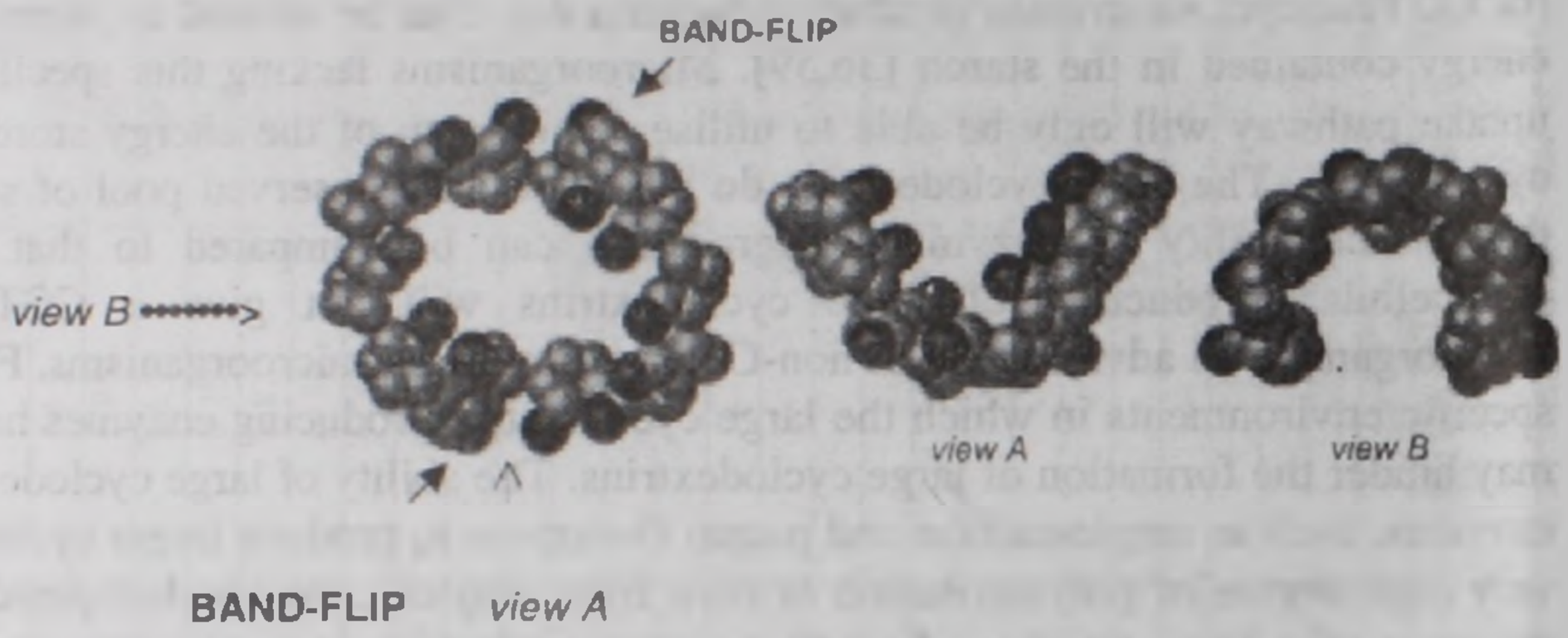

Figure 1. Solid state structure of CD_{14} showing the positions of the band-flips. Hydroxyl groups are omitted for clarity. C6 of glucose is highlighted in black.

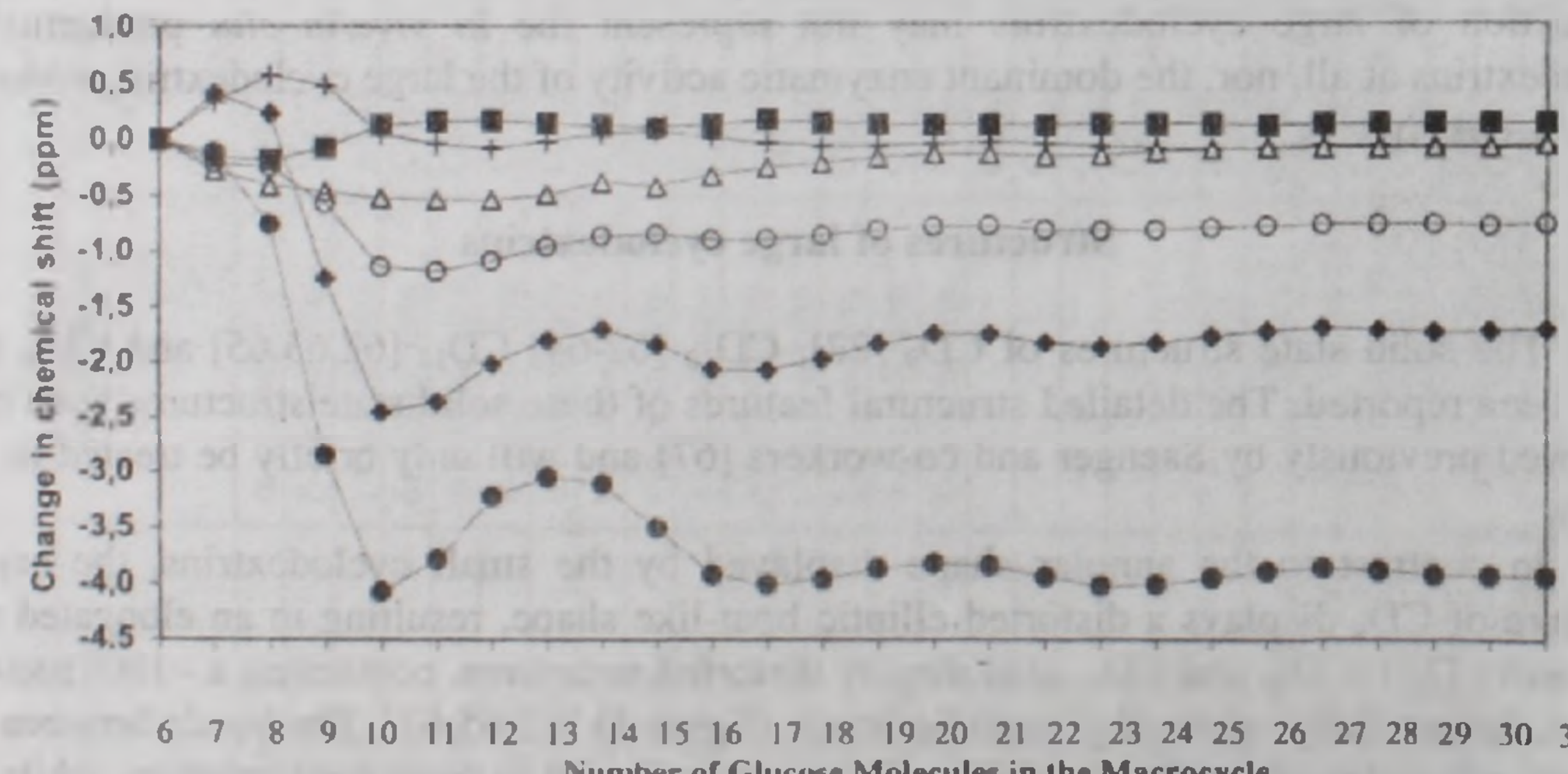

Figure 2. Changes in ^{13}C-NMR chemical shifts of cyclodextrins relative to a-cyclodextrin. •: ^{13}Cl; +: ^{13}C2; Δ: ^{13}C3; ●: ^{13}C4; ❍: ^{13}C5, ■: ^{13}C6. Data obtained from [28].

The small cyclodextrins and CD_{10} and CD_{11} represent two extremes with respect to ^{13}Cl and ^{13}C4 signals, while CD_9 seems to represent an intermediate (figure 2). For larger cyclodextrins these signals shift downward until CD_{14} (^{13}Cl at 100.5 ppm and ^{13}C4 at 78.9

ppm). Hereafter, a slight upward shift is observed until CD_{16} and CD_{17} (^{13}C1 at ~100.1 ppm and ^{13}C4 at ~78.1 ppm) followed by a slight downward shift that stabilises around ~100.4 ppm for ^{13}C1 and ~78.1 ppm for ^{13}C4. The ^{13}C-NMR signals observed for, especially CD_{13} and above, resemble those obtained for amylose (^{13}C1 at ~100.9 ppm and ^{13}C4 at ~78.6 ppm) [68]. This suggests a structural similarity with respect to the conformation of the glycosidic bond in these molecules.

As seen in figure 2, the change in ^{13}C-NMR chemical shifts of the large cyclodextrins above CD_{10}, especially for the ^{13}C1 and ^{13}C4 signals, resembles a damped oscillation with a periodicity of approximately seven. This indicates some structural periodicity in the large cyclodextrins. The major factor determining the structures of these molecules is the torsional angles ϕ and Ψ, describing the rotation around C1 -O4 and O4-C4 bonds in the glucosidic linkage. These angles are rather restricted due to hydrogen bonding between adjacent glycosyi units [67]. Thus, as also suggested by the various models presented on possible structures of large cyclodextrins, including the crystal structures, the large cyclodextrins most likely adopt curvatures in solution resembling those found for the small cyclodextrins and V-amylose. Two proposals for the structures of large cyclodextrins, containing glucosidic bonds in *cis* conformations only, in solution, have been put forward [69]. A circularised single helical and an anti-parallel double helical form with foldbacks at each end (figure 3). Molecular modelling of these two structures indicated that the double helical form was the most likely structure of the two. In contrast, studies on the conformation of CD_{21} in solution by small-angle X-ray scattering with molecular modelling simulations showed that this molecule most likely adopted a circularised three-turn single helical structure with a radius of gyration of 11.5 Å[70]. The circularised single helical form of e.g. CD_{14} in solution, may resemble a twisted eight (8), containing two pseudo-cavities with sizes comparable to those of α- and β-cyclodextrin. If more glucose molecules are introduced in this twisted eight, additional strain will be introduced in the molecule. With the addition of more glucose molecules in the cyclodextrin structure, the molecule will be able to adopt a more unstrained three-clover structure as indicated for CD_{21}, a possible explanation for the periodicity of approximately seven in the ^{13}C NMR chemical shift signals. The accumulated conformational freedom of the torsion angles will prevent conformational strain upon addition of more glucose molecules (glucosidic bonds). This may explain why the ^{13}C NMR chemical shift signals of large cyclodextrins (CD_{24}-CD_{31}) become relatively invariant and equal to those found for their linear counterpart, amylose. Similar arguments may be put forward for the anti-parallel double helical model, as well as models containing band-flips.

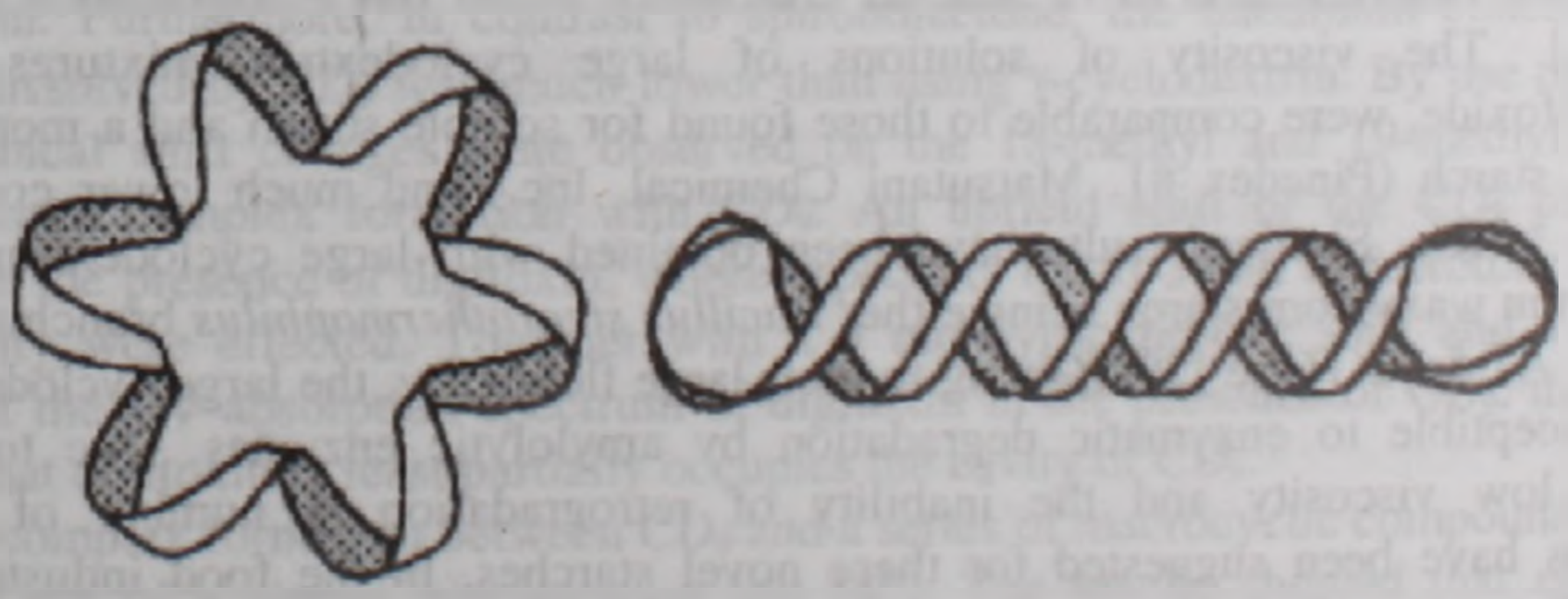

Figure 3. Schematic representations of two possible conformations of CD_{48}. Left: circularised single helical form right: anti-parallel double helical form with foldbacks at each end. Modified from [69].

Whether, the band-flip motif observed in the crystal structure of CD_{10}, CD_{14} and CD_{26} is a special feature resulting from crystal packing effects or it is a common, yet transient, structural feature in α-D-1→4 glucans in solution, remains to be determined. Nevertheless, the studies presented on the large cyclodextrins have revealed that their structures are very different, going from the small rigid, α-, β- and γ-cyclodextrin, over the intermediary types e.g. CD_9-CD_{13} to the very large cyclodextrins, probably resembling circularised amylose. In the few cases where it has been possible to obtain crystal structures of the large cyclodextrins, interesting new structural features have come up. However, in contrast to the crystal structures of the small cyclodextrins, which are often regarded as representative frozen images of their conformation in solution, the crystal structures of CD_9, CD_{10}, CD_{14} and CD_{26} can hardly be taken as representative structures. Owing to the large flexibility of these molecules, a very wide range of structural conformations, possibly including band-flips, can be expected to occur in solution.

Properties of large cyclodextrins

With the exceptions of CD_9, CD_{10} and CD_{14}, the aqueous solubility of the large cyclodextrins is very large compared to their linear counterparts. The solubilities of CD_9, CD_{10}, and CD_{14} are intermediary to those found for α- and β-cyclodextrin (table 1). While the solubility of CD_{11} to CD_{13} and CD_{15} to CD_{17} exceeds 150 g and 120 g per 100 ml, respectively, the solubility of CD_{14} and CD_{10} is very low (2.30 g and 2.82 g per 100 ml, respectively), comparable to that of β-cyclodextrin (1.85 g per 100 ml) [51]. The acid catalysed hydrolysis rate for α-through CD_{17} (table 1), indicates that the stability of the macrocyclic ring decreases with increasing number of glycosyl units, probably owing to increased flexibility and a higher number of decomposition points (α-D-1→4 linkages) [23,51].

The solubility of two different large cyclodextrin fractions, containing only α-D-1→4 glucosidic bonds, (obtained using potato D-enzyme on amylose and amylopectin, respectively) exceeded 1 g per 100 ml at 0, 30, 60 and 100°C [31]. In comparison, only 0.12, 0.58 and 0.08 g per 100 ml of, amylose, waxy corn starch (containing mainly amylopectin) and soluble starch, respectively, could be solubilised at 0°C. These glucans only achieved comparable solubility at 100°C. The large cyclodextrin fractions exhibited no retrogradation after 20 h at 4°C, in comparison to amylose, waxy corn starch and soluble starch where significant retrogradation could be detected after only 3 h. Moreover, the large cyclodextrin mixtures were demonstrated to be able to effectively retard the retrogradation of soluble starch [31]. The viscosity of solutions of large cyclodextrin mixtures in 90% dimethylsulfoxide, were comparable to those found for soluble starch and a more strongly hydrolysed starch (Pinedex #1, Matsutani Chemical, Inc.) and much lower compared to waxy corn starch. Similar results have been obtained with large cyclodextrin fractions prepared from waxy corn starch using either *Bacillus stearothermophilus* branching enzyme or *Bacillus* A2-5a CGTase [29]. Owing to their large flexibility, the large cyclodextrins are readily susceptible to enzymatic degradation by amylolytic enzymes. Due to the high solubility, low viscosity and the inability of retrogradation, a number of industrial applications have been suggested for these novel starches. In the food industry as high energy additive to soft drinks, retrogradation retardant in breads, for bread improvement, freeze resistant jellies and for production of non-sticky rice [31,71-75]. Furthermore, several non-food applications have been suggested, including paper coating material and as starch substitutes in adhesives and biodegradable plastics [29,31,76].

Miyazawa and co-workers revealed the improved toxicological behaviour of CD_9, which in contrast to α-, β- and γ-cyclodextrin did not cause any hemolysis of human erythrocytes at a concentration of 40 mM [23]. In comparison, the zero-effect level for α-, β- and γ-cyclodextrin was approximately 5, 1.5 and 16 mM, respectively. This suggests that CD_9 can be used safely in parenteral dosage formulations. However, the reduced affinity for membrane components may also reflect its general low ability to form inclusion complexes.

Inclusion complex formation

The key to the successful applications of the small cyclodextrins α-, β-, and γ-cyclodextrin) lies in their ability to form inclusion complexes with a large number of molecules [6-8]. In contrast to the numerous studies on the small cyclodextrins, the complex forming properties of the vast range of large cyclodextrins is still to a great extent unknown. Ueda and co-workers have in a series of papers studied the complex forming behaviour between CD_9 and a large range of guest molecules [23,40,44,47]. In their first study [23] it was shown that CD_9 is able to increase the solubility of digitoxin and spironolactone more efficiently than α-cyclodextrin, although to a lesser extent compared to β- and γ-cyclodextrin. Phase solubility studies showed that CD_9 was capable of dissolving more or at least equal amounts of spironolactone, compared to the maximal amount dissolvable by β-, and γ-cyclodextrin, although the concentration of CD_9 needed to achieve this was approximately 10 and 5 times higher, respectively. Assuming a 1:1 complex stochiometry, the stability constants between spironolactone and the cyclodextrins were calculated from the solubility diagrams as 13000 M^{-1}, 3900 M^{-1} and 820 M^{-1} for β-, γ- and CD_9, respectively. Since CD_9 in its crystal structure displays an enlarged cavity compared to γ-cyclodextrin, its solubilising power on a range of relatively large, slightly water-soluble drugs was tested [44]. For the seven drugs tested no significant solubilising power of CD_9 was observed. With the exception of [2,2]-paracyclophane, similar results were observed for α-, β-, and γ-cyclodextrin. A comparative solubility study of digitoxin and six related drugs was performed [44]. Apart from digitoxin, only a slightly enhanced solubility could be observed using CD_9. In most cases, the solubility enhancement of CD_9 was order of magnitudes lower than that obtained with β-, and γ-cyclodextrin. From phase solubility diagrams the complex stability constants between digitoxin and β-, γ- and CD_9 were calculated as 63000 M^{-1}, 33000 M^{-1} and 1700 M^{-1} , respectively. As observed for spironolactone, the stability constant between CD_9 and digitoxin is much lower compared to those obtained with β- and γ-cyclodextrin. Furthermore, in contrast to spironolactone, the maximum concentration of digitoxin dissolved by CD_9 was much lower than using γ-cyclodextrin. By use of ^{1}H-NMR, small chemical shift changes were observed on the 18-methyl and 19-methyl groups of digitoxin upon complex formation with CD_9. An upfield shift of the CD_9 protons was observed in the presence of digitoxin, where especially the H-3 and H-5 protons positioned in the cavity were effected. Together with a 3 nm hypsochromic shift and a decreased intensity of the UV absorption spectrum of digitoxin in the presence of CD_9, these results indicated that digitoxin at least partially occupies the cavity of CD_9.

The complex formation between CD_9 and a series of macrocyclic compounds has been studied by use of a simple precipitation test [47]. The results showed that macrocyclic compounds containing from 11 to 15 carbon atoms (cycloundecanone, cyclododecanone, cyclotridecanone and cyclopentadecanone) were able to precipitate CD_9 under the given experimental conditions. In contrast, α-cyclodextrin only formed precipitates with smaller

macrocyclic compounds containing 8 to 10 carbon atoms (1,5-cyclooctadiene, cyclononanone and cyclodecanone). β- and γ-cyclodextrin formed precipitates with all the studied macrocyclic compounds in higher or comparable amounts than with α-cyclodextrin and CD_9, except for cyclopentadecanone where CD_9 formed of the highest amount of precipitate. However, as the mechanisms of precipitate formation of cyclodextrins with organic molecules as well as their structures are unknown at present, the formation of precipitates can not be taken as evidence for inclusion complex formation. Nevertheless, since cyclodextrins with increasing cavity size form precipitates with macrocycles compounds increasing in size, the role of the cavity as host for these compounds seems obvious. By use of powder X-ray diffraction and differential scanning calorimetry analysis of the complex between CD_9 and cyclododecanone the formation of a solid inclusion complex was substantiated [47].

Furuishi and co-workers reported the formation of a water-soluble complex of C_{70} Buckminsterfullerene with CD_9 [40]. Although, γ-cyclodextrin has been shown to form a water soluble complex with C_{60} Buckminsterfullerene with a 2:1 (γ-cyclodextrin : C_{60}) binding stochiometry [77], only CD_9 was found to effectively solubilise C_{70}.

The complex formation of large cyclodextrins in the range CD_9 to CD_{17} with small aromatic anions has been studied by use of capillary electrophoresis [41,42,46]. Although it was possible to measure reliable stability constants, assuming a 1:1 binding stoichiometry, for a large range of small aromatic anions (mostly benzoic acid derivatives) and the large cyclodextrins, only *4-tert.*-butyl benzoate and the ibuprofen anion yielded stability constants above 50 M^{-1} (Figure 4).

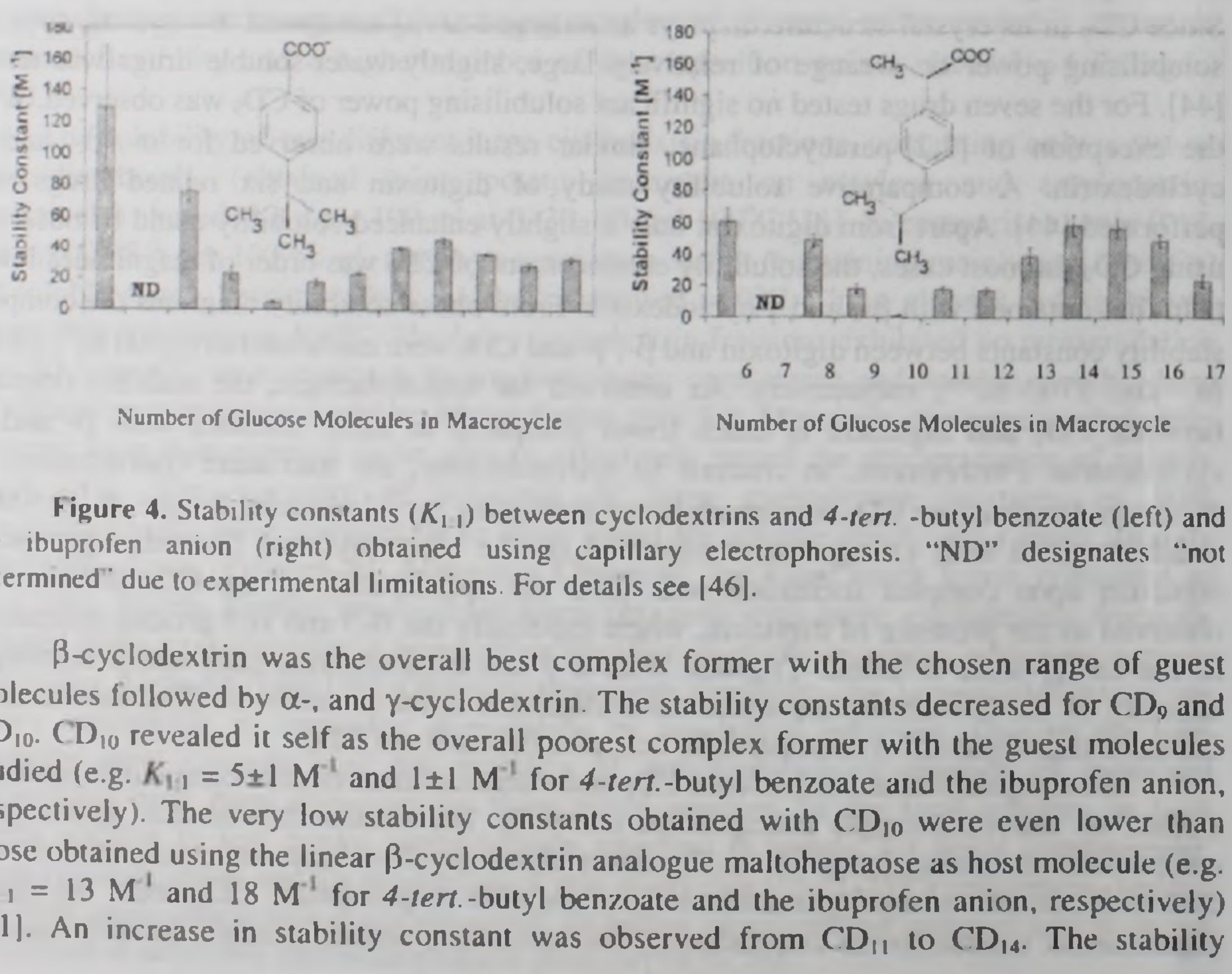

Figure 4. Stability constants ($K_{1:1}$) between cyclodextrins and *4-tert.* -butyl benzoate (left) and the ibuprofen anion (right) obtained using capillary electrophoresis. "ND" designates "not determined" due to experimental limitations. For details see [46].

β-cyclodextrin was the overall best complex former with the chosen range of guest molecules followed by α-, and γ-cyclodextrin. The stability constants decreased for CD_9 and CD_{10}. CD_{10} revealed it self as the overall poorest complex former with the guest molecules studied (e.g. $K_{1:1}$ = 5±1 M^{-1} and 1±1 M^{-1} for *4-tert.*-butyl benzoate and the ibuprofen anion, respectively). The very low stability constants obtained with CD_{10} were even lower than those obtained using the linear β-cyclodextrin analogue maltoheptaose as host molecule (e.g. $K_{1:1}$ = 13 M^{-1} and 18 M^{-1} for *4-tert.*-butyl benzoate and the ibuprofen anion, respectively) [41]. An increase in stability constant was observed from CD_{11} to CD_{14}. The stability

constant observed for the complex between *4-tert.*-butyl benzoate and CD_{14} was approximately one third and half of those obtained with α- and γ-cyclodextrin, respectively. Using the ibuprofen anion as guest molecule both, CD_{14}, CD_{15}, and CD_{16} were able to match the stability constants obtained with α- and γ-cyclodextrin. A slight decrease in stability constant was observed for the complexes between both *4-tert.*-butyl benzoate and the ibuprofen anion and CD_{15}, CD_{16} and CD_{17} compared to CD_{14} (figure 4). A completely different pattern was observed with salicylate as guest molecule. With the exception of β-cyclodextrin ($K_{1:1}$ = 215±54 M^{-1}), all cyclodextrins ranging from α-cyclodextrin to CD_{17} gave weak stability constants ranging from 11 to 17 M^{-1} with a deviation no higher than 2 M^{-1}. This suggests that salicylate forms complexes with these cyclodextrins by a different mechanism than *4-tert.*-butyl benzoate and the ibuprofen anion. It may be that salicylate forms hydrogen bonds with the water-exposed hydroxyl groups of the cyclodextrins, enabling weak interactions irrespective of the size and structure of the cyclodextrin. In contrast, *4-tert.*-butyl benzoate and the ibuprofen anion were unable to form complexes in a similar way, as judged from the lack of an interaction with CD_{10}.

Kitamura and co-workers studied the complex formation of CD_{21} to CD_{31} with iodine using isothermal titration calorimetry [43]. The titration data could not be described assuming 1:1 complex formation. A more elaborate model assuming 1:2 complex formation with identical interacting sites was employed instead. The data suggested that this range of large cyclodextrins is able to accommodate two iodine molecules. The stability constants obtained, K_1 and K_2, defined relative to the progress of saturation, ranged from 700 to 7300 M^{-1} and 3000 to 62600 M^{-1}, respectively. The calorimetric data revealed that the complex formation was accompanied by a large decrease in entropy. This was attributed to a relatively large decrease in conformational flexibility of the large cyclodextrin upon complex formation. For a mixture of larger cyclodextrins with an average degree of polymerisation of 120 very large stability constants, comparable to those found for linear amylose, were obtained (K = 1330000 M^{-1}), using a model assuming independent binding of iodine to multiple sites. This result indicated that very large cyclodextrins would allow a local conformation of the polysaccharide chain comparable to those of long-chain linear amylose.

The presumed resemblance of the complex forming ability of the very large cyclodextrins (DP>50) to that of linear amylose has been further substantiated by Takaha and co-workers [31,36]. By a simple precipitation study it was demonstrated that large cyclodextrins with a degree of polymerisation larger that 50, was able to form complexes with butanol, octanol and oleic acid. Furthermore, a fluorescence enhancement of 8-anilino-1-naphthalene sulfonic acid (ANS) higher than obtainable by α-cyclodextrin, was achieved by use of a mixture of large cyclodextrins [31]. This indicated the formation of an inclusion complex. The inclusion complex forming property of mixtures of very large cyclodextrins (DP 22 to 45 and DP>50, respectively) was demonstrated to be efficient in detergent-mediated refolding of proteins [45]. The large cyclodextrin mixtures, especially the DP>50 mixture, was able to strip detergent molecules of unfolded protein-detergent complexes, thus allowing the protein molecules to refold to their proper, folded, active state. In combination with several detergents, comparable or higher refolding yields could be obtained using the large cyclodextrin mixtures, compared to α-, β- and γ-cyclodextrin. It was shown that the rate of Tween 60 mediated refolding of porcine heart citrate synthase was higher using the large cyclodextrin mixtures as detergent stripping agent compared to β-cyclodextrin. This is not surprising, since β-cyclodextrin can stabilise the unfolded (usually hydrophobic) form of proteins and thus retard the refolding process [78].

The data presented on the complex forming properties of the large cyclodextrins have shown that they, dependent on their size, are able to complex a variety of molecules. With the exception of the highly soluble complex between Buckminsterfullerene C_{70} and CD_9, the complex forming strength of the large cyclodextrins has so far proved to be inferior to the small cyclodextrins, α-, β- and γ-cyclodextrin. Furthermore, direct evidence for inclusion complex formation has so far not been presented. Only in the case of the digitoxin/CD_9 complex, have preliminary NMR results suggested, that the drug molecule reside in the cyclodextrin cavity [44]. Nevertheless, judged on the data presented, the role of the cavity in complex formation by these molecules seems obvious. Since the strength of guest-host complexes relies, at least partially, on multiple weak interactions (e.g. van der Waals interactions, hydrogen bonding, dipole-dipole interactions and hydrophobic effects) size compatibility between the cyclodextrin cavity and the guest molecule is needed in order to form strong inclusion complexes [6,7,13,18,79]. The inclusion complex forming ability of the large cyclodextrins varies greatly according to their size, suggesting that they, just as α-, β- and γ-cyclodextrin, are able to present more or less suitable cavities dependent on the size and structure of the guest molecules. For example, the increased stability constants observed for the complexes formed between 4-*tert*.-butyl benzoate and the ibuprofen anion and CD_{11} to CD_{17}, indicates that these molecules are able to present a more suitable cavity for small guest molecules, compared to CD_9 and CD_{10} [46]. This suggests that an increased flexibility of these molecules allows them either to present a more suitable cavity prior to complex formation or adapt to the guest molecules by an induced fit mechanism. On the other hand, CD_{10} is unable to present a suitable cavity, probably due to limitations in the tortional angles of the glucosidic bonds. The rise and decrease in stability constant observed for the complexes formed between CD_{11} to CD_{17} with 4-*tert*.-butyl benzoate and the ibuprofen anion, respectively, correlates well with the changes in ^{13}C NMR chemical shifts of especially $^{13}C1$ and $^{13}C4$. Both guest molecules have geometries suitable for the β-cyclodextrin cavity. Somehow the large cyclodextrins must be able to present a pseudo-cavity of similar size, in order to form stable inclusion complexes. If we consider the twisted eight model for CD_{14}, two β-cyclodextrin-like pseudo-cavities may be available for complex formation. As discussed above, the introduction of more glycosyl units may increase the strain of the molecule due to tortion angle limitations. This increased strain may cause the formation of a weaker complex, as indicated by the experimental data. A study of the complex formation between even larger cyclodextrin (CD_{18}-CD_{23}) and these or similar guest molecules may confirm this hypothesis, as it may be expected that the 1:1 stability constants will display a local maximum around CD_{20}-CD_{21}.

It has previously been argued that the large cyclodextrins would form very weak complexes due to an anticipated enlarged cavity [6]. Since a large cavity would be occupied by a higher number of water molecules compared to α-, β- and γ-cyclodextrin and thus resemble the bulk water, the presumed driving force for complex formation of "high energy" water residing in the cyclodextrin cavity, would be minimal. Nevertheless, the structural information obtained for the large cyclodextrins indicate that their cavities are only minimally enlarged compared to the small cyclodextrins. Albeit, there is different opinions on whether or not "high energy" water in the cavity should be regarded as a major driving force for complex formation [13,18,79], the reduction of this driving force, going from small to large cyclodextrins, may not be as large as expected.

As demonstrated by Kitamura and co-workers [70] the complex formation between a guest molecule (in this case iodine) and large cyclodextrins involves a large unfavourable entropy change that may be caused in part by a decrease in the conformational flexibility of

the cyclodextrin. Even with the less flexible α-, β-, and γ-CD, complex formation most often result in unfavourable entropy changes, due to the loss of transitional and rotational freedom of both guest and host molecules. However, in the case of the more flexible large cyclodextrins the entropic penalty for complex formation may be even greater, a probable cause for the low stability constants observed. Nevertheless, since the large cyclodextrins are able to present a variety of cavity sizes, compared to the small cyclodextrins, they may be useful for special applications, illustrated by the solubilisation of C_{70} Buckminsterfullerene. Moreover, it is very likely that the large cyclodextrins will be able to display more than one cavity and in the case of very large cyclodextrins even a nanotube-like cavity. The inclusion complex forming properties of the very large cyclodextrins (e.g. DP>50) is very likely comparable to that of their linear counterpart amylose. However, owing to their much higher solubility, lower viscosity and inability to retrograde, they may prove to be valuable for complexation of e.g. long chain fatty acids, alcohols and detergents, as demonstrated by Machida and co-workers [45].

Outlook

The large cyclodextrins represent an interesting class of molecules within the field of macrocyclic and supramolecular chemistry and may be a key to increased understanding of the process of inclusion complex formation, particularly with respect to the effect of flexibility on guest binding. In order to be able to perform comparative studies on properties and applicability of the large cyclodextrins novel production/purification methods are needed. An ideal purification scheme may include the use of selective complexants specific for a particular large cyclodextrin, e.g. CD_{18}. This is however not very likely, since the inclusion complex forming properties of the large cyclodextrins may be regarded as very similar due to the higher flexibility of the macrocycle, compared to the much more rigid α-, β-, and γ-CD. However, one may not rule out the use of selective complexants for the purification of CD_9, since this intermediary form may still retain some rigidity of the macrocycle and thus a more defined and unique cavity. In the case of, particularly, CD_{10} and CD_{14} their low aqueous solubility may be utilised as means of purification. Although purification of the individual large cyclodextrins will pose a great challenge, mixtures of large cyclodextrins may be sufficient in many applications.

The discovery of the production of large cyclodextrins by CGTases shreds new light on the action pattern of these enzymes. Thus, CGTases are not, in contrast to the common belief, exo-acting enzymes. This observation requires a re-evaluation of the enzymatic actions of CGTases on starch, especially on the accumulation of the small cyclodextrins α-, β-, and γ-CD.

Looking beyond the unique properties of the small cyclodextrins with respect to inclusion complex formation, cyclodextrins are still derivatives of one of our prime carbohydrate sources, starch. The small cyclodextrins, especially α- and β-cyclodextrin, have limited use as nutrients because of their toxicological behaviour. In contrast, the present knowledge of the large cyclodextrins suggests that they are without any significant toxicity and that nutritionally they can be regarded as starch. Furthermore, the large cyclodextrins display very high solubility and low viscosity compared to the starch constituents, amylopectin and amylose. These features may be due to the cyclic structure of the large cyclodextrins, which would hinder the formation of stable intermolecular complexes. This effect has also been observed as an inability to retrograde and its ability to prevent the retrogradation of ordinary starches in e.g. wheat bread. These features suggest numerous

uses of large cyclodextrins mixtures in the industry as evidenced by the growing number of patents.

Acknowlegdements. Dr. Marilyn Wiebe, Dr. Niels Thomas Eriksen, Dr. Lars Haastrup Pedersen, Mr. Finn Aackmann and Mr. Brian Mogensen are acknowledged for technical help and helpful suggestions to the manuscript. The atomic coordinates of CD14 was kindly provided by Dr. Katrin Gessler.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

PHYSICOCHEMICAL PROPERTIES OF LARGE-RING CYCLODEXTRINS (CD_{10}~CD_{17}) [1]

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Cyclomaltodecaose (CD_{10}), cyclomaltoundecaose (CD_{11}), cyclomaltododecaose (CD_{12}), cyclomaltotridecaose (CD_{13}), cyclomaltotetradecaose (CD_{14}), cyclomaltopentadecaose (CD_{15}), cyclomaltohexadecaose (CD_{16}) and cyclomaltoheptadecaose (CD_{17}) are cyclic oligosaccharides composed of 10, 11, 12, 13, 14, 15, 16 and 17 D-glucose units, respectively. This report describes the physicochemical properties of CD_{10}, CD_{11}, CD_{12}, CD_{13}, CD_{14}, CD_{15}, CD_{16} and CD_{17}, in terms of aqueous solubility, surface tension, specific rotation and acid-catalyzed hydrolysis.

Цикломальтодекаоза (CD_{10}), цикломальтоундекаоза (CD_{11}), цикломальтододекаоза (CD_{12}), цикломальтотридекаоза (CD_{13}), цикломальтотетрадекаоза (CD_{14}), цикломальтопентадекаоза (CD_{15}), цикломальтогексадекаоза (CD_{16}) и цикломальтогептадекаоза (CD_{17}) - циклические олигосахариды, состоящие из 10, 11, 12, 13, 14, 15, 16 и 17 D-глюкозных единиц соответственно. В сообщении описываются физико-химические свойства CD_{10}, CD_{11}, CD_{12}, CD_{13}, CD_{14}, CD_{15}, CD_{16} и CD_{17} циклодекстринов с точки зрения растворимости в воде, поверхностного напряжения, специфического вращения и кислото-катализируемого гидролиза.

Ցիկլոմալտոդեկաոզան (CD_{10}), ցիկլոմալտոունդեկաոզան (CD_{11}), ցիկլոմալտոդոդեկաոզան (CD_{12}), ցիկլոմալտոտրիդեկաոզան (CD_{13}), ցիկլոմալտոտետրադեկաոզան (CD_{14}), ցիկլոմալտոպենտադեկաոզան (CD_{15}), ցիկլոմալտոհեքսադեկաոզան (CD_{16}) և ցիկլոմալտոհեպտադեկաոզան (CD_{17}) ցիկլիկ օլիգոսախարիդներ են, կազմված համապատասխանաբար 10, 11, 12, 13, 14, 15, 16 և 17 D-գլյուկոզի միավորներից: Այս հաղորդության մեջ նկարագրվում են CD_{10}, CD_{11}, CD_{12}, CD_{13}, CD_{14}, CD_{15}, CD_{16} և CD_{17} ցիկլոդեքստրինների ֆիզիկաքիմիական առանձնահատկությունները ջրում լուծելիության, մակերեսային լարվածության, յուրահատուկ պտտման և թթվով կատալիզվող հիդրոլիզի տեսանկյունից:

Introduction

Cyclodextrin (CD) is a cyclic oligosaccharide produced by cyclodextrin glucanotransferase (CGTase), and α-CD (CD_6), β-CD (CD_7), γ-CD (CD_8) and their derivatives have been well studied. With regard to large-ring CDs (LR-CDs) composed of more than 9 D-glucose units, we have reported a method for preparation and purification from commercially available CD powder [2-5]. In particular, we characterized the physicochemical properties and inclusion complex formation abilities of CD_9 composed of 9 D-glucose units with several guest molecules [2,6,7]. Furthermore, inclusion complex formation constants of CD_{10}, CD_{11}, CD_{12} and CD_{13}, composed of 10, 11, 12 and 13 D-glucose units, respectively, were determined by capillary zone electrophoresis [8]. However, it is difficult to investigate the physicochemical properties and complexation abilities of

LR-CDs with more than 10 D-glucose units in detail, because of the low yields of LR-CDs prepared using commercially available CD powder as the starting material. Recently, it was reported that LR-CDs were preferentially produced in the initial stage of CGTase cyclization reaction and were subsequently converted into smaller CDs [9]. In this study, LR-CD (CD_{10}~CD_{17}) were prepared and purified from CD powder produced by the initial action of CGTase mentioned above. Their physicochemical properties, i. e. aqueous solubility, surface activity, specific rotation, and acid-catalyzed hydrolysis rate, were elucidated in detail in comparison with those of conventional α-, β-, γ-CD and other LR-CDs (CD_9).

Materials and methods

Materials. α- and β-CD were gifts from Nihon Shokuhin Kako Co., Ltd. (Tokyo, Japan). γ-CD was supplied by Wacker Chemie GmbH (Munich, Germany). They were used after recrystallization from water. Other chemicals were obtained from commercial sources and were used without further purification. Milli-Q Water (Milford, MA, USA) was used in all experiments.

Method for production and purification of LR-CD (CD_{10}~CD_{17}). The production of LR-CD powder by the initial action of CGTase on synthetic amylose was carried out as described previously [9]. The CD mixture obtained was dissolved in water. Then, tetrachloroethane and bromobenzene were added and shaken at 4°C for 20h to remove α-, β- and γ-CD. After centrifugation, the supernatant was subjected to HPLC to separate CD_{10}, CD_{11}, CD_{12}, CD_{13}, CD_{14}, CD_{15}, CD_{16} and CD_{17}. HPLC consisted of two main steps using an ODS column and an amino column. The details of the purification conditions and identification of purified products were almost the same as described previously [2]. The yields of each LR-CD (CD_{10}~CD_{17}) were as follows: CD_{10}, 1.33%; CD_{11}, 1.20%; CD_{12}, 1.34%; CD_{13}, 1.01%; CD_{14}, 0.58%; CD_{15}, 0.39%; CD_{16}, 0.36%; CD_{17}, 0.35%.

Physicochemical properties of CDs [2,10,11]. *Solubility.* For the solubility measurement of CD_{10} and CD_{14}, saturated solutions of CD_{10} and CD_{14} were prepared according to the standard procedure at 25°C. The supernatants were subjected to HPLC on an ODS column (YMC-Pack ODS-AQ, 4.6Φ×250mm) with methanol-water as the mobile phase (3:97 for CD_{10}, 4:96 for CD_{14}) at a flow rate of 1.0mL/min at 30°C. The solubilities of CD_{11}, CD_{12}, CD_{13}, CD_{15}, CD_{16} and CD_{17} were determined using other methods. Water was carefully added to a glass vessel containing 100mg of LR-CDs. The quantity of water varied progressively from 0.01 to 0.1mL. The samples were vigorously shaken for 1min at 10min intervals at 25°C. The cycle was continued until CDs had dissolved completely. The total volume of water added was measured, and the saturated solubility was calculated.

Surface tension. Surface tension measurements were taken on a Wilhelmy surface tensiometer (Kyowa Kaimenkagaku Co., Ltd., Tokyo, Japan) with an accuracy of ± 0.2mN/m. The glass vessels used were treated with 20% sulfuric acid before each measurement.

Specific rotation. Specific rotation measurements were taken on a SEPA-200 digital polarimeter (Horiba, Kyoto, Japan) with an accuracy of ± 0.002. The digital polarimeter was calibrated with sucrose solution before measurement.

Acid-catalyzed hydrolysis of CDs. Aliquots of 100mg of each CD were dissolved in 5mL of 1mol/L HCl, and the reaction solution was heated in a water bath at 50°C. Samples of the reaction solution were taken at appropriate intervals and neutralized by addition of 1mol/L NaOH containing an internal standard for HPLC. The samples were determined quantitatively on HPLC. The internal standards used were as follows: γ-CD for measurement of α-CD, β-CD, CD_{10}, CD_{11}, CD_{12}, CD_{13}, CD_{14}, CD_{15}, CD_{16}; α-CD for measurement of CD_{17}; α-CD for measurement of γ-CD; and β-CD for measurement of CD_9. HPLC was conducted under the following conditions: column, YMC-Pack ODS-AQ (4.6Φ×250mm); eluent, methanol-water (3:97 or 4:96); flow rate, 1.0mL/min; column temperature, 30°C.

Results and discussion

Solubility. Table 1 lists the physicochemical properties of α-, β-, γ-CD and LR-CDs. The aqueous solubilities of CDs increased in the order of: β-CD < CD_{14} < CD_{10} < CD_9 < α-CD < γ-CD < CD_{15} ≈ CD_{16} ≈ CD_{17} < CD_{11} ≈ CD_{12} ≈ CD_{13}. The solubility of CD was suggested to be correlated with differences in structural flexibility [12]. The low solubility of β-CD may be a consequence of the rigid structure caused by intramolecular hydrogen bonds and its high crystal lattice energy [13]. In β-CD, all glucose residues are in *syn* orientation, forming systematic interglucose $O(3)_n \cdots O(2)_{n+1}$ hydrogen bonds. On the other hand, the molecular structure of CD_{14} is characterized by typical "band flip" in which diametrically opposed glucose residues are in *anti* rather than in the common *syn* orientation, this conformation being stabilized by interglucose $O(3)_n \cdots O(6)_{n+1}$ hydrogen bonds [5,14]. X-ray crystal structure analysis of CD_{10} also revealed that it had the band flip structure [15]. In addition, CD_{10} and CD_{14} are as readily crystallized from aqueous solution as β-CD [5,14,15]. The crystallization may be caused by intermolecular hydrogen bonds. Therefore, the relatively low solubilities of CD_{10} and CD_{14} may be a consequence of intramolecular and intermolecular hydrogen bonds.

The aqueous solubilities of CD_{15}, CD_{16} and CD_{17} were as high as those of CD_{11}, CD_{12} and CD_{13} in contrast to those of α-, β- and γ-CD. Intra- and intermolecular hydrogen bonds are difficult to form in CD_{11}, CD_{12}, CD_{13}, CD_{15}, CD_{16} and CD_{17}. In general, the nucleation rates, which must precede crystal growth, of sugars are very low, so supersaturated solutions of sugars are often syrup-like liquids [16,17]. The solubilities of CD_{11}, CD_{12}, CD_{13}, CD_{15}, CD_{16} and CD_{17} could not be measured precisely, because the solubility behaviors of CD_{11}, CD_{12}, CD_{13}, CD_{15}, CD_{16} and CD_{17} were similar to those of sugars.

Table 1. Physicochemical Properties of CDs

	Glucose unit	Aqueous[a] solubility (g/100mL)	Surface[a] tension (mN/m)	Specific rotation $[\alpha]_D^{25}$	Half-life of[b] ring opening (h)
α-CD	6	14.5[c]	72	+147.8[d]	33[d]
β-CD	7	1 85[c]	73	+161.1[d]	29[d]
γ-CD	8	23.2[c]	73	+175.9[d]	15[d]
CD_9	9	8 19[c]	73	+187.5[d]	4.2[d]
CD_{10}[d]	10	2.82	72	+204 9	3 2
CD_{11}[d]	11	>150	72	+200 8	3 4
CD_{12}[d]	12	>150	72	+197.3	3.7
CD_{13}[d]	13	>150	72	+198 1	3.7
CD_{14}	14	2 30	73	+199.7	3.6
CD_{15}	15	>120	73	+203.9	2 9
CD_{16}	16	>120	73	+204.2	2.5
CD_{17}	17	>120	72	+201.0	2.5

a) Observed at 25 C
b) In 1mol/L at 50 C
c) I.Miyazawa, H. Ueda, H. Nagase, T. Endo, S. Kobayashi, T. Nagai. Eur. J. Pharm., 3, 153-162 (1995).
d) Presednted in part at the 17th Cyclodextrin Symposium of Japan, Osaka, Japan, October 1999, procceding, p. 69-70.

Surface tension. CD_{10}, CD_{11}, CD_{12}, CD_{13}, CD_{14}, CD_{15}, CD_{16} and CD_{17} showed no surface activity. We assumed that the surface activity of LR-CDs, composed of more than 18 D-glucose units, does not change with increasing D-glucose content.

Specific rotation. The specific rotation increased in the order : α-CD < β-CD < γ-CD < CD_9 < CD_{12} ≈ CD_{13} ≈ CD_{14} ≈ CD_{11} ≈ CD_{17} < CD_{15} ≈ CD_{16} ≈ CD_{10}. In homologous compounds with the different molecular weight, the evaluation of molecular rotation is suited for their rotatory power [18]. Molecular rotation [ϕ] is given by:

$$[\phi]_\lambda^t = \frac{M}{100}[\alpha]_\lambda^t$$

where M is molecular weight, [α] is specific rotation, t is temperature and λ is wavelength. Figure 1 shows the calculated molecular rotations of CDs (CD_6~CD_{17}). van't Hoff proposed "optical superposition", in which the rotation of optically active substances with several asymmetric carbon atoms is expressed with the algebraic sum of optical rotation contributed by individual asymmetric carbon atoms in the molecule. If there are no structural differences affecting optical rotatory power, molecular rotation must increase linearly with the increase in number of glucose units. In Figure 1, the variation in molecular rotation of CDs with number of glucose units is expressed as two straight lines. One is plotted against α-, β-, γ-CD and CD_9, and the other is plotted against CD_{10}~CD_{17}. Therefore, it was suggested that there are specific structural changes such as band flip in the CD_{10}~CD_{17} molecule, that do not occur in α-, β-, γ-CD and CD_9.

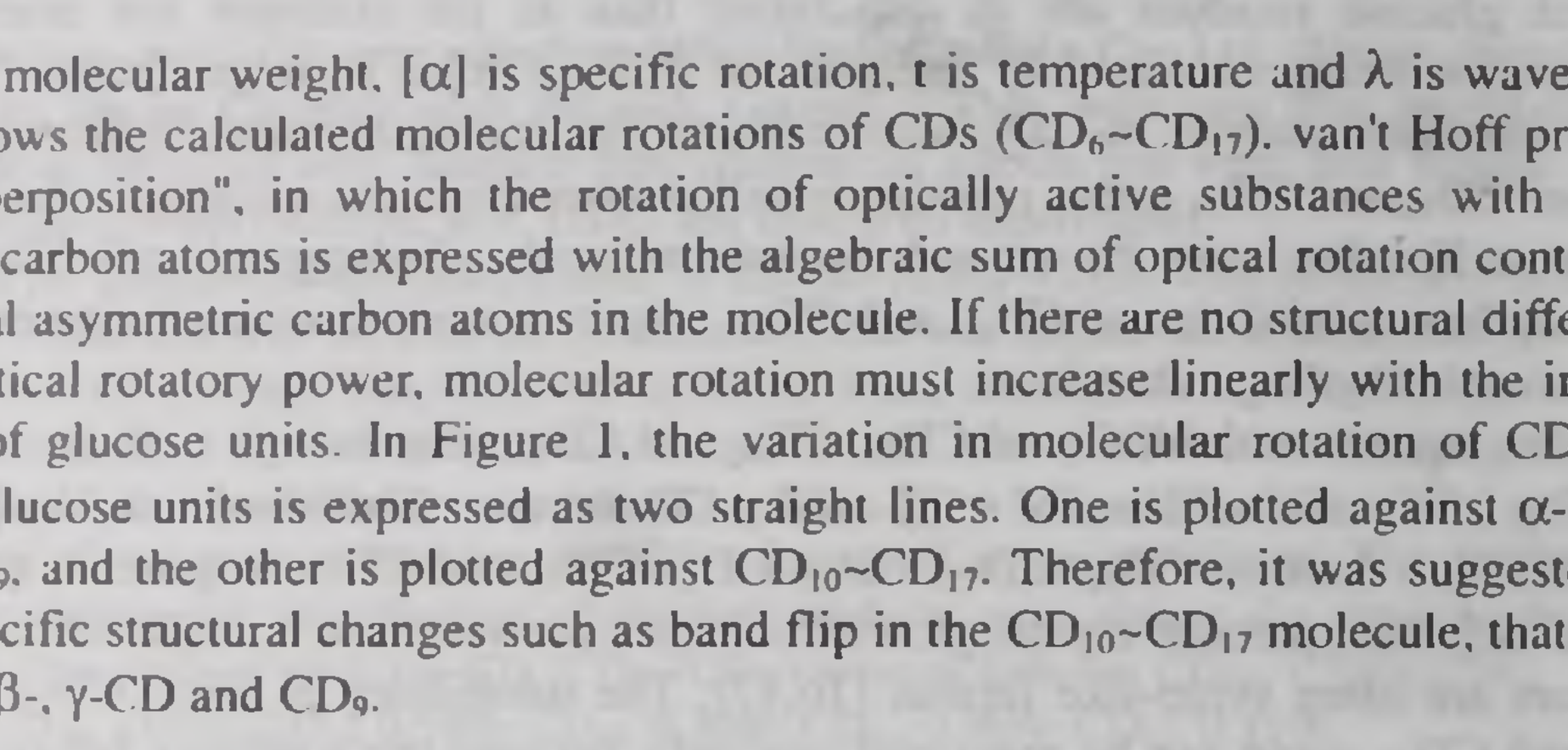

Figure 1. Variation in Molecular Rotation of CDs with Number of Glucose Units

^{13}C-NMR spectroscopy on CD with 6 ~ 17 D-glucose units shows only one sharp signal for each of the six glucose carbon atoms indicating the glucose residues of the individual molecules [3]. In the chemical shifts of C1 and C4 concerned with binding to two glucose residues, the signals for CD with 10 ~ 17 D-glucose units were shifted further upfield than those of α-, β- and γ-CD [3,19]. These results suggested that there are specific structures in CD_{10}~CD_{17}. Furthermore, X-ray analyses showed that there were band flip structures in CD_{10}, CD_{14} and CD_{26} [5,12,15]. Thus, we considered that there are band flip structures in LR-CDs composed of more than 10 D-glucose units, other than CD_{10}, CD_{14} and CD_{26}.

Acid-catalyzed hydrolysis.

Figure 2 shows the acid-catalyzed hydrolysis of CDs. The acid-catalyzed hydrolysis rates of CD_{14}, CD_{15}, CD_{16} and CD_{17} were as fast as those of the other LR-CDs (CD_9~CD_{13}) in contrast to those of α-, β- and γ-CD. There were no marked differences among LR-CDs (CD_{10}~CD_{17}). The above results showed that increases in simple disorder and decomposition sites (α-1,4 linked parts) of their intact rings with increasing number of the D-glucose units did not markedly influence the breakdown of LR-CDs. The hydrolysis rates of CD_{11}, CD_{12}, CD_{13} and CD_{14} were lower than that of CD_{10}. Saenger et al. proposed that the excessive steric strain of the CD ring was relieved by the band flip structures [12], so the steric strains of CD_{11}, CD_{12}, CD_{13} and CD_{14} were probably weaker than that of CD_{10}.

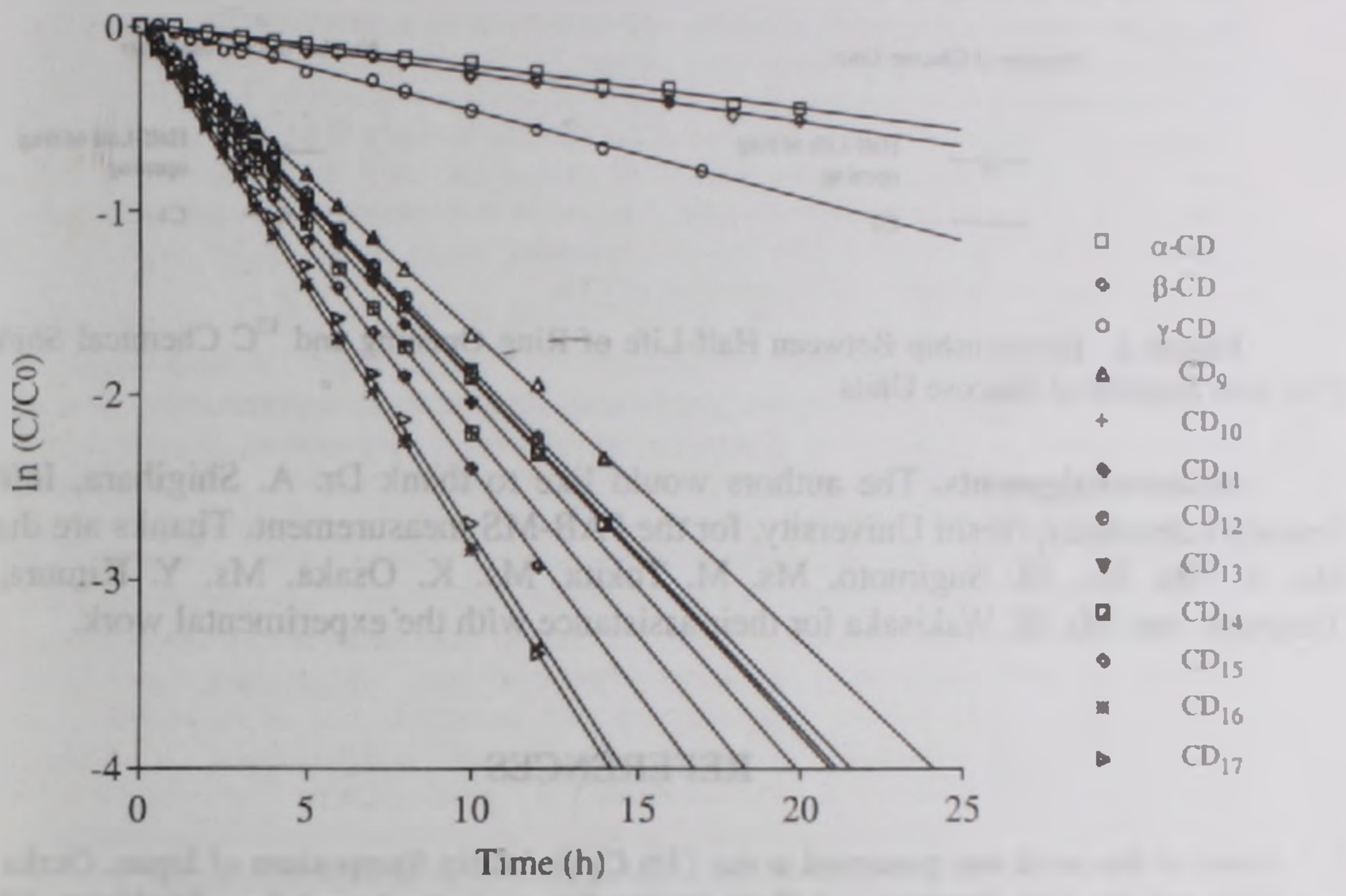

Figure 2. Time-Conversion Profiles of Acid Hydrolysis for CDs in 1mol/L HCl at 50°C

As shown in figure 3, the half-lives of ring opening paralleled ^{13}C chemical shifts of C1 and C4 with number of D-glucose units. We assumed that the signals of C1 and C4 were shifted upfield with the strength of steric strain, and the CD_{10} ring was most distorted among the series of CDs, composed of less than 14 D-glucose units examined in this study. The hydrolysis rates of CD_{15}, CD_{16} and CD_{17} were faster than that of CD_{10} because of the increase in decomposition sites with increasing number of D-glucose units. However, this hypothesis should be confirmed by further experiments using LR-CDs composed of more than 18 D-glucose units.

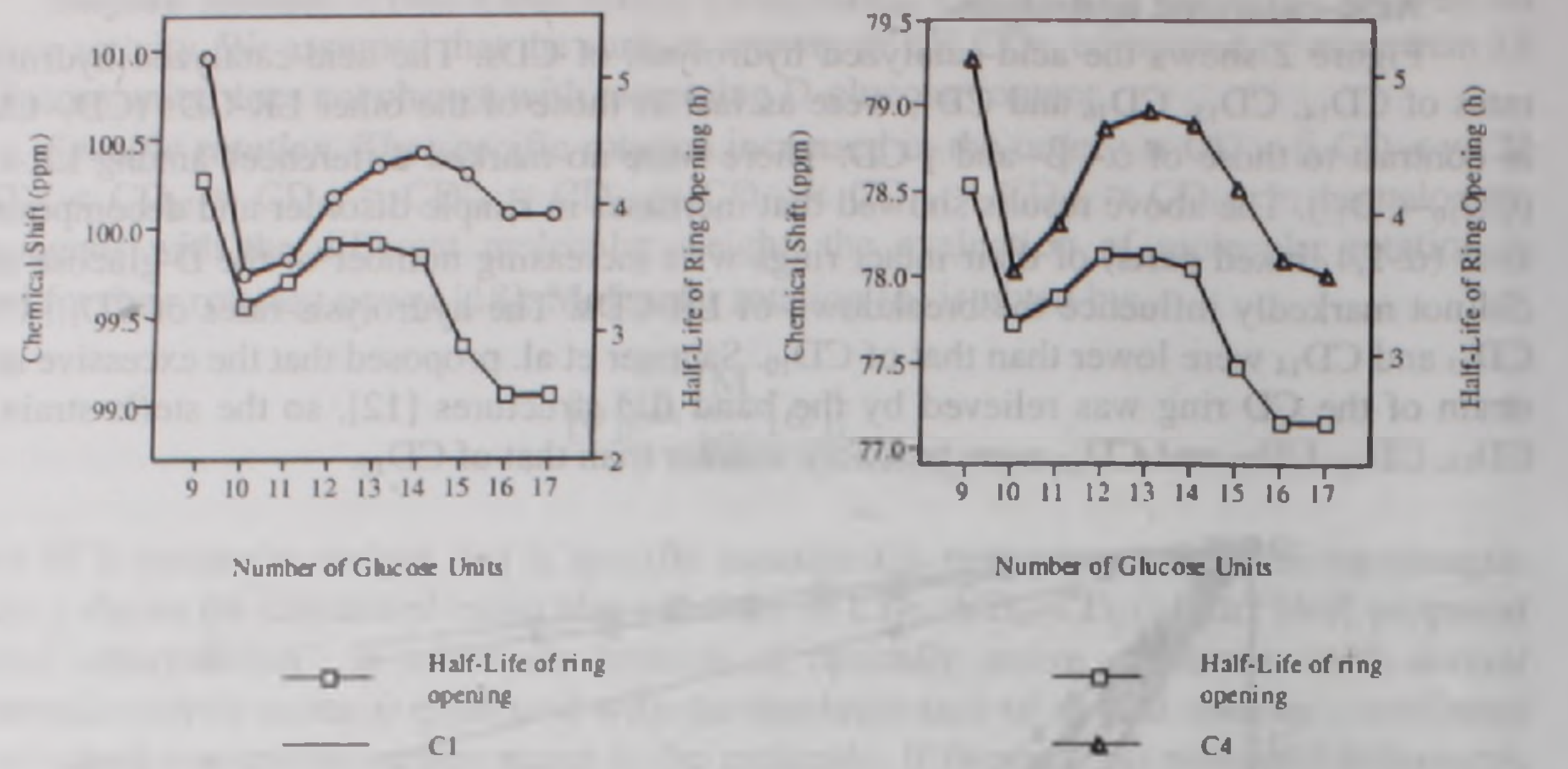

Figure 3. Relationship Between Half-Life of Ring Opening and ^{13}C Chemical Shifts of LR-CDs with Number of Glucose Units

Acknowledgments. The authors would like to thank Dr. A. Shigihara, Information Science Laboratory, Hoshi University, for the FAB-MS measurement. Thanks are due also to Ms. A. Ota, Ms. M. Sugimoto, Ms. M. Tokita, Ms. K. Osaka, Ms. Y. Kimura, Mr. Y. Yonamoto and Mr. M. Wakisaka for their assistance with the experimental work.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

ON THE ACTION PATTERNS OF CYCLODEXTRIN GLUCANOTRANSFERASES

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The action patterns of various cyclodextrin glucanotransferases (CGTase) from different groups of microorganisms have been studied. At the beginning of reaction of CGTases on starch the chaotic splitting of substrate proceeds with simultaneous cyclization. The cyclization and cleavage of the side chain proceeds simultaneously. The formation of LR-CDs may be explained that the enzyme strives to comprise the great piece of amylose or starch for faster transformation to cyclic forms. However, if oligomer binds to the acceptor subsite and no other transfer than to a water molecule is possible, the process of hydrolysis only proceeds, i.e. the linear oligosaccharides are only synthesised what involves in the reactions of disproportionation or cyclization depending on their DP. The low-molecular sugars important for the nutrition of microorganisms are formed by interconversion of CDs.

Изучен механизм действия различных циклодекстрин глюконотрансфераз (ЦГТаза) из различных групп микроорганизмов. В самом начале реакции ЦГТаз на крахмал, протекает совершенно беспорядочное расщепление субстрата с одновременной циклизацией. Циклизация и отщепление боковой цепочки протекает одновременно. Образование ВМ-ЦД может объясняться тем, что фермент стремится охватить как можно большой кусок амилозы или крахмала для более быстрого превращения их в циклическую форму. Однако, если олигомер связывается с акцепторным подцентром без каких-либо возможностей переноса, кроме как на молекулу воды, то протекает только гидролитическая реакция, т.е. образуются только линейные олигосахариды, которые сразу же вовлекаются в реакции диспропорционирования и циклизации, в зависимости от их степени полимеризации. Необходимые для питания микроорганизмов низкомолекулярные сахара образуются взаимопревращением ЦД.

Ուսումնասիրվել է մանրէների տարբեր խմբերից անջատված զանազան ցիկլոդեքստրին գլյուկանոտրանսֆերազների (ՑԳՏազ) ազդման մեխանիզմը: ՑԳՏազների և օսլայի ռեակցիայի սկզբում ընթանում է սուբստրատի կատարյալ անկարգավորված ճեղքում միաժամանակյա ցիկլիզացմամբ: Ցիկլիզացիան և կողքային շղթայի անջատումը ընթանում է միաժամանակ: ԲՄ-ՑԴների առաջացումը կարելի է բացատրել այնպես, որ ֆերմենտը ձգտում է ընդգրկել ամիլոզի և օսլայի որքան հնարավոր է մեծ մաս, ցիկլիկ ձևերի նրանց արագ փոխակերպման համար: Սակայն, եթե օլիգոմերը կապվում է ակցեպտորային ենթակենտրոնի հետ այնպես, որ բացի ջրի մոլեկուլայից բացառվում են այլ հնարավոր փոխակերպումները, ապա ընթանում է միայն հիդրոլիտիկ ռեակցիան, այսինքն առաջանում են միայն գծային օլիգոսախարիդներ, որոնք անմիջապես ընդգրկվում են անհամամասնացման (դիսպրոպորցիոնացման) և ցիկլիզացման ռեակցիաներում կախված նրանց պոլիմերիզացիայի աստիճանից: ՍաՆրէների սննդառության համար անհրաժեշտ ցածրամոլեկուլային շաքարներն առաջանում են ՑԴների միջփոխակերպումների միջոցով:

Introduction

The action patterns of cyclodextrin glucanotransferases (CGTases, EC 2.4.1.19) have been studied for long time [1-17]. In particular, the role of cosubstrates in intermolecular transglycosylation and disproportionation has been investigated [6]. Cyclization was found to involve attack by the enzyme at the reducing end of oligosaccharides [4]. Study of CGTase from *Bacillus circulans* showed that its active site can bind only nine glucose units [3]. This cyclization was found to result from a specific conformational change of the active site of the CGTase [2].The crystallographic and mutagenesis studies have shown that in the case of CGTase from *B.circulans*, three active site residues, Asp-229, Glu-257, and Asp-328, play important roles for cyclization reaction [5, 7, 18, 19]. Characterization of three transglycosylation reactions revealed that they differ in their kinetic mechanisms [12]. Disproportionation is a two substrate reaction and proceeds according to a ping-pong mechanism [20]. Cyclization is a single-substrate reaction with an affinity for the high molecular mass substrate used, which was too high to allow elucidation of the kinetic mechanism. Coupling is the reverse of the cyclization reaction and proceeds according to a random ternary complex mechanism [12]. The time-course analysis revealed that larger cyclic α-1,4-glucans (LR-CD) were preferentially produced in the initial stage of the cyclization reaction catalyzed by alkalophilic *Bacillus sp.* CGTase [13].

However, all investigations have dealt with a single enzyme, and no comparisons have been performed. Besides that, any of CGTases is able to catalyze at least three different reactions: intramolecular transglycosylation; intermolecular transglycosylation and hydrolysis of starch, maltooligosaccharides and CDs. Therefore, when the mechanism of action of the enzyme is considered all these reactions must be taken into the account

We previously described the main properties and mode of action of CGTases produced by some alkalophilic, thermophilic, halophilic, and mesophilic bacilli, as well as actinomycetes [15-17, 21]. It has been revealed that each CGTase possesses unique properties, which may be similar within the species range of the producers. This paper deals on action patterns of CGTases from different groups of microorganisms with simultaneous investigation of all aboveindicated reactions.

Material and Methods

The mesophilic bacilli *Bacillus macerans BIO-2m*, *Bacillus circulans BIO-3m*, *Bacillus coagulans BIO-13m*, *Bacillus licheniformis BIO-9m*, thermophilic *Bacillus stearothermophilus B-4905*, *Bacillus circulans B-4018*, *Bacillus licheniformis B-4025*, alkaliphilic *Bacillus alkaliphilus B-3103* and *BA-4229*, halophilic *Bacillus halophilus BIO-01H* and *BIO-12H* strains, and also *Thermoactinomyces vulgaris Tac-3554* have been used as objects in the recent investigations.

The nutrition media for cultivation of strains are follows:

Mesophiles (%%):

Starch-(1,0), corn steep liquor -(0,25), $(NH_4)_2SO_4$-(0,5) and $CaCO_3$ –(0,2) (pH 7,0-7,5; 39°C, 24 hrs) [21].

Alkalophiles (%%):

Soluble starch –(1,0), corn steep liquor –(0,5), K_2HPO_4-(0,1), $MgSO_4$ x$7H_2O$ -(0,02) and Na_2CO_3 -(1,0) (37°C; 48 hrs) [22].

Halophiles (g/l):

Potato starch–20.0, peptone –10,0, yeast extract –1,0, NaCl –120.0, KCl –2,0, $MsSO_4$x$7H_2O$ – 20,0 (pH 7,0-7,2; 37°C; 24 hrs) [23].

Thermophiles (%%):

Starch –0,7; corn steep liquor–0,5; NH_4Cl –0,53; $CaCO_3$ –0,2 (pH 5,7; 56°C; 18-20 hrs) [22].

Actinomycetes (g/l):

Starch –20,0; corn steep liquor –10,0; peptone –10,0; NaCl –10,0; $CaCO_3$ –10,0 (pH 7,0-7,2; 49°C; 20-24 hrs) [21].

CDs and maltooligosaccharides were determined by HPLC in an HPP 4001 instrument (Czechoslovakia) with a Separon SGX-NH_2 column (150 x 3,3 mm) and eluted with acetonitrile-water (70:30 v/v) at 2 ml/min. The flow cell was set at 30°C, and products were detected with a refractive index detector [15].

The amount of LR-CDs was calculated by subtracting the amount of glucose released by glucoamylase from that of total glucan in the reaction mixture [13] or by subtracting the amount of glucose released by glucoamylase and α-amylase from that released by glucoamylase only [24].

The cells were separated by centrifugation at 5000 g for 20 min.

The filtrate of the culture broth was concentrated by ultrafiltration on hollow fiber membranes AR-0,2.

Total and reducing sugars and protein were assayed by standard methods.

Photoinactivation of enzymes was developed by [25, 26]; modification by diethylpyrocarbonate - according to [27]; cyclizing activity – by [12,28]; dextrinizing activity – by [29]; α-CD specific activity – by [30]; β-CD specific activity – by [31]; coupling activity – by [12]; disproportionation activity – according to [8].

The ability of enzymes to form LR-CDs was identified according to the modified method [32]. For this purpose CGTases (1.0 ед/mL) were incubated with 5 % of soluble potato starch solution (optimum pH and optimum temperature for the enzyme). Through the certain intervals of time the samples were taken and reaction was terminated by boiling the solution for 10 min. Then, pH was adjusted to 5,5 and the reaction mixture was incubated with glucoamylase (0,4 units) for 18 hrs at 40°C. The resulting solution was subjected to the ethanol fermentation for 60 hrs at 30°C and after centrifugation the α-, β- and γ-CDs were removed from the supernatant as insoluble complexes with trichloroethylene and bromobenzene. The residual dextrins were precipitated by ethanol and removed by centrifugation. The solution was concentrated under vacuum up to syrup state, then dissolved in water and again incubated with 0,2 units of glucoamylase for 20 hrs at 40°C. The solution obtained was subjected to deionization by ion-exchange resins KU-2-8 (H^+) and Amberlite IR (OH^-) and decolorization by active carbon. After filtration and concentration up to 50-55 % of dry substances, the glucoamylase-resistant carbohydrates were precipitated by acetone. The dried precipitate was dissolved in distilled water (10 mg/mL) and subjected to the gel-filtration on Biogel P-2. The fractions containing of non-reducing sugars were collected and lyophilized. The further purification of LR-CDs was carried out on a column with the modified active carbon [33]. 1 mL of mixture containing 25 mg of dry material was passed through the column (2 x 20 cm) and eluted consistently with distilled water, then 3%, 7%, 12 %, 20% and 50% of methanol solutions. Each fraction was collected separately, evaporated up to syrup state and lyophilized. Their further purification was carried out by paper and thin-layer chromatography [33].

For faster definition of LR-CDs the enzyme reaction was stopped by addition of three volumes of cold methanol and placed at 4°C for 3 hrs. After this time the precipitated material have been removed by centrifugation, the supernatant was concentrated and dried by lyophilization.

CGTases have been purified to an electrophoreticall homogeneous state using polymeric CDs [34].

Results and Discussion

Some characteristics of the CGTases produced by abovementioned microorganisms are summarized in the Table 1.

Table 1. Some properties of CGTases

Microbial strains	Mol. weight. kD	Optimum pH	pH-stability	Thermo-stability, °C	Major CD
Mesophiles					
B.macerans BIO-2m	65	6.5	6.0-9.0	50	β>α>γ
B.circulans BIO-3m	82	5,5-5,8	6.0-9.5	60	β>α>γ
B.coagulans BIO-13m	62	6.0-6.5	6.0-10.0	70	β>α>γ
B.licheniformis BIO-9m	70	5.0-6.0	5.5-9.5	60	α>β>γ
Thermophiles					
B.stearothermophilus B-4905	69	6.5	6.0-9.0	60	β>α>γ
B.circulans B-4018	80	5.5-7.0	6.0-9.5	75	β>α>γ
B.licheniformis B-4025	74	5.0-6.5	5.5-9.0	65	β>α>γ
Alkalophiles					
B.alkalophilus B-3103	82	8.5	6.0-10.0	85	β>>γ
B.alkalophilus BA-4229	85	8.0-9.0	7.0-10.0	60	β>>γ
Halophiles					
B.halophilus BIO-12H	70	6.5-7.0	6.0-9.0	50	β>>γ
B.halophilus BIO-01H	67	7.0-7.5	6.0-8.5	60	β>>γ
Thermoactinomycetes					
Thermoactinomyces sp. A554	66	6.0-7.0	5.5-8.5	80	α/β

The studies on interactions of active site and different glycosides with ability to inhibit the cyclization reaction and/or stimulate the intermolecular transglycosylation are an important part of investigations of the CGTase action patterns.

Based on the observation that N-acetylglucosamine, 3-O-methylglucose, and glucose-6-phosphate exhibit no or only weak inhibition of cyclization, relative to glucose, it was resumed that the C2, C3, and C6 hydroxyl groups of the terminal glucopyranosyl cycle are essential for binding to the active site of CGTase. Isomerization at C2 (mannose) or C4 (galactose) produced similar effects. On the other hand, the inhibition became more expressed upon blockage of semiacetal hydroxyl groups (methyl-α- and methyl-β-D-glucosides), and in the case of low-molecular weight acceptors, the nature of the anomer bond and non-carbohydrate part does not affect on the CGTase activity (Table 2).

In addition, the equatorial hydroxyl group at the position C4 of the acceptor must be free for the interaction, in accordance with the earlier data [2, 15,35,36].

It should be noted that the major products of intermolecular transglycosylation have acceptor residues at the terminus. On the other hand, studied CGTases produce CDs up to certain level and certain ratios of α-, β-, and γ-CDs in the initial reaction stage in the presence of any acceptor. The CDs yield and ratio are characteristic for given CGTase. Thereafter their amounts drop because of decyclization and intermolecular transglycosylation until they are completely converted into linear oligosaccharides. So, though various CDs can occupy the CGTase active site, this is not sufficient for the reaction to proceed with a high rate. Various acceptors must be also present. Thus, CDs were not converted at all on being incubated for 5 days with low amounts of highly purified CGTases (0,1 U/g CD). However, the hydrolysis and disproportionation reactions proceeded rapidly upon addition of acceptor (glucose, maltose, etc.).

Table 2. Inhibition of Cyclization by Various Acceptors
(A mixture of 10 mg soluble starch, 50 mM acceptor, and 2.0 U CGTase in 1.0 ml buffer (optimum pH) was incubated at 50°C for 10 min)

Acceptors	Inhibition, %				
	1	2	3	4	5
Glucose	43	44	43	37	44
Galactose	20	8	10	0	1
Mannose	0	2	9	25	12
Fructose	10	5	7	15	2
Xylose	7	11	7	5	4
Maltose	88	91	86	86	81
Saccharose	25	25	28	22	27
N-Acetylglucosamine	16	14	16	16	20
Methyl-α-D-glucoside	86	85	80	79	81
Methyl-β-D-glucoside	72	72	78	76	78
3-O-Methylglucoside	25	25	24	21	23
Glucose-6-phosphate	0	0	0	0	0

(1). *B.macerans BIO-2m*; (2). *B.stearothermophilus B-4905*; (3). *B.alkalophilus B-3103*; (4). *B.halophilus BIO-12H*; (5). *Thermoactinomyces vulgaris Tac-3554*.

On the other hand, at ten times greater CGTase concentrations, CDs conversion was observed also in the absence of acceptors. Initially, β-CD yielded α-CD and vice versa, while formation of γ-CD was observed only after accumulation of a sufficient amount of maltooligosaccharides with degree of polymerization (DP) more than eight (Figure 1). The final molar ratios of newly produced α-, β- and γ-CD when they were used as substrates were approximately same as for starch. At reaction times exceeding more than 7 hrs, CDs were completely converted into linear maltooligosaccharides, i.e. CDs themself may inhibit cyclization.

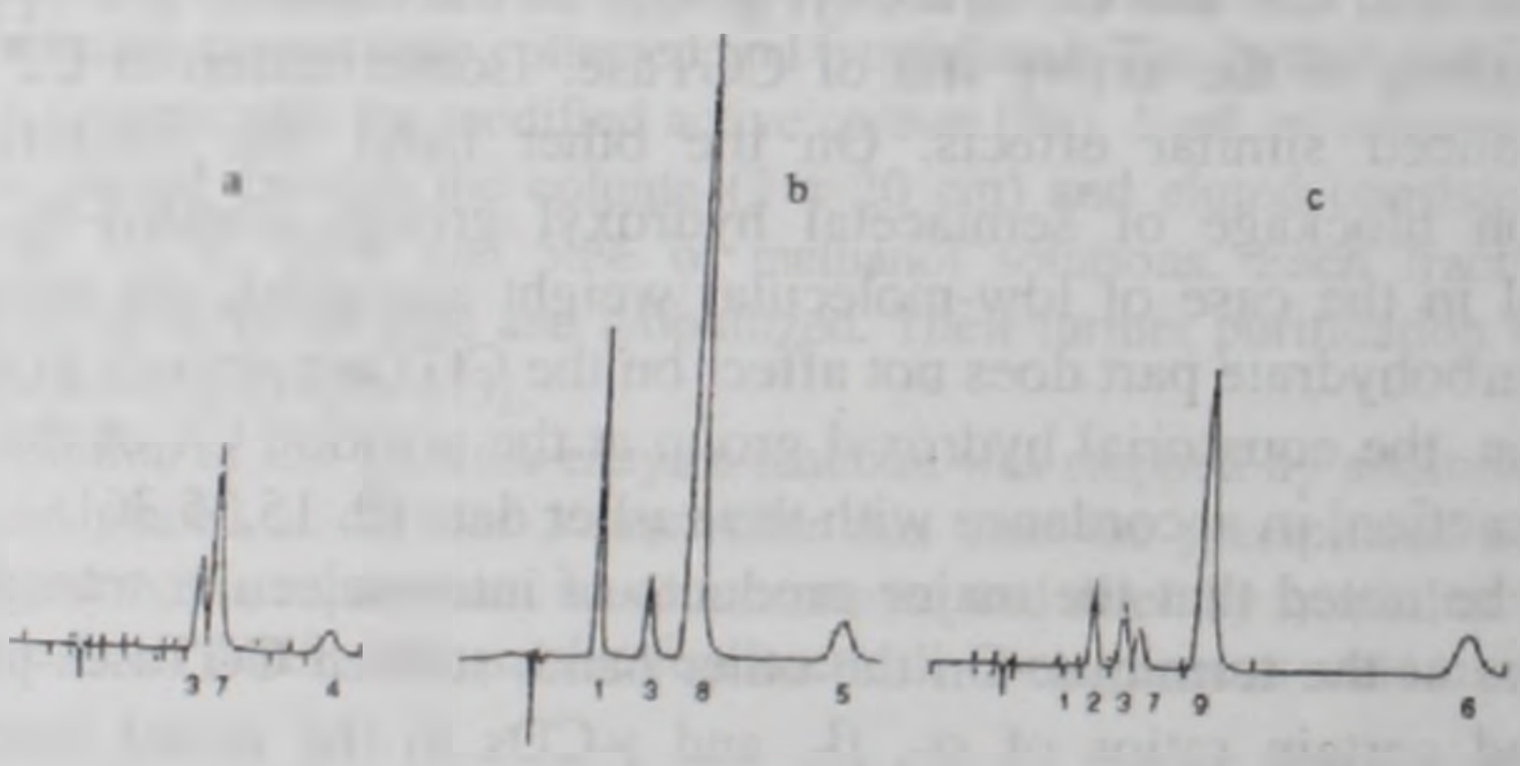

Figure 1. Interconversion of α-(a), β-(b), and γ-CD (c) catalyzed by CGTase of *B.stearothermophilus* B-4905. 1) glucose; 2) maltose; 3) maltotriose; 4) maltohexaose; 5) maltoheptaose; 6) maltodecaose, 7) α-CD; 8) β-CD, 9) γ-CD

At the very early stage of the reaction (30 sec, 50°C) CGTases produce maltotriose and maltohexaose from α-CD (Figure 1a). Accumulation of glucose and maltose is observed only after formation of CDs. Initially, from α-CD forms β-CD and vice versa, while

synthesis of γ-CD was observed only after accumulation of a sufficient amount of maltooligosaccharides with DP more than eight. The enzyme produced glucose, maltotriose, and maltoheptaose form β-CD (Figure 1b) and maltotriose, maltooctaose, β-CD, and small amount of glucose from γ-CD (Figure 1c). Thus, the reaction with CDs is apparently related with formation of linear maltooligosaccharides, which are further hydrolyzed and spliced by intermolecular transglycosylation.

At the initial stage of the reaction on CDs the interconversion rate was low, but then it is increased with the accumulation of low-molecular weight sugars. Probably, the frequency of decyclization depends from the presence of acceptors with small DP – lower than maltooctaose. The yield and ratio of CDs formed are considerably affected by quantity and nature of acceptors, i.e. a substantial influence on these reactions may be exerted by various low-molecular-weight sugars. It is worthy to note that the ring opening and inerconversion activities of CGTases from mesophilic and thermophilic bacilli, and thermoactinomycetes are considerably higher with compared with alkalophilic and halophilic enzymes. In the case with alkaliphilic and halophilic CGTases γ-CD was the most labile, and α- and β-CDs practically did not involved in reactions of interconversion in the absence of any acceptors.

Thus, it has been shown that various CDs can participate in interconversion reactions. They can be formed from one another in the absence of starch, which is a new side mechanism of CDs formation (Figure 2).

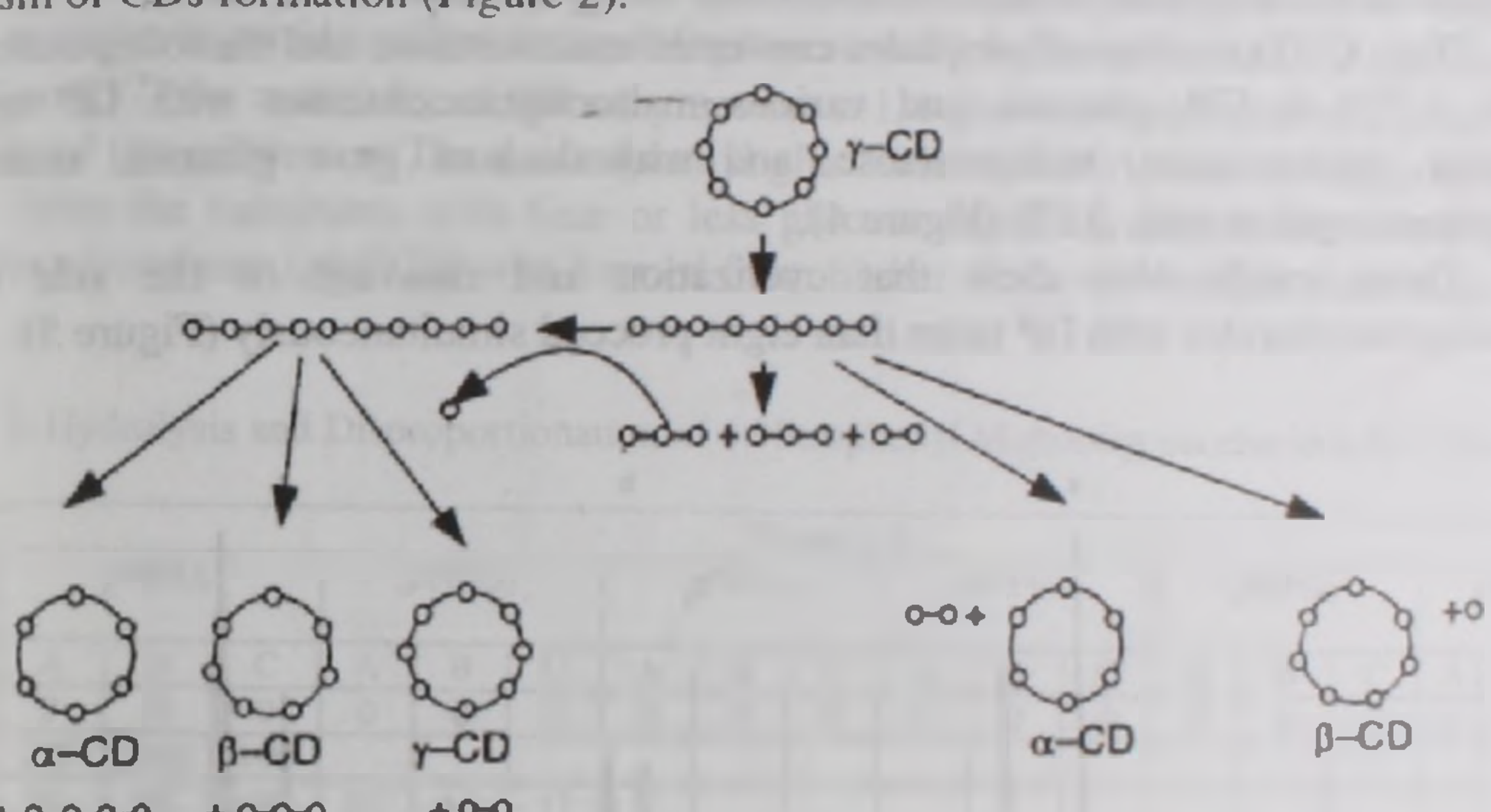

Figure 2. Scheme of Interconversion of CDs Exemplified with γ-CD

All the CGTases purified produce CDs mixture not only from starch, but also from different maltooligosaccharides. The yield of CDs was found to depend significantly on the size of substrate, and the higher degree of polymerization of the maltooligosaccharides and higher total yield of CDs (Figure 3). The α-, β- and γ-CD ratios were shown to depend on the type of the substrate and vary significantly in time. However, at the initial stage of the reaction, CGTases from the thermophilic, mesophilic bacilli and thermoactinomycetes produce mainly α-CD, and the enzymes from the alkalophilic and halophilic strains produce β-CD, while other CDs were formed only later.

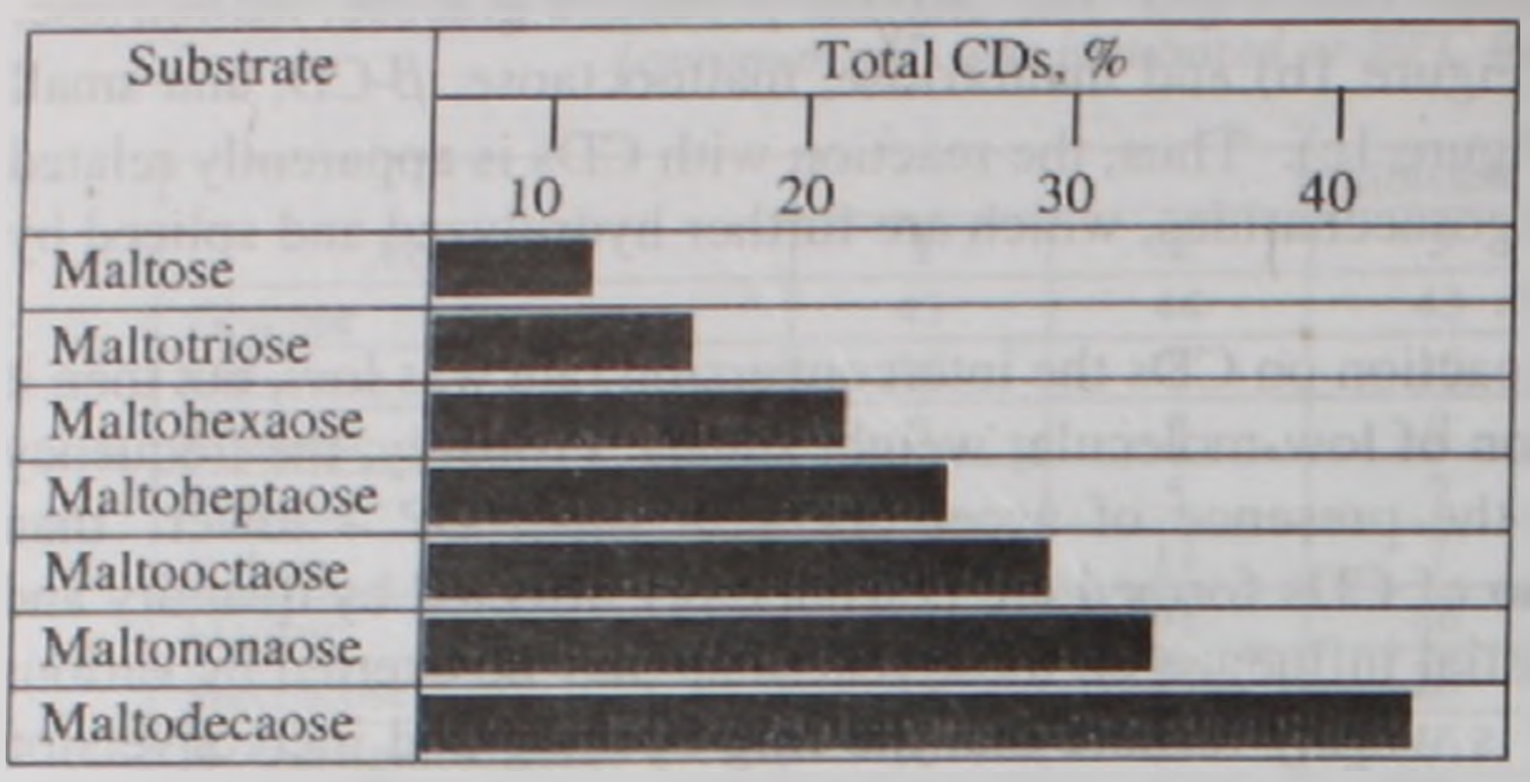

Figure 3. Formation of CDs from Various Maltooligosaccharides

At the beginning of the reaction (30 sec, 40°C) all the enzyme preparations did not produce CDs from maltooligosaccharides with DP up to 7. However, after one min of incubation maltooligosaccharides with DP more than seven are formed, and then CDs appeared. It seems likely that maltooligosaccharides with DP less than eight first undergo intermolecular transglycosylation to give new maltooligosaccharides with higher DP, which can serve as direct substrates for cyclization. Only maltooligosaccharides with DP more than seven could be used as direct substrates in intermolecular transglycosylation, maltooctaose being the smallest of them.

Thus, CGTases from alkalophiles converted maltohexaose and maltoheptaose (pH 8,5; 2 min; 37°C) to CD, glucose, and various maltooligosaccharides with DP up to ten. However, maltooctaose, maltononaose, and maltodecaose gave glucose, maltose, and maltotriose together with β-CD (Figure 4).

These results also show that cyclization and cleavage of the side chain of maltooligosaccharides with DP more than eight proceed simultaneously (Figure 5).

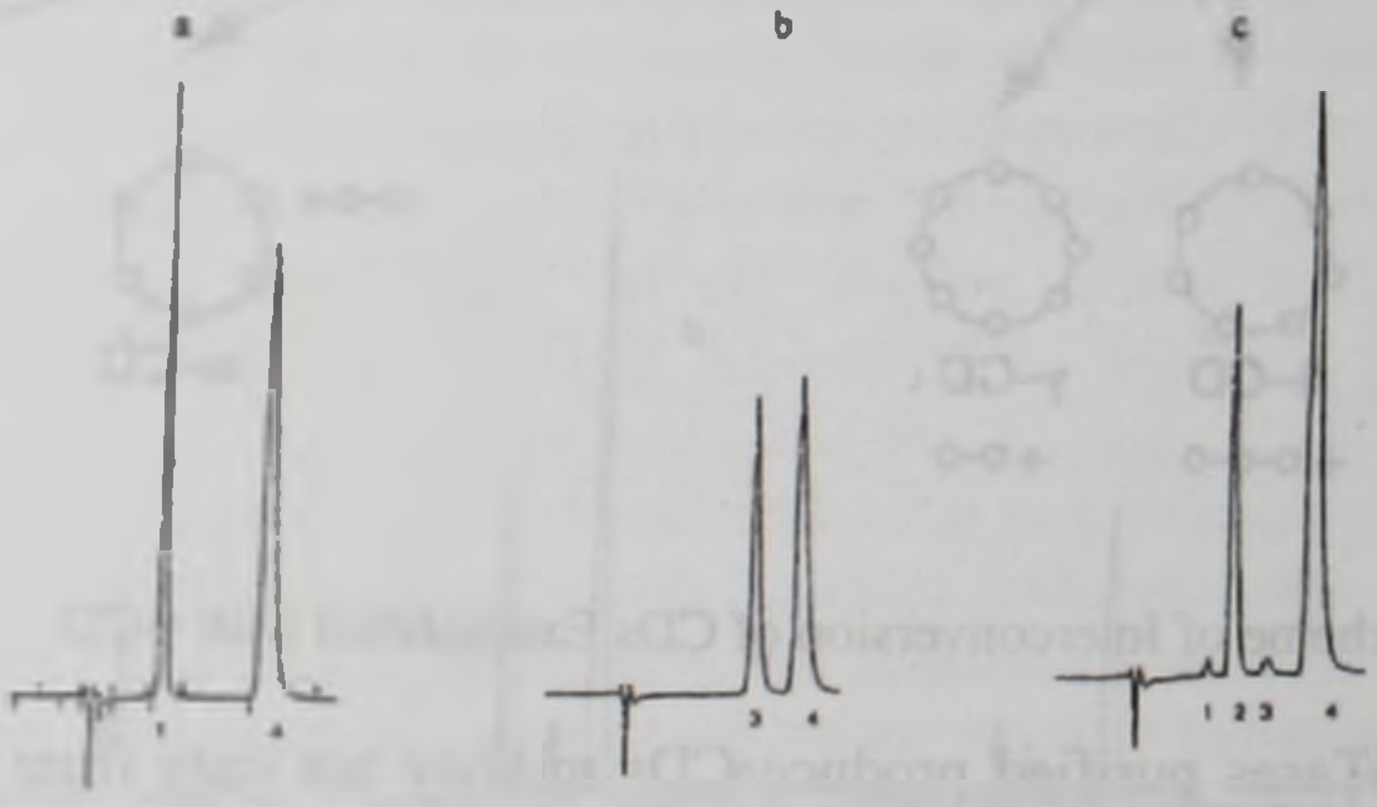

Figure 4. Formation of CDs from maltotriose (a), maltononaose (b), and maltodecaose (c) catalyzed by the enzyme from B.alkalophilus B-3103 (2 min, 37°C). 1) glucose; 2)maltose; 3) maltotriose; 4) β-CD.

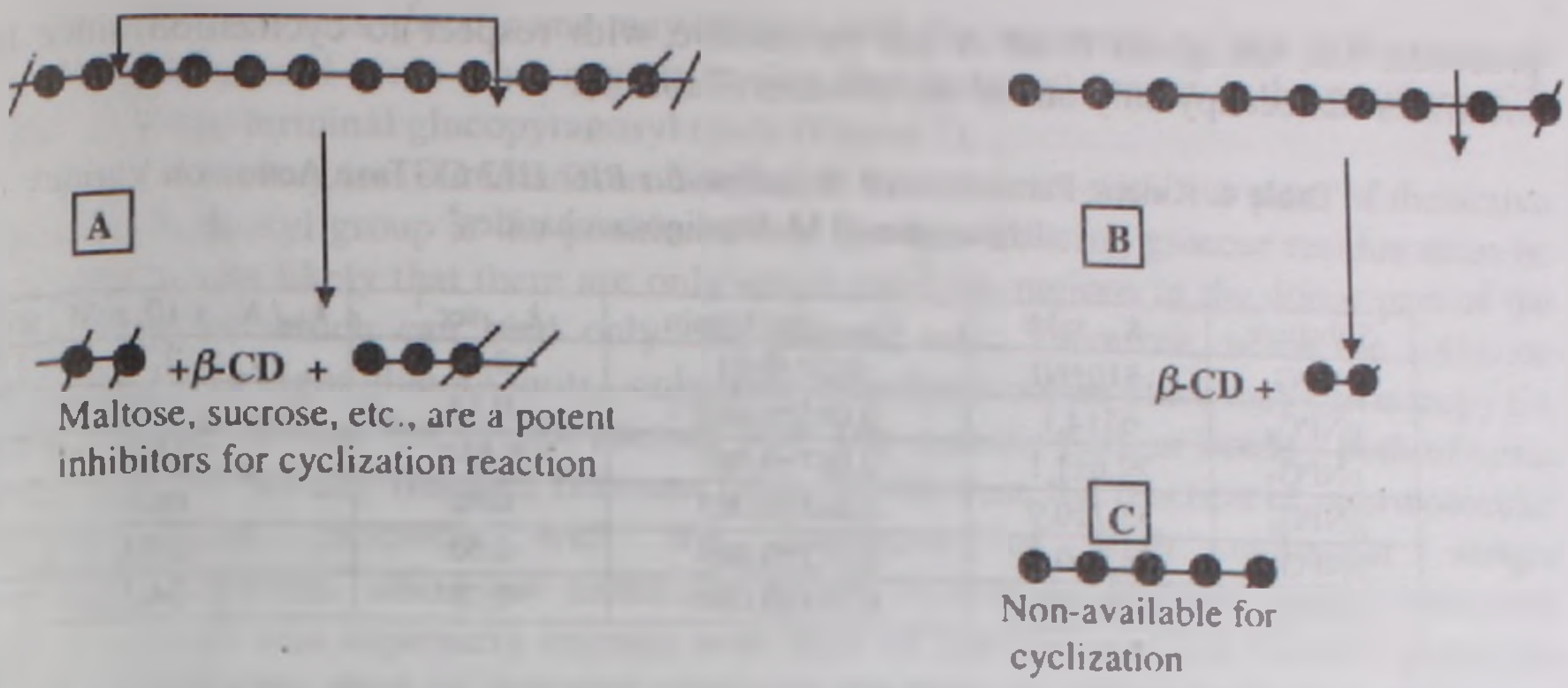

Figure 5. Formation of β-CD from Starch or α-1,4-Maltooligosaccharides.

The similar results were obtained with the use of various substituted maltooligosaccharides as substrates. In the reactions of the CGTases with 4-nitrophenyl-α-maltooligosaccharides, only products of hydrolysis during a short period of time were studied in order to avoid cyclization and disproportionation. As in the case of CGTase from *B.circulans* [37] the reactivity of the enzymes on α-1,4-glucans was found to depend on the chain size of the substrates. The 4-nitrophenyl glucosides (*p*NPG1) was the main hydrolysis product from the substrates with four or less glucose residues, meanwhile 4-nitrophenyl glucosides of maltose (*p*NPG2) was formed from higher molecular weight substrates (Table 3).

Table 3. Hydrolysis and Disproportionation of 4-Nitrophenyl Maltooligosaccharides by CGTases[a]

Sub-strate	Product, %																	
	*p*NPG$_1$			*p*NPG$_2$			*p*NPG$_3$			*p*NPG$_4$			*p*NPG$_5$			*p*NPG$_6$		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
*p*NPG$_1$	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*p*NPG$_2$	100	100	100															
*p*NPG$_3$	81	85	84	21	15	17												
*p*NPG$_4$	46	51	52	29	30	37	21	19	20									
*p*NPG$_5$	33	28	30	50	51	56	14	16	16	6	5	7						
*p*NPG$_6$	18	20	22	53	55	53	20	18	17	7	7	8						
*p*NPG$_7$	34	30	33	26	33	32	15	17	16	6	5	6	16	15	13			
*p*NPG$_8$	16	15	17	52	54	55	11	12	12	11	13	12	4	3	2	4	3	2

(A), CGTase from *B.macerans BIO-2m*; (B), *B.halophilus BIO-12H*; (C), *T. vulgaris Tac-3554*.

[a] A mixture of 20 mM substrate and 2 nM CGTase in 1 mL of 0,16 M Tris-HCl, pH 7.0, was incubated for 10 min at 50°C.

The affinity to substrate increase from nitrophenyl-α-maltose (pNPG2) to maltopentaose (pNPG5), somewhat decreased for pNPG6, and reached the maximum with pNPG8. The maximum reaction rate slightly depends on the size of the maltooligosaccharides from pNPG4 to pNPG7, but decreases significantly for pNPG8, which seems to be able to interact with both sites simultaneously, a necessary condition for CD formation. In the case of other substrates used, only intermolecular transglycosylation

proceeds, i.e., the given bond is not productive with respect to cyclization since these substrates can occupy only one of the subsites (Table 4).

Table 4. Kinetic Parameters of *B.halophilus BIO-H12* CGTase Action on Various 4-Nitrophenyl Maltooligosaccharides[a]

Substrate	K_m, mM	V_{max}, μmole/min	k_{cat}, sec^{-1}	k_{cat}/K_m x 10, mM^{-1} sec^{-1}
pNPG$_3$	810±60	0,27±0,01	2,25	2,77
pNPG$_4$	65±4.1	0,065±0,002	0,54	8,33
pNPG$_5$	22,6±1.1	0,067±0,003	0,558	24,70
pNPG$_6$	33,7±0.9	0,068±0,003	0,56	16,81
pNPG$_7$	25,5±0.5	0,072±0,003	0,60	23,5
pNPG$_8$	8,3±0.2	0,034±0,001	0,28	34,1

[a]For experimental conditions, see Table 3.

The maximum affinity of all the enzymes studied to substrate was observed for the subsite 2. The affinities of the subsites 1, 4 and 5 were also positive, while those of subsites 6 an 7 were close to zero and that of the subsite 3 was negative. According to the theory [38], this indicates that the site of cleavage of the substrates is located between the subsites 2 and 3, i.e., at the site with the lowest affinity (Figure 6). The similar results have been obtained for other CGTases tested.

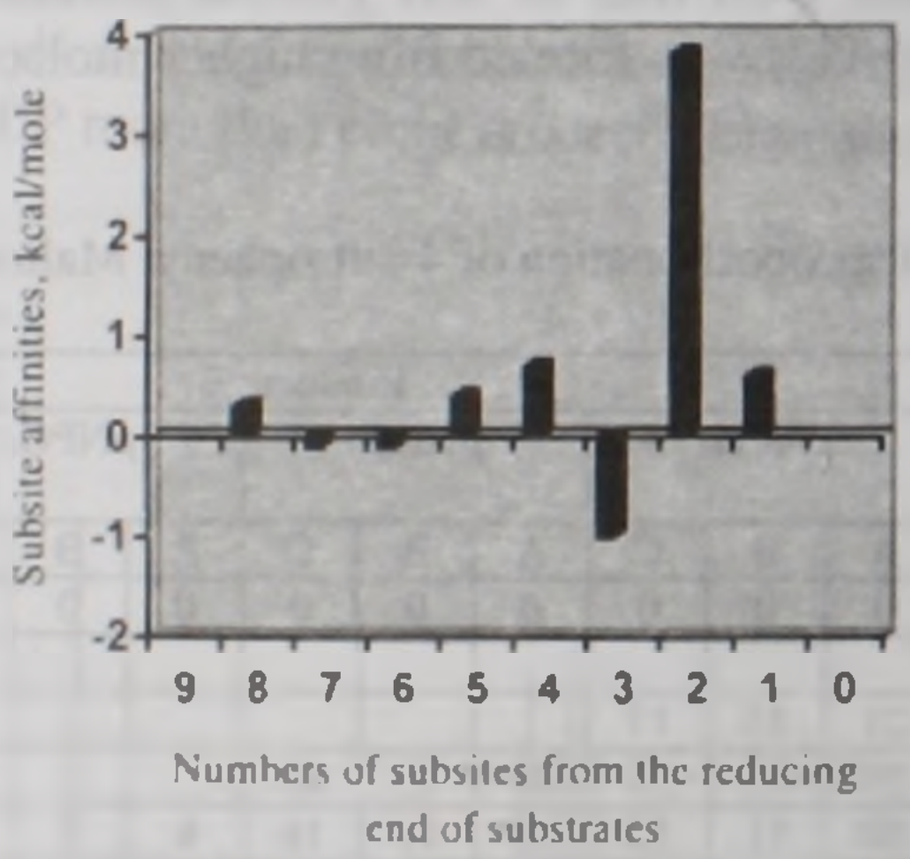

Figure 6. Affinity Histogram of The Subsites *T.vulgaris Tac-3554*. The arrow indicates the site of the catalytic cleavage.

The CGTases of high purity were undergone to photoinactivation and modification by diethylpyrocarbonate in the presence of salicin, acarbose and β-CD-polymer. The values of pK and ionization heat of the groups located in active site of the enzymes have shown that the carboxyl and two imidazole groups of histidine which play the role of nucleophilic-electrophilic pair are very important in the catalytic action of CGTase.

Based on these data, one can speculate that the active site of CGTases of halophilic, mesophilic, thermophilic, alkalophilic bacteria as well as thermoactinomycetes are similar and can be divided into two sectors responsible for acceptor and donor binding, respectively. The donor subsite is strictly specific to α-1,4-linked maltooligosaccharides and the other one

(acceptor subsite) is less specific and may interact with the non-reducing end of the terminal D-glucose residue and some other sugars having free hydroxyl groups at the positions C2, C3 and C6 of the terminal glucopyranosyl cycle (Figure 7).

Cyclization requires simultaneous binding of the substrate with two parts of the active site and the hydroxyl group at the position C4 of the non-reducing glucose residue must be free. It also seems likely that there are only seven catalytic regions in the donor part of the enzyme, each of which can bind only one glucose unit. Therefore, when the substrate contains less than eight glucose units, only their hydrolysis occurs since they can occupy the specific part of CGTase but do not interact with non-specific part, or occupy both of sites, however there is no any linkages between them. In this case the reaction of intermolecular transglycosylation proceeds with the formation of high molecular weight maltooligosaccharides, since low molecular weight hydrolysis products or low-molecular weight acceptors can separately interact with both of specific and non-specific parts. For example, saccharose used as acceptor produces non-reducing oligosaccharides terminated with fructose.

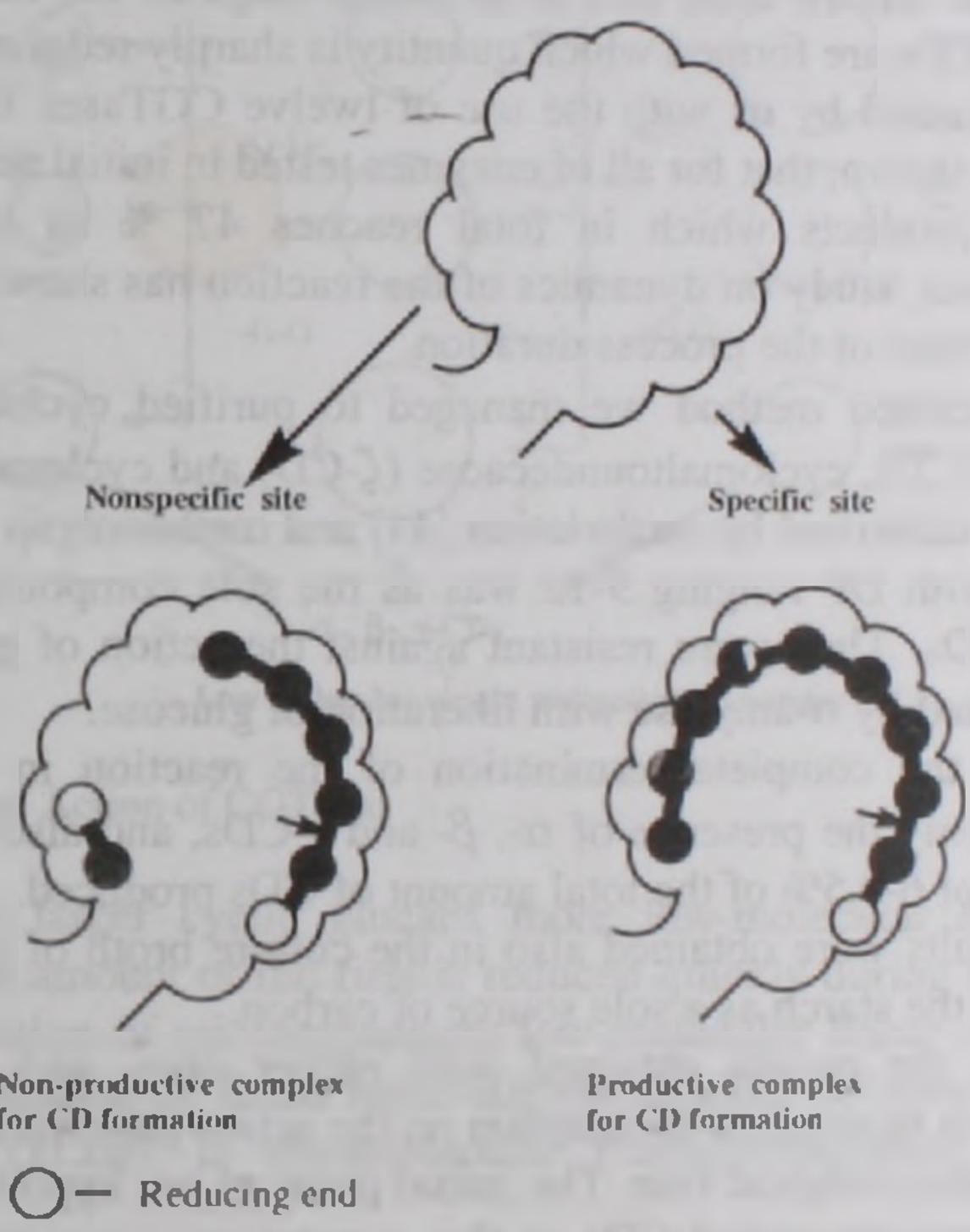

Figure 7. Proposed Structure of the Active Site of CGTase. The arrow indicates the site of the catalytic cleavage.

It is presumed that the acceptor-binding site has preliminary binding function, which can induce a change in active site conformation and favor substrate interaction with the donor-binding site in a way required for transglycosylation. In other words, initial binding of substrate with the acceptor site determines its interaction with the donor site and conformation of the latter. The mere fact of CDs hydrolysis counts in its favor. Should CGTase be only a transglycosylase, it would not have converted CDs in the absence of acceptors. If, however, CDs bind to the acceptor subsite and no transfer other than to a water molecule is possible, the hydrolysis may proceeds at the donor subsite.

By the mechanism presented it is possible to explain all the reactions catalyzed by CGTases on maltooligosaccharides as substrates.

However these results may not explain the formation of LR-CDs which are synthesized in the cases of amylose and starch as substrates. Their presence in the commercial preparations of CDs [39-41], and also in the reaction mixtures of CGTases from *B. macerans* [41,14], *B. circulans* [14], alkaliphilic *Bacillus sp.* [13], and also *Paenibacillus sp.* [14] has been shown.

To explain the formation of LR-CDs, the structure of the substrate has to be taken into consideration. In its crystal structure, amylose can occur as double-stranded helices or, in the presence of organic compounds (e.g. fatty acids and long-chain alcohols), as a single helix with 6-8 glucose molecules in one helical turn [14,42]. According to the most widespeared hypothesis, amylose in solution has an interrupted coil-like helical structure composed of helical and non-helical segments [43]. The production of α-, β- and γ-CDs could be a consequence of a preferential helical structure of the amylose in solution, while LR-CDs are produced as random "mistakes" due to the flexibility of the amylose chain [14].

However, it was shown also, that at an initial stage of the reaction of CGTases on starch first of all LR-CDs are formed which quantity is sharply reduced in time [13].

The results obtained by us with the use of twelve CGTases from various groups of microorganisms have shown, that for all of enzymes tested in initial stage of the reaction LR-CDs are the main products which in total reaches 47 % in the mixture of cyclic carbohydrates. However, study on dynamics of the reaction has shown, that their quantity is reduced with the increase of the process duration.

The above described method we managed to purified cyclomaltononaose (δ-CD), cyclomaltodecaose (ε-CD), cyclomaltoundecaose (ζ-CD) and cyclomaltododecaose (η-CD). Their structures were identified by methylation [44] and methanolysis [45]. Methyl-2,3,6-tri-O-methyl-glucoside with DP ranging 9-12 was as the sole compound in the methanolysis products for all of CDs. They were resistant against the action of glucoamylase, however were quickly hydrolyzed by α-amylase with liberation of glucose.

However after the complete termination of the reaction in the final mixture we managed to identify only the presence of α-, β- and γ-CDs, and also δ-CD which quantity was within the limits of 6-9.5% of the total amount of CDs produced.

The similar results were obtained also in the culture broth of microorganisms during the fermentation with the starch as a sole source of carbon.

Comparison of the results obtained with earlier ones, and also with the known literature data indicates more exact assumption on the action patterns of CGTases and allows to explain also their physiological role. The initial point of our hypothesis is the supposition that the microorganisms are stored CDs as the reserve energy materials – sources of the inaccessible carbohydrates for other organisms.

Just at the beginning of reaction of CGTases on starch, proceeds chaotic splitting of substrate with their simultaneous cyclization. The cyclization and cleavage proceed simultaneously [15]. At this stage the formation of LR-CDs may be explained that the enzyme is aspiring to covered the more piece of amylose or starch for their faster transformation into the cyclic form. However, if oligomer binds to the acceptor subsite and no transfer other than to a water molecule is possible, the hydrolysis may only proceed, i.e. the linear oligosaccharides are synthesized which at once are involving in the reactions of disproportionation or cyclization depending of their DP.

For the nutrition of microorganisms low-molecular sugars are formed by interconversion of CDs. First of all, the cycle opening reaction is realized by coupling or

hydrolysis, and the resulting maltooligosaccharides, depending from their DP again are involved in the cyclization or disproportionation reactions. In the first case the CD with smaller cycle and low-molecular weight linear sugar are formed, and in the second case – the higher-molecular weight linear maltooligosaccharides which are able to form a cycle (maltooctaose is smallest of them) are synthesized. In both cases a part of low-molecular weight carbohydrates are used for nutrition of microorganisms, and other part represents itself as acceptors in the disproportionation and coupling reactions (Figure 8).

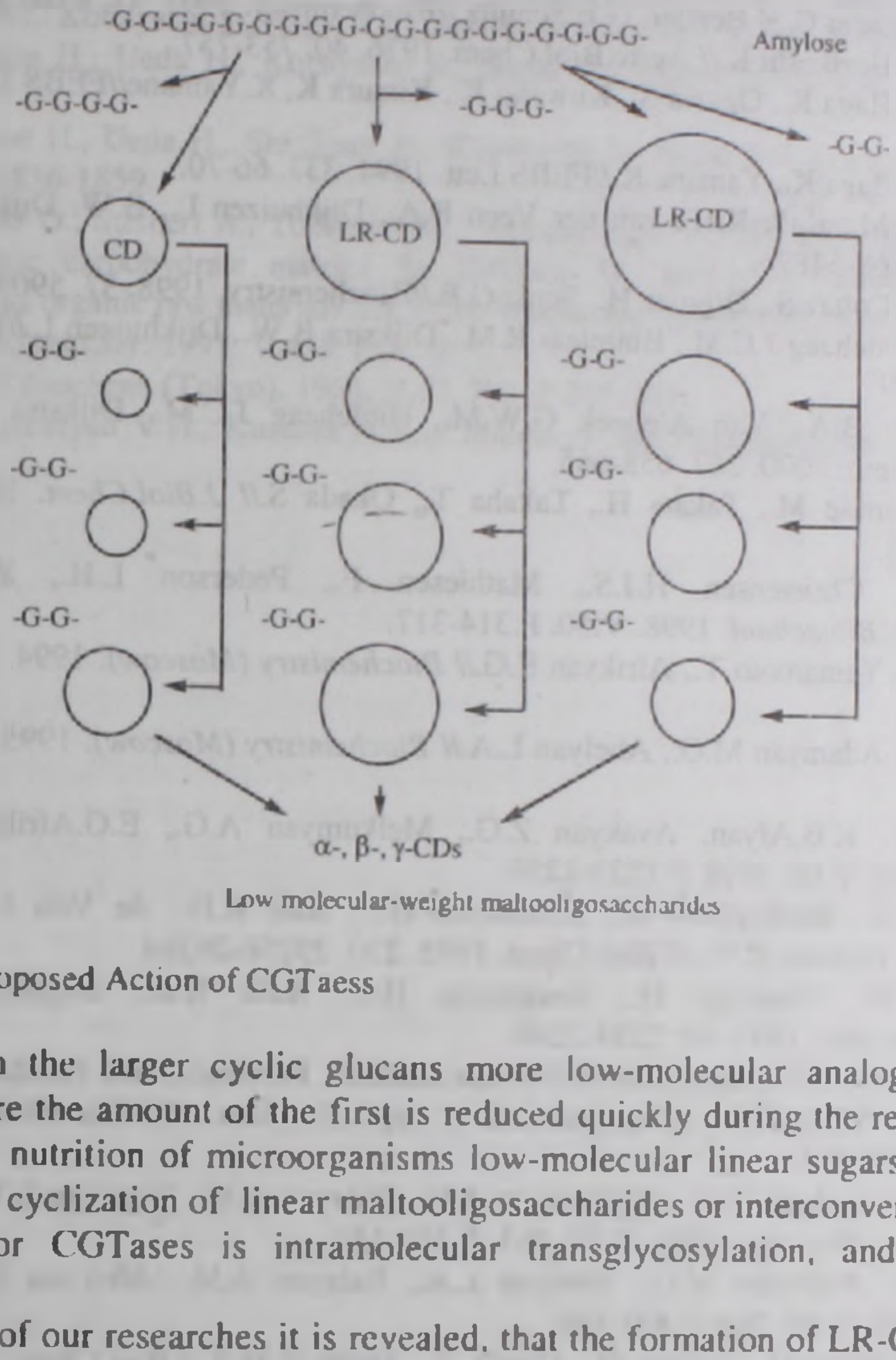

Figure 8. Proposed Action of CGTaess

Thus, from the larger cyclic glucans more low-molecular analogues are formed basically, therefore the amount of the first is reduced quickly during the reaction. Anyhow, but necessary for nutrition of microorganisms low-molecular linear sugars are synthesized only by means of cyclization of linear maltooligosaccharides or interconversion of CDs, i.e. basic reaction for CGTases is intramolecular transglycosylation, and all others are subordinated to it.

As a result of our researches it is revealed, that the formation of LR-CDs at the initial stage of the reaction is the prominent feature of CGTases of the investigated types of microorganisms that follows from the physiological features of the strains-producers. They are only intermediate spare nutrients - sources of glucose and/or low-molecular linear oligosaccharides. It concerns also to conventional CDs. Therefore in the nutrition media with the content of glucose as a sole carbon source, any of investigated strains did not produce CGTase. Besides with the increase of reaction duration all CDs finally are transformed to the low-molecular linear forms.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRIN GLUCANOTRANSFERASE AND INDUSTRIAL PROCESSES FOR PRODUCTION OF CYCLODEXTRIN

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Various approaches for industrial production of CDs have been presented. In order to increase the bioavailable of the substrate, the raw starch was extruded instead of liquefaction. The processes of CDs production using raw starch as substrate, liquefied starch and swollen extrusion starch were compared. The highest yield of CDs (54 g/l) was obtained after 24 hours for extrusion starch, compared to 45 g/l of that of liquefied starch and only 6 g/l CDs from raw starch (starch concentration was 100 g/l).

Представлены различные подходы промышленного получения ЦД. Для увеличения биодоступности субстрата, сырой крахмал подвергали экструзии вместо разжижения. Сравнились процессы получения ЦД с использованием сырого, разжиженного и разбухшего экструзионного крахмала в качестве субстратов. Наибольшой выход ЦД (54 г/л) получен после 24ч в случае экструзионным крахмалом, по сравнению с 45 г/л из разжиженного крахмала и только 6 г/л из сырого крахмала (концентрация крахмала 100 г/л).

Ներկայացված են ՑԴների արդյունաբերական ստացման տարբեր մոտեցումներ: Սուբստրատի կենսահասանելիությունը բարձրացնելու նպատակով, մասնակի հիդրոլիզի փոխարեն, չմշակված օսլան ենթարկել են էկստրուզիայի: Համեմատվել են ՑԴների ստացման եղանակների արդյունավետությունը չմշակված, հիդրոլիզացված և էկստրուզիոն օսլայի դեպքում: ՑԴների ամենաբարձր քանակություն (54գ/լ) ստացվել է 24ժամ հետո էկստրուզիոն օսլայից, համեմատած 45գ/լ ջրիկացված օսլայի և միայն 6գ/լ չմշակված օսլայի դեպքերի հետ:

Introduction

Cyclodextrins (cyclomaltooligosaccharides, cyclic(1→4)-α-D-glucans, CDs) are produced by the action of cyclodexrin glucanotransferase(CGTase) from starch, amylose, amylopectin, and other long-chain maltooligosaccharides, and posses a hydrophilic outside and hydrophobic central cavity. The formation of inclusion complex between CDs and dye was discovered by Cramer *et al.* at the beginning of the 1950s, and a patent covering practical application of CD in drug formulations was filed by Freudenberg *et al.* Thereafter, the enzymatic production of CDs, fractionation of CDs, and characterization of their chemical and physical properties had been intensively studied, and the commercialization of CD was initiated in Japan at 1970s [1].

CDs have been widely utilized in food, pharmaceutical, chemical, cosmetic, and agricultural industries because of their ability to form inclusion complexes with a wide variety of chemicals by partially encapsulating them into their cavity, thereby, altering the physical and chemical properties of these compounds. The utilization of CDs in Korea was stated at 1984 using the import β-CD, and their utilization has been gradually increased in pharmaceutical, food, and cosmetic industry. CD mixture comprising α-CD as a major CD as well as pure β-CD are manufactured by Sewon Inc. since the end of the 1980s [2].

Cyclodextrin Glucanotransferase (CGTase)

CGTase enzymes are able to produce CDs from starch via the cyclization reaction, which is the basis of their industrial application. Recent developments, however, also concentrate on the use of the CGTase catalyzed coupling and disproportionation reactions for synthesis of modified oligosaccharides by using alternative acceptor substrates.

(1) Reactions catalyzed by CGTase

The synthesis of CDs can easily be performed by enzymatic reaction of starch or other CGTase. CGTase are able to catalyze several reactions [3]. As shown in Figure 1, there is the unique cyclization reaction that is only efficient if long-chain substrates are used (dextrose equivalent (DE) < 20). According to Bender, optimal cyclization rate are achieved with 1,4-α-D-glycopyranosyl chins at a length of 16-80 glucose residues. Chain > G8 can be cyclized directly [6]. The cyclization reaction is initiated by an exo-attack of suitable substrates from their nonreducing chain ends.

CGTase can catalyze the reverse reaction of cyclization called "coupling". This reaction explains the time-dependent shift of CD-products during conversion of starch to CDs. For example, during the enzymatic reaction of a α-CGTase, a high initial peak of α-CD can be transformed to a significant extent to β-CD as shown in Figure 1 via the coupling-cyclization equilibrium. The reverse coupling reaction is favored at higher concentrations of maltooligosaccharieds of glucose [7].

The disproportionation reaction occurs mainly at the beginning of the CGTase reaction, if longer chain starch substrates are used. This is apparent from the rapid and drastic decrease of the viscosity of substrate. It is assumed that the disproportionation reaction does not influence the synthesis of CDs any further. The difference between the cyclization reaction and disproportionation is that cyclization is a one-substrate and disproportionation is a two-substrate reaction [7,8]

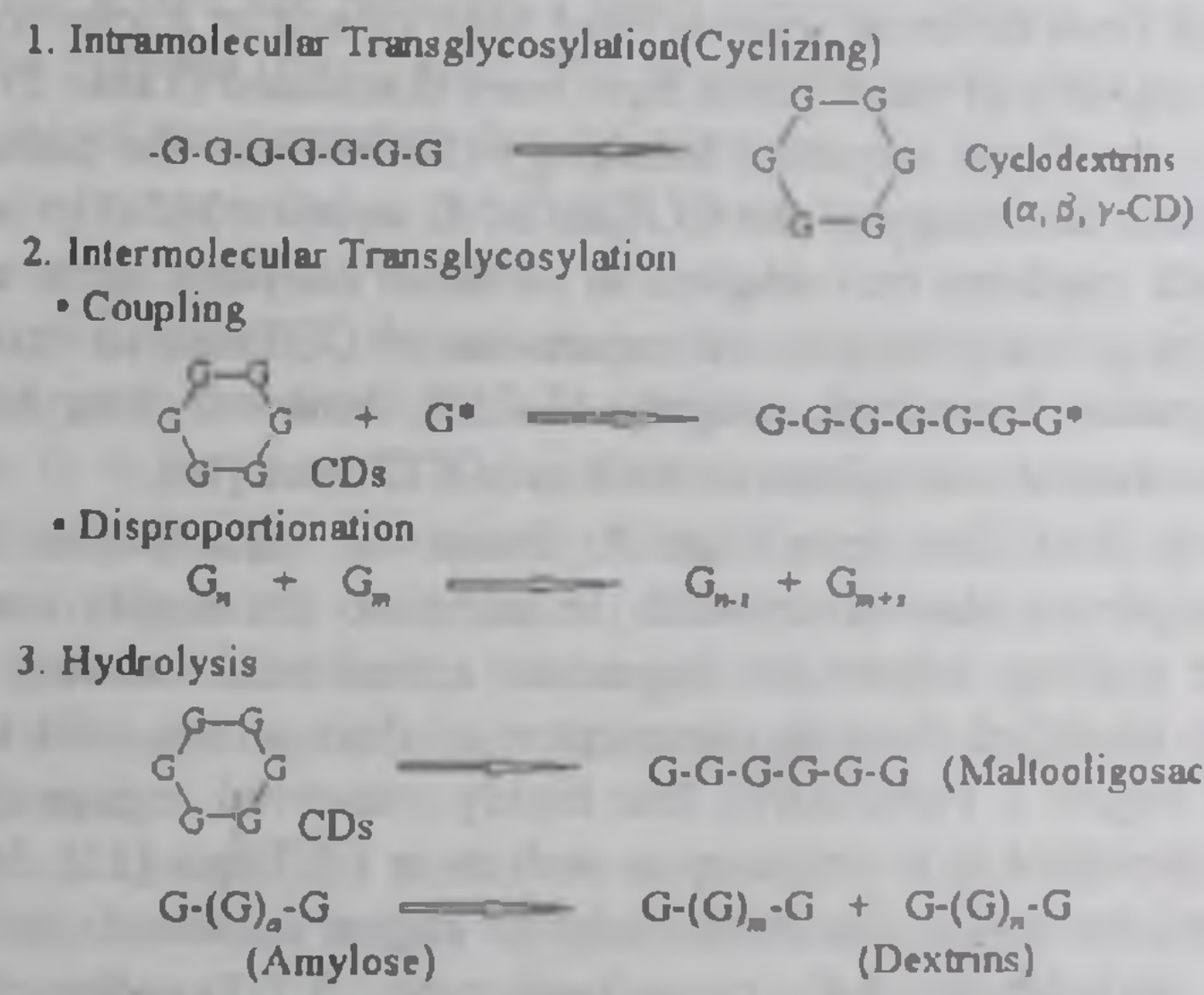

Figure 1. Various catalytic reactions of CGTase.

(2) Classification and source

CGTase are divided into three types (α, β, and γ) according to the type of CD formed initially. As the enzymatic reaction continues, the two other forms of CD are usually also

synthesized. When the reaction is allowed to reach equilibrium, β-CD is the main end-product; formation of β-CD is thermodynamically more favorable than that of α- and γ-CD [7].

Table 1 classifies microorganisms producing CGTase according to its main product from starch. Hence, α-CGTase produces (initially) α-CD. Typical representatives are the CGTase of *B. macerans*, *B. stearothermophilus*, *Thermoacaerobacter* sp., and *Klebsiella oxytoca* M5al. β–CGTase have been analyzed from *B. circulans* and a variety of alkalophilic bacteria. Examples of unique enzyme are the β-CGTase of the alkalophilic strains, *B. ohbensis*, *Bacillus* 1-1, and *B. firmus* var. *alkalophilus*. Both enzymes produce high amounts of β-CD, some γ-CD, but no α-CD under standard reaction conditions [4, 5, 7].

Table 1. Microorganisms producing cyclodextrin glucanotransferase (CGTase)

Main product from starch	Microorganisms
α-CD	*Bacillus macerans*, *B. stearothermophilus*, *Klebsiella pneumoniae*, *Thermoanaerobacter sp.*
β–CD	*B. megaterium*, *B. circulans*, *B. ohbensis*, Alkalophilic *Bacilli*, *B. firmus* var. *alkalophilus*
γ–CD	*Bacillus sp* AL6, *B. subtilis* No. 313, *Brevibacterium* sp. No. 9605

(3) Biochemical and genetic features

Typically, CGTase have a molecular weight of about 70-75 kDa, which is significantly higher than that of α-amylases (45-55 kDa). Most of them require Ca^{2+} for optimal activity and stability. They show activity over a broad pH-range, often from pH 5 to pH 10. The thermostability of the mesophilic enzymes is limited to about 60°C. An exception is the CGTase of *Theroanaerobacter* sp. which shows highest activity at 95°C [10].

At least 18 genes from different sources have been cloned in *Escherichia coli* and the DNA sequence of the majority of these genes have been elucidated (Table 2) [4]. Among the *Bacillus* sp. CGTase a significant sequence homology (50-70%) of the deduced amino acid sequence exists. Sequence homology of the CGTase of *K. oxytoca* M5al to other CGTases is only about 30%, which confirms the uniqueness of these enzymes from a gram-negative bacterium. Interestingly, primary sequence comparisons of CGTases to various α-amylases indicate moderate sequence homology, roughly 15-25%, however, four highly conserved regions have been identified in α-amylases as well as in CGTases [10].

In our laboratory, β-CGTase gene from *B. firmus* var. *alkalophilus* has been cloned and it s nucleotide sequence also determined. In addition, ten highly conserved regions including above four regions, which are important amino acid residues in catalysis of CGTase, were further identified through comparison of their amino acid sequence among CGTases as shown in Figure 2. Particularly, four highly conserved regions (region II, V, VI, and VIII) have been identified in α-amylases as well as in CGTases [12]. In addition, other enzymes were analyzed containing this conserved four-region sequence motif. The so called α-amylase family includes-besides α-amylase and CGTase-branching enzymes, neopullulanase, cyclodextrinase, and amylomaltase. β-Amylase and glucoamylase do not possess these sequence motifs and belong to another protein family [10]. Region IX and X are belonged to putative raw starch-binding motif, and important in CGTase catalysis.

Table 2. Bacterial sources and enzymatic properties of CGTases

Enzyme source	M.W.	CGTase type	Opt. PH	pH stability	T. S.	Gene cloned
Bacillus megatrium	75,000	β-type	5.2-6.2	7.0-10.0	<55°C	-
Bacillus circulans	-	β-type	5.2-5.7	7.0-9.0	<55°C	+
B. stearothermophilus	68,000	α-type	6.0	8.0-10.0	<50°C	+
Bacillus macerans	75,000	α-type	5.2-5.7	8.0-10.0	<55°C	+
Klebsiella pneumoniae	-	α-type	5.2	5.0-7.5	-	+
Alkalophilic *Bacillus* sp. 38-2	88,000	β-type	4.5-4.7	6.0-10.0	<65°C	+
B. firmus var. *alkalophilus*	75,000	β-type	6.0	5.5-10.0	<60°C	+
Bacillus ohbensis	35,000	β-type	5.5	6.5-9.5	<55°C	-
Bacillus sp. AL6	45,000	γ-type	7.0	6.0-10.7	<55°C	-
Bacillus subtilis	64,000	γ-type	8.0	6.0-8.0	<50°C	+
Thermoanaerobacter sp.	75,291	α-type	6.0	5.0-6.7	100°C	+
Brevibacterium sp	75,000	γ-type	10.0	6.0-8.0	<50°C	-

M.W.: molecular weight, T.S. : temperature stability

(4) Crystallography, active site, and site directed mutagenesis

Crystallographic structural data confirm that four highly conserved regions are involved in the active site (substrate - binding catalytic center) and the Ca^{2+}-ion binding. Despite the low sequence homology between α-amylase and CGTase they show a common overall folding pattern and active arrangements [9]

In both cases the amino-terminal domain (A) is a TIM barrel structure, consisting of eight inner, parallel β-sheets, surrounded by eight α-helixes (β/α) 8-barrel). This crystal structure is now known for 21 proteins. Characteristic for the α-amylase family is the arrangement of the active site between a small separate domain (B-domain) in between β-strand 3 and helix 3 of the (β/α)8 fold. The B-domain is supposed to be responsible for the functional diversity of the α-amylase family enzymes.

A C-domain is succeeded to the (β/α)8 barrel, approximately 100 amino acid residues ling and folds as eight parallel β-sheets in a Geek key topology. The function of the C-domain is unknown, although mutations in the C-domain of α-amylases inactivate the enzymes which suggests that the domain is necessary for enzyme activity.

In contrast to α-amylses, CGTase exhibit two further domains (D and E) which are anti-parallel β-Sheets at the C-terminal end of the protein and which cause the higher molecular weight compared to α-amylases (~25kDa) as shown in Figure 3. The D-domain following the C-domain is similar to immunoglobulin topology [11]. No function has been suggested for this domain up to now. Surprisingly, the α-CGTase of *K.oxytoca* M5al is missing this D-domain.

The E-domain found in the tertiary structure of CGTases has a raw starch binding site and shows high similarity to region of glucoamylase enzyme which are responsible for binding starch granules, and also contained two maltose-binding sites (MBS); one would seems to be involved in raw starch binding (MBS1) and the other guides the starch chain into the active site as well as playing an inhibitory role in CD production through accessibility control of substrates (MBS2) from X-ray crystallographic studies [9,11].

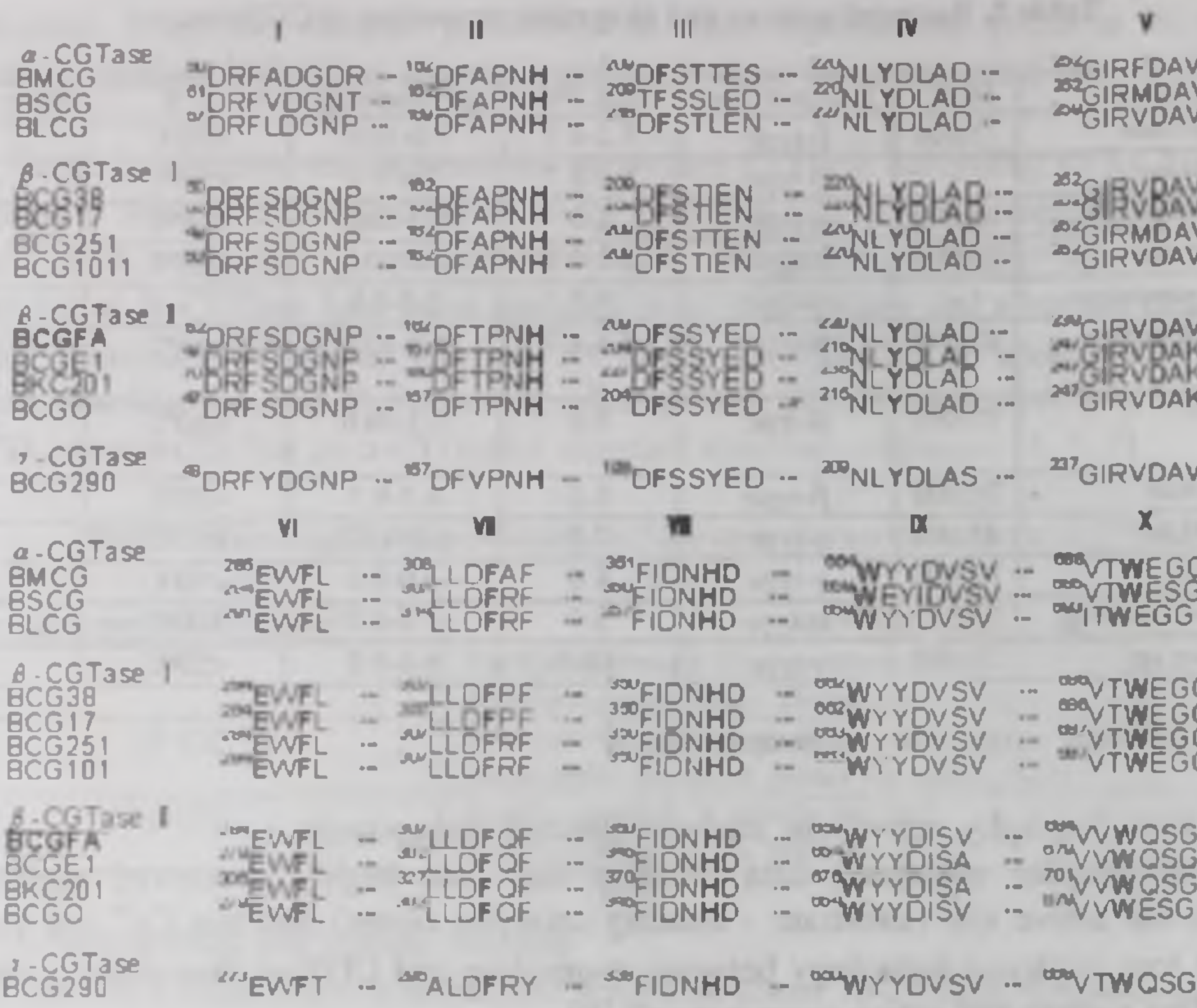

Figure 2. Comparison of the deduced amino acid sequences of the CGTase gene from *Bacillus firmus* var. *alkalophilus* with other CGTases.

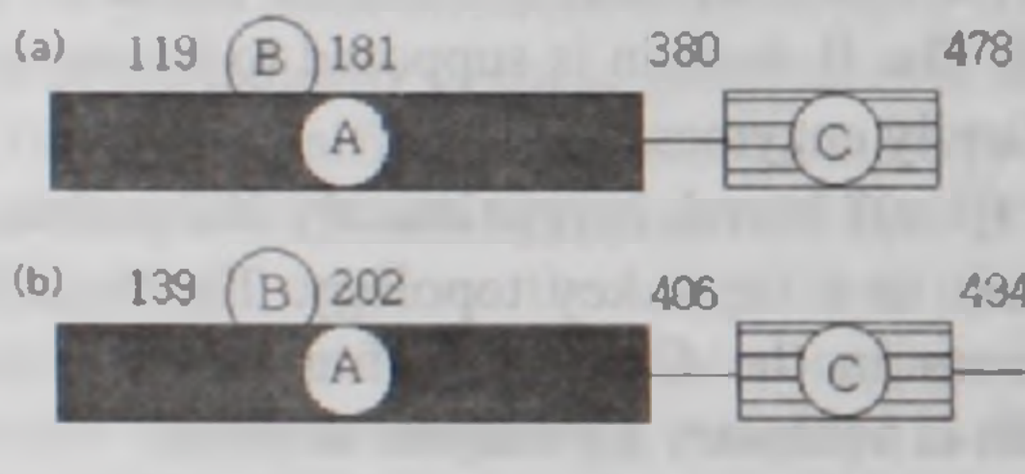

Figure 3. Schematic protein domain arrangement of (a) taka-amylase and (b) CGTase

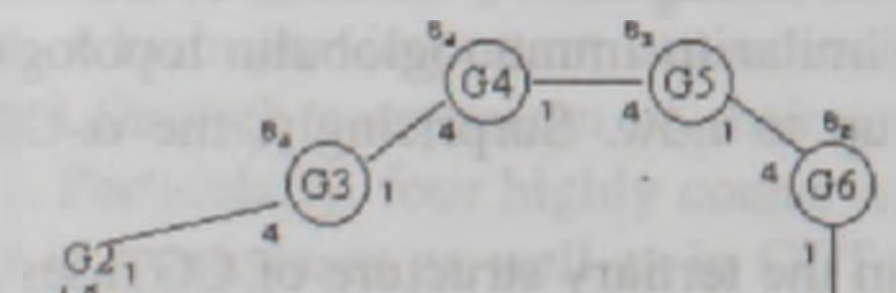

Figure 4. Model for the active site of CGTase: S refers to the subsites for binding of a glucopyranosyl residue (G) which are α-1,4-linked. The "cleavage" and "coupling" site is between subsites S_1 and S_1'.

In our laboratory, in order to identify the catalytic functions of E-domain, especially MBS1, site-directed mutation on MBS1 of E-domain from β-CGTases gene of *B. firmus* var. *alkalophilius* was cared out, thereby several mutant β-CGTases was obtained. Our recent work using site-directed mutagenesis showed that MBS1 of E-domain plays a more critical role in the cyclization reaction forming CDs from starch than in the coupling or starch-hydrolysis reactions as shown in Figure 11 [12].

Three-dimensional structures of α-amylase and CGTases on X-ray crystallographic data reveal a common principle in the set up of their catalytic domains. The N-terminal domain A, a(β/α)8 barrel. This common structure motif and the four highly conserved primary sequences, which are part of the active site, implies that both enzymes are a common catalytic mechanism. However, distinct variations in β-strand-loop structures are believed to be responsible for different product specificity in different enzymes.

Klein *et al.*[13] have proposed that the active center of CGTase contains seven subsites designated from the nonreducing end to the reducing end, as S5 through S2` as shown in Figure 4. The cleavage point is located between sub-site S1 and S1`. The acidic amino acids Asp-229, Glu-328 are arranged next to the cleavage point and play an essential role in catalysis.

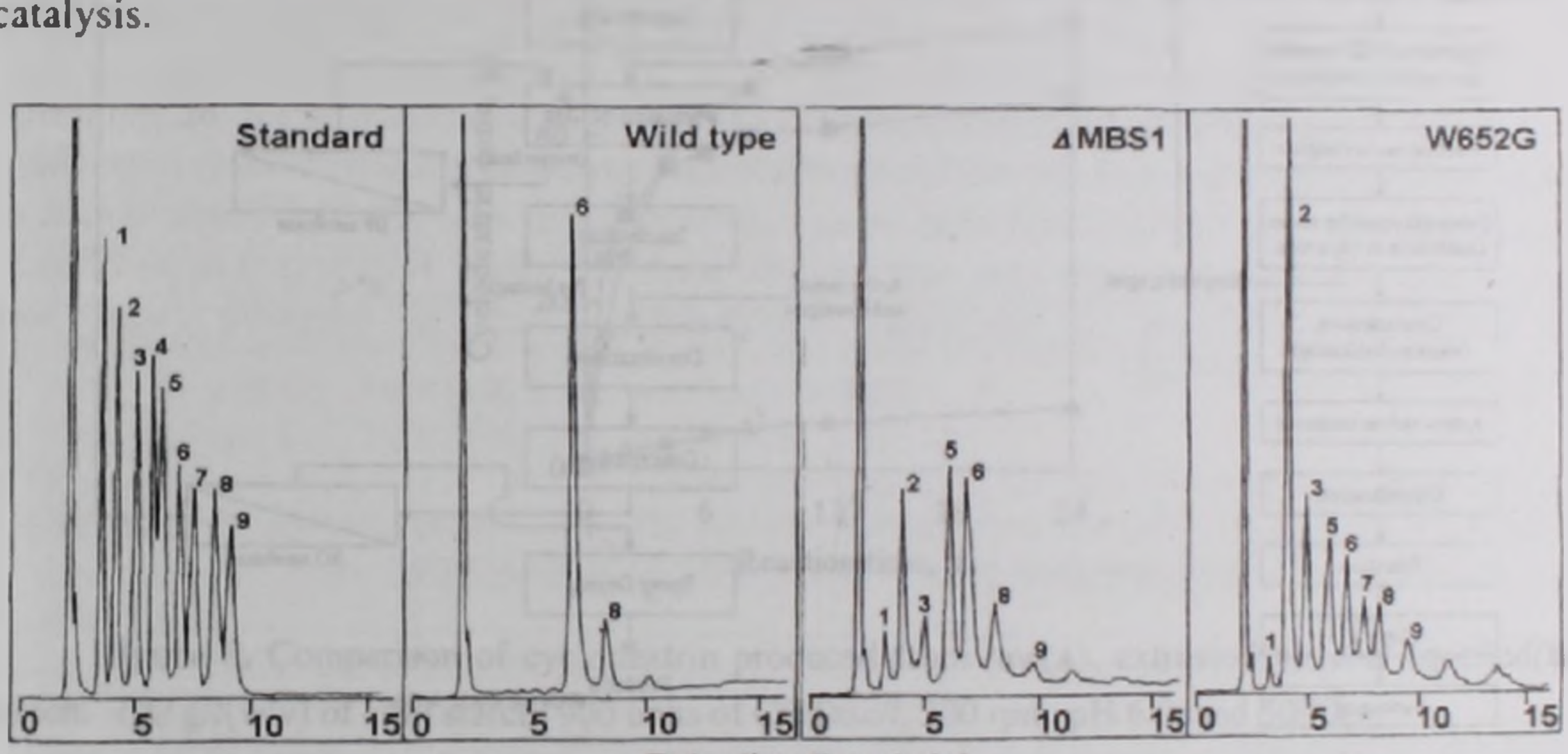

Figure 5. HPLC chromatogram of CDs and maltooligosaccharides produced from soluble starch by wild-type, MBS1, and W652G β-CGTases.

Wild-type, MBS1, and W652G β-CGTases (0.1 unit/ml cyclization activity) were reacted with 5.0 %(w/v) of soluble starch dissolved in 50 mM Tris-Maleic acid-NaOH buffer (pH 6.0) at 50°C for 12 hours. Peak 1 : Glucose (G1), 2 : maltose (G2), 3 : Maltotriose (G3), 4 : α-CD, 5 : maltotetraose (G4), 6 : β-CD, 7 : maltopentaose (G5), 8 : γ-CD, 9 : maltohexaose (G6)

Industrial Processes for Production of Cyclodextrins

Production of CDs derived from starch has been carried out by two stages; liquefaction or partial cyclization of starch by liquefying amylase or CGTase and then cyclization of the liquefied starch by CGTase [14]. And also, surfactant, solvents, and PEG etc. as complexants are added into reaction in order to enhance the CD conversion yield and production rate in solvent process developed in the late 1960s from work by Corn Products Corporation, a US-based food company. Figure 6. shows the scheme of a typical solvent process for production of CDs.

However, the addition of solvents may be cause the safety in foods and pharmaceutical applications and need the complicated process to recycle the solvent. Therefore, CDs produced by solvent process are restricted the application of certain field because remained the solvent in DE cavity. In the beginning of the 1970s, to overcome the problems of solvent process, a process for manufacturing CDs using chromatography or ultrafiltration system instead of the use of any organic solvent in Japan [15,16]. Fig .7. shows manufacturing procedure for cyclodextrin using ultrafiltration and reverse osmosis membrane of Dnsuiko Co. in Japan.

However, above traditional enzyme reaction system utilizing liquefied starch as the substrate for follow-up enzyme reaction by various carbohydrase has the following shortcomings, such as, high energy consumption, low product yield, and complexities of separation and purification of products because the residual oligosaccharides formed at liquefaction step and remained after main reaction by various carbohydrases.

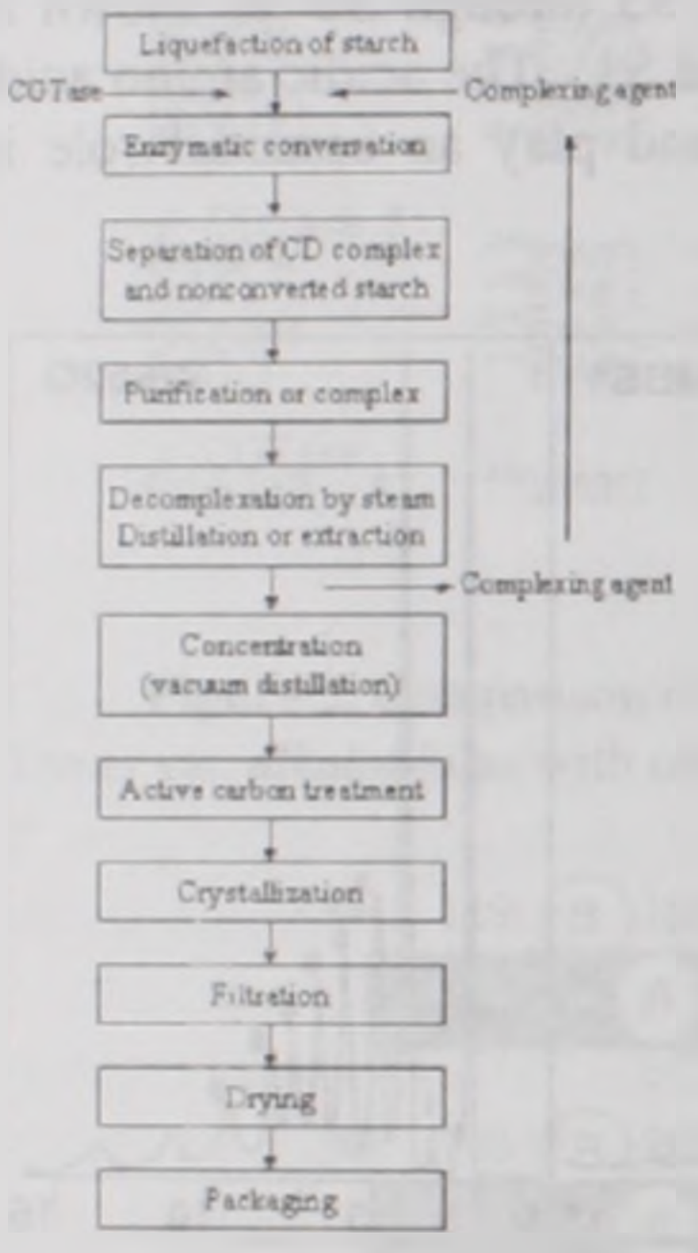

Figure 6. Solvent process for production of cyclodextrin

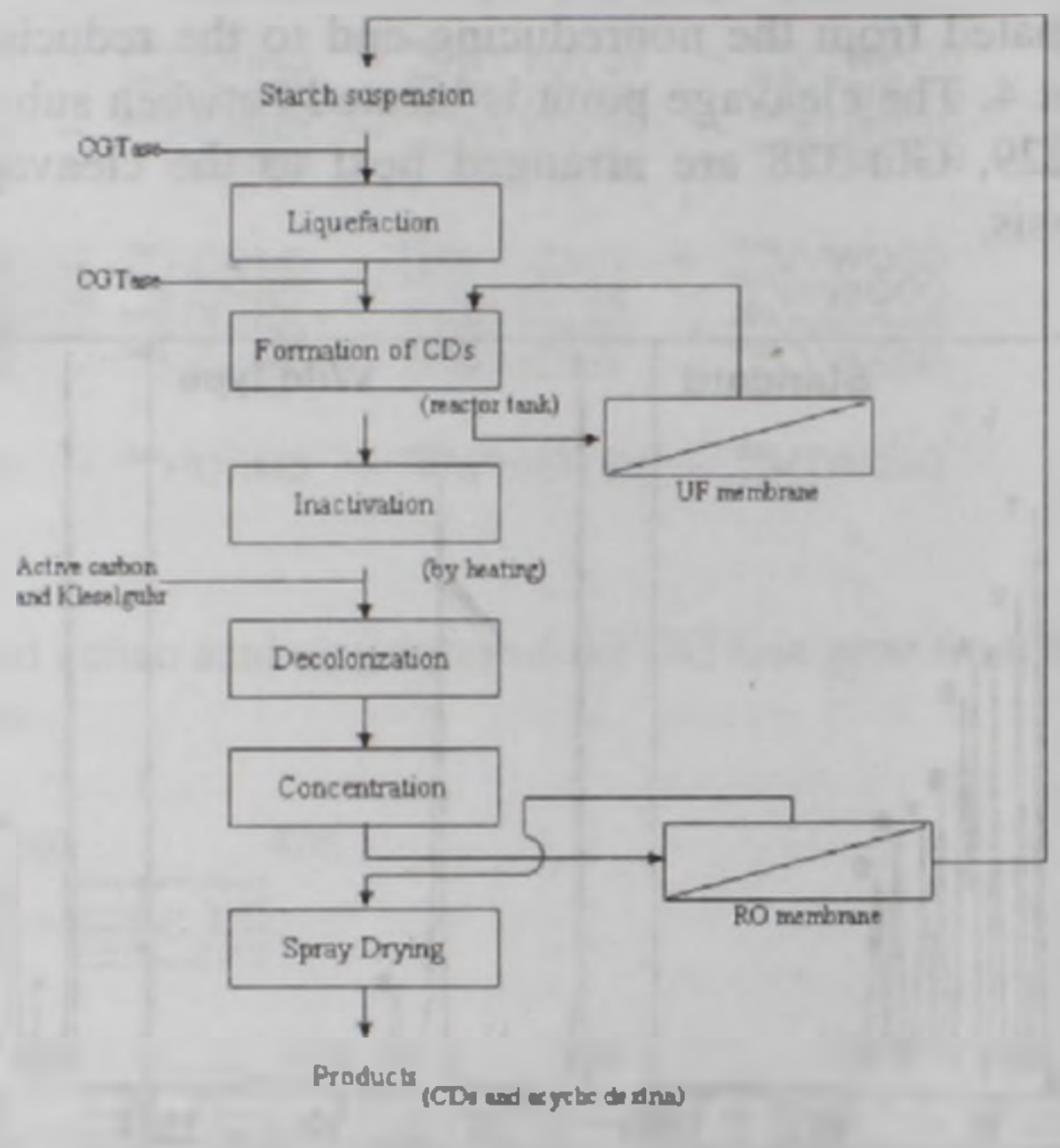

Figure 7. Manufacturing procedure for cyclodextrin using ultrafiltration and reverse osmosis membrane

If the above products can be produced from insoluble raw starch directly without liquefaction of starch, it can be expected that the high purity of products can be obtained without accumulation of undesirable oligosaccharides, for enzyme reaction may be carried out directly from the glycosyl residues in the surface of raw starch. Also, the easy separation of residual insoluble starch by simple separation process will facilitate the purification of various products. However, because the raw starch exists as the compact crystalline structure, the enzyme reaction rate and yield of products from raw starch would be too low for industrial implication. Therefore the structure modification of raw starch is required to increase the susceptibility to enzymes.

In order to increase the susceptibility to various carbohydrases, the raw starch was extruded instead of liquefaction by cooking and liquefying enzyme. The extrusion starch

exists as nearly water insoluble suspension state, the intermediate states between water soluble liquefied and insoluble raw starch, therefore the practical heterogeneous enzyme reaction system composed of soluble enzyme and nearly insoluble substrate could be maintained. In this way the advantages of direct production of CD, maltose, and transglycosylated stevioside from surface of swollen extrusion starch may be achieved.

In our laboratory, characteristics of carbohydrase reaction in heterogeneous enzyme reaction system utilizing swollen extrusion starch were investigated [17-23]. The progresses of enzyme reaction of CD production using raw starch, liquefied starch, and swollen extrusion starch, were compared. The highest concentration of 54 g/l CDs (total of α-, β-, and γ-CD) was obtained after 24hours for extrusion starch, compared to 45 g/l of that of liquefied starch. Meanwhile only 6 g/l of CD was produced after 24 hours from raw starch indicating that CD production reaction from raw starch is very limited. Figure 8 also showed that the rate of CD synthesis from swollen extrusion starch was slightly lower at the initial stage of reaction compared with that of liquefied starch, however increased steadily, and them exceeded from after 4 hours of reaction.

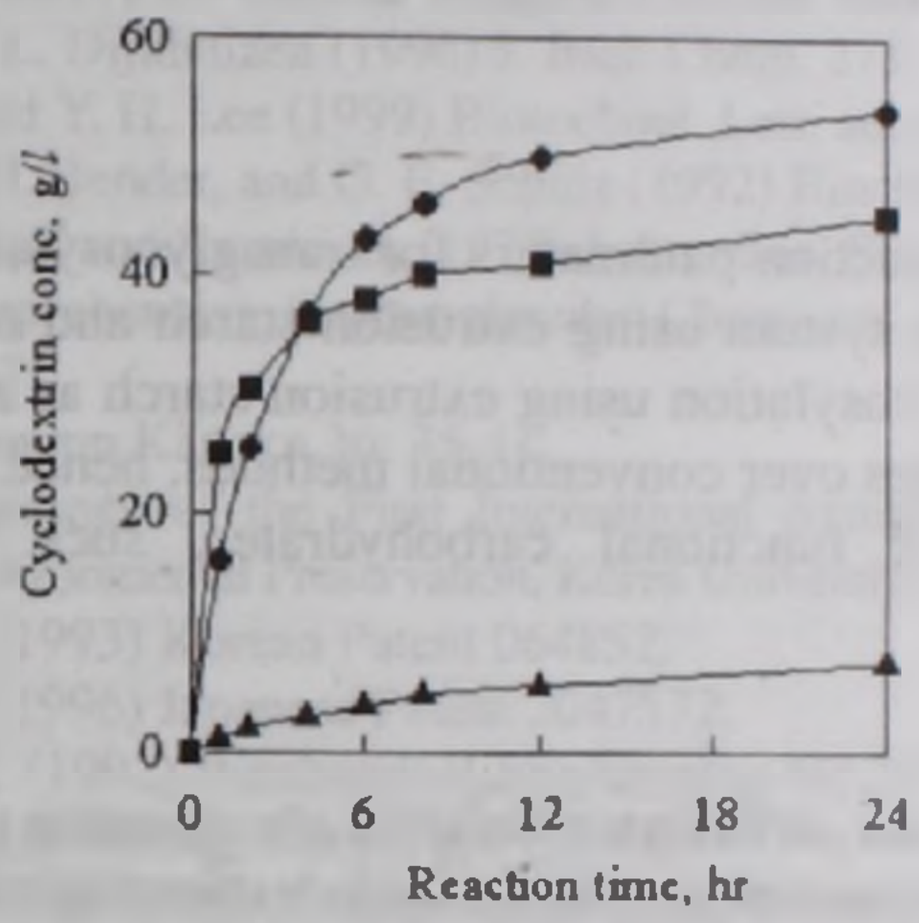

Figure 8. Comparison of cyclodextrin produced from raw(▲), extrusion(●), and liquefied(■) starch. 100 g/l(w/v) of corn starch, 900 units of CGTase/l, 200 rpm, pH 6.0, and 50°C.

As shown in Figure 9 in the case of swollen extrusion starch, CD was mainly produced without accumulation of any significant amount of maltooligosacchries as can be seen in HPLC chromatogram. Above characteristics may be explained by the action of CGTase that synthesize CD from the non-reducing ends of extrusion starch, and the extrusion starch existed in swollen granular structure without significant fragmentation, so the formation of other maltooligosaccharides was depressed.

Also the un-reacted residual starch could be easily separated by simple unit operation, such as certifugation which will facilitate recovery and purification of CD produced after enzyme reaction. It was also suggested that the spent CGTase remained in the reaction mixture could be recovered for reutilization by adsorbing on fresh swollen extrusion starch. Above facts suggested that the CD production in heterogeneous enzyme reaction system utilizing the swollen extrusion starch seems to have potential advantages for CD production in industrial scale, and further study needed to be conducted.

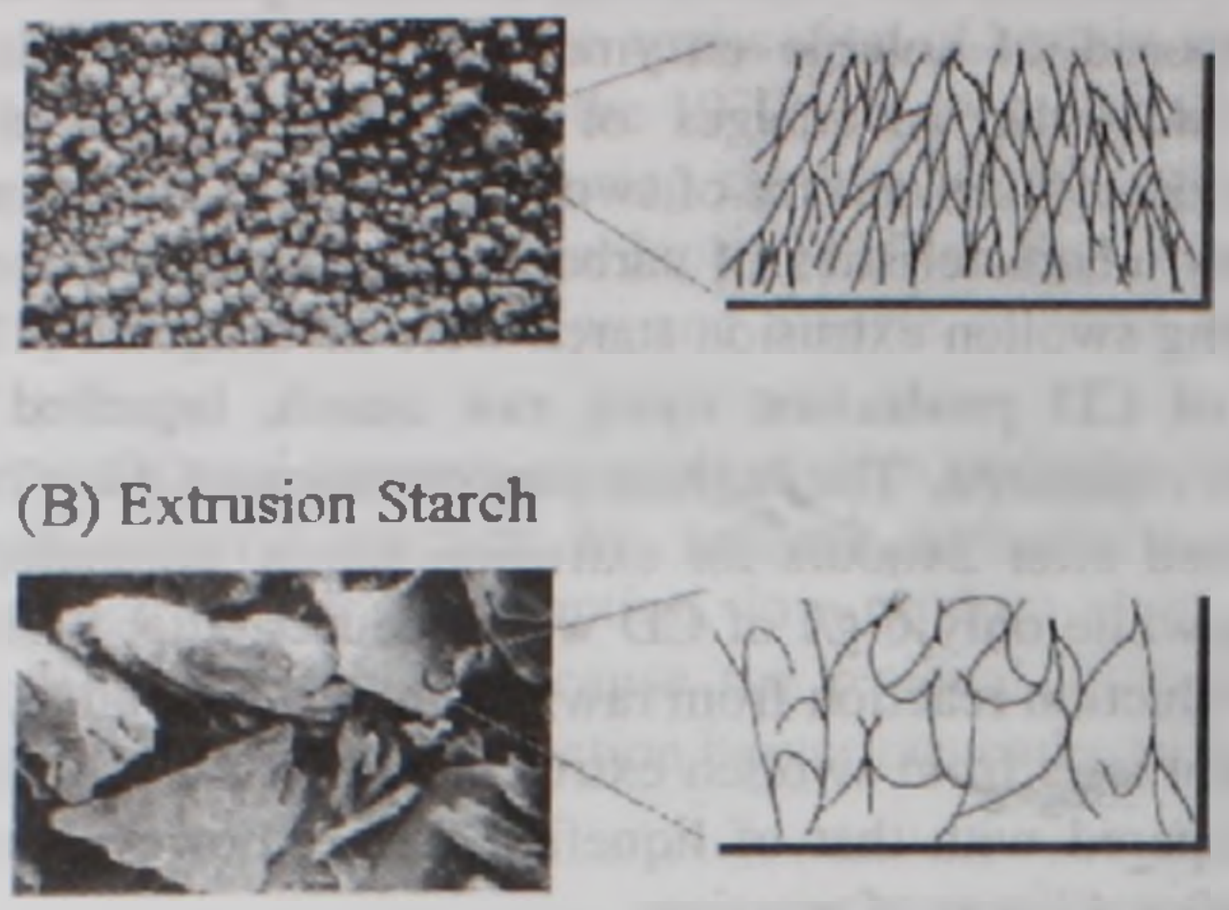

Figure 9. Scanning electron microscopic photograms and hypothetical micellar structures of raw and extrusion starch.

Table 3 compares the reaction parameters for transglycosylation reaction of stevioside in the heterogeneous reaction system using extrusion starch and conventional system using liquefied starch. The transglycosylation using extrusion starch as a glycosyl donor seems to have many potential advantages over conventional methods, hence, is expected to be utilized for industrial production of functional carbohydrates, such as coupling sugar and transglycosylated stevioside.

Table 3. Comparison of the main performance variables of convention process and heterogeneous enzyme reaction systems for cyclodextrin production

	Conventional process	Heterogeneous enzyme reaction system	
		Bioattritor	Extrusion starch
Starch concentration (g/l)	100	100	100
Cyclodextrin concentration (g/l)	45	48	54
Yield of cyclodextrin	0 45	0.48	0.54
Half reaction time (hr)*	2 0	6.2	3.2
Separable residual starch (g/l_	-	51	43
Purity of total cyclodextrin (%)	65	>95	>95
Glucose concentration (g/l)	16	<1	<1
Maltooligosaccharide concentration (g/l)	8	<1	<1
α-CD: β-CD: γ-CD ratio	1 9:2.6:1.0	1.6:3.9:1.0	1.9:4 3:1.0
Separation of residual maltooligosaccharides	Required	Not required	Not required
Separation of residual starch	Difficult	Easy	Easy
Separation and purification of cyclodextrin	Difficult	Easy	Easy

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRINS AND POLYMERS TOWARDS APPLICATIONS

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Miscellaneous possibilities are known to bring cyclodextrins and polymers together. The polymerization of cyclodextrins leads to polymeric materials that can be used for several applications. The formation of polyrotaxanes due to threading of cyclodextrin molecules onto a polymeric backbone or side chains alters the behaviour of polymers. The permanent fixation of cyclodextrins on the surface of different polymeric materials enables the creation of textile materials with new properties.

Известны разнообразные возможности связывания циклодекстринов и полимеров. Полимеризация циклодекстринов ведет к образованию полимерных материалов, которые могут быть использованы для нескольких целей. Образование полиротаксанов, благодаря волокнистости молекул циклодекстрина на полимерной основе или на боковых цепях, изменяет поведение полимеров. Постоянная фиксация циклодекстринов на поверхности разных полимерных материалов дает возможность создавать текстильные материалы с новыми свойствами.

Հայտնի են ցիկլոդեքստրինների և պոլիմերների կապակցման բազմազան հնարավորություններ: Ցիկլոդեքստրինների պոլիմերիզացիան առաջ է բերում պոլիմերային նյութեր, որոնք կարող են օգտագործվել որոշ նպատակներով: Պոլիոռոտաքսանների առաջացումը, շնորհիվ ցիկլոդեքստրինի մոլեկուլի թելատվության պոլիմերային հիմքի կամ կողքային շղթայի վրա, փոխում է պոլիմերների վարքագիծը: Ցիկլոդեքստրինների մշտական ֆիքսացիան տարբեր պոլիմերային նյութերի մակերեսին թույլ է տալիս ստեղծել նոր առանձնահատկություններով մանածագործական նյութեր:

Introduction

The degradation of starch results in formation of cyclodextrins, a process that was presented in the literature more than 100 years ago [1,2]. The structure of the degradation products was determined by Freudenberg [3,4]. Cramer was the first who realized the formation of inclusion compounds in solution with cyclodextrins [5]. At the beginning of the 50th this idea was rather revolutionary. In a lecture given at the First International Symposium on Cyclodextrins Cramer described this state of affairs as follows: "A whole maffia of physicochemists was united against the young greenhorn from Heidelberg. I believe the concept was finally successful." [6].

The formation of complexes with cyclodextrins has been studied with increasing interest since that time [7]. Beside the complex formation, the chemical modification of cyclodextrins also received much interest. Thus a large number of substituted cyclodextrin molecules are already known [8].

Polymeric cyclodextrins. Chemical substances with two or more covalently bounded cyclodextrins are described as polymers. They are synthesized from the reaction of

cyclodextrins with bifunctional or multifunctional substances. The synthesis of different polymers with cyclodextrins has already been reviewed in detail [7,8]. Some industrial applications of cyclodextrin containing polymers have also been described [7,9].

The synthesis of cyclodextrin polymers is relatively easy. The hydroxyl groups of the cyclodextrins are located at the upper and lower rim of the torus, see Figure 1. Mainly the reaction of cyclodextrins with epichlorhydrin has been studied.

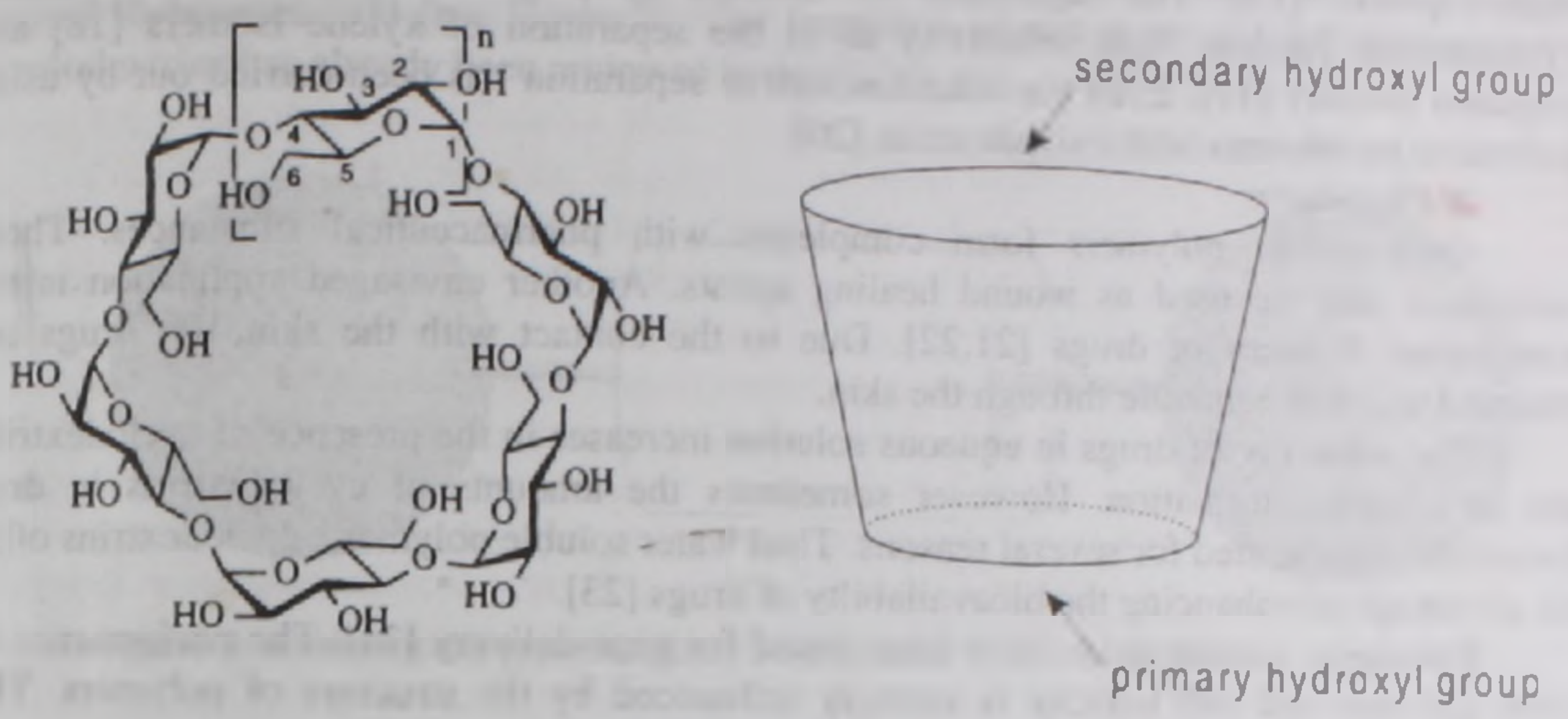

Figure 1. Chemical structure of cyclodextrins (α-CD: n=1, β-CD: n=2; γ-CD: n=3) and the position of the hydroxyl groups.

The behaviour of cyclodextrin polymers varies extremely. They can be arranged in the following manner:

soluble in water,
unsoluble but strong swelling in water,
weak swelling,
solid (foils, fibre, foam).

Even in the cavities of the polymeric cyclodextrins other organic substances are included. In solution the stability of the complexes formed with cyclodextrins in polymers are higher as with the monomeric cyclodextrin. This is due to the simultaneous interactions of at least two cyclodextrin molecules of the polymer with the guest molecule [10,11]. The complex formation between solid cyclodextrin polymers and molecules in solution results in more stable complexes compared with the reaction of cyclodextrins in solution. This effect is caused by the reaction kinetic. The rates of complex formation in homogeneous and heterogeneous systems are similar. However, the kinetics of the dissociation reaction in the heterogeneous system is several orders of magnitude smaller as in solution [12].

Polymers with cyclodextrins are already used in some applications.

1. Analytical chemistry:

Differences of the complex stability of cyclodextrins with organic substances are used for their chromatographic separation. The polymeric cyclodextrins are employed as stationary phase in liquid chromatography [13,14]. The thermal stability of cyclodextrin resins is high enough to allow them acting as stationary phase in gas chromatography [14,15].

Immobilized cyclodextrins are used in affinity chromatography. Using a stationary phase of β-cyclodextrin tetradecasulfate polymer mixed with Cu-Sepharose the purification of fibroblast growth factor has been performed [16].

A significant improvement of separation and catalytic ability of membrane has been achieved by the introduction of cyclodextrin oligomer in the polymeric membrane. In this respect, the preparation of a poly(vinyl alcohol) membrane containing β-cyclodextrin oligomer and its pervaporation characteristics for water-alcohol mixture separation have been reported [17]. The separation of isomers by polymeric membranes containing cyclodextrins features high selectivity as in the separation of xylene isomers [18] and propanol isomers [19]. Even the enantioselective separation has been carried out by using polymeric membranes with cyclodextrins [20].

2. Pharmacy:

Cyclodextrin polymers form complexes with pharmaceutical substances. These complexes may be used as wound healing agents. Another envisaged application is the transdermal delivery of drugs [21,22]. Due to the contact with the skin, the drugs are liberated and will penetrate through the skin.

The solubility of drugs in aqueous solution increases in the presence of cyclodextrins due to complex formation. However sometimes the amounts of cyclodextrins in drug formulation are limited for several reasons. Thus water soluble polymeric cyclodextrins offer the advantage of enhancing the bioavailabilty of drugs [23].

Polymeric cyclodextrins have been tested for gene delivery [24]. The performance in DNA delivery and cell toxicity is strongly influenced by the structure of polymers. The incorporation of β-cyclodextrin in the polymer backbone decreases significantly the toxicity of polymers.

3. Food Science:

Since insoluble cyclodextrin polymers can easily be removed from food, they are not really food additives. Insoluble cyclodextrin polymers are used to remove the bitter component of orange and grapefruit juices [25]. The browning of food products is caused by the enzymatic oxidation (e.g., chlorogenic acid). In order to prevent this undesired reaction, the chlorogenic acid may be removed from juices using polymeric cyclodextrins [26].

4. Environmental:

The formation of dye complexes with cyclodextrins is well known [27]. Using insoluble polymer resins with cyclodextrins makes possible the removal of dye molecules from aqueous solution [28]. The polymeric cyclodextrins form complexes with different types of dyes. Consequently, such a method has been proposed for the decolorization of waste water from the dye house.

The removal of other organic compounds from aqueous solution is possible too. Thus aromatic substances like benzene or phenol [29], different substituted chlorophenols and nitrophenol [30] or even trichloroethylene [31] are complexed by insoluble cyclodextrin polymers. Even the removal of the polar urea from aqueous solution has been presented in the literature [32].

Nonionic surfactants like alkylphenol ethoxylates have been employed in many industrial processes. Their biodegradation leads to the formation of several biorefractory metabilites. Some of them are acute toxic or endocrinic [33]. The removal of alkylphenol ethoxylates from aqueous solution is difficult. Polymeric cyclodextrins offer simple means for complexation of nonionic surfactants. The polymeric material can easily be regenerated by extraction with alcohols [34].

Rotaxanes and polyrotaxanes with cyclodextrins. Another way to synthesize polymers containing cyclodextrins was accomplished much later. The formation of a complex between α-cyclodextrin and polyethyleneglycol bisamines followed by a reaction of the amino end groups with 2,4-dinitrofluorobenzene leads to polymeric materials with threaded cyclodextrin molecules [35,36]. The cyclodextrins are arranged like pearls on a string formed by the polymer chain. These cyclodextrins are unable to thread from the polymer chain although they are not fixed by chemical bonds. According to Schill these molecules formed from an axis and a wheel held together without any chemical bonds are called **Rotaxanes** [37] (see Figure 2). The formation of rotaxanes and polyrotaxanes with cyclodextrins has already been reviewed in detail [38-41].

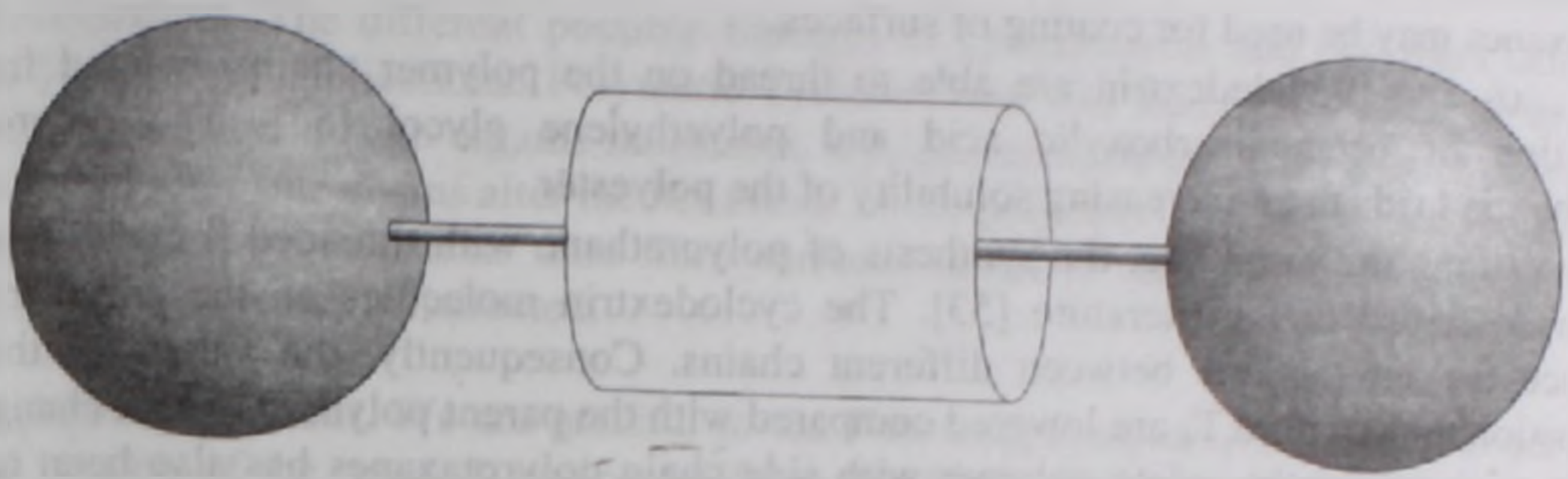

Figure 2. Schematically presentation of a rotaxane.

Mixing polyethylene glycols with α-cyclodextrin already results in the precipitation of the corresponding complexes [42]. β-Cyclodextrin does not form insoluble complexes with polyethylene glycols [43]. However, a complex formation takes place in solution [44]. Polypropylene glycols form solid complexes with β-cyclodextrin [45].

Using α-cyclodextrin polyrotaxenes with polyethylene glycol the cyclodextrin molecules can be crosslinked using epichlorohydrin. After dethreading from the polyethylene glycol chain pure cyclodextrin tubes are obtained [46]. Their schematic structure is given in Figure 3.

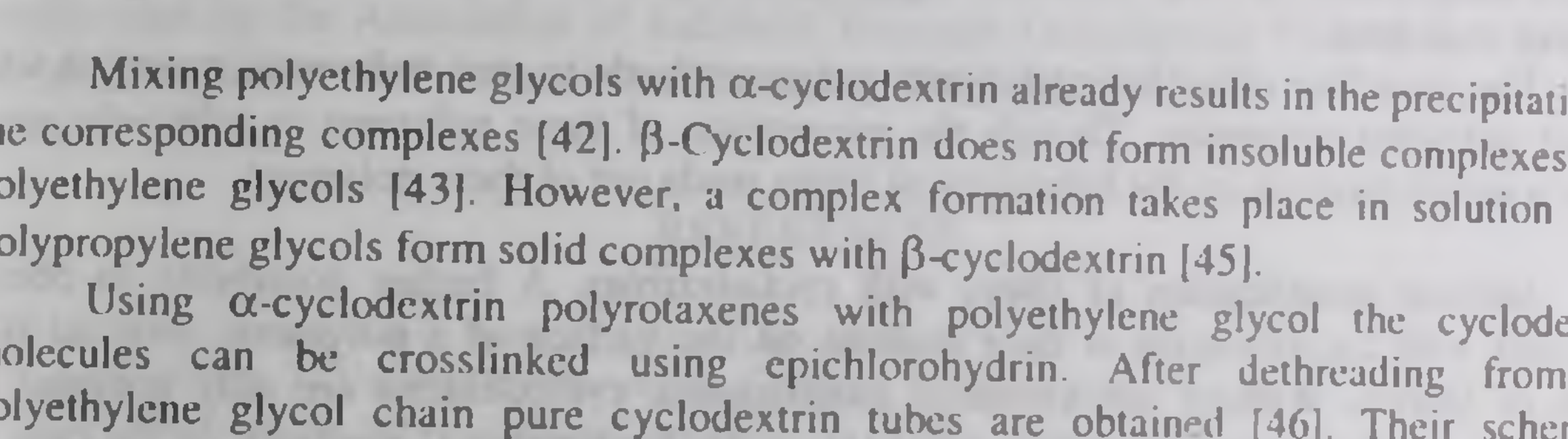

Figure 3. Schematic structure of cyclodextrin tubes.

These tubes may be considered as a new class of cyclodextrin polymers. These cyclodextrin tubes are expected to form complexes. Only recently, the reactions between the tubes from α-cyclodextrin and alkyl sulfonates have thoroughly been studied [47]. The

stability of the complexes formed depends upon the number of the methylene groups of the alkyl chain.

In general, the formation of two different types of polymeric rotaxanes with cyclodextrins are possible. The cyclodextrins are located on the polymer main chain [48] or in side chains [49]. The presence of threaded cyclodextrin molecules changes the physical behaviour of the polymeric material.

Thus polyamide rotaxanes obtained by solid state polycondensation of α-cyclodextrin complexes with different amino acids are soluble in water [50,51]. After dissolution of these polyrotaxanes in few cases a slow formation of a precipitate is observed depending on the chemical structure of the amino acids. Bulky substituents at the amino acids prevent the cyclodextrin molecules from dethreading. Due to their solubility in water these polyamid rotaxanes may be used for coating of surfaces.

α- and β-cyclodextrin are able to thread on the polymer chain obtained from the reaction of octanedicarboxylic acid and polyethylene glycol [52]. The polyrotaxane formation leads to an increasing solubility of the polyester.

Along the same line, the synthesis of polyurethane with threaded β-cyclodextrin has been described in the literature [53]. The cyclodextrin molecules on the polymer chains reduce the interactions between different chains. Consequently, the values of the glass transition temperature T_g are lowered compared with the parent polyurethane. A change in T_g value of a polymethacrylate polymer with side chain polyrotaxanes has also been reported [54]. The presence of the threaded cyclodextrin molecules reduces the mobility of the polymer segments.

The threading of cyclodextrins onto polymers leads to new polymeric materials with almost unknown properties. Though the preparation of these polymers is relatively easy, there is no information on the behaviour of fibres made out of these polymers.

Surface modification of fibres with cyclodextrins. A further possibility to obtain polymers with cyclodextrins is their fixation on the surface of a polymeric material like fibres or fabrics. Without any chemical modification, cyclodextrins are only bounded to cellulosic materials using epichlorhydrin [21] or other bifunctional reactants. In the case of all other synthetic polymers, either cyclodextrin derivatives have to be used or the fibre surface has to be modified chemically to enable the fixation of cyclodextrins. Different strategies for the fixation of cyclodextrins on polymeric materials are presented in Table 1.

Table 1. Strategies for the fixation of cyclodextrins onto the surface of different fibres [55].

	Fibre material				
Fixation Mode	Cellulose	Wool	Polyamid Fibre	Polyester Fibre	Polyacrylonitrile Fibre
Cross-linking agent	+	-	-	-	-
Ionic interactions	-	+	+	-	+
Covalent bonds	+	+	+	-	-
Van der Waals interactions	-	-	+	+	+

A cyclodextrin derivative with a reactive group (e.g., the monochlorotriazinyl group) is able to react with the hydroxyl groups of cellulosic fibres like a reactive dye [56, 57]. Permanent fixation on fibres made from polyester is only possible with cyclodextrin derivatives with long alkyl chains or other hydrophobic groups. Comparable with disperse dyeing, the hydrophobic part of the substituted cyclodextrins migrates into the fibre above

the glass transition temperature. The polar cyclodextrin molecules remain on top of the fibre surface [58]. After graft polymerization of glycidyl methacrylate onto polypropylene fabrics α-, β- and γ-cyclodextrin are coupled with the epoxide group [59,60].

With cyclodextrins fixed onto the fibre surfaces, such textile materials get new properties. As for instance the comfort of clothing increases. The organic components of sweat are complexed and therefore the possibility of body odour development is reduced. The chemical analysis of sweat components can also be used for medical applications. The identification of organic compounds from patients enables new ways in medical diagnosis. Various aspects of textile materials permanently finished with cyclodextrins have been reviewed very recently [61].

Conclusions. The different possible linkages of cyclodextrins and polymers offer a large varity of new applications. Polymeric cyclodextrins will mainly be used as strong complex forming agents with organic molecules. Cyclodextrins threaded on a main polymer chain or in polymer side chains alter the behaviour of the polymers compared with polymers. The attachment of cyclodextrins onto fibre surfaces modifies only the surface area. The polymer properties remain unaffected.

Acknowledgement. We are grateful to the Forschungskuratorium Textil e.V. for the financial support of the research project (AiF-Nr. 12850N). This support was granted from resources of the Federal Ministry of Economics and Technology (BMWi) via supplementary contributions by the Association of Industrial Research Organization "Otto-von-Guericke" e.V. (Arbeitsgemeinschaft industrieller Forschungsvereinigungen, AiF).

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

MOLECULAR IMPRINTING OF CYCLODEXTRIN IN HOMOGENEOUS SOLUTIONS

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In homogeneous and alkaline aqueous solutions, cyclodextrin was reacted with epichlorohydrin in the presence of various templates. The molecularly imprinted polymers, which involve 2-4 cyclodextrin residues and are completely soluble in water, satisfactorily recogndasdasdaized the template molecules and selectively bound them in water. New methodology to prepare receptors for nanometer-scaled guests in a tailor-made fashion has been developed.

В однородных и щелочных водных растворах циклодекстрин вступает в реакцию с эпихлоргидрином в присутствии разных темплетов (эталонов). Молекулярно распечатанные полимеры, которые включают 2-4 остатка циклодекстринов и полностью растворимы в воде, соответственно узнают молекулы темплета и избирательно связывают их в воде. Разработана новая методология приготовления рецепторов в виде "мужского" покроя для нанометр-масштабированных "гостей".

Սիատարր և հիմնային ջրային լուծույթներում ցիկլոդեքստրինը ռեակցիայի մեջ է մտնում էպիքլորհիդրինի հետ տարբեր թեմփլեթների (էտալոնների) ներկայությամբ: Մոլեկուլյար բացված պոլիմերները, որոնք ներառում են ցիկլոդեքստրինի 2-4 մնացորդներ և լիովին լուծելի են ջրում, համապատասխանաբար ճանաչում են թեմփլեթի մոլեկուլները և ընտրողաբար կապում են դրանք ջրի մեջ: Նանոմետր-մասշտաբային «հյուրերի» համար մշակվել է ռեցեպտորների պատրաստման նոր մեթոդոլոգիա տղամարդու ձևածքի տեսքով.

Introduction

Host-guest chemistry has made such a great progress that we can now prepare very good receptors rather easily and successfully as long as the target guest is small (e.g., the diameter < 5 Å) [1]. Cyclodextrins (CDs) and their derivatives are typical examples [2]. However, the design of receptors for large guest molecules (10 Å diameter or greater) has not yet been very successful. Thus there still remains a big gap between naturally occurring receptors and artificial ones [3]. These receptors are regarded as the key for the future science and technology, in which wide spectrum of large molecules must be precisely differentiated from each other. Recently, we presented a new methodology for the preparation of artificial receptors which selectively and efficiently bind nanometer-scaled guests in water [3-6]. By using molecular-imprinting technique [7], CD molecules are built up in a predetermined way (Figure 1). Each of the CD molecules in the ordered assembly binds the predetermined portion of the target guest, and thus the assembly as a whole recognizes this guest very exclusively [8].

Figure 1. Molecular-imprinting of CD to prepare polymeric receptors for nanometer-scaled guests.

As described previously [3-6], the molecular imprinting of CDs (their crosslinking) was achieved either in DMSO or in water in the presence of the target guest. By these procedures, receptors for steroids [4], peptides [5], and antibiotics [6] were successfully prepared. In the cholesterol receptors, for example, the binding-sites are formed from two CD molecules which are connected at the secondary hydroxyl sides [4]. The regulation of mutual conformation of CDs by the molecular-imprinting effect has been concretely evidenced by MALDI-TOFMS. Under the imprinting conditions, the template places two or more CD molecules in a close proximity and accelerates the crosslinking between them. These arguments were supported by the NMR analysis.

As described above, the molecular-imprinting of CD can provide very selective receptors for many nanometer-scaled guests in a tailor-made fashion, and thus is highly promising for future applications. The receptors obtained were highly crosslinked polymers which are useful for practical purposes. However, water-soluble receptors are also useful for applications, although it has not yet been clear if the polymeric receptors must be water-insoluble or not. Another question is if the molecular-imprinting must be achieved in heterogeneous systems, as usually done before, or it is also accomplished in homogeneous mixtures Information on these points is important for further developments.

In this paper, we show that the molecular imprinting of CD can be successfully achieved even in homogeneous solutions. The CD polymers as tailor-made receptors for nanometer-scaled guests are obtained either in homogeneous states or in heterogeneous states. One can easily choose either of these two methods, depending on the purpose. This finding should greatly widen the scope of application of this novel methodology.

Materials and Methods

β-Cyclodextrin (β-CD) and other chemicals were commercially obtained. Molecular imprinting of β-CD was achieved by reacting β-CD with epichlorohydrin in aqueous NaOH solutions in the presence of appropriate template. Typical reaction conditions for the molecular imprinting were as follows: [β-CD] = 0.2 M, [epichlorohydrin] = 1.4 M, [NaOH] = 2 M, and [the template] =0.1 M. After the reactions at 25°C for 8 h, the mixtures were dialyzed with water for 3 days by using a seamless cellulose membrane (UC 16-32-100 (Sanko Junyaku Co., Ltd.)). Complete removal of the template, the crosslinking agent, and others from the polymers by this procedure was confirmed by using UV-visible absorption spectroscopy. Circular dichromism (CD) spectra were obtained at pH 8 (50 mM phosphate buffer) on a Jasco J-725 spectropolarimeter. MALDI-TOFMS was run on a Shimadzu/KRATOS KOMPACT TYPE I spectrometer.

Results and Discussion

When L-Phe-L-Phe was used as the template, the molecular imprinting of β-CD under the above conditions proceeded in completely homogeneous solutions (see Figure 1). On the other hand, with the use of Z-Trp-Phe as the template, the reaction mixtures were heterogeneous in the early stage of the reactions. However, the mixtures gradually became homogeneous as the reaction proceeded, and totally homogeneous solutions were obtained at the end of the reactions. According to MALDI-TOFMS spectroscopy, the products, obtained after the dialysis with a cellulose membrane (see the Materials and Methods section), are composed of monomeric, dimeric, trimeric, and tetrameric β-CDs, in which the corresponding number of β-CD molecules are connected by epichlorohydrin-derived residues. Furthermore, several 2,3-dihydroxyethyl residues are bound to these β-CD oligomers. These residues come from the epichlorohydrin molecules, which react with the hydroxyl group of one β-CD molecule and the other ends react with water molecules (or hydroxide ion) instead of another β-CD molecule. As expected, the degree of substitution of β-CD by the 2,3-dihydroxyethyl residues showed a normal distribution. In these homogeneous solutions, β-CD molecules were efficiently crosslinked with each other by epichlorohydrin, and totally water-soluble polymeric receptors towards the template molecules have been successfully obtained.

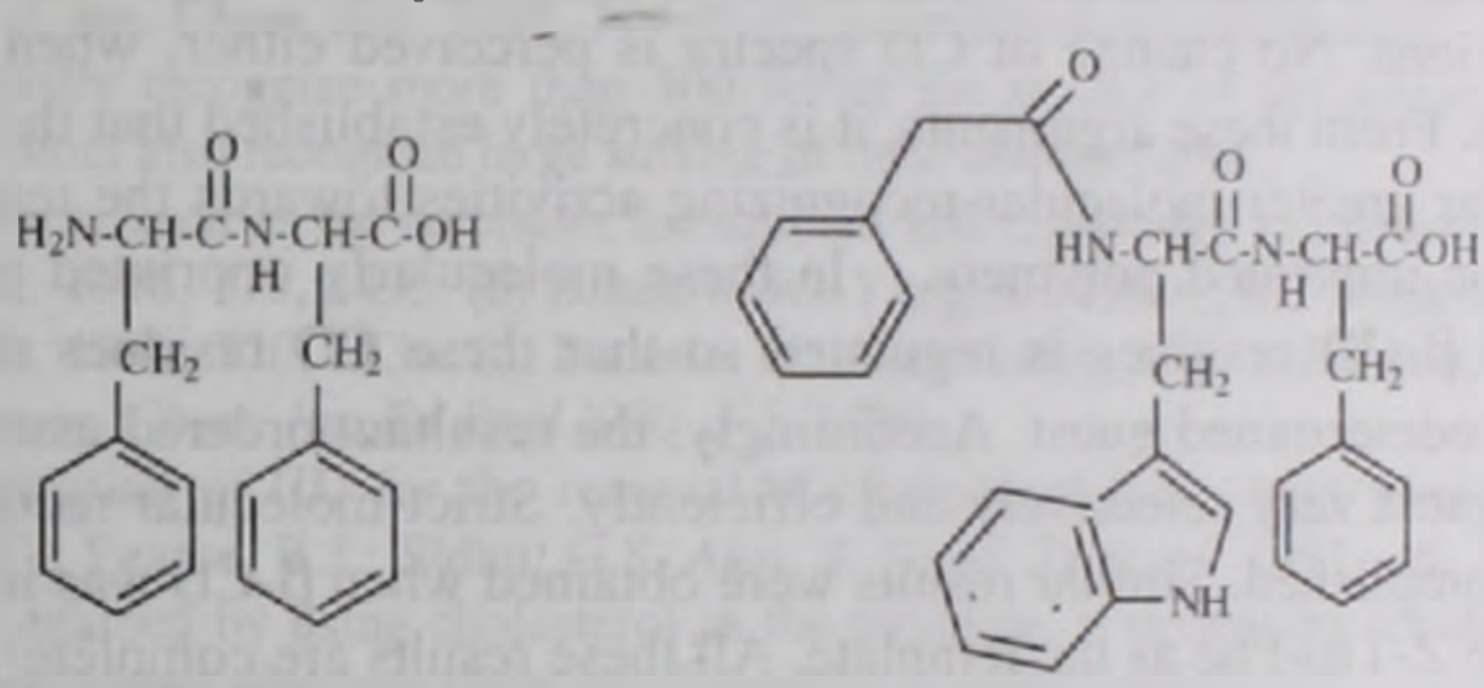

Figure 2. CD spectra of L-Phe-L-Phe in the presence of (A) the imprinted β-CD polymer obtained by using L-Phe-L-Phe as the template and (B) non-imprinted β-CD polymer: the concentrations of β-CD residue in the polymer are (a) 0.0, (b) 1.0, (c) 2.0, (d) 5.0, and (e) 10.0 mM. [L-Phe-L-Phe] = 0.9 mM at pH 8 (phosphate buffer).

Figure 2 (A) shows the CD spectra of L-Phe-L-Phe (guest) in the pH 8 solutions which contain the water-soluble imprinted β-CD polymers. The polymers were prepared by using L-Phe-L-Phe as template (in this case, L-Phe-L-Phe is used as both the template and the guest). As the amount of the imprinted β-CD polymer in the solutions is increased, the CD signal due to the L-Phe-L-Phe gradually and monotonically shifts towards longer wavelength. Apparently, L-Phe-L-Phe is clearly recognized by the imprinted β-CD polymer. In contrast, only marginal change of CD spectrum is observed when D-Phe-D-Phe is used as the guest in place of L-Phe-L-Phe. Thus, the induced CD is specific to L-Phe-L-Phe as the guest (note that this polymer was obtained by using L-Phe-L-Phe as the template). The present imprinted β-CD polymers exhibit sufficient enantio-selectivity.

In contrast with the efficient molecular recognition of L-Phe-L-Phe by the L-Phe-L-Phe-imprinted β-CD polymers, non-imprinted β-CD polymers, prepared by crosslinking β-CD in the absence of the template under the same conditions, show no changes in the CD spectra of L-Phe-L-Phe (Figure 2 (B)). These non-imprinted polymers are also composed of monomeric, dimeric, trimeric, and tetrameric β-CDs. Even at higher concentrations of β-CD residue in the polymer, the change of spectrum is only marginal. Furthermore, the parent β-CD itself (in the monomer form) does not induce any change in the CD spectra, when it is mixed with L-Phe-L-Phe in the solutions of pH 8. The formation constant of the β-CD/L-Phe-L-Phe complex is rather small (around 10 M^{-1}) so that this complex is hardly formed in the sample solutions. No change of CD spectra is perceived either, when D-Phe-D-Phe is used as the guest. From these arguments, it is concretely established that the imprinted β-CD polymers have far greater molecular-recognizing activities towards the template molecules than those of non-imprinted polymers. In these molecularly imprinted polymers, mutual conformation of β-CD residues is regulated so that these CD residues show cooperative binding to the predetermined guest. Accordingly, the resultant ordered assemblies of β-CD bind the target guest very selectively and efficiently. Strict molecular recognition has been successfully accomplished. Similar results were obtained when β-CD was imprinted towards another dipeptide Z-Trp-Phe as the template. All these results are completely consistent with our previous results on water-insoluble β-CD polymers as tailor-made receptors[3-6].

In conclusion, the present study has confirmed the following two facts. (1) The molecular imprinting of β-CD is successfully achieved even in homogeneous solutions, as well as in heterogeneous mixtures. (2) Homogenous receptors for nanometer-scaled guests can be obtained in a tailor-made fashion by imprinting β-CD towards the target guest. Both of these factors further substantiate that the molecular imprinting of CD is one of the most promising methods to prepare artificial receptors for large guest molecules, and that this method is applicable to versatile guests. They are certainly applicable to separation of desired products, removal of impurities, and other purposes in chemical industry and biotechnology [10]. Preparation of artificial antibodies and artificial catalytic antibodies by the molecular imprinting are certainly the next targets. These attempts are currently under way in our laboratory.

Acknowledgements. This study was partially supported by the Grant from "Research for the Future" Program of the Japan Society for the Promotion of Science (JSPS-RFTF97I00301) and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan. The support by JSPS Research Fellowships for Young Scientists (for T. H.) is also acknowledged.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRIN NEOGLYCOCONJUGATES

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Progress towards the use of cyclodextrins as drug targetting devices is summarized.

Обобщено развитие работ, ведущих к использованию циклодекстринов в качестве целевых мишеней лекарственных веществ.

Ամփոփված են այն աշխատանքները, որոնք նպաստում են ցիկլոդեքստրինների որպես դեղամիջոցների նպատակային թիրախի օգտագործմանը:

Introduction

Because of their unique structure, cyclodextrins (cyclomaltooligosaccharides, CDs) [1-4] have found applications as molecular encapsulating agents in various fields, including drug delivery [5-8]. Their truncated cone-shaped hydrophobic cavity can accommodate other organic "guest" molecules which, eventually, can be solubilised and stabilised in water. Yet, the anomalous low water solubility of the most interesting representative at least from the industrial point of view (and of its inclusion complexes), namely β-cyclodextrin (cyclomaltoheptaose, β-CD), and its relatively high haemolytic character are important drawbacks to pharmacological uses. To overcome these limitations, several chemically modified CDs, such as hydroxypropyl and methyl ethers, have been proposed [9]. Albeit these so-called "second generation CDs" exhibit a much higher water solubility while still keeping reasonable inclusion ability, they are generally obtained as mixtures of compounds differing in their substitution pattern. In any case, drug transport using either the native (first generation) or the second generation CDs is, essentially, site-unspecific, since CDs do not possess the capability of molecular recognition within the organism.

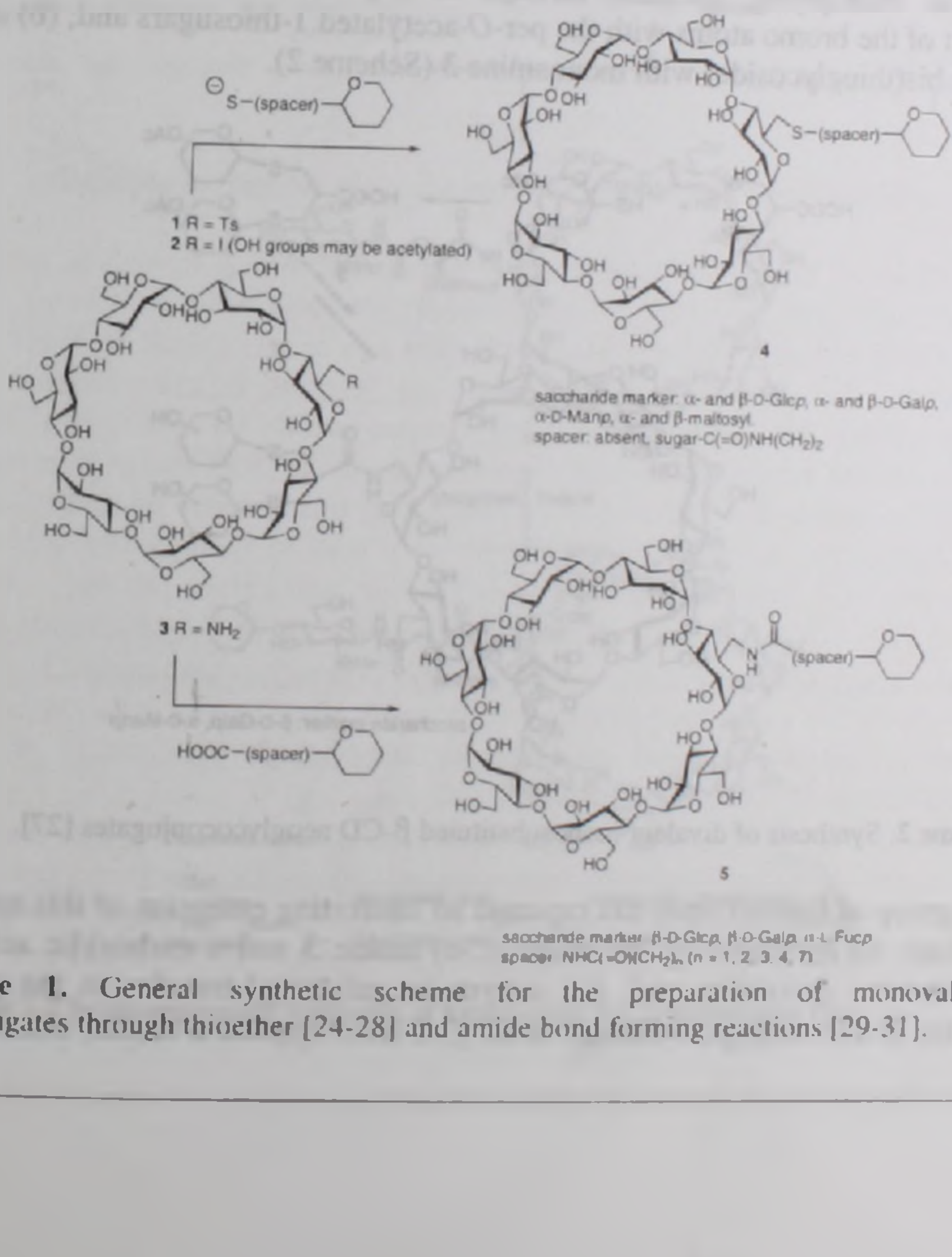
In the last few years an intense effort has been directed towards the design of drug delivery systems able to target pharmacologically active molecules to specific cells, tissues or organs. Since interactions between carbohydrates and carbohydrate-binding proteins (lectins) play important roles in numerous cell recognition processes [10-13], grafting biorecognisable saccharide epitopes onto suitable molecular carriers appears as an attractive approach for the design of new systems for site-specific delivery of therapeutics. Several examples of oligosaccharide-branched CDs have been reported for this purpose. In a first approach, enzymatic transglycosylation of industrially produced glucosyl- or maltosyl-branched CDs was put forward for the preparation of heterogeneously branched CDs incorporating galactose [14-17] or mannose ligands [18]. Although specific interactions of these conjugates with complementary lectins was demonstrated, the heterogeneity of the reaction mixtures precludes a systematic study of the inclusion and recognition properties.

On the other hand, the chemical glycosylation of the native CDs requires long protection/deprotection sequences and is unpractical [19]. As an alternative, substitution of the *O*-glycosidic linkages at the branching points by other atoms or group of atoms has been proposed for the preparation of CD-carbohydrate conjugates (CD neoglycoconjugates), a field in which our laboratories have been actively involved. The development of such strategies runs parallel to the development of efficient methodologies for the chemical functionalisation of CDs [20].

In this article, we summarize the progress that we and others have made to the design of CD-neoglycoconjugates as drug carriers. For reasons of applicability, these works concentrate on β-CD derivatives. Emphasis is placed in the versatility of the synthetic approaches and the lectin-binding and drug inclusion capabilities of the resulting conjugates.

Monovalent Cyclodextrin Neoglycoconjugates

The key precursor for the preparation of monobranched β-CD neoglycoconjugates is the 6^I-deoxy-6^I-tosylcyclomaltoheptaose **1** [21], for which we have recently reported an improved synthesis [22, 23]. This compound can experience S_N2 displacement reactions by suitable nucleophiles in high yield. This reactivity was first exploited by the group of Defaye in the preparation 6^I-*S*-glycosyl-6^I-thiocyclomaltoheptaose derivatives (**4**) by using the sodium salt of 1-thio-α- and β-D-glucopyranose as nucleophile [24] and was further extended to the synthesis of the analogous monovalent α- and β-thiomaltosyl β-CD conjugates [25] (Scheme 1).

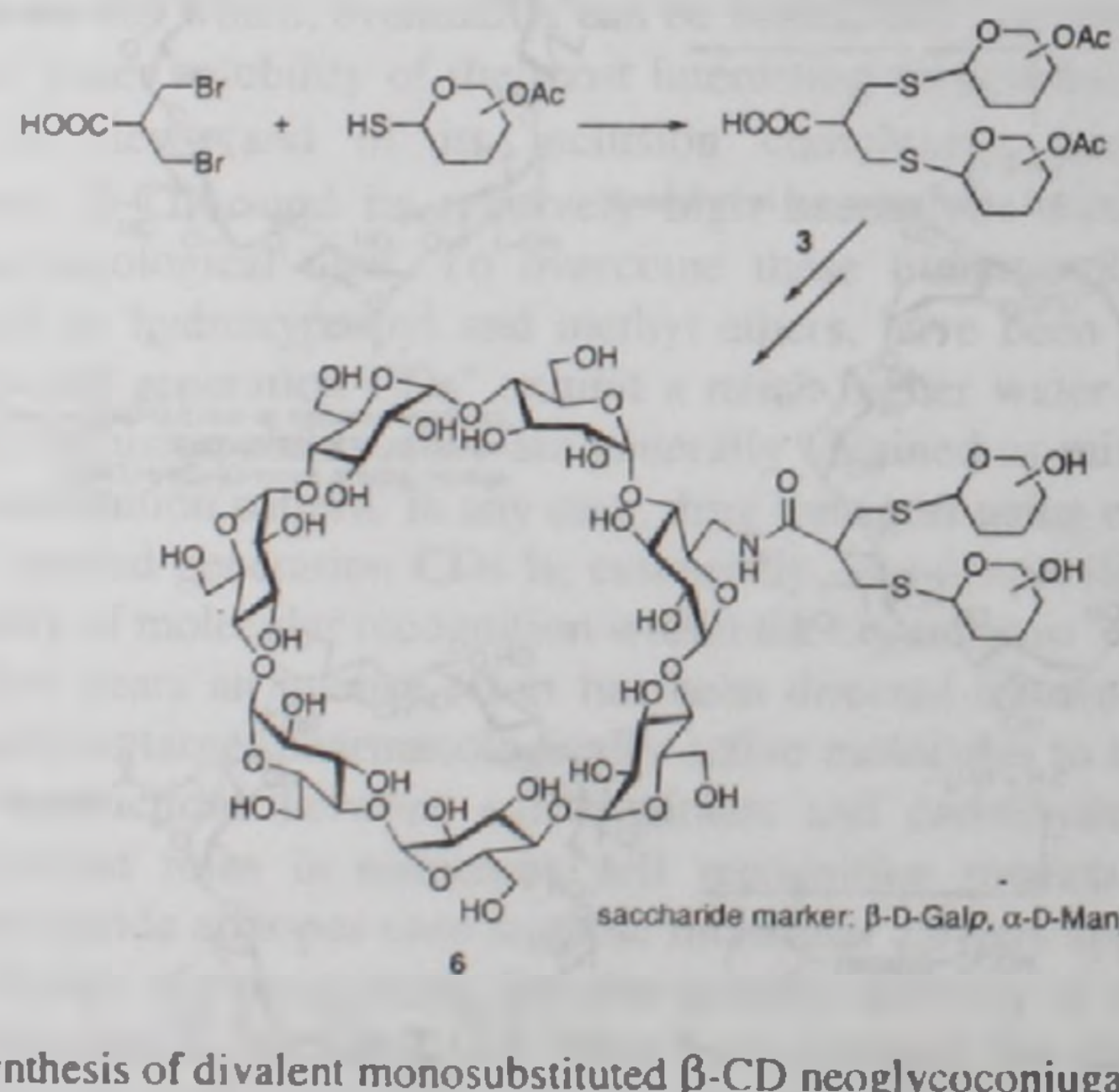


Scheme 1. General synthetic scheme for the preparation of monovalent β-CD neoglycoconjugates through thioether [24-28] and amide bond forming reactions [29-31].

Driguez et al. have used a similar approach that employs the peracetylated mono-(C-6)-iodo-β-CD derivative **2**, obtained from the monotosylate **1**, as precursor [26, 27]. The peracetylated 1-thioglycose (β-D-glucopyranose, β-D-galactopyranose, α-D-mannopyranose) is then used as the nucleophile. A final deacetylation step afforded the target fully unprotected neoglycoconjugates. More recently, Hattori et al. [28] have reported the incorporation of a spacer arm between the CD core and the saccharide marker by: (i) reacting lactonolactone and aminoethanol and; (ii) introducing the resulting galactosyl-gluconoamide-ethanethiol fragment at the 6^1 position of β-CD following the above general synthetic scheme (Scheme 1).

A second general approach for the preparation of monovalent β-CD-neoglycoconjugates consists in the amidation reaction between 6^1-amino-6^1-deoxycyclomaltoheptaose (**3**), readily available from **1** via the corresponding azide, and saccharide markers bearing a carboxylic acid functional group. This strategy has been widely developed by the group of Parrot-Lopez [29-31]. First, a monoester of a dicarboxylic acid was coupled to a glycosyl isothiocyanate to give a glycosylamide. After hydrolysis of the ester group, the resulting carboxylic acid was allowed to react with amine **3** to give adducts **5** (Scheme 1). The length of the spacer can be easily modified just by changing the starting dicarboxylic acid. Alternatively, aldonolactones can be used as amidating agents [32].

Driguez et al. [27] have applied the amidation reaction to the synthesis of divalent monosubstituted β-CD-neoglycoconjugates bearing β-D-galactopyranosyl and α-D-mannopyranosyl ligands (**6**). Commercially available 3-bromo-2-(bromoethyl)propionic acid was used as multiplying element through a sequence involving: (a) nucleophilic displacement of the bromo atoms with the per-*O*-acetylated 1-thiosugars and; (b) coupling of the resulting bis(thioglycoside) with monoamine **3** (Scheme 2).



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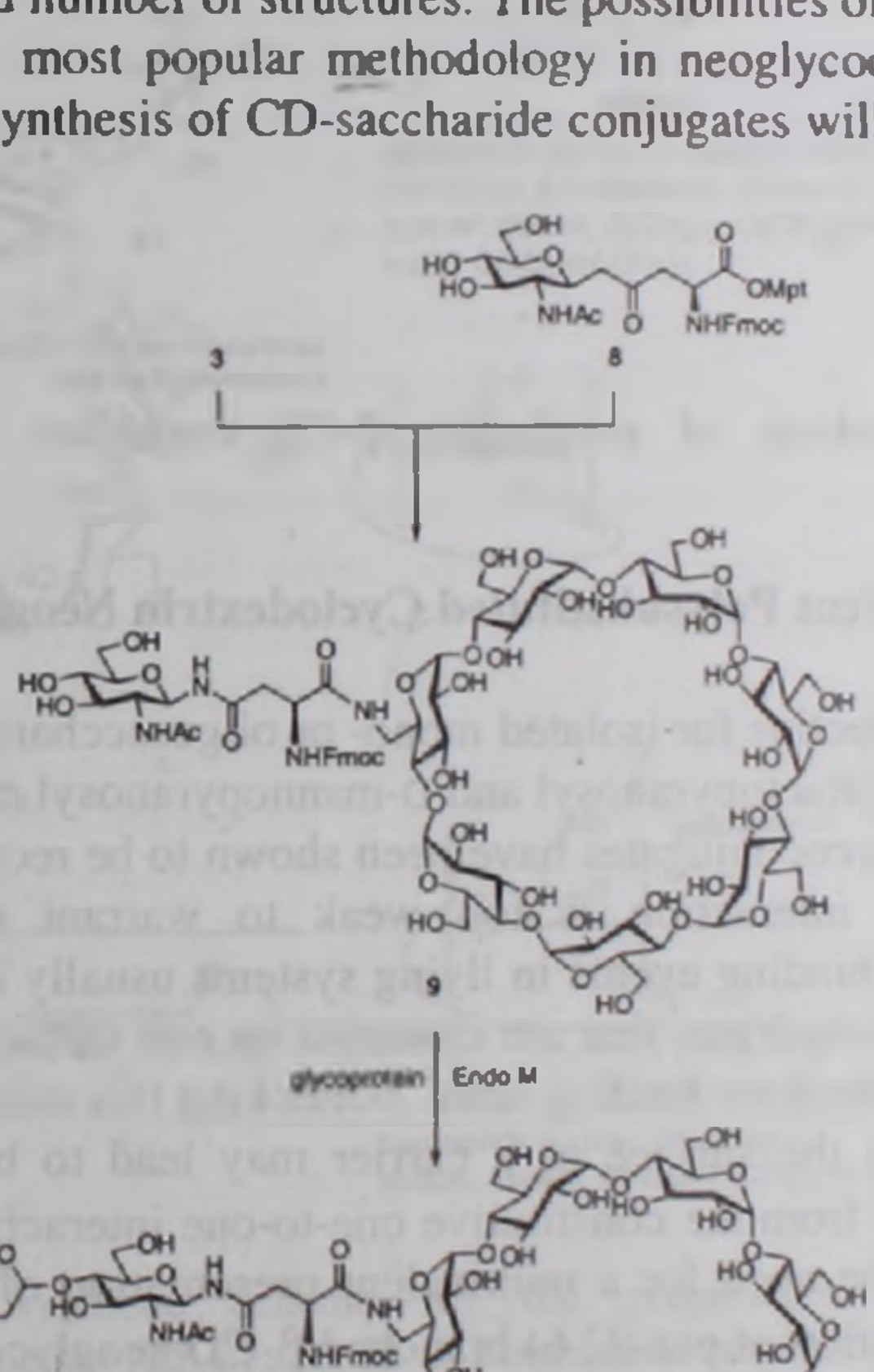
Scheme 2. Synthesis of divalent monosubstituted β-CD neoglycoconjugates [27].

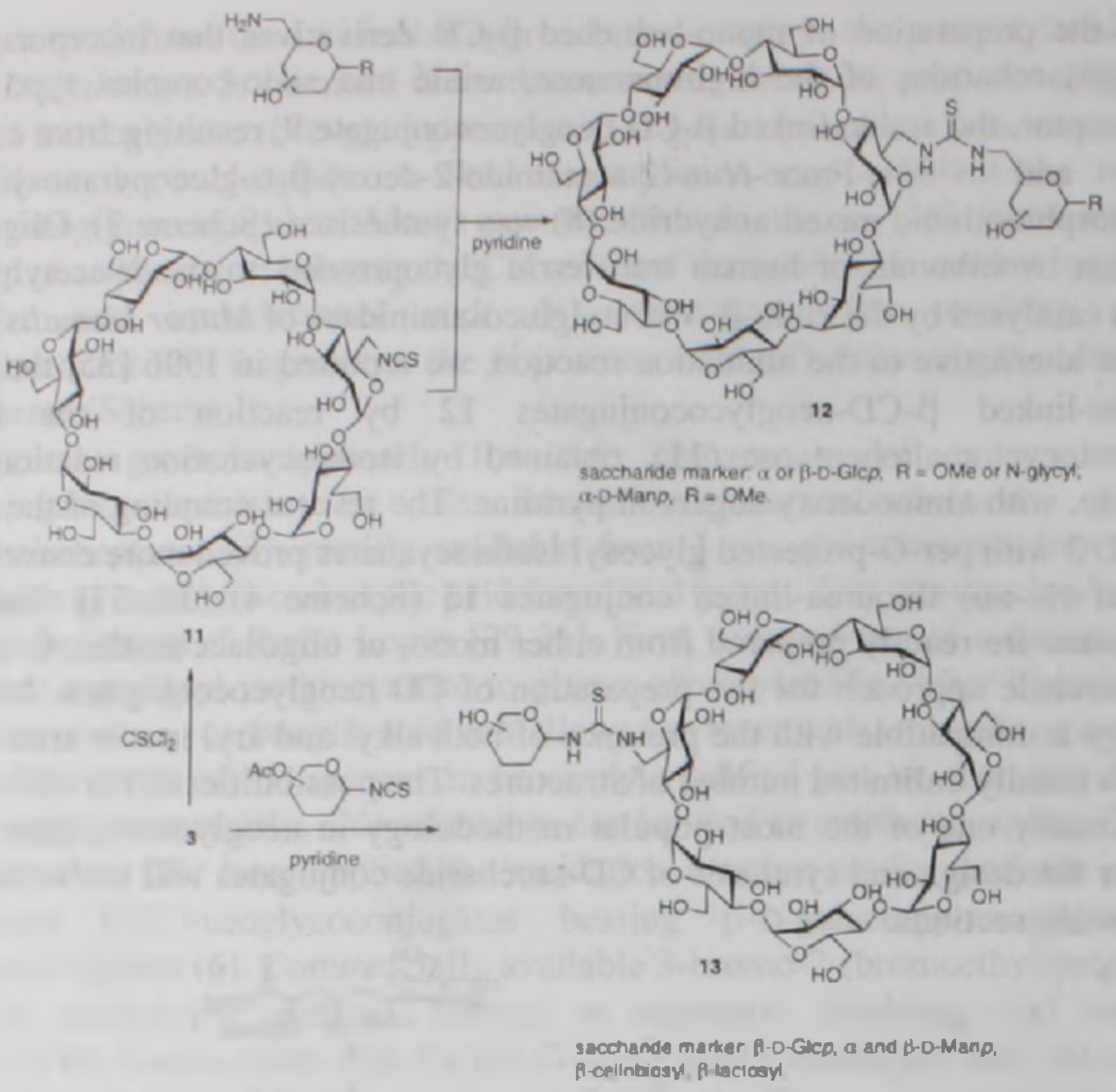
The group of Parrot-Lopez has reported an interesting extension of this approach [33] that combines: (i) amidation of the mono-(C-6) amine **3** and a carboxylic acid-armed *N*-acetylglucosamine derivative and; (ii) enzymatic galactosyl-transfer to the glucosamine moiety in the β-CD-conjugate. Hattori et al. [34] have applied a similar chemo-enzymatic

strategy to the preparation of mono-branched β-CD derivatives that incorporate complex natural oligosaccharides of the high-mannose, asialo and sialo-complex type (**10**). As a suitable acceptor, the amide-linked β-CD neoglycoconjugate **9**, resulting from condensation of **3** and *N*-α-Fmoc-*N*-ω-(2-acetamido-2-deoxy-β-D-glucopyranosyl)asparagine dimethylphosphinothioic mixed anhydride (**8**) was synthesised (Scheme 3). Oligosaccharide transfer from ovoalbumin or human transferrin glycoproteins to the *N*-acetylglucosamine moiety was catalysed by the endo-β-*N*-acetylglucosaminidase of *Mucor hiemalis* (Endo M).

As an alternative to the amidation reaction, we reported in 1996 [35] the preparation of thiourea-linked β-CD-neoglycoconjugates **12** by reaction of the 6^{I}-deoxy-6^{I}-isothiocyanatocyclomaltoheptaose (**11**), obtained by isothiocyanation reaction of **3** with thiophosgene, with aminodeoxy sugars in pyridine. The reverse coupling of the mono-(C-6) amine β-CD **3** with per-*O*-protected glycosyl isothiocyanates proved more convenient for the synthesis of (1-->6) thiourea-linked conjugates **13** (Scheme 4) [36, 37]. Since glycosyl isothiocyanates are readily prepared from either mono- or oligosaccharides, this is probably the most versatile approach for the preparation of CD neoglycoconjugates. Moreover, the methodology is compatible with the presence of both alkyl and aryl spacer arms [36], giving access to a virtually unlimited number of structures. The possibilities of the thiourea-forming strategy, actually one of the most popular methodology in neoglycoconjugate preparation [38, 39], for the design and synthesis of CD-saccharide conjugates will be further illustrated in the following sections.



Scheme 3. Chemo-enzymatic synthesis of monovalent β-CD conjugates [34].



Scheme 4. Synthesis of monovalent β-CD conjugates through the thiourea-forming reaction [35-37].

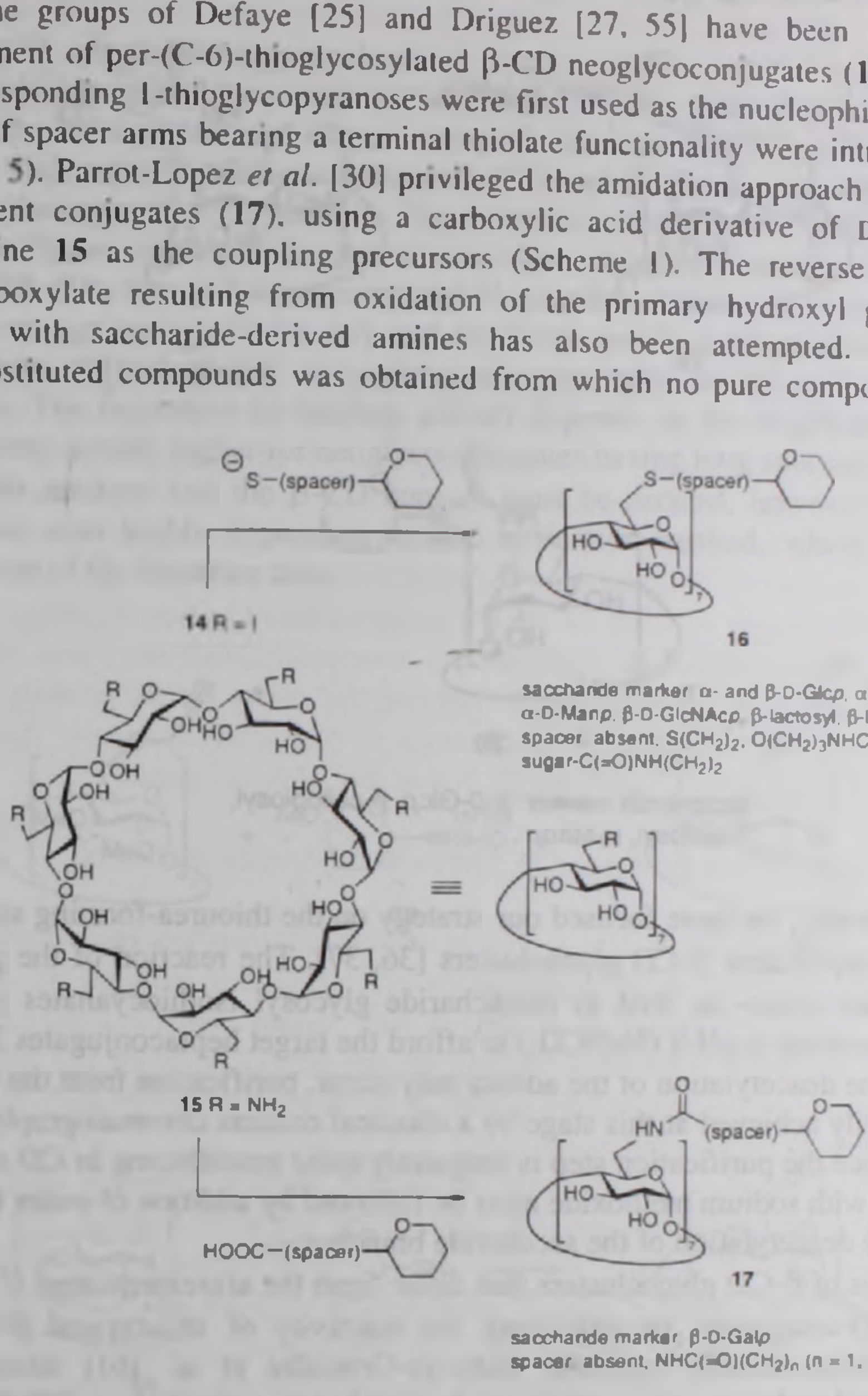
Multivalent Polysubstituted Cyclodextrin Neoglycoconjugates

The affinity of lectins for isolated mono- or oligosaccharide ligands is, generally, low. Actually although D-galactopyranosyl and D-mannopyranosyl moieties in some of the above monovalent CD neoglycoconjugates have been shown to be recognised by specific lectins in in vitro assays, the interaction is too weak to warrant efficient targeting in vivo. Carbohydrate-protein binding events in living systems usually involve several simultaneous contacts between carbohydrates that are clustered on cell surfaces and protein receptors that contain multiple carbohydrate binding-sites. Mimicking this scenario by multiplication of the saccharide epitope on the surface of a carrier may lead to biologically useful affinities, greater than predicted from the constitutive one-to-one interactions [40-49] — the so-called cluster effect [50]. The need for a multivalent presentation of the saccharide markers has stimulated the preparation of per-(C-6) branched β-CD neoglycoconjugates. Highly efficient coupling reactions are a prerequisite for such prospect, since partial substitution would lead to a mixture of undersubstituted regioisomers from which purification of the desired persubstituted compound becomes a difficult task.

Basically, the preparation of per-(C-6) substituted β-CD derivatives bearing carbohydrate appendages relies on the same synthetic methodologies previously commented for the synthesis of monovalent conjugates, i.e. thiol displacement of a good leaving group (I or Br; **14**), amidation of the per-(C-6)-amino β-CD (**15**), or reaction of the latter with sugar isothiocyanates. The development of very efficient procedures for the preparation of the key

per-(C-6)-halo β-CD precursors by the group of Defaye has been, not surprisingly, a turning point in the chemistry of these derivatives [51-54].

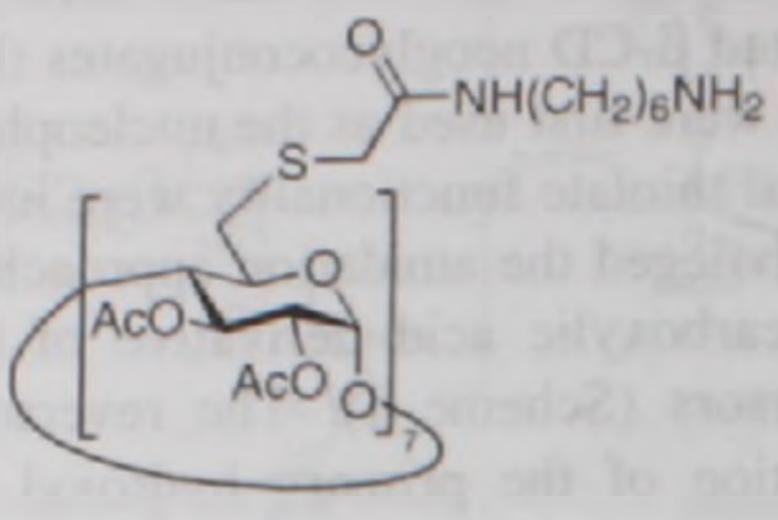
The groups of Defaye [25] and Driguez [27, 55] have been instrumental in the development of per-(C-6)-thioglycosylated β-CD neoglycoconjugates (**16**). The thiolates of the corresponding 1-thioglycopyranoses were first used as the nucleophiles. Alternatively, a variety of spacer arms bearing a terminal thiolate functionality were introduced [28, 55-57] (Scheme 5). Parrot-Lopez *et al.* [30] privileged the amidation approach for the synthesis of heptavalent conjugates (**17**), using a carboxylic acid derivative of D-galactose and the heptaamine **15** as the coupling precursors (Scheme 1). The reverse strategy using the heptacarboxylate resulting from oxidation of the primary hydroxyl groups of β-CD in reaction with saccharide-derived amines has also been attempted. Yet, a mixture of undersubstituted compounds was obtained from which no pure compounds were isolated [58].



Scheme 5. General synthetic scheme for the preparation of heptavalent β-CD neoglycoconjugates through thioether [25, 27, 28, 55-57] and amide bond forming reactions [30].

Recently, the groups of Santoyo-González and Vargas-Berenguel [59, 60] have reported some modifications of this general scheme aimed at diversifying the nature of the linkers while keeping the coupling efficiency, including: (i) the use of sugar derived thiouronium salts as nucleophilic agents instead of the corresponding thiolates, (ii) the preparation of the heptakis(6-chloroacetamido-6-deoxy)cyclomaltoheptaose **18** and its use as precursor in coupling reactions with the aforementioned nucleophiles, and (iii) the synthesis of the per-(C-6)-thioether β-CD derivative **19** bearing a terminal amino group that can be

used in coupling reactions with sugar isothiocyanates. Yet, all these approaches need the protection of the hydroxyl groups both in the β-CD precursor and in the functionalised saccharide marker.



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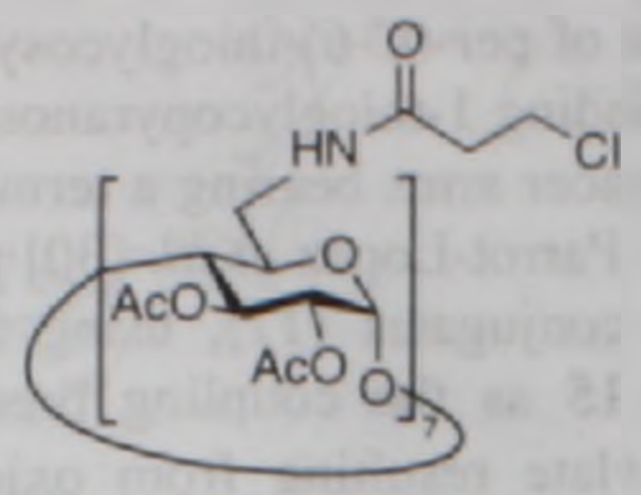

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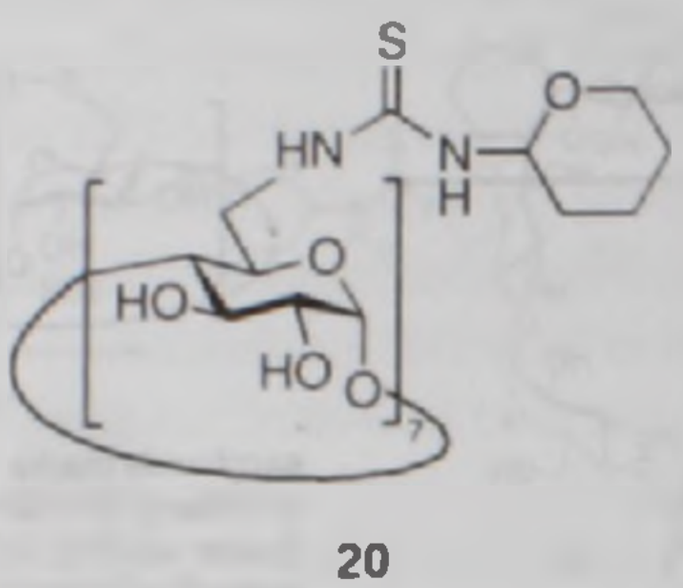

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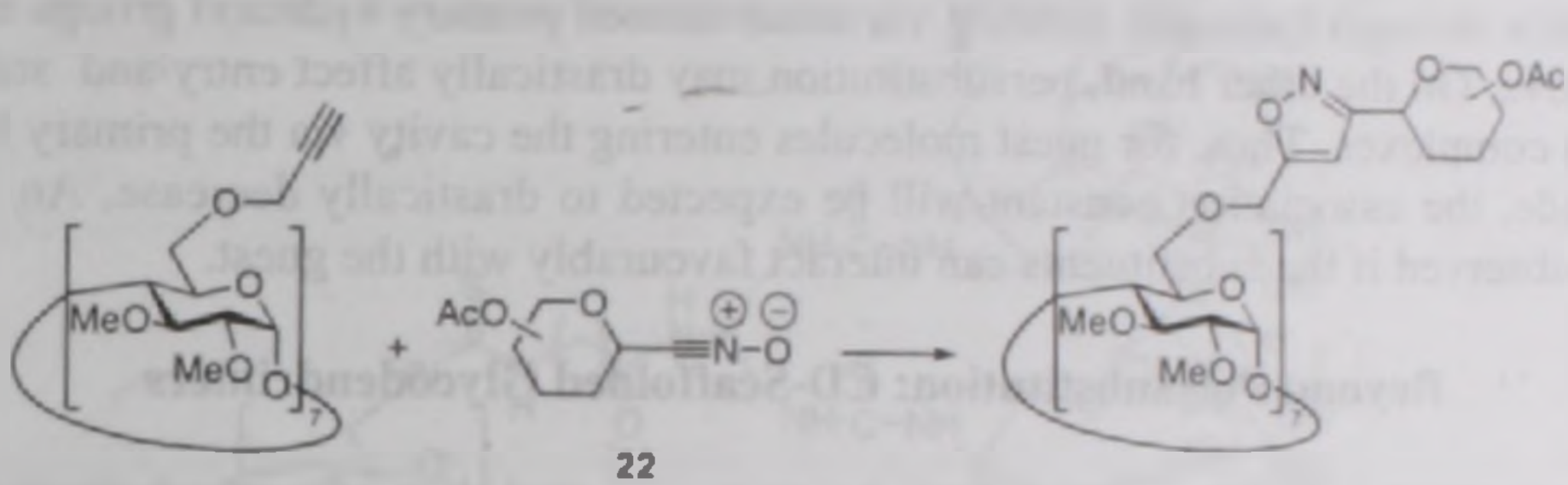
saccharide marker: β-D-Glc*p*, β-cellobiosyl, β-lactosyl, D-Man*p* .

In our laboratories, we have focused our strategy on the thiourea-forming strategy for the preparation of heptavalent β-CD glycoclusters [36, 37]. The reaction of the per-(C-6)-amine β-CD **15** with mono- as well as disaccharide glycosyl isothiocyanates proceeded smoothly in water-acetone at pH 8 ($NaHCO_3$) to afford the target heptaconjugates **20** in high yield. Although some deacetylation of the adduct may occur, purification from the unreacted precursors was readily achieved at this stage by a classical column chromatography step, an important aspect since the purification step is frequently quite troublesome in CD chemistry. The final treatment with sodium methoxide must be followed by addition of water to warrant full and quantitative deacetylation of the saccharide branches.

Two examples of β-CD glycoclusters that differ from the aforementioned (C-6)-*S* or -*N* substituted β-CD conjugates by exploiting the reactivity of unsaturated β-CD ether derivatives have been recently reported. Santoyo-Gonzalez et al. [61] described the preparation of an heptavalent neoglycoconjugate having heterocyclic linkers (**23**) by the 1,3-dipolar cycloaddition of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl nitrile oxide **22** and the per-(2,3-di-*O*-methyl-6-*O*-propargyl)-β-CD **21** (Scheme 6). Stoddart and Fulton [62] prepared the per-(6-*O*-allyl-2,3-di-*O*-methyl-)-β-CD derivative and effected the photochemical (anti-Markovnikov) addition of 2,3,4,6- tetra-*O*-acetyl-1-thio-β-D-glucopyranose to the double-bonds. Interestingly, this strategy was also extended to β-CD derivatives per-*O*-allylated at O-2 and per-(di-*O*-allylated) at O-2 and O-6 positions (**24**), giving rise to the first examples of an heptavalent β-CD neoglycoconjugate branched at the secondary face and of a 14-valent (**25**) β-CD conjugate (Scheme 6).

Lectin-Binding and inclusion ability of Monovalent *versus* Multivalent Polysubstituted Cyclodextrin Neoglycoconjugates

β-CD neoglycoconjugates bearing biorecognisable saccharide markers have been shown to be recognised by complementary lectins in vitro. Thus, D-galactopyranosyl ligands binds to the cell wall lectin of *Kluyveromyces bulgaricus* (KbCWL) [29-31, 33, 63], *Arachis hypogea* (peanut) [60], *Ricinus communis* [27] and *Griffonia simplicifolia* I (GSI) lectins [58]; D-glucopyranosyl ligands to *Pisum sativum* (pea) lectin [60], D-mannopyranosyl ligands to *Pisum sativum* [60] and *Concanavalia ensiformis* (concanavalin A, Con A) lectins [32, 34, 58, 60]; *N*-acetylglucosamine and N-acetyllactosamine to *Triticum vulgaris* (WGA, wheat germ agglutinin) [56-58, 60], and *Erythrina corallodendron* (EcorL) lectins [57, 58]. Multivalent polysubstituted conjugates are generally bound with higher association constants. The increment on binding affinity depends on the length and the nature of the linker, being usually higher for neoglycoconjugates having long spacers between the external saccharide markers and the β-CD core. It must be noticed, however, that lectin binding results are also highly depending on the evaluation method, which hampers a reliable comparison of the literature data.



Scheme 6. Synthesis of multivalent b-CD neoglycoconjugates from unsaturated ether derivatives [61, 62].

Concanavalin A is one of the most popular lectin for the study of carbohydrate-protein interactions. Its commercial availability and extensive structural knowledge make it particularly attractive for preliminary evaluation of targeting devices. Moreover, several reports on Con A-mannosyl clusters associations have recently been reported using the enzyme-linked lectin assay (ELLA) protocol [64-68]. For these reasons, we have undertaken a systematic evaluation of thiourea-linked mannose-β-CD conjugates using this technique, including both monovalent and heptavalent adducts, in comparison with model compounds

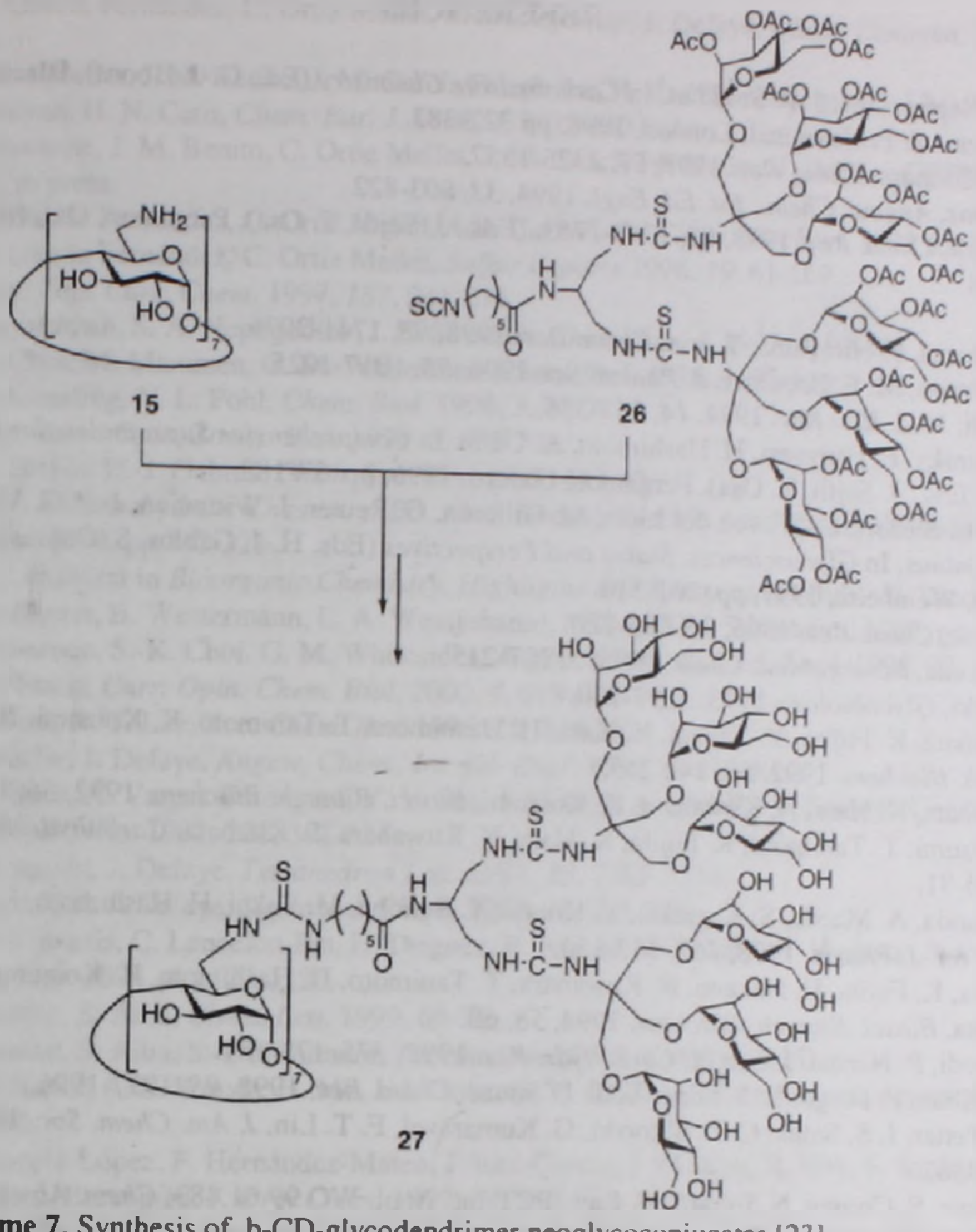
lacking the CD moiety [37]. Several interesting conclusions have been drawn: (i) The thiourea linker slightly decreases the binding affinity in comparison with classical *O*-glycosidic linkages and totally abolishes anomeric specificity (in contrast with the known 10-fold higher affinity for the α-anomer in the *O*-mannoside series); (ii) the β-CD aglycon in monovalent conjugates interacts with the protein stabilising the complex by about -0.35 kcal; (iii) surprisingly, heptasubstitution at the primary hydroxyls rim with mannopyranosylthioureido ligands fully abolishes Con A binding, probably due to an unfavorable steric effect. The expected increase in binding affinity was observed after intercalation of a C_5 spacer.

Only a few data on the inclusion ability of β-CD neoglycoconjugates is available in the literature. Defaye and coworkers [25] have shown that the stability of inclusion complexes of monovalent (C-6)-*S*-linked β-CD neoglycoconjugates is strongly dependent on the inclusion dynamic and complex stabilisation mechanism. Complexes with guest molecules entering the cavity through the narrower rim may experience some decrease in the association constant as compared with the corresponding native β-CD due to the steric hindrance imposed by the substituent. No significant difference was observed, however, for molecules entering the cavity through the wider secondary hydroxyls rim and complex stabilisation through hydrogen bonding via unsubstituted primary hydroxyl groups may then be operative. On the other hand, persubstitution may drastically affect entry and stability of inclusion complexes. Thus, for guest molecules entering the cavity via the primary hydroxyl groups side, the association constant will be expected to drastically decrease. An opposite effect is observed if the substituents can interact favourably with the guest.

Beyond Polysubstitution: CD-Scaffolded Glycodendrimers

From all the above reported data, it can be concluded that heptasubstitution at the primary hydroxyls rim of β-CD with incorporation of saccharides markers has beneficial effects from the biological standpoint as compared with monovalent conjugates, provided that longer enough spacer arms are used to warrant the accessibility of the glycocluster structure to carbohydrate-lectin recognition events. Yet, heptasubstitution may also impair inclusion and stabilisation of potential guests.

Ideal CD-carriers for use in drug targeting should combine both the advantages of monosubstitution regarding efficient inclusion capabilities and a multivalent display of the required saccharide epitope to comply with the need of high biological receptor binding affinity. Towards this aim, we have recently reported the preparation of β-CD-scaffolded glycodendrimers (e.g. **27**) as a new class of multivalent monosubstituted β-CD neoglycoconjugates [23]. A modular strategy for the preparation of isothiocyanate-armed mannosyl-coated dendritic wedges (e.g. **26**) was devised and these structures were attached to the β-CD monoamine **3** in a final step (Scheme 7). Evaluation of the Con A binding ability by ELLA tests for a series of derivatives going from mono to hexavalent indicated a dramatic increase in binding efficiency for the higher-valent conjugates. Moreover, the solubilisation experiments using the anticancer drug Taxotère® as model guest indicated solubility values similar to those previously encountered for monovalent monobranched β-CD conjugates, about 20% higher as compared with per-(C-6) substituted analogues.



Scheme 7. Synthesis of b-CD-glycodendrimer neoglycoconjugates [23].

Concluding remarks

From the present body of results, it becomes clear that combination of the ability to form host-guest inclusion complexes characteristic of CDs and the biorecognition capabilities of oligosaccharide appendage makes CD neoglycoconjugates promising candidates as site-specific drug delivery systems. Efficient and high yielding synthetic methodologies involving a limited number of protection/deprotection sequences have been settled, opening the way to unlimited possibilities of biorecognition structures which could result from the combination of chemical and enzymatic methods as well.

Acknowledgements. Part of the results from the authors laboratories herein reported have been obtained with the support of the European Commission DG XII, under the FAIR programme CT95-0300.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

SYNTHESIS OF SUGAR-BRANCHED CYCLODEXTRINS AND THEIR DUAL ASSOCIATION WITH PROTEINS AND DRUGS OBSERVED BY SPR ASSAY

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The efficient synthesis of the sugar-branched cyclodextrins was carried out. Evaluation of the dual association consisting of saccharide-recognition with concanavalin A and PNA of the lectin protein, and the inclusion interactions with immobilized cholic acid and doxorubicin as a model drug analyzed by surface plasmon resonance (SPR) assay. The high mannosyl type oligosaccharide-branched cyclodextrins showed a tight interaction both with concanavalin A and cholic acid, showing a K_a of 10^7 M^{-1} or more. It was observed that the number of sugar antennas and the length of the spacer arm are quite important for the dual association with proteins and drugs. We can foresee applications of the sugar-branched cyclodextrins to a targeting drug delivery system.

Осуществлен эффективный синтез сахар-разветвленных циклодекстринов. Методом поверхностного плазмон резонанса (ППР) произведена оценка двойственной ассоциации, содержащей сахарид-познающий участок с конканавалином А и с ПНК лектин - протеином, а также анализ реакции включения между иммобилизованной холиновой кислотой и доксорубицином, как модели лекарства. Олигосахарид-разветвленные циклодекстрины с высокой степенью типа манозил показали тесное взаимодействие как с конканавалином, так и с холиновой кислотой, представляя значения Ка 10^7 M^{-1} или более. Выявлено, что количество антен и длина пространственной ветви сахаров весьма важны для двойственной ассоциации с протеинами и лекарствами. Можно предположить, что сахар-разветвленные циклодекстрины могут применяться в качестве мишени для изыскания лекарств.

Իրականացվել է շաքար-ճյուղավորված ցիկլոդեքստրինների արդյունավետ սինթեզը: Սակերեսային պլազմոն ռեզոնանսի մեթոդով (ՍՊՄ) կատարվել է երկակի համակեցության գնահատումը, կազմված կոնկանավալին A-ի և լեկտին-պրոտեինի ՊՆԹ -ի հետ սախարիդ-ճանաչող հատվածից, ինչպես նաև իմոբիլիզացված խոլինաթթվի և դոկսոռուբիցինի որպես դեղերի մոդելի, ներառման միջռեակցիաների անալիզը: Հիմնականում մանոզիլ տիպի օլիգոսախարիդ-ճյուղավորված ցիկլոդեքստրինները ցուցաբերել են սերտ փոխազդեցություն ինչպես կոնկանավալինի, այնպես էլ խոլինաթթվի հետ, ցուցաբերելով Ka-ի 10^7 M^{-1} և ավելի արժեքներ: Բացահայտվել է, որ շաքարների անտենաների քանակը և տարածական արմունկի երկարությունը չափազանց կարևոր են պրոտեինների և դեղերի հետ երկակի համակեցության համար: Կարել է կանխատեսել, որ շաքար-ճյուղավորված ցիկլոդեքստրինները կարող են կիրառվել որպես թիրախ դեղերի որոնման համար:

Introduction

Among so many biological events, the interaction between a saccharide and protein has an important role in recognition phenomena [1-4]. The driving force for the association of saccharides with proteins is water solvation, van der Waals force and hydrogen bonding. A typical example of a structure scheme was known between the PNA lectin protein and a saccharide, galactose-β-1-3-*N*-acetylgalacosamine [5]. The saccharides bind with specific

lectin proteins through hydrogen bonding, sometimes using water. PNA lectin forms a tetramer of the subunit having a molecular weight of 26 kD [6]. The binding site with saccharide is at the four corners.

By the wide application of the saccharide-protein interaction, there are many examples of saccharide-conjugates in the field of polymers [7], oligomers [8], dendrimers [9-15], calixarenes [16-22], surfactants [23] and cyclodextrins (CDs) [24-39]. Table 1 shows the previous studies on the synthesis of sugar-branched CDs.

Table 1. Previous studies on the synthesis of sugar-branched cyclodextrins

Authors	Oligosaccharide-branched CD	References
1) Parrot-Lopez, Leray, Col" eman (1993)	*Mono*-Glc, Gal, Man, Fucose-CD	26
2) Hattori, Takahashi, Kosikawa (1994)	*Bi*-Gal-CD	24
3) Robertis, Lancelon-Pin, Driguez, Attioui, Bonaly, Marsura(1994)	*Hepta*-Gal-CD	36
4) Matsuda, Inazu, Haneda, Mizuno, Yamanoi, Hattori, Yamamoto, Kumagai (1997)	High mannose type oligosaccharide-CD Srato complex type oligosaccharide-CD Asialo complex type oligosaccharide-CD	31, 32, 33
5) Imata, Kubota, Hattori, Aoyagi, Jindoh (1997)	*Mono*-Glc-CD, *Mono*-Gal-CD	34, 35
6) Mellet, Fernabdez, Benito, Law, Chmurskie, Defaye (1998)	*Hepta*-Gal-CD	30
7) Ikuta, Tanimoto, Koizumi, Murata, Usui, Kitahata, Fujita, Hashimoto, Nakagawa, Shimoda, Kagatani, Maeda, Konnno, Hara, Fujito, Sonobe (1999)	*Bi*-Gal-Lac-CD *Bi*-Man_3-CD *Mono*-Gal-CD *Mono*-Glc-CD	28, 29, 59
8) García-Lopez, Hernández-Mateo, Isac-García, Kim, Roy, Santoyo-Gonzalez, Vargas-Berenguel (1999)	*Hepta*-Gal-CD *Hepta*-Glc-CD *Hepta*-GlcNAc-CD	25
9) Fruike, Aiba(1999):	*Hepta*-Gal-CD	38
10) Yasuda, Aoki, Abe, Hattori (2000)	*Hepta*-Gal-CD	37
11) Fulton, Stoddart (2000)	*Tetradeca*-Glc-CD *Hepta*-Glc-CD	39

Koizumi's group prepared glycosyl-branched CDs enzymatically and purified by HPLC. Many glycosylated CDs were obtained. These compounds are very interesting because they showed the binding to corresponding lectin to be 10^4-10^5 M^{-1} [28]. Galactose-branched CD was proved *in vivo* to be a good carrier to the liver for the practical drugs [29].

In recent years, *multi*antennary sugar-cluster type of cyclodextrins have been synthesized and evaluated. Hattori [37], Furuike [38] and Stoddart [39] prepared various *per*-glycosylated CDs having galactose at the terminal unit. Garcia-Lopez and the colleague did recent study about the synthesis of *per*-glycosylated CD and interaction with lectins. Glucose, galactose, N-acetylglucosamine, and mannose were attached on β-CD with spacer of sulfur atom, sufomethleneamide, and oxyphenylene-hexamethylenediamide-methylenesulfur. These spacer arms were consisted one, four and 18 atoms between sugar and C-6 of β-CD [25]. They showed the relative association with lectins by the inhibition assay.

Generally speaking, a galactosylated carrier will target the liver parenchymal cell carrying drug to cure an illness. Also, the mannosylated carrier will target the liver Kuffer cell, the macrophage and other cells [40-42].

We can suggest a schematic scheme of the targeting DDS by the application of the sugar-branched CD on a specific cell surface as shown in Figure 1. The lectin proteins or

receptor proteins is recognizing the saccharide, and is associating with a specific sugar-branched CD. CD cavity shows the inclusion of a drug as a guest compound [53-57].

Also, we can imagine another case where the lectin is connected between the sugar-branched CD and the saccharide on the cell surface as in the left side in the Figure.

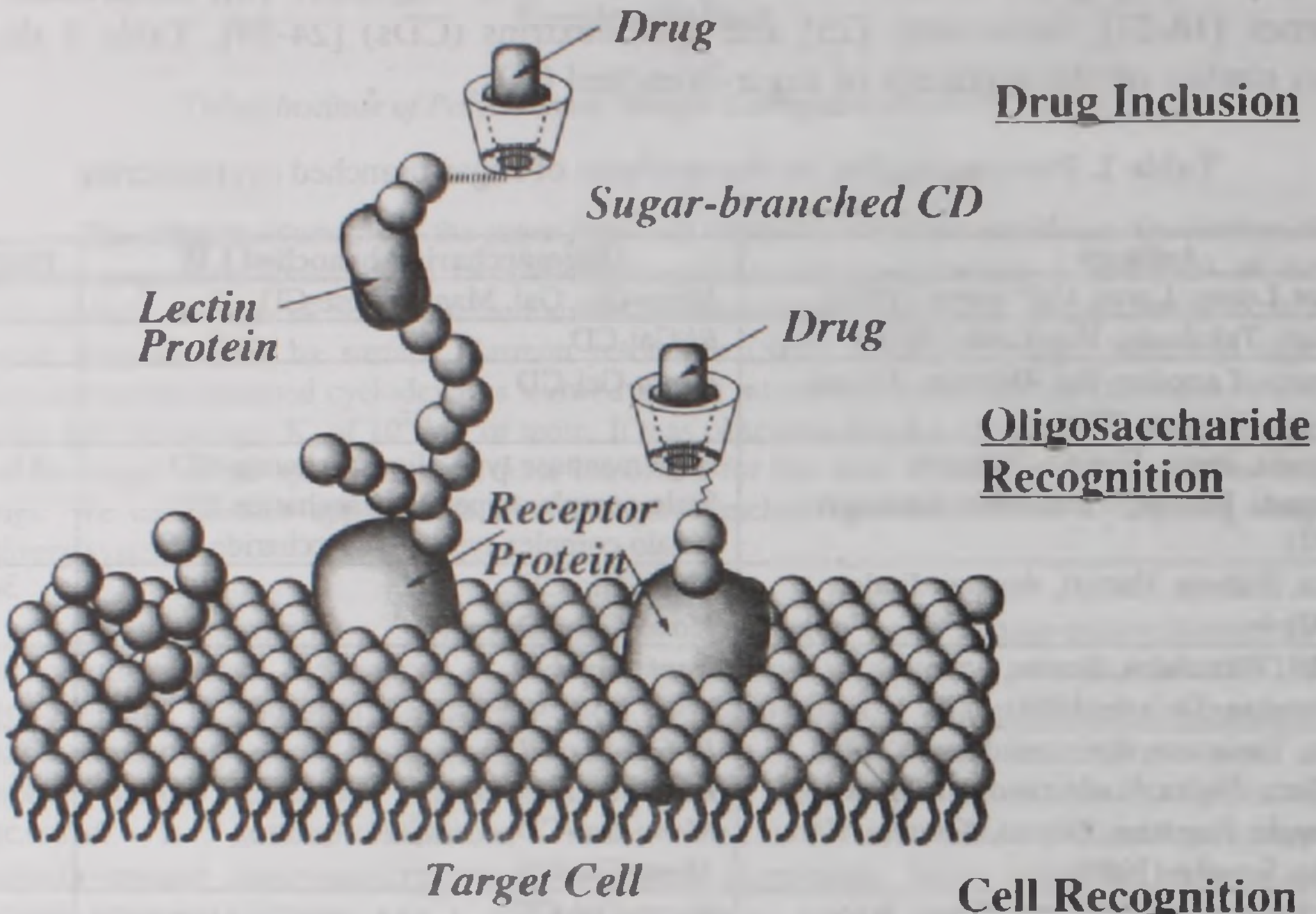

Figure 1. A concept of sugar-branched cyclodextrins for targeting drug delivery system.

The main purposes of the present review are focused on the following items.

1) The efficient synthesis of the sugar-branched CD.

2) Evaluation of the dual molecular recognition consisting of saccharide-recognition with lectin proteins, and the inclusion interactions with model drugs observed by SPR apparatus.

3) The observation results for the number of sugar antennas and the length of the spacer arm between sugar and CD in the structure of sugar-branched CDs shows quite important for the dual association with proteins and drugs.

4) The applications of the dual recognition of sugar-branched CD to a targeting drug delivery system is hopeful.

Synthesis of sugar-branched cyclodextrins

The *hexa*mannosyl asparagine (M6) was obtained according to the literature [43]. Attachment of fluorenyl-methylene-oxycarboxylate (Fmoc) group on asparagine of the oligosaccharides gave an 88% conversion for the high mannose type. The connection reaction of the Fmoc-mannosyl asparagine and *mono*amino-β-CD was tested in various condensing agents and solvents. The best reaction condition was to use PyBOP in *N*-methylpyrrolidinone solvent. We succeeded in the uptake of $Man_6GlcNAc_2$FmocAsn-CD (M6CD) and $Man_7GlcNAc_2$FmocAsn-CD (M7CD) in a similar manner. The HPLC and TOF-MS spectrum supported the structure. Three kinds of aminosugars (glucosamin,

galactosamin and mannosamin) were attached to glucuronylgluconyl-β-CD (GUG-CD). Other sugar-branched CDs (*bi*-antennary galactose-branched CD, *mono*-antennary galactose-branched CD, and *hpta*antennary galactose-branched CD) were prepared starting from the corresponding amino- or iodo-β-CDs.

Evaluation method for the association

SPR assay for the association kinetics.

The selection of the analytical method for the interaction analysis was considered. Various approaches are known for the interaction analysis. There are three broad categories. The indirect method such as competition inhibition and ELISA assay is used to define the relative binding activity. However, it does not provide direct information about the association constants. The kinetic method gives the association constant. Especially the SPR technique has the following advantages [4].

1) large range for analyte
2) association and dissociation rate measurement
3) small immobilized ligand sample
4) small analyte concentration
5) rapid measurement

SPR is a technique for the analysis of the association of free analytes with an immobilized ligand on a sensor metal that induces a change in the refractive index of the biosensor surface [44-46]. Changes in the refractive index (which is termed Response in arc sec unit) depend on the interacting mass with a ligand at the surface irrespective of the type of molecules. The information about the association and dissociation kinetics of the binding and the overall K_a is obtained.

We selected SPR and the immobilization of the protein and the drug on the biosensor cuvette were done.

Immobilization of ligands on the SPR optical biosensor [34,35]

The immobilization of concanavalin A (ConA) on the sensor cuvette was carried out by the reaction of a reactive linker molecule with the cuvette surface having an aminobutyl group. ConA in an acetate buffer of *p*H 5.3 was added to the cuvette. From the increase in response, 11 ng/mm^2 of ConA in dimeric subunit were immobilized. The immobilization of cholic acid on the cuvette surface was done with *N*-hydroxysuccinic amide, and a water-soluble carbodiimide in water. The amount of immobilized cholic acid and DXR was 1.23 and 0.67 ng/mm^2, respectively.

Interaction analysis by association kinetic assay [47,48]

The specific binding of a saccharide to a protein is governed by this equation. K_a is defined as the association constant in M^{-1} unit. K_a is equal to k_a/k_d, where k_a is the association rate constant and k_d is the dissociation rate constant. K_a is related to the standard free energy change in the binding.

In case to obtain the kinetic plots, several time-response curves are determined by changing the analyte concentration. The association kinetic constants were obtained from the linear plots between on-rate-constant, k_{on} and the concentration of the sugar-branched CD. The slope of the line gave k_a and they-intercept gave k_d. The k_{on}, was obtained from the slope of the plots between dR/dt *vs.* R from the saturation curves [54-55].

Natural oligosaccharide-branched cyclodextrins

Synthesis of *hexa*mannosyl and *hepta*mannosyl saccharide-branched CDs [31-33]

The synthesis of the natural oligosaccharide-branched CD were done using the procedure:

A) preparation of natural oligosaccharide sugar chain from chicken

B) protection of *N*-position of asparagine

C) connection of the oligosaccharide sugar with CD

From the egg white, ovalbumin was extracted by phenol and then centrifuged. To cut the peptide thoroughly, protease hydrolysis and then gel and ion-exchange chromatographies were repeated. From ovalbumin, *hexa*mannosyl asparagine was obtained according to the literature [43]. From egg yolk, the sialo-type oligosaccharide-asparagines, was obtained [60]. Enzymatic removal of the sialic acid group produced the asialo type oligosaccharide-asparagine.

Attachment of Fmoc group on asparagine of the oligosaccharides with Fmoc-OSu and hydrophobic adsorbent gave an 88 % in conversion for the high mannose type, 74 % for the sialo type and 88 % for the asialo type oligosaccharides.

We tried two route for the preparation of the high mannose oligosaccharide (termed FmocM6). The old route is straight separation in the first step. It gave only 8 % yield. However, the new route to attach Fmoc group on the *N*-position of the mixture was tried. It gave 30 % separation yield of Fmoc-M6 from the mixed Fmoc-oligosaccharides. Finally, 16.7 mg of the FmocM6 was able to prepare from one egg [32-33].

The connection reaction of the Fmoc-M6 and *mono-C6*-amino-β-CD was tested in various condensing reagents and solvents. The best reaction condition was to use PyBOP in *N*-methylpyrrolidinone solvent. This gave a quantitative yield of the M6CD ($Man_6GlcNAc_2$FmocAsn-β-CD). Other sialo-type and asialo-type oligosaccharides gave conversion yields of 30 % and 97 %, respectively. The HPLC chromatogram of M6CD showed it to be quite pure. The TOF-MS spectrum also supported the structure.

It was suggested that synthesizing the M7 oligosaccharide-CD in place of M6CD in hope of better association. M7CD ($Man_7GlcNAc_2$FmocAsn-β-CD) has an longer mannosyl oligosaccharide. Additional work on a *hepta*mannosyl derivative was carried out. The analytical HPLC chromatogram of mixed Fmoc-mannosylasparagines using an ODS column contains at least six fractions. We succeeded in the uptake of the *hepta*mannosyl type oligosaccharide, FmocM7 in 18 % yield from a peak after ODS column and amide column chromatographies. In a similar manner as the M6CD, also M7CD was synthesized and identified by TOF-MS.

Competitive inhibition assay.

The interaction of the synthesized sugar-branched CDs with the immobilized ConA was observed in the acetate buffer of *p*H 5.3 using the SPR apparatus (IAsys). The amount of association on the ligand was indicated when the response R increased with time and showed a saturation curve. In the presence of a mannose molecule as an epitope which blocks the binding site of ConA, the curve showed only the physiochemical adsorption at the short initial period and no saturation curve due to the competitive inhibition between the oligosaccharide and ConA. This result demonstrates that ConA recognized the mannosyl residues of the oligosaccharide-CD. Similarly, the response curve between the immobilized cholic acid and the oligosaccharide-CD was observed. In the presence of cyclohexanol, and

because of a competitive inhibitor only during the initial period, a physiochemical adsorption was observed, and not the saturation curve based on the inclusion association. This proved that the inclusion phenomena between the cyclodextrin cavity and the cholic acid.

Dual association of *hexa*mannosyl-branched cyclodextrin (M6CD)

M6CD shows the association constant K_1 on the order of 1.3×10^7 M^{-1} during the binding with ConA. This is reasonable in comparison with the reported value using the equilibrium dialysis method [49,50]. K_2 between the immobilized ConA in the presence of excess cholic acid is 4.7×10^6 M^{-1}. K_3 is the association constant of 1.3×10^7 M^{-1} between M6CD and the immobilized cholic acid. The unexpected large association constant of K_3 may be due to the induced fit effect by the branched group such as Fmoc and high mannosyl oligosaccharide. The association constant of K_4 between the complexed M6CD with ConA and free cholic acid was calculated to be 4.7×10^6 M^{-1} from the relation using rough assumptions. Also, the association constant with DXR showed a larger value of 4×10^7 M^{-1}. These values seem to be expected to provide sufficient adhesion to interact with a receptor on biomaterials and indicate the release of the drug on the receptor protein.

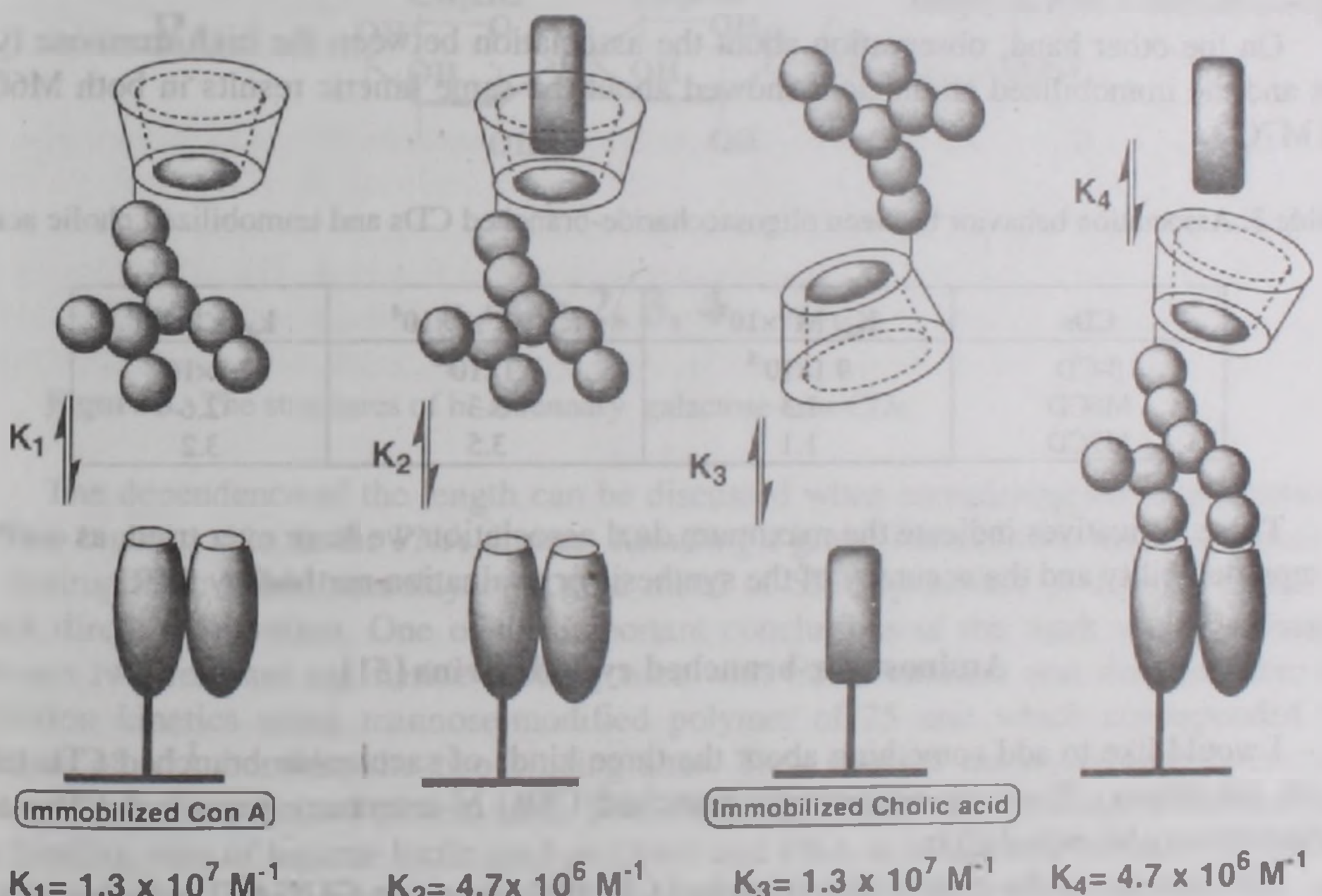

Figure 2. A schematic scheme of the association constants concerning dual association of hexamannosyl oligosaccharide-branched CD with Con A and cholic acid.

Comparison of heptamannosyl CD with hexamannosyl CD.

The results of the association constant, association rate constant, and the dissociation rate constant of two kinds of oligosaccharide-CDs having 6 and 7 mannose residues with ConA are summarized in the Table 2.

Table 2. Association behavior between oligosaccharide-branched CDs and immobilized ConA

CDs	K_a / $M^{-1}\times10^7$	k_a / $M^{-1}s^{-1}\times10^4$	k_d / $s^{-1}\times10^{-3}$
β-CD	0	0	0
FmocM6	1.2	5.8	5.0
M6CD	1.3	2.3	1.8
FmocM7	8.4	6.8	0.81
M7CD	8.1	5.4	0.67

Without a sugar branch, β-CD itself does not show any association with the ConA protein. The M6 type and M7 oligosaccharide itself without a CD cavity shows almost the same values of M6CD and M7CD, respectively. This means that the CD moiety is not involved with the interaction. The association constant of M7CD shows approximately a 7-fold increase due to the decrease in k_d. Naimith and his colleague[61] obtained an X-ray diffraction of the complex between ConA and trimannose. The recognition was induced by a number of hydrogen bonds between the amino acid residues of ConA and trimannose. These facts may demonstrate that the larger mannosyl oligosaccharide will resist removal from the oligosaccharide-ConA complex.

On the other hand, observation about the association between the high mannose type CDs and the immobilized cholic acid showed about the same kinetic results in both M6CD and M7CD.

Table 3. Association behavior between oligosaccharide-branched CDs and immobilized cholic acid

CDs	K_a / $M^{-1}\times10^7$	k_a / $M^{-1}s^{-1}\times10^4$	k_d / $s^{-1}\times10^{-3}$
β-CD	9.1×10^{-5}	7.1×10^{-6}	7.8×10^{-2}
M6CD	1.3	3.3	2.6
M7CD	1.1	3.5	3.2

These derivatives indicate the maximum dual association we have ever tried, as well as the reproducibility and the accuracy of the synthesis or evaluation method by SPR.

Aminosugar-branched cyclodextrins [51]

I would like to add something about the three kinds of saccharide-branched CDs tried in our laboratory. They are aminosugar-branched CDs, *bi*-antennary branched CDs, and *hepta*antennary branched CDs.

The first one is the aminosugar-branched CD made by using GUG-CD, which contains a carboxylic acid in the terminal glucose unit of *mono*-C6 maltosyl-β-CD. In quantitative yields, three kinds of aminosugars, that are mannosamine, glucosamine, and galactosamine, are attached to GUG-CD.

The evaluation of dual recognition with the corresponding lectin, cholic acid and anticancer DXR was done. Moderate K_a values of around 10^4 M^{-1} were obtained from the association with lectins. The association constants for the inclusion of cholic acid were also on the order of around 10^4 M^{-1}. Association with the anticancer drug, DXR, was also in the same order of 10^4 M^{-1}.

Bi-Antennary galactose-branched cyclodextrin [51]

Bi-antennary galactose-branched β-CD at *A, D* positions of the CD ring unit having various lengths of the spacer arm were prepared. The number of aminohexanoic acid units ranged from two to four as spacer arm between galactose and CD cavity. In the association constant with the PNA lectin, the K_a values increased depending on the arm length as 1.0, 2.6 and 4.6 ($\times 10^6$ M^{-1}) corresponding the arm length (n= 2, 3 and 4, respectively).

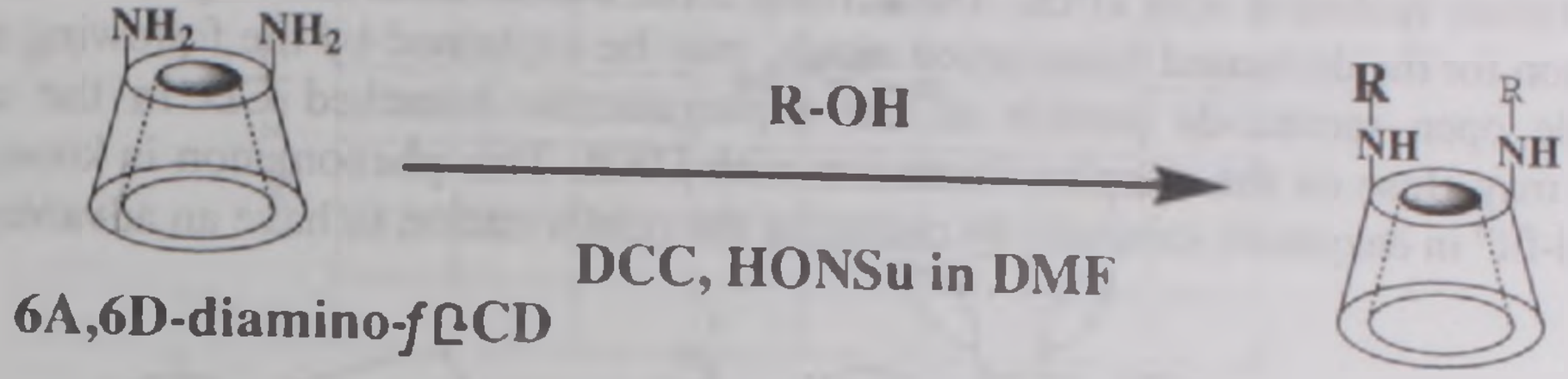

Figure 3. The structures of bi-antennary galactose-arm-CDs.

The dependence of the length can be discussed when considering the length between the two binding sites in the PNA dimers. Kiessling's group immobilized mannose-lipoid on the hydrophobic lipoid monolayer on gold metal of SPR tip surface as a probe ligand for ConA direct observation. One of the important conclusions of the work was the distance between two relevant saccharide binding sites with ConA tetramer was detected from the inhibition kinetics using mannose-modified polymer of 25 unit which corresponded the distance of 65Å between the two binding sites. They stressed the multivalent interaction between carbohydrate and protein [52]. The X-ray data suggested the distance between the two binding sites of legume lectin such as ConA and PNA to be about 65 Å [61]. Therefore, the *bi*-antennary galactose-branched CD derivative having a larger arm between the two galactoses may reach two binding sites on PNA, and the sugar-cluster effect [58] may appear in this case. Compounds with an arm shorter than 65 Å may bind at only one binding site. However, the depth of the binding site should need a minimum length to fit and form stable enough complexes. The association constant for the inclusion of DXR indicated that the cavity associated with DXR tighter than the *mono*antennary derivatives at the level of $3.4.10^5$ M^{-1}.

HEPTA-Antennary galactose-branched cyclodextrins [51]

The third experiment of the derivative is the comparison of the *hepta*antennary galactose-CD with the *mono*antennary galactose-branched CD. We prepared these structures starting *mono*- and *hepta*-C6-iodo-β-CDs using a thiol derivative of galactose unit. The K_a value of *hepta*galactose-branched CD with PNA lectin showed a 16-fold larger value than the *mono*antennary CD. In this case, the association constant of *hepta*galactose CD showed a 20-fold tighter inclusion with DXR. The driving force comes from the large decrease in k_d. The reason for the decreased dissociation rate k_a may be explained by the following scheme. The wide open saccharide portion of the *hepta*galactose-branched CD in the aqueous solution may close on the complex formation with DXR. This phenomenon is known as an "induced-fit" in enzymatic catalysis by changing the conformation to have an advantage.

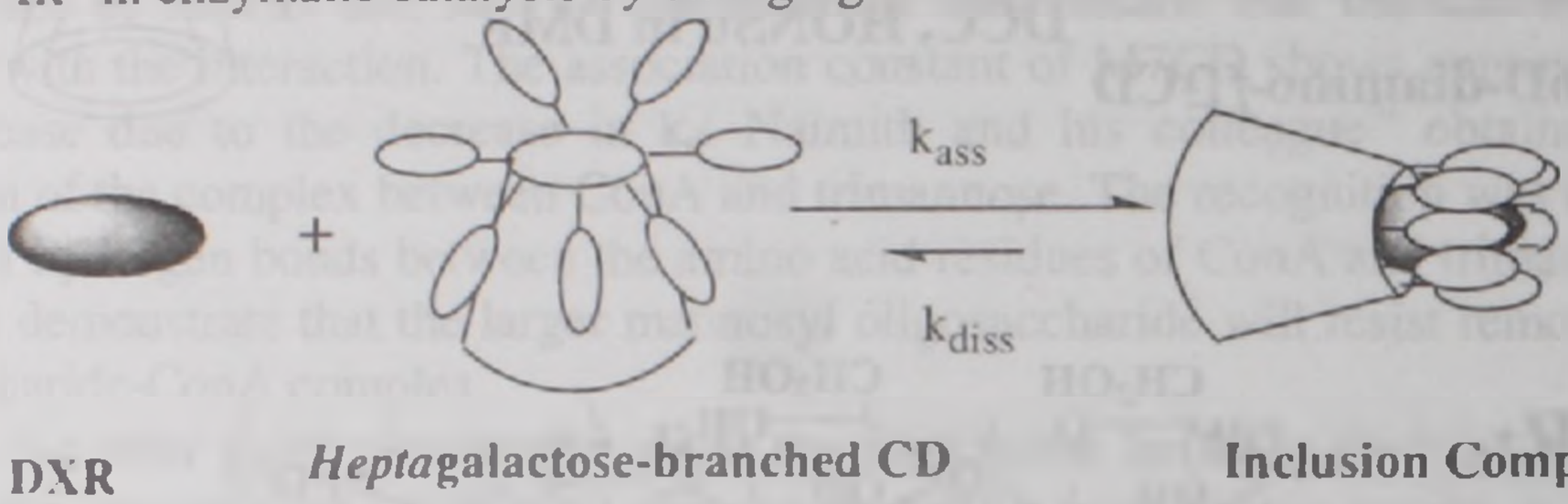

Figure 4. Induced-fit type conformational change at the association of heptagalactose-branched CD with DXR.

The computer-assisted molecular modeling supported this idea. The status before the complex formation may exist in a minimum potential energy. For the status of the complex formation with DXR, the conformation of the branch became closed with a decreased steric energy.

A two dimensional map for dual association of sugar-branched CD [51]

In this map Figure 5, the x-axis is log K_a of inclusion and, the y-axis is log K_a of oligosaccharide recognition. Almost all the aminosugar-branched CD exists in the area around 3-5 for the inclusion constant and around 3-5 for the saccharide recognition. The *mono*antennary galactose-branched CD goes up to the right and increase in this map when changed to the *hepta*antennary galactose-branched CD. The longer arm of *bi*antennary galactose-branched CD (n= 3) made the position upward (x= 4.6, y= 6.4). However, a natural high mannosyl CD existed in such an advantageous position (x= 7, y= 7-8) of a higher association constant with both the lectin and drug.

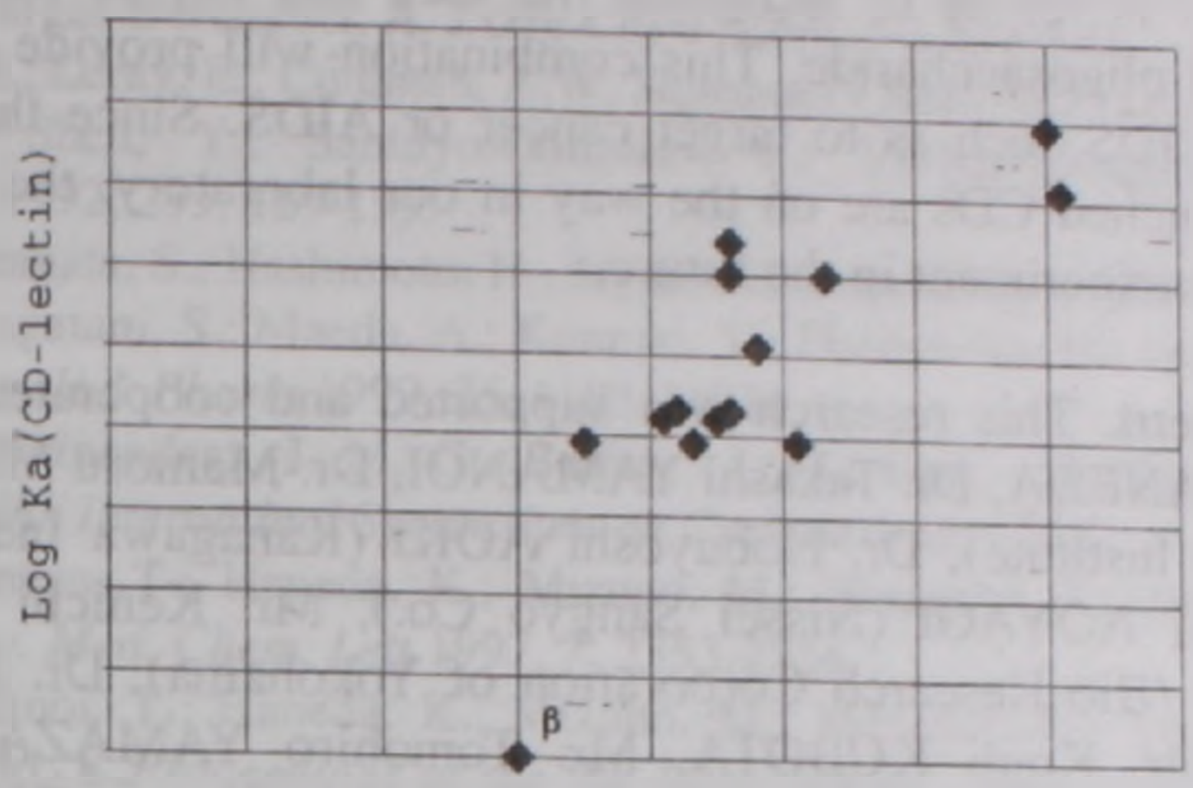

Figure 5. A two-dimensional map for dual association. K_a(CD-protein) means the association constant between oligosaccharide-branched CD and lectin protein. K_a(CD-drug) means the association constant between oligosaccharide-branched CD and cholic acid or DXR. *Octa*-Glc-calixarene means an *octa*glucose-branched calixarene [31].

In comparison with other carrier systems such as the calixarene-saccharide conjugates made by Aoyama's group, their compound is positioned around (x= 5.4, y= 6.0). They synthesized *octa*antennary glucose cluster also that has alkyl chain.[22] This associate with ConA at 10^6 M^{-1} and guest compound dye eosin around 1.8×10^5 M^{-1}.

All the other systems in the literature were not evaluated by the dual association constant, so they can not be exactly compared in this map.

Conclusion

In conclusion, we verified the following points.

A) The high mannosyl type oligosaccharide-branched CD (that is, M6CD and M7CD) showed a tight interaction both with lectin and a drug, showing a K_a of 10^7 M^{-1} or more by SPR assay.

B) The number of antennas attached on CD and the length of the spacer arm between two recognizing sites are quite important for the dual association with protein and drug.

C) In order to obtain an overview of the dual association, a two-dimensional map can be suggested.

Scope

I would like to add the scope of this review.

A) This will make the better universal carrier for various drugs ever studied. The sugar-branch itself may enforce the complex formation by a sugar-cluster effect and make a stable inclusion with drugs accompanying the induced-fit type conformational change.

B) The mannosyl group will associate with the receptors on the Kuffer cell in the liver, or on a macrophage. Also the galactosyl unit can target the liver parenchymal cell. As a step in the development of this research, we have to find the *in vitro* interaction with liver

cells, bacteria, and viruses. It will then, be necessary to proceed to the practical tests *in vivo*, followed by clinical tests.

C) Another key is for us to recognize the lock and key in the structural relation between a protein and oligosaccharide. This combination will provide us new strategy to design for effective TDDS such as to target cancer or AIDS. Since the study on various structures of sugar-branched CDs are on the way in our laboratory, the hopeful possibility should be proven in the experiment in the future.

Acknowlwdgement. This research was supported and cooperated by Dr. Toshiyuki INAZU, Dr. Katsuji HANEDA, Dr. Takashi YAMANOI, Dr. Mamoru MIZUNO, Dr. Hideki ISHIDA (the Noguchi Institute), Dr. Nobuyoshi AOKI (Kanagawa Industrial Technology Institute), Mr. Masaaki AOYAGI (Nissei Sangyo Co.), Mr. Kenichi HAMAYASU, Dr. Hitoshi HASHIMOTO (Bio Research Corporation of Yokohama), Dr. Hideo IMATA, Mr. Keisuke MATUDA, Mr. Kouji KUBOTA, Mr. Tomohiro YAMAZAKI, Mr. Naoyoshi YASUDA, Mr. Hideyoshi ABE, Mr. Hidenobu KOUZU, Ms. Yasuyo TURUI, Mr. Takayuki NAGAI and Associate Professor Keiko TAKAHASHI (Tokyo Institute of Polytechnics). I owed so much to them.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

NOVEL AMPHIPHILIC CYCLODEXTRINS: SYNTHESIS, CHARACTERIZATION AND PROPERTIES

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A new class of amphiphilic cyclodextrins have been obtained by grafting one single cholesterol moiety on the primary face of cyclodextrins. The properties of these new derivatives strongly depend on the structure of the adduct with a very specific effect of the methylation of the cyclodextrin core. The properties of these compounds have been investigated in details in the absence and in the presence of a synthetic phospholipidic matrix by Nuclear Magnetic Resonance (NMR) and scattering techniques (light, X-rays, neutrons). It has been shown that a high diversity of structures (liposomes, vesicles, micelles) can be obtained depending on the structures of the molecule.

Получен новый класс амфифилических циклодекстринов с помощью пересаживания одной единственной сердцевины холестерола на первичной лицевой стороне (полости) циклодекстринов. Особенности этих новых производных строго зависят от структуры аддукта и очень специфического эффекта метилирования ядра циклодекстрина. Детально изучены особенности этих соединений в отсутствии и в присутствии синтетического фосфолипидного матрикса с помощью ядерного магнитного резонанса (ЯМР) и светорассеивания (свет, рентгеновские лучи, нейтроны). Показано, что большое разнообразие структур (липосомы, полости, мицеллы) может быть получено в зависимости от структур молекул.

Ստացվել է ամֆիֆիլային ցիկլոդեքստրինների նոր դաս խոլեստերոլի միայն մեկ միջուկի ցիկլոդեքստրինների առաջնային երեսի վրա պատվաստման միջոցով: Այս նոր ածանցյալների առանձնահատկությունները խիստ կախված են հաղորդակցող տարրի (ադդուկտի) կառուցվածքից ցիկլոդեքստրինի միջուկի մեթիլացման շատ յուրահատուկ ազդեցությունից: Այս միացությունների առանձնահատկությունները մանրամասն ուսումնասիրվել են սինթետիկ ֆոսֆոլիպիդային մատրիքսի առկայությամբ և բացակայությամբ միջուկային մագնիսական ռեզոնանսի (ՄՄՌ) և լուսացրման միջոցով (լույս, ռենտգենյային ճառագայթներ, նեյտրոններ): Ցույց է տրվել, որ կառուցվածքների մեծ բազմազանություն (լիպոսոմներ, խոռոչներ, միցելներ) կարող է ստացվել կախված մոլեկուլների կառուցվածքներից:

Introduction

Since a number of years, a special attention has been given to the preparation of amphiphilic cyclodextrins in order to use them as new compounds for the preparation of novel materials (nanoparticules for example) or to insert them in a preformed lipidic matrix such as liposomes. In both cases, the main objective of this approach was to combine the size specificity of cyclodextrins for hydrophobic guests and the transport properties of a structure of liposomes or analogs. Before making any attempt to prepare new amphiphilic

cyclodextrins two main questions must be addressed i.e. first, how many lipophilic groups will be grafted and second, on which side (primary or secondary or both) the grafting will be achieved. All situations have been encountered in the literature (see refs. 1-7 in [1]). We decided to focus in all cases depicted here on a mono-substitution on a primary hydroxyl site of the cyclodextrin. Although the chemistry of single site modification is sometimes more tedious to afford highly pure compounds, it can be achieved rather easily in reasonable yields. The selection of substitution on the primary side (on one of the CH_2OH group) offers two advantages : First, the wider secondary face is not modified and it is well documented that most guest molecules enter through this face. Second, since we have developed the production of antibodies against natural and modified cyclodextrins [2] which allow a very sensitive and selective method for the quantitative determination of these hosts and since the epitope in the recognition process is the secondary face without any effect the modification of primary hydroxyls side, the latter is the most appropriate for mono-substitution. Our first attempt to investigate the properties of amphiphilic cyclodextrins was made by grafting an aliphatic chain of various length through an amide bond on one single primary position [3]. This led to the family of "lollipops". Unfortunately, although these compounds exhibit interesting properties, it was shown by NMR that the aliphatic chain shows a strong tendency to include in the cavity of its own cyclodextrin carrier, therefore limiting its potential application. (see figure 1a). This problem of self-inclusion of the aliphatic chain could be avoided by using an aliphatic chain carrying a very bulky end-group in place of the methyl end. This was achieved using a *t*-butyloxycarbonyl moiety which cannot include in the cavity of the CD. This new class of amphiphilic compounds has been investigated in details [4] in terms of insertion in model liposomes and of inclusion of guests in the cavity of the CD. These derivatives were called "Cup and Ball cyclodextrins". It was indeed shown that in the absence of a guest the bulky end-group attempts to enter the cavity through the primary face (Fig. 1b). However, when a guest compound is included in the cavity through the secondary cavity, the chain is expelled as displayed in figure 1c.

Figure 1. Schematic structures of "Lollipops" (a) and "cup and ball molecules" without (b) and with an included guest (c).

The liberated chain is then able to be inserted in a phospholipid bilayer matrix. In the present case as well as all other cases considered later in the present paper, the liposome model selected was made of di-myristoyl-phosphatidylcholine (DMPC) the molecular structure of which is displayed in Figure 2.

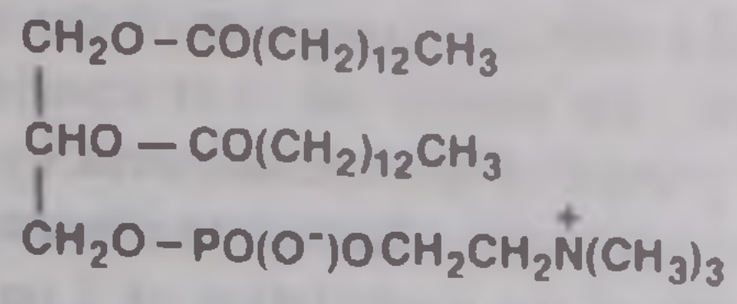
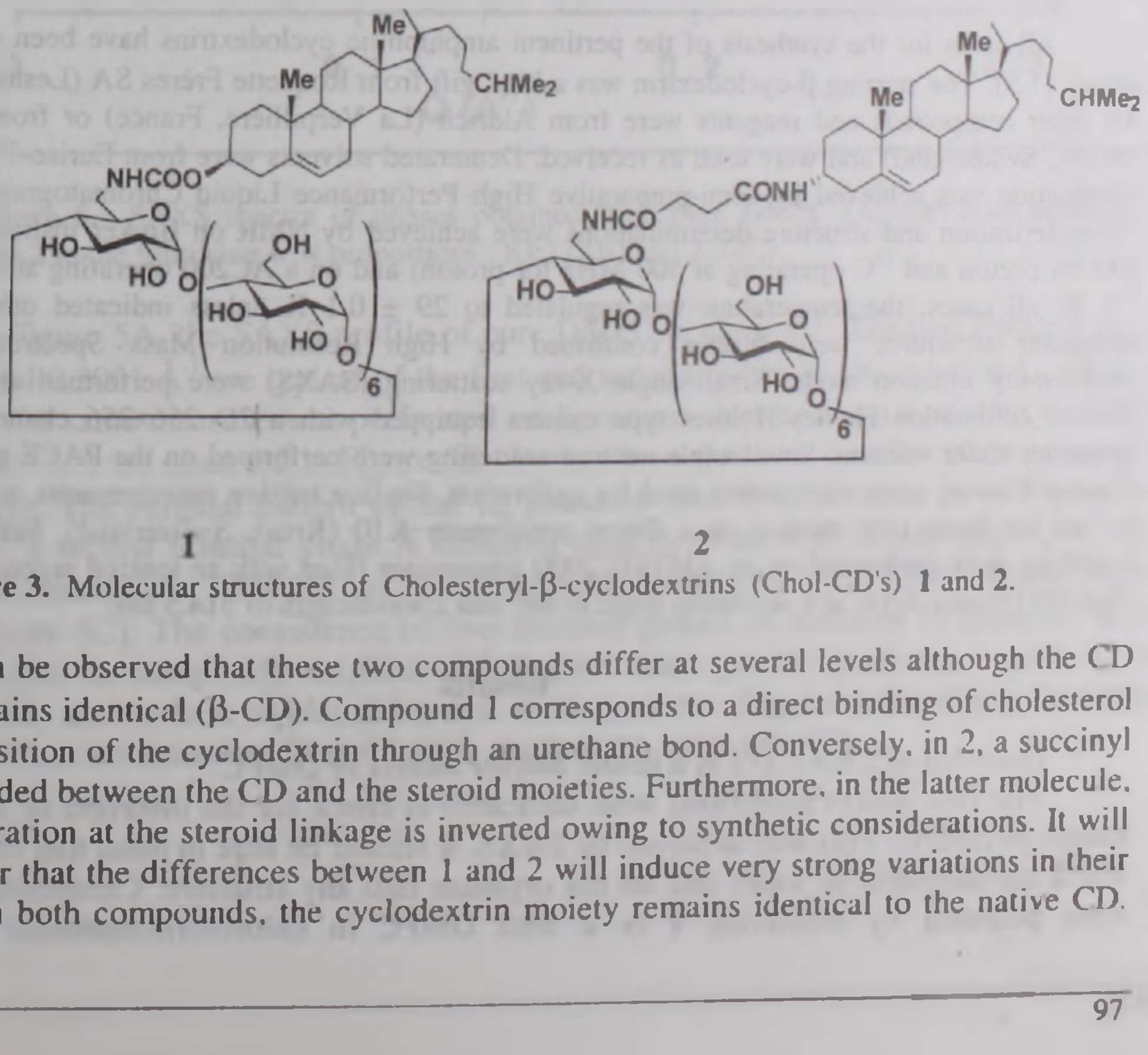


Figure 2. Structure of Dimyristoyl-phosphatidyl-Choline (DMPC).

This matrix was selected for several reasons :

- Hydration leads to the formation of large liposomes formed by well characterized bilayers.
- Sonication leads to small (single-layered) vesicles (average diameter 30 nm)
- The transition temperature between the gel (frozen chains) and the fluid states (molten chains) is ca. 23°C. This allows to make all experiments in a reasonable temperature range to retain the more biologically relevant fluid state.

The use of various techniques indeed allowed to show that the "Cup and Ball" molecules, when loaded by a guest, include in DMPC liposomes or vesicles [4]. The insertion is characterized by a partition coefficient indicating the proportion of amphiphilic complex inserted in the DMPC matrix. However, the insertion level did not reach very high values whatever the length of the chain. We then attempted to overcome these limitations by using a different nature for the hydrophobic moiety of the amphiphilic cyclodextrin. The basic principle of a single grafting at the primary position was retained. The choice was made to use cholesterol or derivatives as hydrophobic part since these compounds are well known to insert easily into phospholipid bilayers and play a key role in the properties of living cell membranes. In a first step two types of molecules were prepared as shown in Figure 3.



1 2

Figure 3. Molecular structures of Cholesteryl-β-cyclodextrins (Chol-CD's) **1** and **2**.

It can be observed that these two compounds differ at several levels although the CD moiety remains identical (β-CD). Compound 1 corresponds to a direct binding of cholesterol on the 6 position of the cyclodextrin through an urethane bond. Conversely, in 2, a succinyl spacer is added between the CD and the steroid moieties. Furthermore, in the latter molecule, the configuration at the steroid linkage is inverted owing to synthetic considerations. It will be seen later that the differences between 1 and 2 will induce very strong variations in their behavior. In both compounds, the cyclodextrin moiety remains identical to the native CD.

All OH groups are unmodified with the exception of the single primary one used for the grafting. In a second step of the results we will consider the effects of the selective replacement of the hydroxyl groups of the cyclodextrin by methoxy groups and it will be shown that very different and surprising effects are observed. We therefore found of very important to examine the effects of the methylation of 2 on its properties. The synthesis of the derivative to be considered further has been described in details in [5]. It corresponds to the structure of 2 but all OH groups in positions C2 and C6 have been methylated. The molecular structure of this compound further labeled as 3 is displayed on Figure 4.

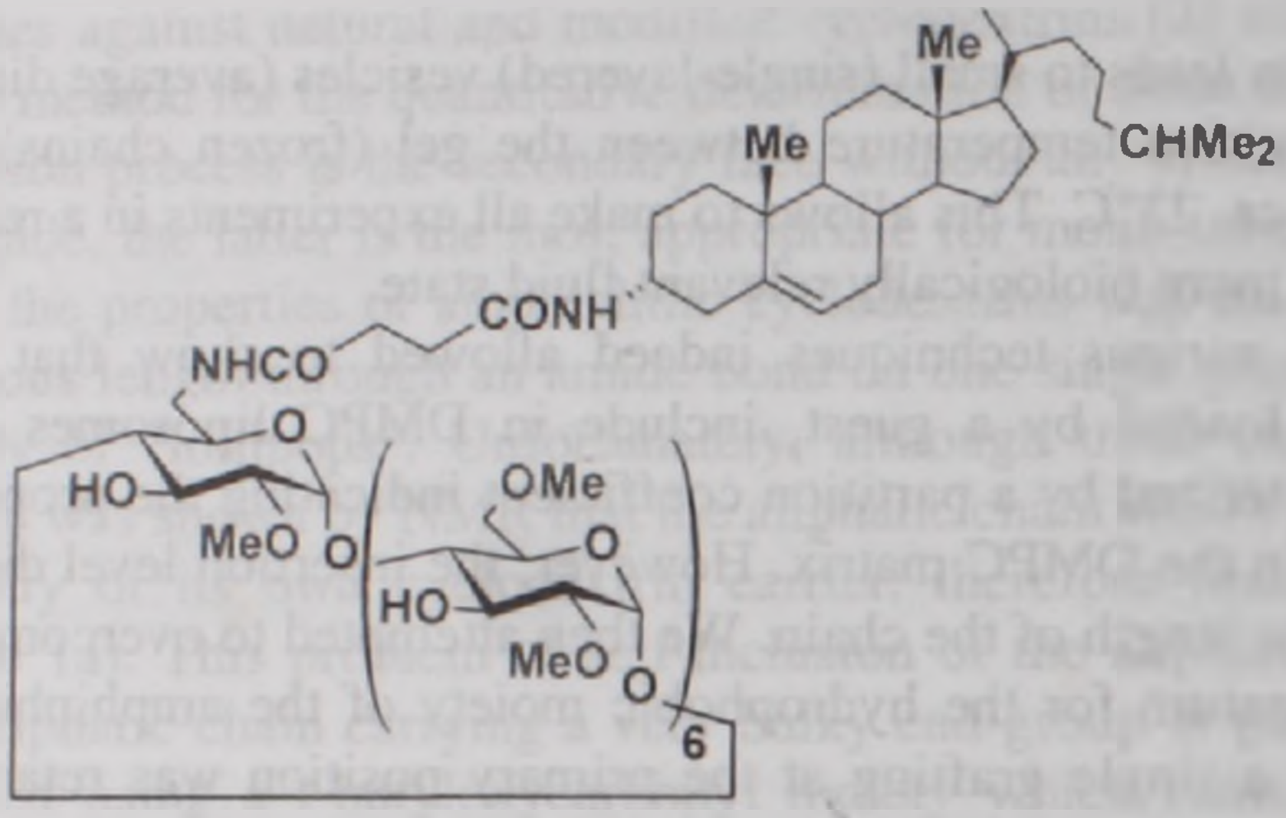


Figure 4. Molecular structure of **3** (Chol-dimeb).

Materials and methods

All steps for the synthesis of the pertinent amphiphilic cyclodextrins have been described in details [1,5]. The starting β-cyclodextrin was a kind gift from Roquette Frères SA (Lestrem, France). All other compounds and reagents were from Aldrich (La Verpillière, France) or from Fluka AG (Buchs, Switzerland) and were used as received. Deuterated solvents were from Euriso-Top (France). Purification was achieved by semi-preparative High Performance Liquid Chromatography (HPLC). Characterization and structure determinations were achieved by NMR on Bruker instruments (DRX 500 for proton and ^{13}C operating at 500 MHz for proton) and on a AC200 operating at 81 MHz for ^{31}P. In all cases, the temperature was regulated to 29 ± 0.1 K unless indicated otherwise. The molecular structures were further confirmed by High Resolution Mass Spectrometry using electrospray infusion mode. Small angle X-ray scattering (SAXS) were performed at 30°C on a pinhole collimation Huxley-Holmes type camera equipped with a 2D 256x256 channels detector operating under vacuum. Small angle neutron scattering were performed on the PACE setup at LLB (Saclay, France) pure water being used for calibration. Surface tension measurements were achieved by the Du Nouy ring method on a digital tensiometer K10 (Krüss, Switzerland). Static and light scattering were performed on an AMTEC 2000 goniometer fitted with an ionized argon laser source (Spectra Physics 2016) at a scattering angle of 90° and a wavelength of 514.5 nm.

Results

Insertion of Chol-CD's in a model bilayer matrix of DMPC

The first assays performed were dedicated to check for the insertion of **1** and **2** in a matrix of DMPC. This was achieved by SAXS. It should be kept in mind that compounds **1** and **2** are insoluble in water and do not organize into any structure. Composite liposomes were prepared by dissolving **1** or **2** with DMPC in chloroform-methanol (2:1, v:v).

evaporating to dryness under vacuum to a glassy film, hydration in excess water and vortexing to obtain a milky suspension of the liposomes. This was done using various ratios of **1** or **2** vs. DMPC in order to follow the evolution of the structure(s). In all cases presented in this work, the total lipid:water ratio was set to 1:4

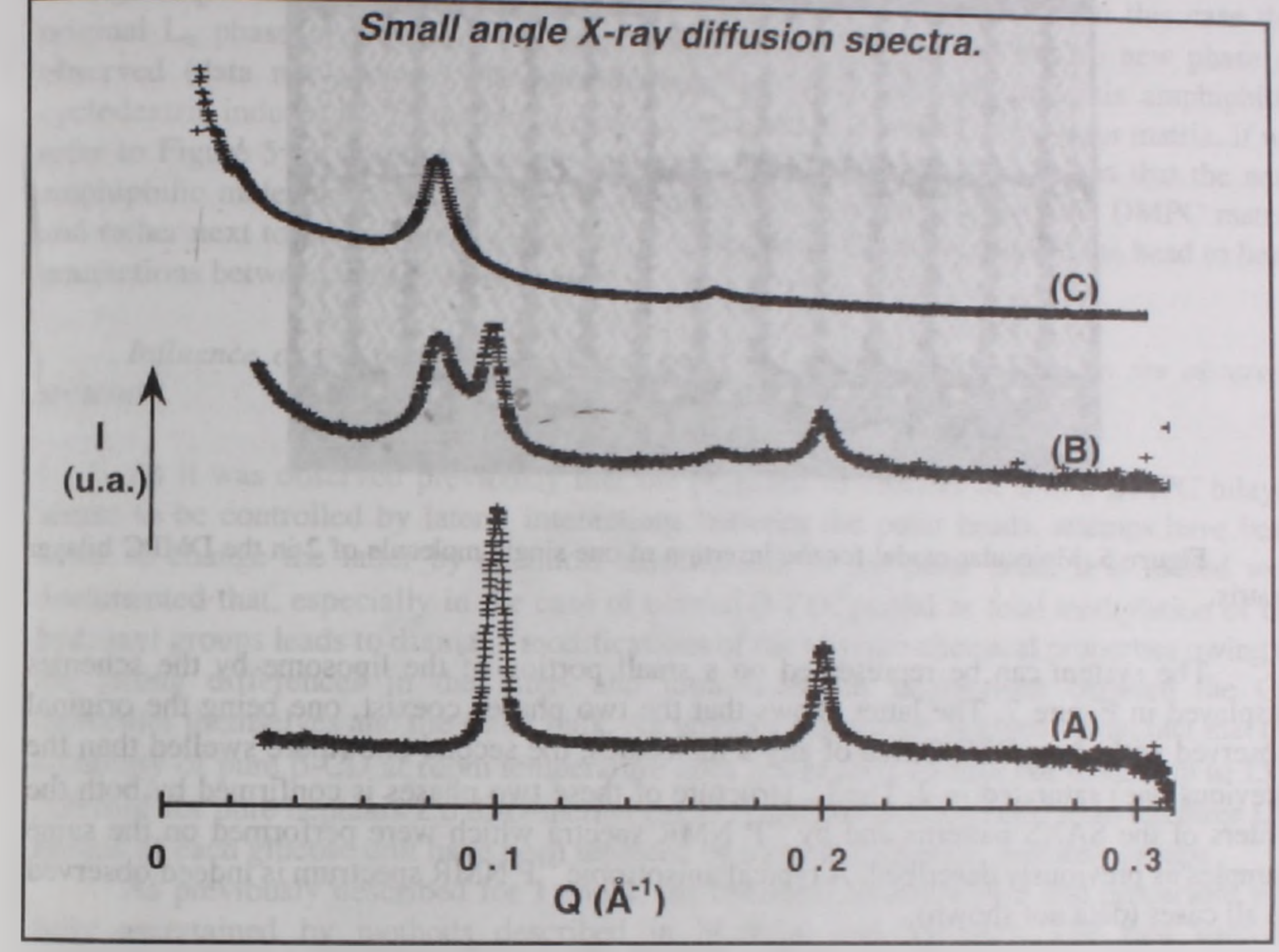


Figure 5. SAXS spectra of phases obtained from pure DMPC (A) and from mixtures of DMPC and **2** in the following w:w proportions : 83:17 (B) and 2:3 (C).

In Figure 5A, the SAXS profile of pure DMPC is observed The two Bragg peaks at 0.1005 and 0.2001 $Å^{-1}$ are typical of the first and second orders of the lamellar L_α phase of DMPC. The same measurement performed on liposomes derived from a mixture of DMPC and **2** (83:17, w:w) observed in Figure 5B clearly shows that two lamellar phases coexist in the sample. The original pattern of the L_α phase of DMPC is retained as in Figure 5A. However, a second lamellar phase is observed with two orders at 0.0845 and 0.1672 $Å^{-1}$. Further increasing the DMPC:**2** to 2:3 (w:w) leads to the pure "new phase" further called $L_{\alpha CD}$ (Figure 5C). The coexistence of two lamellar phases of different thicknesses in the same sample is completely original and implies that specific processes involving the cyclodextrin moiety have to play a key role in this process. The insertion of **2** in the matrix of DMPC is schematized on Figure 6 for a single guest molecule of **2**.

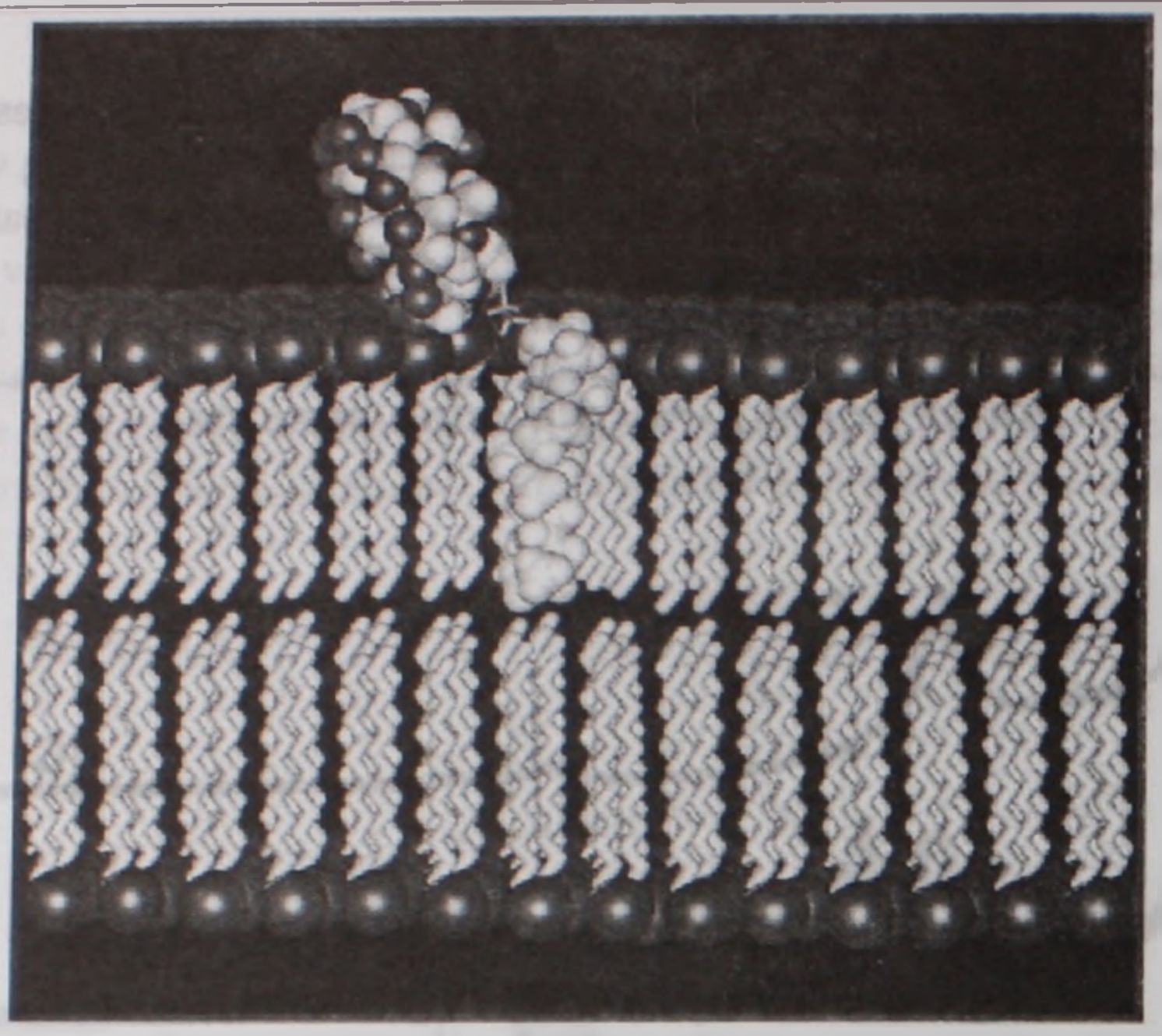


Figure 6. Molecular model for the insertion of one single molecule of **2** in the DMPC bilayer matrix.

The system can be represented on a small portion of the liposome by the schemes displayed in Figure 7. The latter shows that the two phases coexist, one being the original observed with pure DMPC free of any **2** molecules, the second one (more swelled than the previous one) saturated in **2**. The L_α structure of these two phases is confirmed by both the orders of the SAXS patterns and by ^{31}P NMR spectra which were performed on the same samples as previously described. A typical anisotropic ^{31}P NMR spectrum is indeed observed in all cases (data not shown).

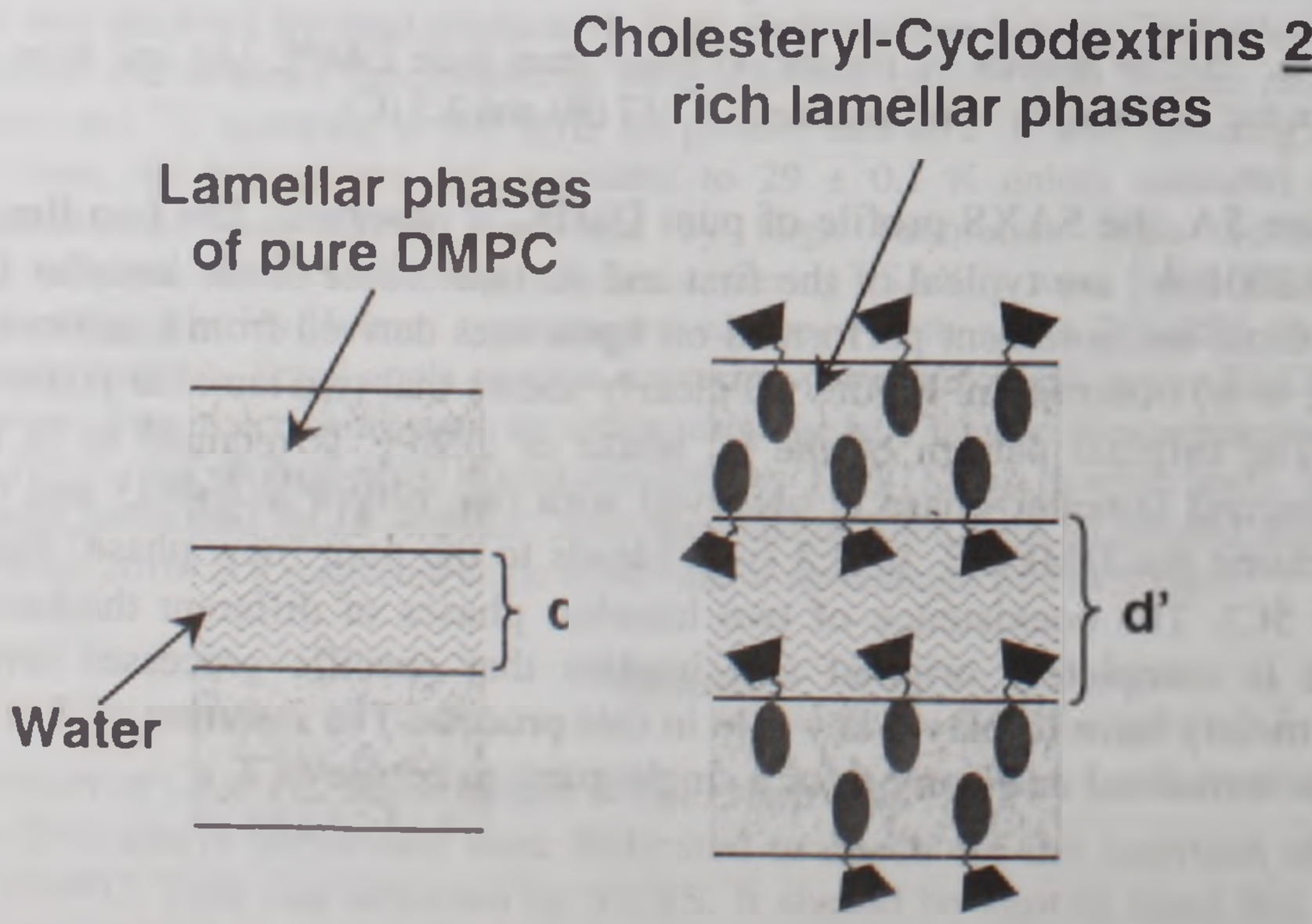


Figure 7. Structures of the two coexisting phases in mixtures of DMPC and **2**

The original behavior of these samples implies that the CD moiety plays a very important role in the structure of the mixed samples. It can be explained by strong interactions between the CD polar heads leading to clusters saturated in **2** in the presence of normal pure DMPC bilayer domains. These "lateral interactions" will be confirmed later in this paper. The presence of a spacing group between the CD and the cholesterol derivative is of high importance since the same phenomenon is not observed with **1**. In this case the original L_α phase of DMPC is strongly perturbed and less ordered but no new phase is observed (data not shown). The specific behavior of **2** indicates that this amphiphilic cyclodextrin induces the formation of clusters saturated in **2** in the DMPC layer matrix. If we refer to Figure 5 in which one single molecule of **2** is represented, it means that the next amphiphilic molecule will also insert in the matrix but not anywhere in the DMPC matrix and rather next to the previously installed **2** molecule in order to optimize the head to head interactions between the CD head-groups.

Influence of the chemical modification of the cyclodextrin moiety on the observed structure.

As it was observed previously that the presence of clusters of **2** in a DMPC bilayer seems to be controlled by lateral interactions between the polar heads, attemps have been made to change the latter by chemical modification of the polar head. It is indeed well documented that, especially in the case of normal β-CD, partial or total methylation of the hydroxyl groups leads to dramatic modifications of the physico-chemical properties owing to the strong differences in the inter- and intra-molecular interactions between the CD molecules themselves and the water bulk. An obvious illustration is given by the fact that the solubility of pure β-CD at room temperature does not exceed 15 mM but climbs up to 150-200 mM for pure heptakis 2,6 di-*O*-methyl β-CD (DIMEB) although two from the three OH groups of each glucose unit have been replaced by more hydrophobic methoxy groups

As previously described for **1** and **2**, the chemical structure of **3** and precursors was fully ascertained by methods described in Material and Methods. The first surprise concerning this compound is that it is highly soluble in water reaching 800g/L in water at 25°C. It gives a single isotropic line in ^{31}P NMR indicating the absence of very large objects. This encouraged us to attempt surface tension measurements to look for the presence of a critical micellar concentration (CMC). It was indeed found [5] that a clear transition is observed vs. concentration of **3** in water indicating a CMC of ca. $5\ 10^{-6}$ M at 25°C; Dynamic and static light scattering confirmed that the observed objects are not small vesicles but compact objects with an average radius of 30 Å. The exact shape of the latter was confirmed by SANS and the experimental data fitted with several models. The SANS profile and fittings are reported on Figure 8.

It clearly appears from this figure that the objects obtained in water with **3** correspond to almost monodisperse spherical micelles with an average number of 24±4 molecules per micelle and an average diameter of 5 nm. A schematic representation of the latter object (containing 24 monomers) is displayed on Figure 9.

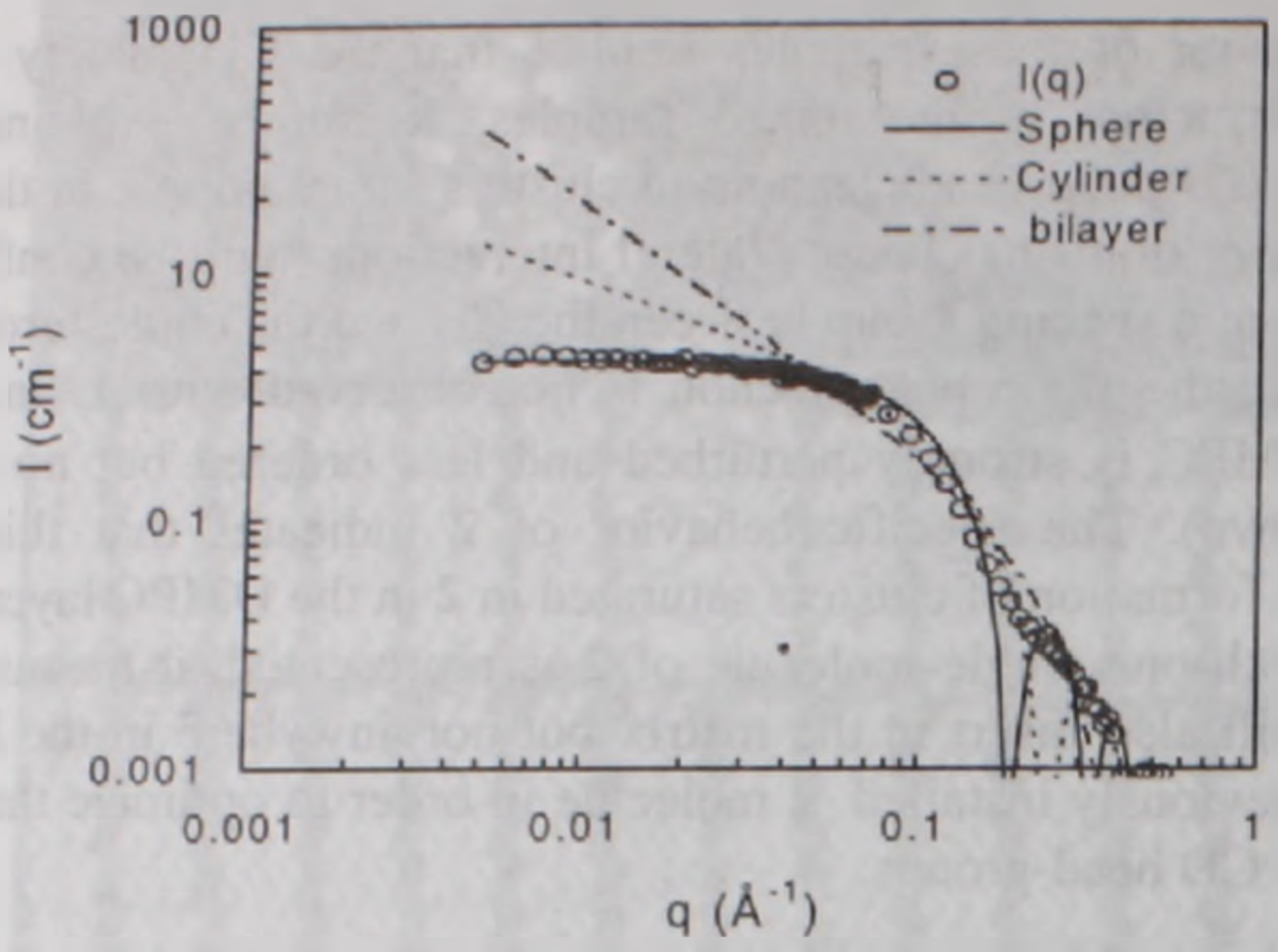


Figure 8. SANS of micelles obtained from **3** in water and demonstration of the spherical shape by attempts to fit the results with other models (cylinders and bilayers)

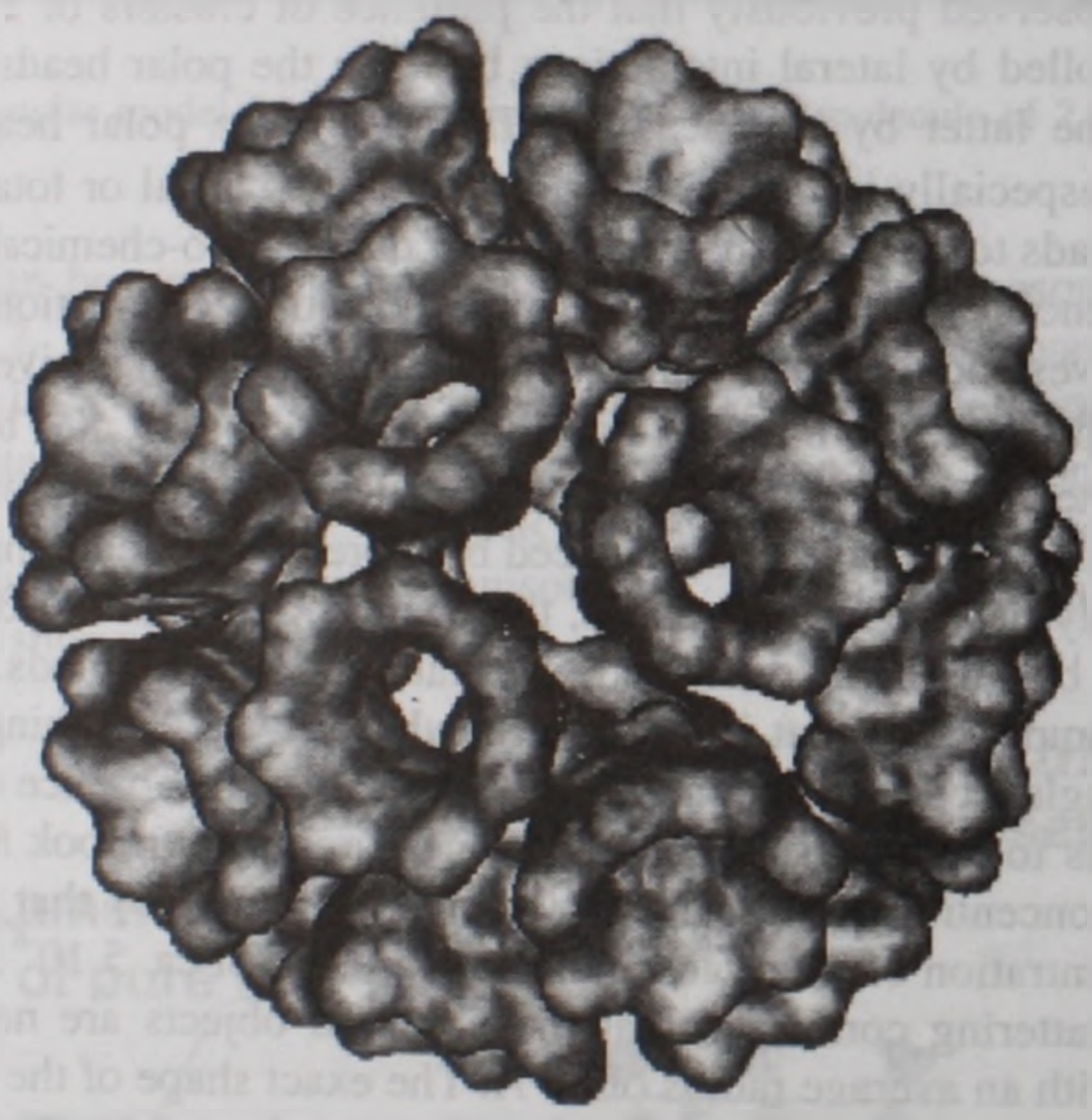


Figure 9. Molecular modeling of a micelle formed by **3** in water, the inner core formed by cholesterol being not visible on this picture.

The density profiles afforded by both X-Ray and Neutron scattering fully support that the CD cavities are exposed to the water interface as shown in Figure 9. Since these structures are clearly defined, two further questions must be addressed :

- Does the cavity of the cyclodextrin retains its ability to include guests ?
- How do these micelles behave in the presence of a liposome matrix as observed for **1** and **2** with bilayers of DMPC ?

To provide answers to the question, a selection of guests well identified to include in the cavity of DIMEB was assayed on **3** in the micellar form using dedicated NMR techniques combining diffusion methods and dipolar interactions (nOe pumping). These procedures are described in details in [6]. Furthermore, the SAXS and SANS methods used previously for the micelles alone were repeated in the presence of potential guests for the cavity of the cyclodextrin. These techniques were assayed for both charged and uncharged guests. It was shown that the inclusion of charged guests (inducing the presence of electric charges on the surface did not modify in a significant way the size and shape of the micelles and that even strong lateral interactions could not change the area per molecule of **3** from its original value (340 $Å^2$). This implies that the packing of **3** molecules into homogeneous micelles is exceptionally robust.

Concerning the second point, the investigation of the interaction of the previously described micelles with DMPC liposomes showed that mixed micelles between **3** and DMPC can be obtained and that isotropic solutions can be attained indicating a completed destruction of the bilayer phase. This implies that these compounds have strong detergent properties and could be used to destroy phospholipidic bilayers in order to isolate membrane proteins. This point is clearly evidenced by ^{31}P NMR on vesicles of DMPC as displayed on Figure 10.

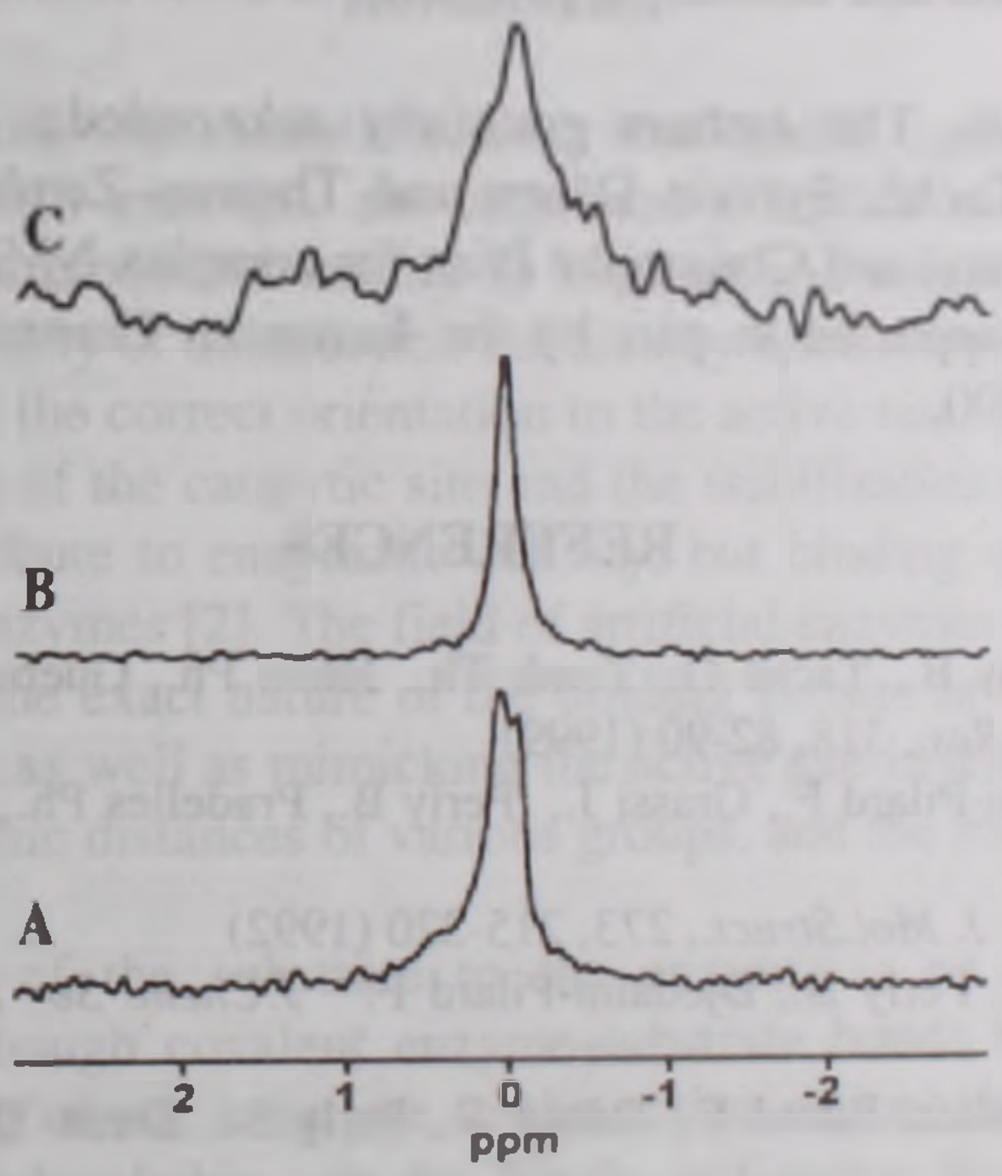


Figure 10. ^{31}P NMR Spectra of small unilamellar vesicles of pure DMPC 15 mM (A) and in the presence of DIMEB (C) or Chol-dimeb **3** (B) For B and C the ratio between DMPC and the CD derivatives is 15:3 (mM:mM).

In the case of Figure 10A, a sharp doublet is observed. The two lines correspond to the ^{31}P signals from the outer and inner lipids in the vesicle. This pattern is typical of small unilamellar vesicles. The corresponding solution is translucent. In Figure 10B, a clear solution is observed and a single sharp line is observed on the spectrum as expected for micelles. Complementary light scattering experiments showed that the mixed micelles have an average diameter of 13 nm. In 10C, the addition of the CD moiety alone (DIMEB) leads to a milky biphasic solution and to a very broad spectrum indicating that the original

vesicles are destroyed leading to large objects. The solubilization properties of these novel amphiphilic cyclodextins are under further investigation and will be described in the future.

Conclusions

A novel class of amphiphilic cyclodextrins has been presented here. They have all been fully characterized in terms of chemical structures and purity. Their interactions with preformed model phospholipid bilayers (DMPC liposomes) have been investigated using a very large variety of techniques which all converge to identical results. A number of different structures has been encountered (liposomes with or without lateral phase separation, spontaneous and mixed micelles). It was also shown that the cavity of the CD moiety retains a full capacity to include external guests which is of considerable importance for potential applications. The key-point in this study is that going from one type of structure to another one is achieved by very small modifications of the cyclodextrin moiety (presence or absence of a spacing arm, methylation or not of the OH groups ...) Further investigations involving the same strategies and techniques but replacing the cholesterol by another highly hydrophobic structure are on the way and will be presented in a near future. The main conclusion is that this quite novel class of amphiphilic molecules open a very large field for basic research as well as for applications in a wide variety of domains ranging from pharmacy, cosmetics, food and household processes to basic chemistry and physics.

Acknowledgments. The authors gratefully acknowledge Pierre Guenot for Mass Spectrometry, Olivier Taché, Sylvain Désert and Thomas Zemb for diffusion techniques (light, X-rays and neutrons) and Christophe Péan for complex NMR experiments.

This work was supported in part by the European Commission (DGXII) under the FAIR program CT95-0300.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

ARTIFICIAL REDOX ENZYMES

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A general method for synthesis of artificial redox enzymes has been offered. Some of the catalytic properties of these systems have been studied and presented.

Предложен общий метод синтеза искусственных окислительно-восстановительных ферментов. Изучены и представлены некоторые каталитические свойства этих систем.

Առաջարկվել է ընդհանուր մեթոդ արհեստական օքսիդա-վերականգնող ֆերմենտների սինթեզի համար: Ուսումնասիրվել և ներկայացվել են այդ սիստեմների որոշ կատալիտիկ հատկանիշները:

Introduction

Enzymes are proteins with catalytic activity that exhibit high specificity and large rate accelerations [1]. Although enzymes are large and complex molecules, their power to catalyze reactions can he attributed mainly to binding and catalysis. Binding is not only responsible for the specificity of the reaction but also by stereochemistry brings the substrate in close proximity and in the correct orientation to the active site. Other factors, such as the microscopic environment of the catalytic site and the stabilization of the transition state by hydrogen bonding, contribute to enzymatic activity, but binding and catalysis are the two essential features of all enzymes [2]. The field of artificial enzymes deals with the science of synthetically mimicking the exact nature of the binding subsite in terms of shape, size, and microscopic environment as well as mimicking the active site in terms of identity of groups, stereochemistry, interatomic distances of various groups, and the mechanism of action of the enzyme [3].

Binding: Binding of the substrate to the enzyme is an essential feature of all enzymatic reactions. Although covalent enzyme-substrate bonds are formed during some reactions, usually binding of the substrate to the active site of the enzyme involves non-covalent forces such as hydrophobic, van der Waals, or London dispersion forces, hydrogen bonding, and electrostatic interactions. Cumulative effects of such forces produce tight binding if the binding subsite is complementary to the structure of the substrate or if conformational changes occur in the enzyme during the binding process [4]. Cyclodextrins, consisting of 6, 7, or 8 units of α-1,4- linked D-glucopyranoses, have played an important role as binding sites in artificial enzymes. They are doughnut shaped molecules with secondary hydroxyl groups at the 2- and 3-positions of glucose units arranged in the more open end and primary hydroxyl groups at the 6-position at the other end. The interior of the cavity, consisting of a ring of C-H groups, a ring of glycosidic oxygen atoms, and another ring of C-H groups, is hydrophobic in nature, similar to the binding subsite of many enzymes. The inner diameter of the cavities are approximately 4.5 Å in α-cyclodextrin, 7.0 Å in β-cyclodextrin, and 8.5 Å in γ-cyclodextrin. α- and β-cyclodextrins fit an aromatic ring snugly with dissociation constants varying from 10^{-2} to 10^{-3} M, depending on the

substituent on the ring [5].

Catalysis: Enzymes use a variety of catalytic groups depending on the type of reaction that needs to be catalyzed [6]. These catalytic groups act intra-molecularly with the bound substrate to bring about large rate accelerations. The importance of intra-molecularity in enzymatic catalysis has been well established. There are several reviews which details the reasons for the large rate accelerations in intra-molecular reactions [7]. One of the widely used catalytic group for redox reactions is riboflavin. Flavoenzymes consisting of a riboflavin cofactor bound non-covalently to a substrate binding site play key roles in electron transport, respiration, photosynthesis, bioluminescence, and other oxidation-reduction systems [8,9,10].

Synergism of Binding and Catalytic Groups To Produce an Artificial Redox Enzyme: The design of the artificial redox enzyme consists of a cyclodextrin molecule acting as a binding site covalently attached to a flavin molecule acting as a catalytic site as shown in the Figure 1.

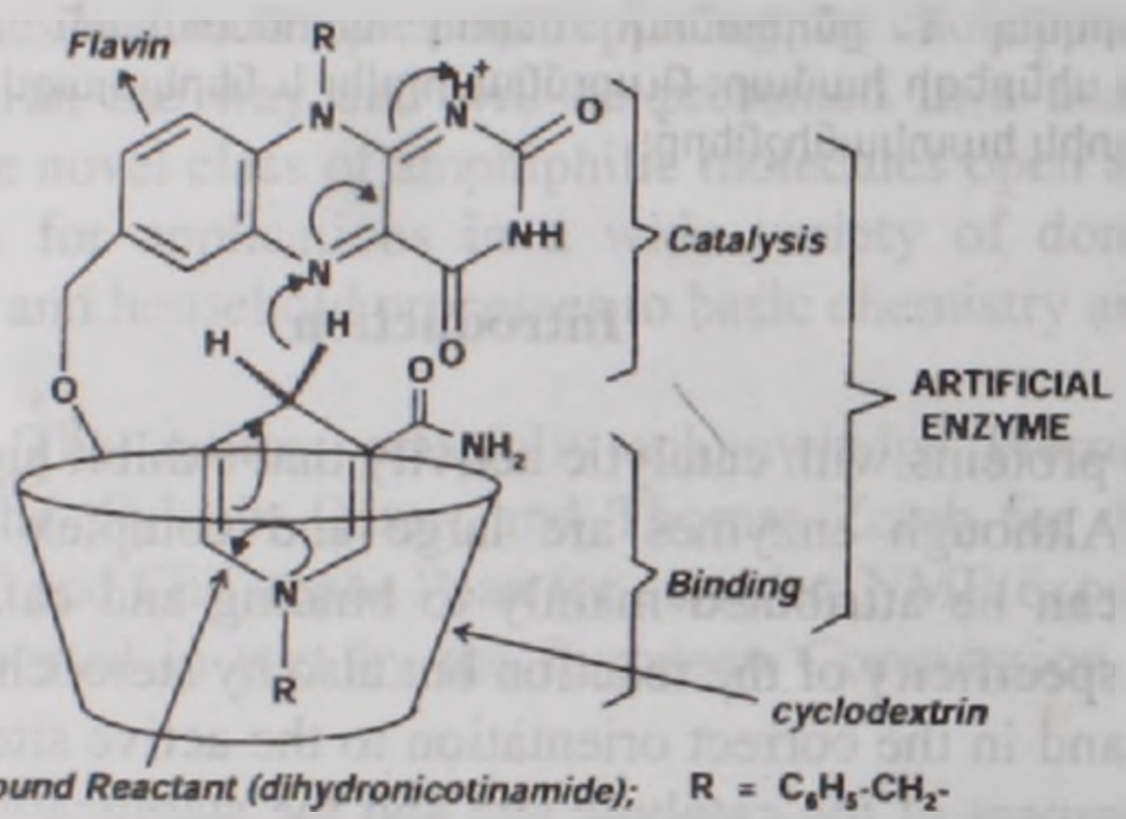


Figure 1. Schematic representation of an artificial redox enzyme.

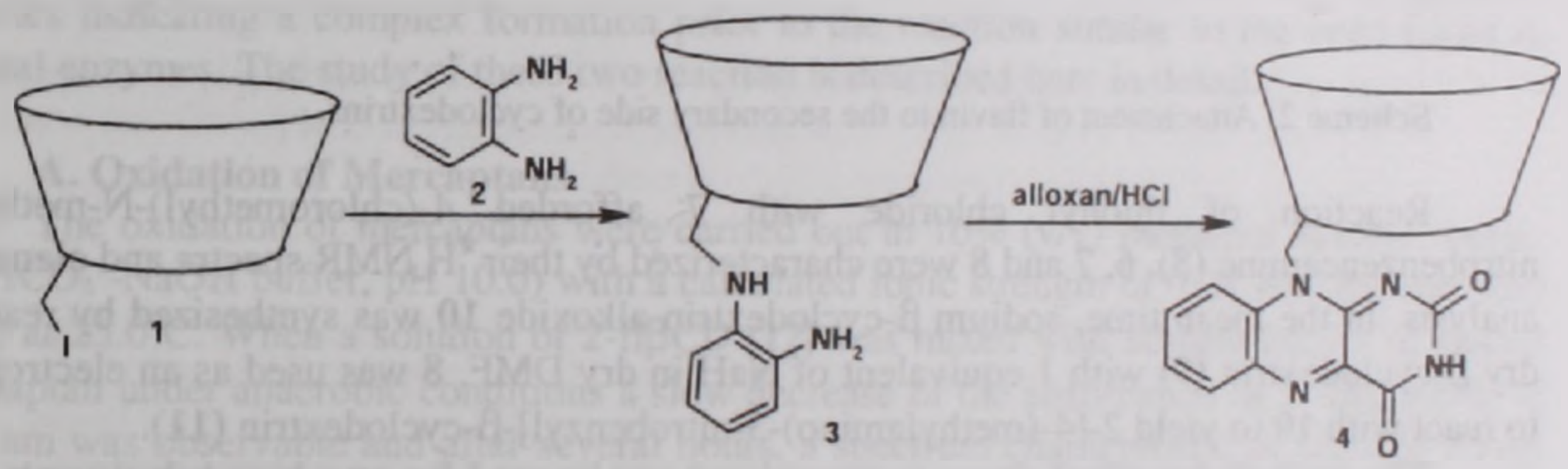
Synthesis of Artificial Redox Enzymes

Synthesis of artificial enzyme consists of attachment of a flavin derivative to cyclodextrins. Cyclodextrins have hydroxyl groups on its primary as well as secondary sides. Potentially, the flavin can be attached to either of these sides and the efficiency of the artificial enzyme depends on the orientation of the substrate bound in the cavity of cyclodextrin.

Attachment of Flavin to the Primary Side of Cyclodextrin. An approach for synthesis of artificial enzyme with flavin attached to the primary side of cyclodextrin involves synthesis of an o-phenylenediamine derivative of cyclodextrin and followed by its condensation with alloxan to give the desired flavocyclodextrin [11]. However, in the synthesis of flavins, the condensations of alloxan with o-phenylenediamine derivatives are carried out at high temperature [12] in the presence of acids such as sulfuric acid, hydrochloric acid, acetic acid, and boric acid [13]. Since cyclodextrins hydrolyze under acidic conditions at high temperature this approach can be successful only if the o-phenylenediamine derivative of cyclodextrin can be condensed with alloxan under conditions mild enough to minimize acid induced decomposition of cyclodextrin. Approximately one percent of the ring of p-cyclodextrin are hydrolyzed in 1.15N HCl at

60°C in 30 minutes [14]. Assuming that the rate of cleavage of mono-functionalized P-cyclodextrin is the same as that of the β-cyclodextrin, the amount of cyclodextrin hydrolyzed under these conditions can be tolerated in a reaction sequence.

Following Scheme 1, the reaction of 6-iodocyclodextrin (**1**) with a large excess of o-phenylenediamine (**2**) gave 6-(2-aminoanilino)-β-cyclodextrin (**3**). Upon work up, the TLC showed a single spot which had UV absorbance and charred upon H_2S0_4/heat treatment indicating the presence of both the phenylenediamine moiety as well as the cyclodextrin moiety in the product. ^1H and ^{13}C NMR spectra indicated that one o-phenylenediamine molecule was attached to the 6-position of β-cyclodextrin. The ^1H NMR spectrum showed all the peaks of β-cyclodextrin and multiplets of aromatic peaks of o-phenylenediamino moiety in the range between 6.3 - 6.6 ppm. The ^{13}C NMR peaks at 45.3, 70.0 and 84.5 ppm for C'6, C'5 and C'4 of the substituted glucose unit are shifted 15.1 and 2.0 ppm upfield and 2.8 ppm downfield respectively from the original peaks for C6, C5 and C4 of β-cyclodextrin indicating that the substiutent is at the 6-position [15]. However, the color of an aqueous solution of **3** turns dark brown when exposed to air for 1 day, suggesting the oxidation of the o-phenylenediamine moiety. Therefore, freshly prepared product was placed in vacuum for 1 hr at room temperature and then used immediately in the following reaction to avoid such oxidation.

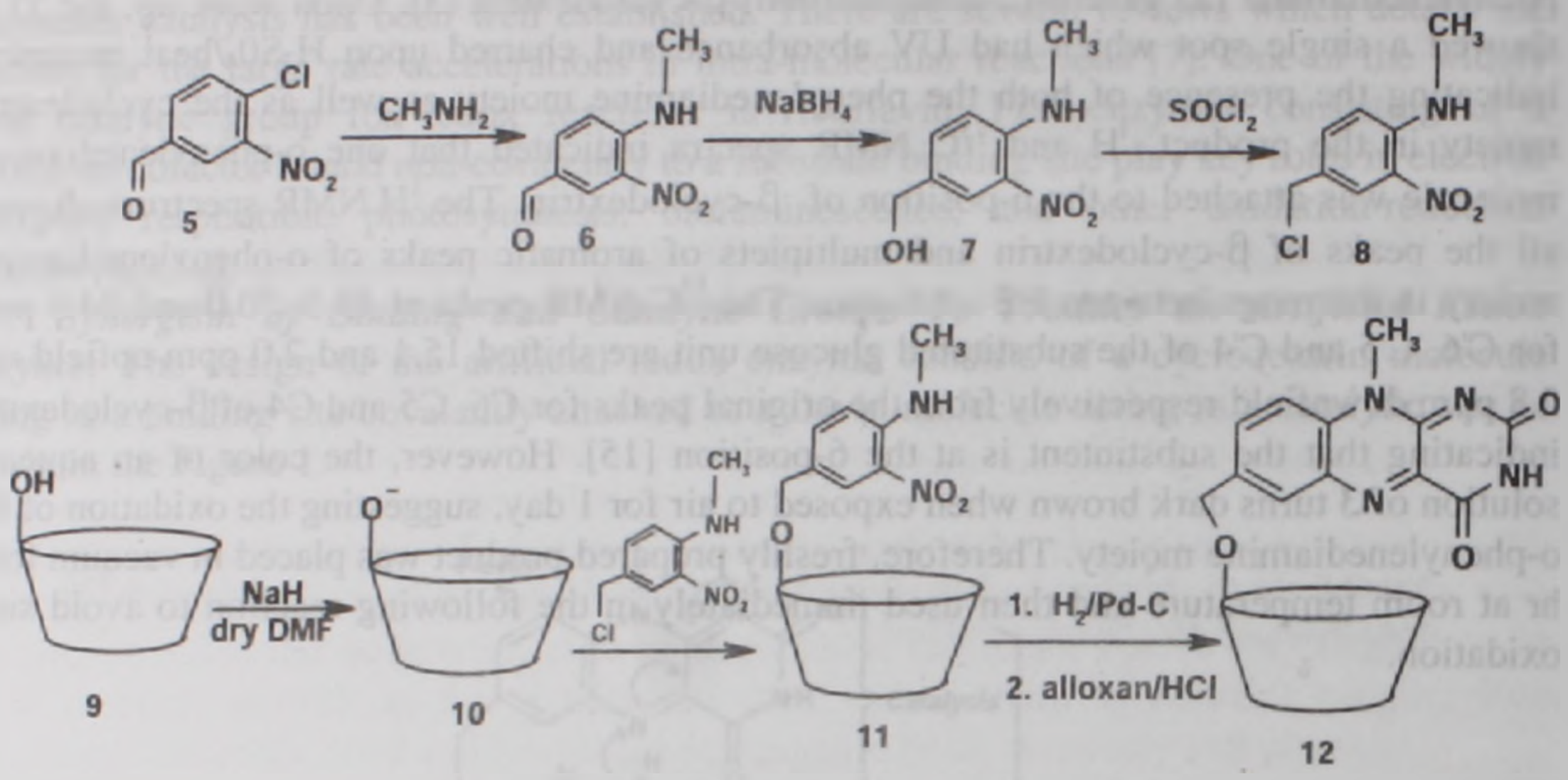


Scheme 1. Attachment of flavin to the primary side of cyclodextrin.

A mixture of **3** with alloxan monohydrate was dissolved in aqueous 1 N HCl and heated in a refluxing acetone bath for 15 minutes when TLC indicated the completion of more than 95% of the reaction. Reverse phase chromatography using C_{18} column and 10% aqueous acetonitrile as the eluent gave pure 6-(10-N-isoalloxazino)-β-cyclodextrin (**4**) as indicated by a single yellow TLC spot which charred on H_2S0_4/heat treatment and confirmed by ^1H and ^{13}C NMR and elemental analysis. The catalytic properties of this artifical redox enzyme (referred to as 6-flβCD) is reported in a later section.

Attachment of Flavin to the Secondary Side of Cyclodextrins. Binding studies have shown that the secondary side of cyclodextrin is more important than the primary side in enzyme mimic chemistry because the substrates generally bind to cyclodextrins with their functional groups oriented towards this side [16]. Thus, catalytic groups attached to the secondary side cyclodextrins should be more effective enzyme mimics. Conventional methods to modify this side of cyclodextrin give low yield of the product [17] and distort the cyclodextrin cavity [18]. Our approach for building organic models of flavoenzymes illustrated here by the synthesis of 2-(7α-O-7,10-dimethylisoalloxazino)-β-cyclodextrin (**12**)

(Scheme 2) overcomes these problems. 4-Chloro-3-nitrobenzaldehyde (**5**) was reacted with methylamine to yield 4-(methylamino)-3-nitrobenzaldehyde (**6**) which was then reduced by $NaBH_4$ to 4-(methylamino)-3-nitrobenzenemethanol (**7**).



Scheme 2. Attachment of flavin to the secondary side of cyclodextrins.

Reaction of thionyl chloride with 7 afforded 4-(chloromethyl)-N-methyl-2-nitrobenzeneamine (**8**). **6**, **7** and **8** were characterized by their ^1H NMR spectra and elemental analysis. In the mean time, sodium β-cyclodextrin alkoxide **10** was synthesized by reacting dry β-cyclodextrin (**9**) with 1 equivalent of NaH in dry DMF. **8** was used as an electrophile to react with **10** to yield 2-[4-(methylamino)-3-nitrobenzyl]-β-cyclodextrin (**11**).

The method described above is general and can be used for attaching substituents onto the secondary side of cyclodextrins without the stereochemical inversions and the ring distortion caused by the conventional methods for modification of the secondary side of cyclodextrin.

Compound **11** was hydrogenated and then condensed with alloxan monohydrate to yield **12**. The structure of **12** was elucidated by ^1H and ^{13}C NMR. The ^1H NMR in spectrum in D_2O exhibits all the normal peaks for β-cyclodextrin in the range of 4.4-3.0 ppm; and the peaks in the range of 7.2-7.45 can be assigned to aromatic protons of the flavin moiety by comparison with the spectrum of flavins [19]. The absence of any resonance at > 7.5 in ^1H NMR in D_2O and presence of a broad peak at 11.41 in the ^1H NMR in DMSO-d_6 indicate the presence of the acidic N-H(3) in this compound. In ^{13}C NMR (D_2O) the ten peaks at > 116 ppm can be assigned to the aromatic carbons of the flavin moiety in comparison to the spectrum of flavins [20]. These assignments are further supported by INEPT ^{13}C NMR in D_2O which shows three positive peaks corresponding to three methine carbons and seven negative peaks corresponding to quaternary carbons in the structure of the flavin moiety. The six normal peaks for the β-cyclodextrin moiety in ^{13}C NMR in D_2O of 14 can be assigned [21] as 60.1 (C6), 71.6 (C2), 71.9 (C5), 72.9 (C3), 80.93 (C4) and 101.6 (C1). The peak at 33.0 can be assigned to CH_3 because its chemical shift falls in the expected range for a methyl groups and it's appearance as a positive peak on INEPT ^{13}C NMR spectrum supports

the fact that it has three protons attached. The peaks at 79.8 and 99.6 can be assigned to C'2, and C'1 respectively of the substituted glucose unit because the flavin group, like the tosyl group, is an electron withdrawing group and when attached to the hydroxyl group at the 2-position of β-cyclodextrin, it will cause a large downfield chemical shift on C'2 and a significant upfield chemical shifts on C'1 of the substituted glucose unit15. These two peaks are shown as positive peaks in INEPT 13C NMR (D_2O) indicating that they correspond to methine carbons. The procedure described above is a general method for synthesis artificial redox enzymes. We have also synthesized an artificial enzymes based a-cyclodextrin using this method. The catalytic properties of these artifical redox enzyme (referred to as 2-flβCD and 2-flαCD) is reported in the following section.

Catalytic Reaction of Artificial Redox Enzymes

Flavoenzymes catalyze a variety of reactions using flavin co-enzyme as the catalytic group. Similarly, the artificial redox enzyme whose synthesis is described above catalyzes all these reaction since it has a flavin as the co-enzyme. Most of these reactions were found to follow a second order kinetics indicating that the reaction catalyzed is not of the substrate bound in the cavity. These reactions were comparable to reactions of riboflavin (ribloflavin is referred to as Rfl) However, oxidation of benzyl mercaptans to corresponding disulfide and photo-oxidation of benzyl alcohols to benzaldehyde were found to follow saturation kinetics indicating a complex formation prior to the reaction similar to the ones found in natural enzymes. The study of these two reaction is described here in detail.

A. Oxidation of Mercaptans.

The oxidation of mercaptans were carried out in 10% (v/v) methanol aqueous buffer ($NaHCO_3$ -NaOH buffer, pH 10.0) with a calculated ionic strength of 0.24 M (adjusted with KCl) at 25.0°C. When a solution of 2-flβCD **(12)** was mixed with 80-fold excess of benzyl mercaptan under anaerobic conditions a slow decrease of the absorbance of 2-flβCD **(12)** at 440 nm was observable and after several hours, a spectrum characteristic of reduced flavin remained. The spectrum of 2-flβCD can be completely restored by re-oxidation of the reaction mixture with air. Compared with the oxidation of mercaptans by flavins, the decomposition of flavins is so slow that it can be ignored. With excess benzyl mercaptan, the absorbance of 2-flβCD and Rfl as a function of time gave good first order plots under anaerobic conditions, but the reaction with 2-flβCD was faster than that with Rfl. When the concentration of benzyl mercaptan (still in excess) was varied a plot of the initial rates for the reaction of Rfl with benzyl mercaptan vs. the concentrations of mercaptan gave a straight line showing the reaction to be overall second order. The second order rate constant then can be obtained from this plot and shown in Table 1. In contrast to Rfl, similar plots for the reactions of 2-fl"CD and 2-flβCD with benzyl mercaptan showed the saturation kinetics. The K_{diss} and k_{cat} calculated from these plot s are listed in Table 1 [22].

The oxidations of substituted benzyl mercaptans and α-naphthyl mercaptan by Rfl and flavocyclodextrins were monitored by the decrease in absorbance at 440 nm due to flavin moiety. The kinetic measurements were performed in $NaHCO_3$ -NaOH buffer (pH 10.0) containing 30% (v/v) methanol at 25.0°C at a calculated ionic strength of 0.24 M. The oxidation of substituted benzyl mercaptans catalyzed by Rfl followed second order kinetics, while the saturation kinetics was observed for the oxidation catalyzed by flavocyclodextrins. The rate constants for these reactions are listed in Table 1.

Table 1. The Rate Constants for the Oxidation of Benzyl Mercaptan and Substituted Benzyl Mercaptans by Flavins*

	Substrate	Flavin	$K_{diss}x10^3$ (M)	$k_{cat}x10^3$ (s^{-1})	k_{cat}/K_{diss} ($s^{-1}M^{-1}$)	k_2x10^2 ($M^{-1}s^{-1}$)	$(k_{cat}/K_{diss})/k_2$***
1	Benzyl mercaptan**	2-flβCD	1.89± 0.23	1.11±0.13	0.587		53
2	Benzyl mercaptan**	2-flαCD	2.38±0.09	0.585±0.022	0.246		22
3	Benzyl mercaptan**	Rfl				1.11±0.06	
4	*p*-Chlorobenzyl mercaptan	2-flβCD	2.91±0.17	1.20±0.07	0.412		21
5	*p*-Chlorobenzyl mercaptan	6-flβCD				11.8±0.8	
6	*p*-Chlorobenzyl mercaptan	Rfl				1.93±0.1	
7	*o*-Chlorobenzyl mercaptan	2-flβCD	1.53±0.11	0.178±0.013	0.116		4
8	*o*-Chlorobenzyl mercaptan	Rfl				2.82±0.12	
9	*m*-Chlorobenzyl mercaptan	2-flβCD	8.85±1.02	3.53±0.40	0.400		16
10	*m*-Chlorobenzyl mercaptan	Rfl				2.45	
11	α-naphthyl mercaptan	2-flβCD				13.2±1.8	

* The kinetics measurements were carried out in $NaHCO_3$-NaOH buffer (pH 10.0) containing 30% methanol (v/v) at 25.0±0.1°C at calculated ionicstrength of 0.24 M.

** $NaHCO_3$-NaOH buffer containing 10% (v/v) methanol.

*** Second-order rate constants of the oxidation of mercaptans by Rfl.

The oxidations of benzenethiol, phenethyl mercaptan and cyclohexyl mercaptan by Rfl and flavocyclodextrins were also investigated in a similar manner. The oxidations either by Rfl or by flavocyclodextrins were either very slow, or did not proceed at all because the plots of absorbance vs. time for these reactions were almost the same as the decomposition of flavins.

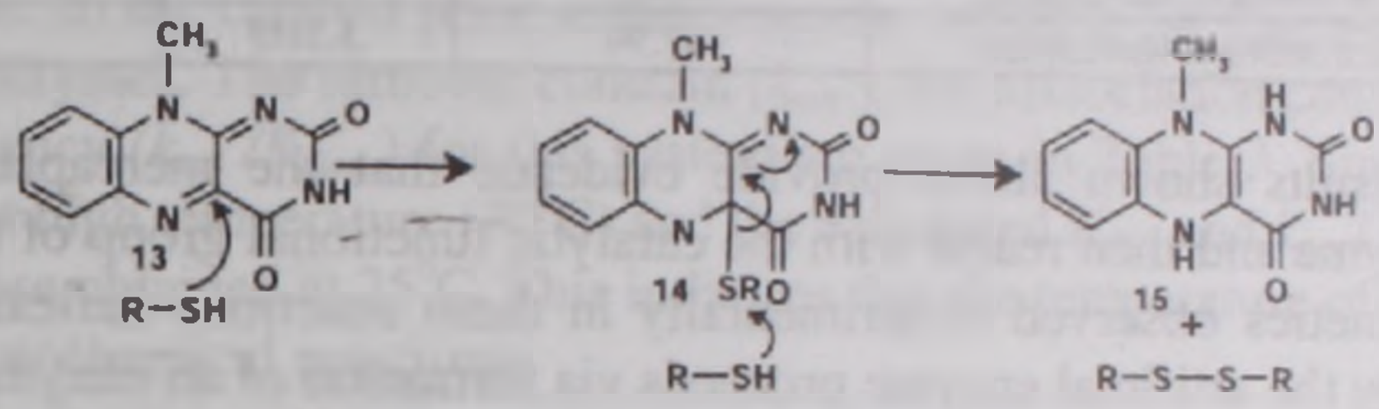
Table I gives all results for the oxidation of benzyl mercaptans by flavins. First of all, the oxidation of benzyl and substituted benzyl mercaptans by 2-flavocyclodextrins shows saturation kinetics, i.e., reactions proceed by the complex formation between 2-flavocyclodextrins and mercaptans. In contrast to 2-flavocyclodextrins, 6-flavo-β-cyclodextrin (6-flβCD) gives the second order kinetics in the oxidation of p-chlorobenzyl mercaptan (entry 5 in Table I), which is similar to the oxidation of mercaptans by Rfl. A computational chemistry study [23] of the conformations of the inclusion complexes between benzyl mercaptan and 2-flβCD indicates that conformation in which the thiol group is oriented towards the secondary side of cyclodextrin, is more stable than conformation where the thiol group is oriented towards the primary side of cyclodextrin.

The difference in energy between these two conformations is 44 kcal/mol. The preferred conformation 23 brings the thiol group of the substrate close to the catalytic functional group of 2-flavocyclodextrins. In the case of 6-flβCD, the complex formation brings the thiol group of the substrate away from the catalytic functional group. Therefore, the flavin moiety of 6-flβCD only reacts with unbound p-chlorobenzyl mercaptan.

Entries 1, 4 and 9 in Table I give the rate constants of the oxidation of benzyl mercaptan, *p*-chlorobenzyl mercaptan and An-chlorobenzyl mercaptan by 2-flβCD. The

stability of the complexes formed between benzyl mercaptans and 2-flβCD is directly related to the reaction rate. The more stable the complex (smaller K_{dis}), the higher the rate acceleration factor.

The oxidation of benzyl mercaptan by 2-flβCD (entry 1) is faster than by 2-flαCD (entry 2). Because the dissociation constants of these two reactions are close, the difference in rate is caused mainly by the difference of catalytic rate constant k_{cat}. Model studies for the oxidation of mercaptans to disulfides by oxidized flavins suggest that thiolate anions add to C(4a)-position of oxidized flavins, producing an adduct (**14**) as shown in scheme 3. The reaction of a second molecule of mercaptan with the adduct gives the disulfide and reduced flavin [24,25]. It has been shown that the rate-determining step in the oxidation of thiols by flavins is the formation of the C(4a) adduct.



Scheme 3. Mechanism for oxidation of mercaptans by flavins.

Therefore, the distance between C(4a) of catalytic functional group of the host and the sulfur atom of the thiol group of the guest [*d*6-C(4a)] (Figure 2) is very important for the catalytic reaction.

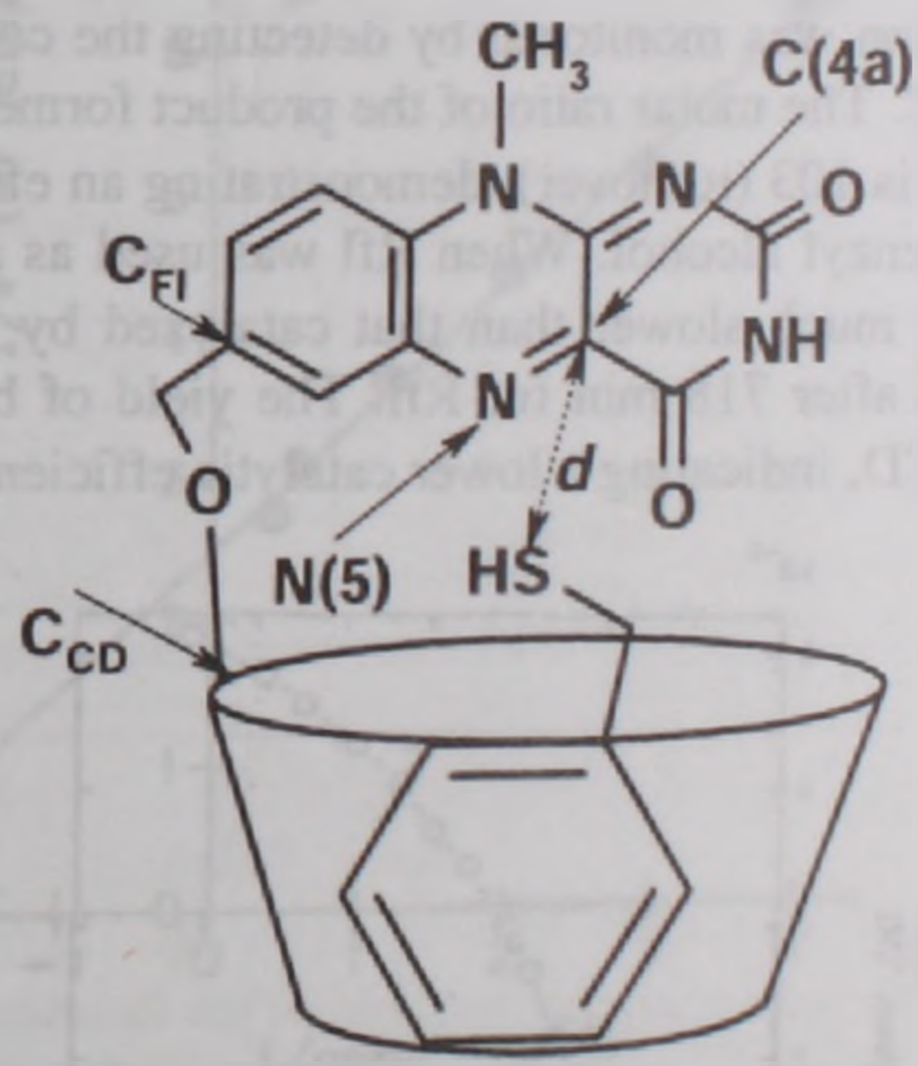


Figure 2. Important distances in the oxidation of mercaptans by flavins.

Because the cavity size of α-cyclodextrin and β-cyclodextrin differs, the *d*S-C(4a) will be different in the complexes of benzyl mercaptan with 2-flαCD and 2-flβCD. This difference is further transferred to the reaction rate. For the same reason, the oxidation of α-naphthyl mercaptan by 2-flβCD shows second order kinetics. Although α-naphthyl mercaptan can form a complex with β-cyclodextrin [26], the *d*S-C(4a) of the complex with

2-flβCD is too long to undergo reaction. The artificial enzyme 2-flβCD only reacts with unbound α-naphthyl mercaptan and the reaction follows the second order kinetics.

The reaction rates for the oxidation of different benzyl mercaptans by 2-flβCD can be alternately explained by the *d*S-C(4a) distance. Computational chemistry studies were performed to determine the relationship between the experimental catalytic rates and the dS-C(4a) distance. The results shown in Table 2 leads to the conclusion that the shorter the distance *d*S-C(4a), the faster the oxidation of the mercaptans by the artificial enzyme.

Table 2. The Relationship Between *d*S-C(4a) and the Catalytic Rates

Substrate	ΔE (Kcal/mol)	$d_{S\text{-}C(4a)}$ (Å)	k_{cat} xIO^{3} (s^{-1})
Benzyl mercaptan	-24.77	3.531	1.11
p-Chlorobenzyl mercaptan	-26.87	3.322	1.20
m-Chlorobenzyl mercaptan	-47.94	3.300	3.53

The results shown above provide evidence that the mercaptan first binds to the artificial enzyme and then reacts with the catalytic functional group of the artificial enzyme. Saturation kinetics observed experimentally in these reactions indicates that oxidation of mercaptans by the artificial enzyme proceeds via formation of an enzyme-substrate complex. This reaction path allows the artificial enzyme to catalyze the reaction 53 times faster than riboflavin.

B. Photo-oxidation of benzyl alcohols

Irradiation of an air-saturated aqueous solution containing 2-flβCD, benzyl alcohol, and $HClO_4$ with visible light of 360 nm < λ < 440 nm resulted in the formation of benzaldehyde. The reaction was monitored by detecting the concentration of benzaldehyde with reverse phase HPLC. The molar ratio of the product formed after 674 min to the initial amount of 2-flβCD used is 103 (turnover), demonstrating an efficient recycle of 2-flβCD in the photo-oxidation of benzyl alcohol. When Rfl was used as a photocatalyst instead of 2-flβCD, the reaction was much slower than that catalyzed by 2-flβCD (Figure 3). Only 6 turnovers were observed after 718 min for Rfl. The yield of benzaldehyde is lower in the case of Rfl than of 2-flβCD, indicating a lower catalytic efficiency of the former.

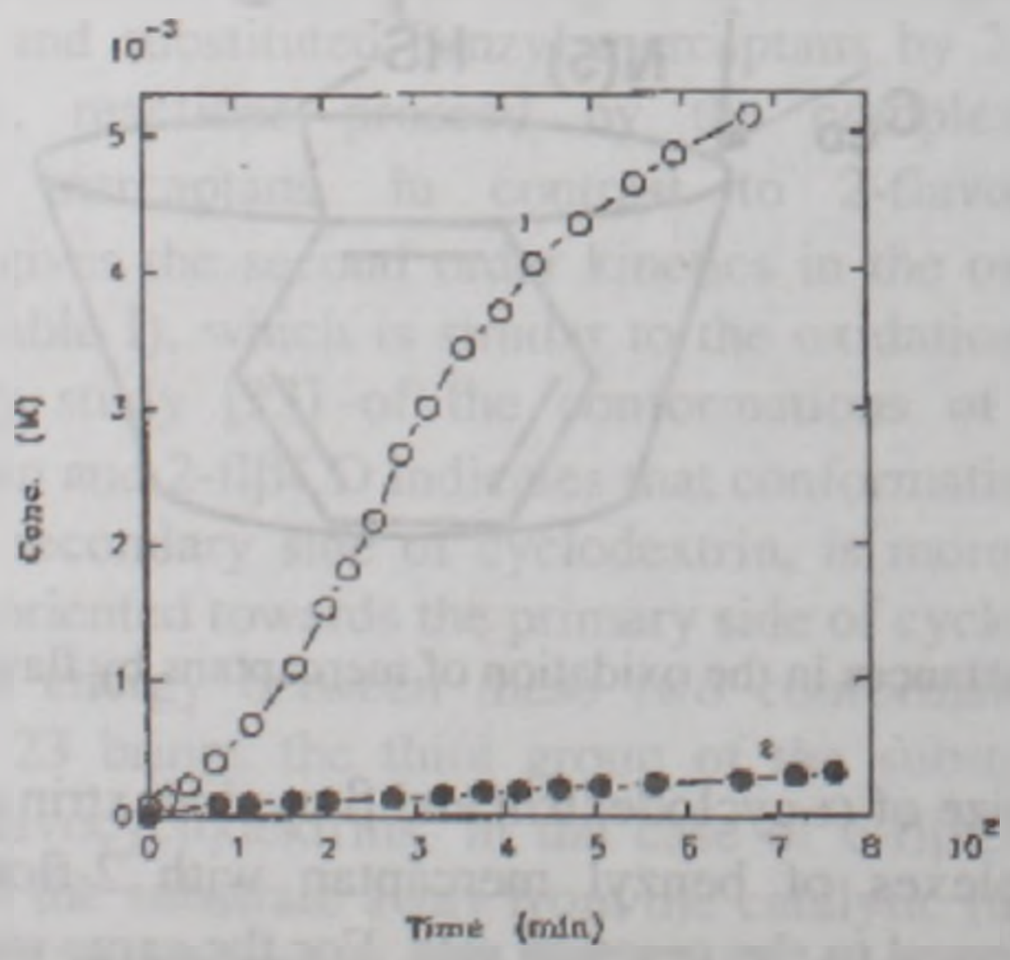


Figure 3. The Concentration of Benzaldehyde vs. Irradiation time for the Oxidation of Benzyl Alcohol by Oxygen Catalyzed by 1) 2-flβCD and 2) Rfl.

***Complex Formation between 2-FlβCD and p-t-Butylbenzyl Alcoho*l.** Cyclodextrins form inclusion complexes in solution with a variety of organic substances, and it is likely that increase in the rate of oxidation of substituted benzyl alcohols catalyzed by 2-flβCD compared with those catalyzed by Rfl involved the formation of 2-flβCD-benzyl alcohol complex. Efforts to get direct experimental evidences for the formation of complexes were made. The dependence of the catalytic action of flavins on the concentration of the substrate was examined. A plot of the initial rates vs substrate concentration for the oxidation of p-t-butylbenzyl alcohol catalyzed by Rfl gives a straight line with zero intercept, indicating a first order dependence on the substrate [27]. A similar plot for the same reaction catalyzed by the artificial enzyme [i.e., 2-flβCD] shows saturation kinetics. Lineweaver-Burk treatment of these data gives an excellent straight line with a slope of K_{diss}/V_{max} = 88.92 min and a Y intercept equal to $1/V_{max}$ = 2.46x105 M^{-1} min (Figure 4). This result indicates binding of the substrate to the catalyst prior to the reaction, which is similar to the reaction scheme followed by enzymes. The turnover constant (k_{cat}), the dissociation constant (K_{diss}), and the enzyme efficiency (k_{cat}/K_{diss}) for this system are given in Table 3. Similar studies were also performed at high temperature (70°C) and the calculated K_{diss} and k_{cat} (Table 3) are almost the same as those obtained at 25°C. This indicates that the temperature of the reaction is not important in photochemical reactions.

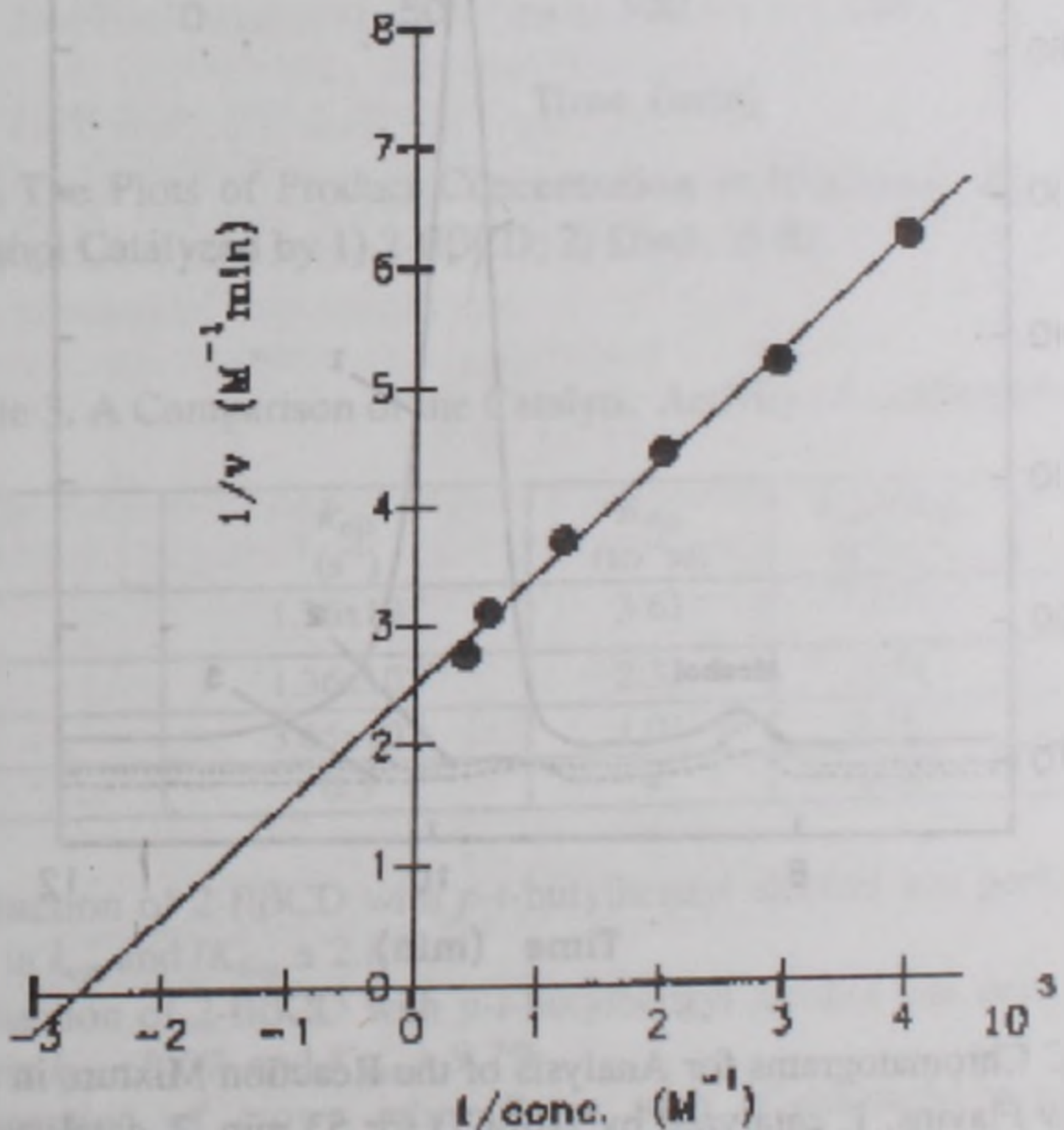


Figure 4. Double Reciprocal Plot of the Oxidation of p-t-Butylbenzyl Alcohol by the Artificial Enzyme.

The oxidation of p-t-butylbenzyl alcohol catalyzed by 2-flβCD was compared with that catalyzed by Rfl and 7,10-dimethylflavin (referred to as Dmfl) to determine the contribution of structural differences (the differences of redox potentials) between Rfl and 2-flβCD. The HPLC chromatograms of the reaction mixtures (Figure 5) and the plots of

product concentrations vs irradiation time (Figure 6) indicate that the oxidation catalyzed by 2-flβCD is much faster than those catalyzed by Rfl and Dmfl . Because the structure of the flavin moiety of 2-flβCD is identical with the structure of Dmfl, these results demonstrate clearly that the rate acceleration by 2-flβCD is due to complex formation. The differences in reaction rates caused by change in flavin structure (the difference of redox potentials) as in the cases of Rfl and Dmfl is very small (plots 2 and 3 in figure 6).

The oxidation of p-t-butylbenzyl alcohol under irradiation conditions catalyzed by 2-flαCD is only slightly slower than that catalyzed by 2-flβCD but is considerably faster than that catalyzed by Rfl and Dmfl. We interprets this by the fact that both 2-flαCD and 2-flβCD have binding sites (cyclodextrins) to form complexes with the substrate and thus this accelerates the reaction. The flavin moiety of 2-flαCD and 2-flβCD is identical, the difference of the reaction rates is caused by the different binding energies between 2-flαCD and 2-flβCD with the substrate. This observation further supports the conclusion that the rates of the oxidation of benzyl alcohols are accelerated by the complex formation between flavocyclodextrins and substrates.

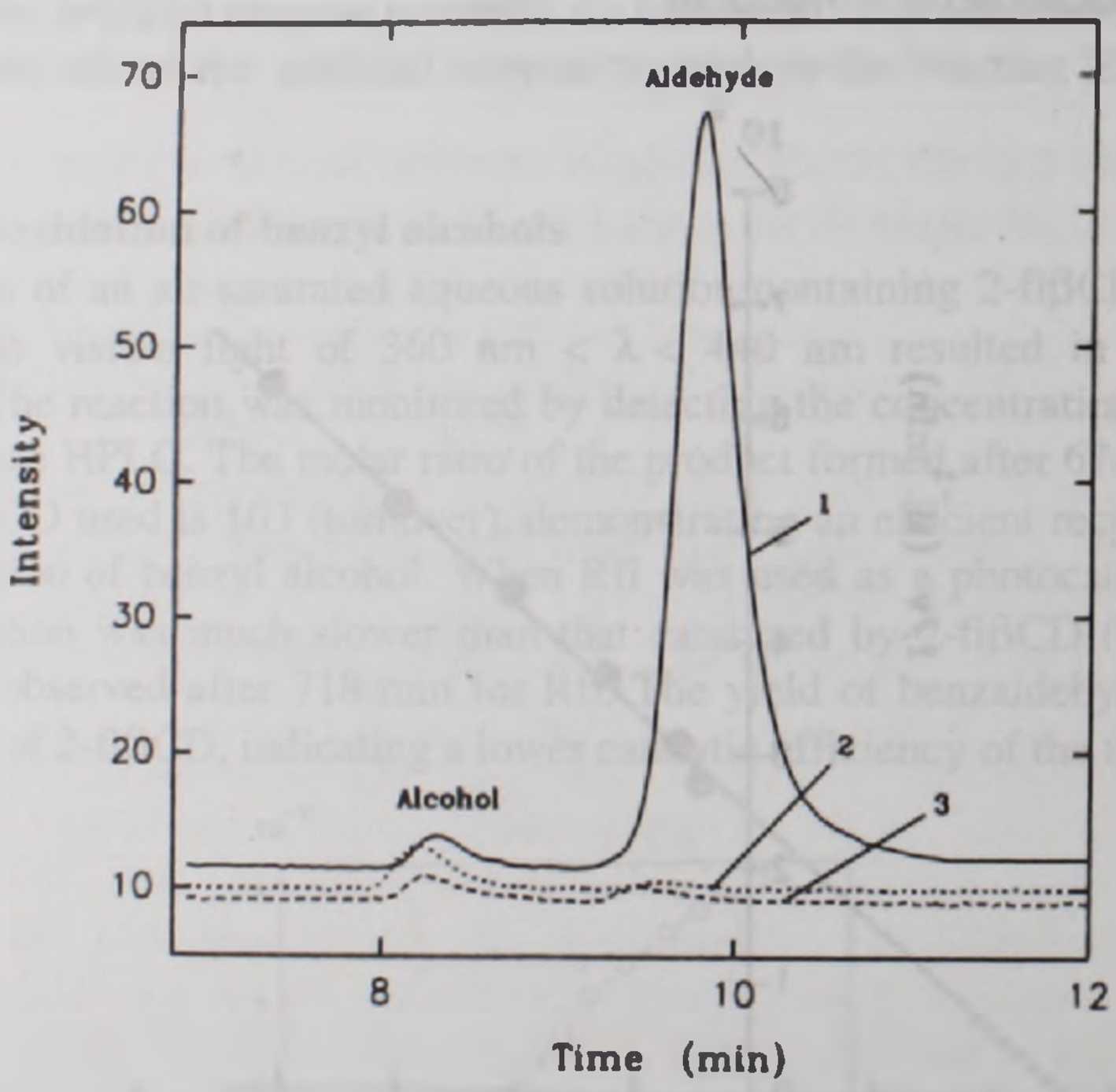


Figure 5. HPLC Chromatograms for Analysis of the Reaction Mixture in the Oxidation of p-t-Butylbenzyl Alcohol by Flavins. 1. catalyzed by 2-flβCD for 53 min. 2. catalyzed by Rfl for 51 min; 3. catalyzed by Dmfl for 76 min.

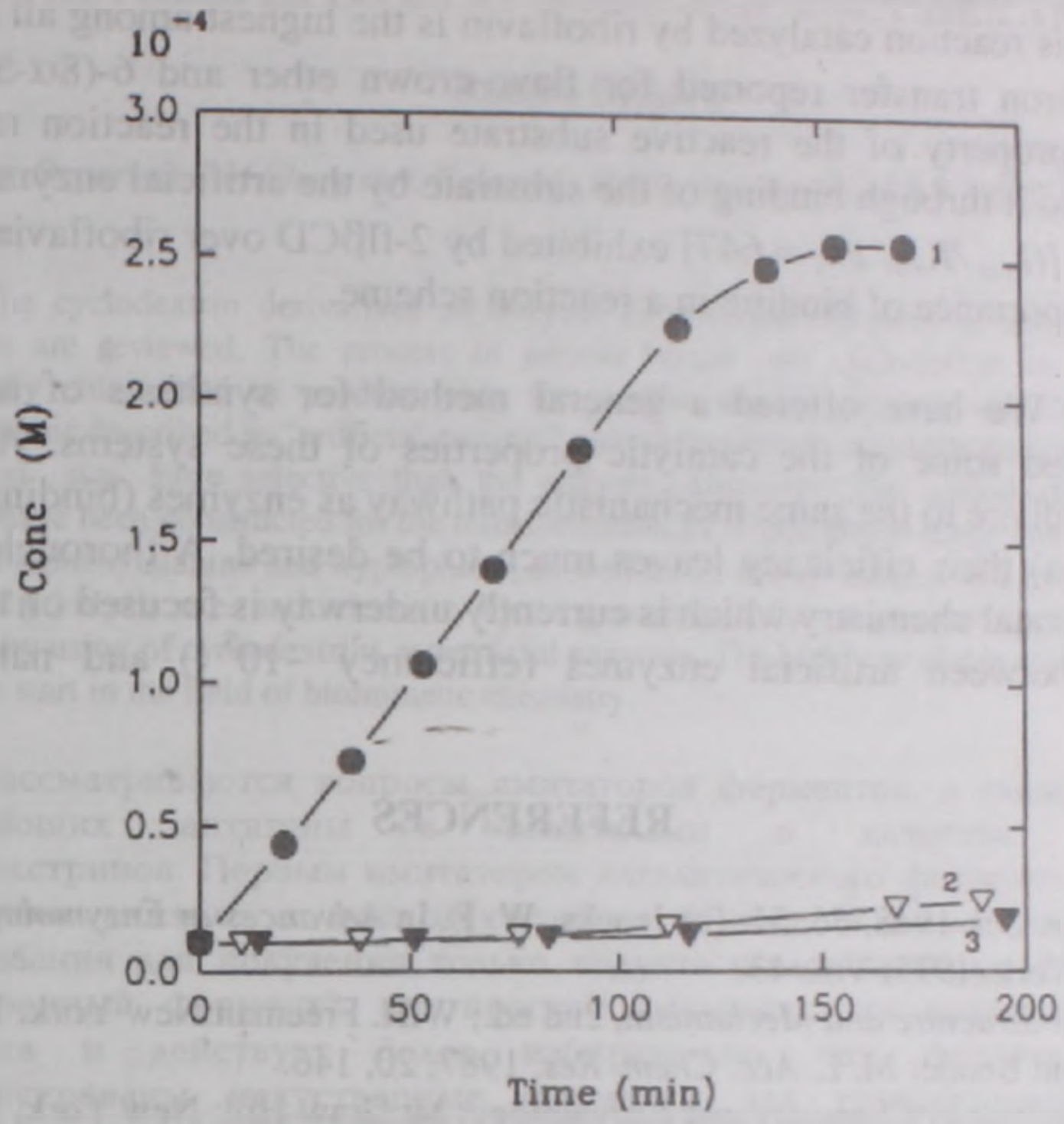


Figure 6. The Plots of Product Concentration vs Irradiation Time for the Oxidation of p-t-Butylbenzyl Alcohol Catalyzed by 1) 2-flβCD; 2) Dmfl; 3) Rfl.

Table 3. A Comparison of the Catalytic Activity of Artificial Redox Enzymes

Enzyme	k_{cat} (s^{-1})	K_{diss} (10^{-4}M)	$k_{cat}/<K_{diss}$ ($s^{-1}M^{-1}$)	k_2 ($s^{-1}M^{-1}$)	(k_{cat}/K_{diss}) $/k_2$
2-flβCD[a]	$1.36x10^{-3}$	3.61	3.77	$5.83x10^{-3}$	647
2-flβCD[b]	$1.36x10^{-3}$	2.32	5.86		
flavo-crown ether[c]	$3.65x10^{-5}$	1.01	0.361	$1.26x10^{-2}$	29
flavocyclodextr[d]	0.5	4	1250	117.1[e]	11

a. The reaction of 2-flβCD with *p-t*-butylbenzyl alcohol was performed at 25.0°C with error limits in k_{cat} and $/K_{diss}$ ± 2.8%.
b. The reaction of 2-flβCD with *p-t*-butylbenzyl alcohol was performed at 70.0°C with error limits in k_{cat} ±5.6% and K_{diss} ± 9.7%.
c. The reaction of crown ether flavin with N^1-dodecyl-l-(*p*-ammoniomethy)benzy)-l .4-dihydronicotinamide is from ref 23.
d. The reaction of 6-(8α-S-riboflavo)-α-cyclodextrin with 1-(1-hexyl)1.4-dihydronicotinamide is from ref 25.
e. pH7.0, 25°C.

It is interesting to compare this artificial enzyme with previously published non-proteinic enzyme models (Table 3): flavo-crown ether and 6-(8α-S-riboflavo)-α-cyclodextrin[28]. While the dissociation constants (K_{diss}) for all these systems are in the same

range, the turnover constant (k_{cat} for 2-flβCD is higher than flavo-crown ether. The turnover constant for 6-(8α-S-riboflavo)-α-cyclodextrin is the highest because the bimolecular rate constant (k_2) for this reaction catalyzed by riboflavin is the highest among all these systems. The efficient electron transfer reported for flavo-crown ether and 6-(8α-S-riboflavo)-α-cyclodextrin is a property of the reactive substrate used in the reaction rather than the advantage offered to it through binding of the substrate by the artificial enzyme. The highest acceleration factor [$(k_{cat}/K_{diss})/k_2 = 647$] exhibited by 2-flβCD over riboflavin offers a clear evidence for the importance of binding in a reaction scheme.

Conclusion: We have offered a general method for synthesis of artificial redox enzymes and studied some of the catalytic properties of these systems. Although these artificial enzymes adhere to the same mechanistic pathway as enzymes (binding the substrate prior to the reaction) their efficiency leaves much to be desired. A thorough investigation including computational chemistry which is currently underway is focused on the reasons for such a disparity between artificial enzymes (efficiency ~10^3) and natural enzymes (efficiency 10^{12}).

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRIN DERIVATIVES AS MIMICS OF ENZYMES AND ANTIBODIES

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The cyclodextrin derivatives as enzyme mimics and imitators of antigens binding by antibodies are reviewed. The process of anisole bound into cyclodextrin in water and then catalytically chlorinated to produce only the p-chloroanisole product was the first catalytic enzyme mimic described as "artificial enzyme", where the simple cyclodextrin acted as an enzyme mimic and was more selective than the enzyme chlorinase. The cyclodextrins as artificial enzymes have been constructed for the transformation by which amino acids were biosynthesized, particularly phenylalanine and tryptophane, as well as for the cyclization and hydrolysis of RNA. The high hydrolysis rates and selectivity by the geometry of the enzyme-substrate complex can be achieved by using of cyclodextrins as artificial enzymes. The highly available cyclodextrins are an important start in the field of biomimetic chemistry.

Рассматриваются вопросы имитаторов ферментов, а также имитаторов связывающих антигены с антителами в качестве производных циклодекстринов. Первым имитатором каталитического фермента был процесс связывания анизола с циклодекстрином в воде и далее каталитического хлорирования для получения только подукта р-хлороанизола, описанный как "искуственный фермент", где простой циклодекстрин является имитатором фермента и действует более избирательно, чем фермент хлориназа. Сконструированы искуственные ферменты для трансформации с целью получения аминокислот, в частности фенилаланина и триптофана, а также для циклизации и гидролиза РНК. Высокие скорости гидролиза и селективность достигаются по геометрическим показателям фермент-субстратного комплекса с использованием циклодекстринов в качестве искуственных ферментов. Имеющиеся циклодекстрины позволяют сделать важный шаг в области биомиметической химии.

Քննարկվում են ցիկլոդեքստրինների ածանցյալները որպես ֆերմենտների նմանակիչների, ինչպես նաև որպես հակագենների հետ հակամարմինների կապման նմանակիչների վերաբերյալ խնդիրները: Ցիկլոդեքստրինի հետ անիզոլի կապակցման պրոցեսը ջրում և հետագայում կատալիտիկ քլորացումը միայն p-քլորոանիզոլի ստացմամբ, եղել է կատալիտիկ ֆերմենտի առաջին նմանակիչը (իմիտատորը), նկարագրված որպես «արհեստական ֆերմենտ», երբ սովորական ցիկլոդեքստրինը դառնում է ֆերմենտի նմանակիչ և ազդում է առավել ընտրողաբար, քան քրոլինազ ֆերմենտը: Ստեղծվել են արհեստական ֆերմենտներ տրանսֆորմացիայի միջոցով ամինաթթուների կենսասինթեզի, ինչպես նաև ՌՆԹ-ի ցիկլիզացիայի և հիդրոլիզի համար: Օգտագործելով ցիկլոդեքստրինները որպես արհեստական ֆերմենտներ կարելի է հասնել հիդրոլիզի բարձր արագությունների և ընտրողականության, շնորհիվ ֆերմենտ-սուբստրատային համալիրի երկրաչափական ցուցանիշների: Առկա ցիկլոդեքստրինները թույլ են տալիս կատարել կարևոր առաջընթաց բիոմիմետիկ քիմիայի բնագավառում:

The field of Biomimetic Chemistry has grown in interest ever since I first used this name in the literature [1]. Although mimics of enzymes include various partial mimics that use coenzyme derivatives and other catalytic groups, sometimes in intramolecular reactions, the most interesting enzyme mimics are those that bind a substrate, perform a catalytic process, and then release the products. There is also particular interest in enzyme mimics that function in water solution, among other reasons because water is an environmentally

benign solvent. Thus cyclodextrins are quite appealing as components of enzyme mimics, since they reversibly bind hydrophobic species in a reasonably well-defined geometry, and the binding is sufficiently reversible that the products can be released.

Cyclodextrin binding can also be considered as biomimetic in itself, imitating the binding of antigens by antibodies. However, simple cyclodextrin binding is often quite weak. For example, in water the hydrophobic sidechain of phenylalanine leads to a binding constant of only 20 M^{-1} or so, while antibodies bind complementary antigens with association constants ranging from 10^4 M^{-1} up to 10^{15} M^{-1}, and perhaps even higher. For this reason, we and others have examined the binding by dimers and even trimers and tetramers of cyclodextrins. In an appropriate case, where the cyclodextrin dimer is joined by two linking strands so that it is held in the correct geometry for substrate binding, we observed an association constant greater than 10^{11} M^{-1} for the correct substrate, while a close analog with not quite the right geometry lost a factor of at least 4000 in binding [2].

In this brief comment it will not be possible to describe all the work that has been done in this very large field. However, we have reviewed cyclodextrin derivatives as enzyme mimics very recently [3], so we will try just to indicate some of the high points in the development of this area.

The impetus for the field came from a publication by Cramer [4], describing his work and that of his predecessors on inclusion compounds in water between cyclodextrins and hydrophobic substrates. This stimulated our work, and at the same time Bender carried out some interesting studies showing that reactions of bound substrates with the cyclodextrins could be understood in geometric terms [5]. His reactions were not catalytic. We examined the first catalytic enzyme mimic, in which anisole bound into cycldextrin in water and then was catalytically chlorinated to produce only the p-chloroanisole product [6, 7]. The binding promoted a process in which the chlorine atom was passed from a hydroxyl group of the cyclodextrin to the accessible para position of the bound anisole, but not the otherwise reactive ortho position that was inaccessible in the complex. There was catalytic turnover as the product released from the catalyst and was replaced by more substrate.

In this process simple cyclodextrin was acting as an enzyme mimic, and indeed it was more selective than is the enzyme chlorinase that also catalyzes the chlorination of anisole--in the enzyme case to a mixture of ortho and para chlorinated products. However, better enzyme mimics need not just binding near a cyclodextrin hydroxyl group, but binding near other catalytic groups. Thus we incorporated a metal binding ligand into the cyclodextrin, and saw that the resulting metal complex would catalyze the hydrolysis of substrate esters that bound to the cyclodextrin [8]. This was the first catalyst that was described in the chemical literature as an "artificial enzyme."

Many enzymes use coenzymes, not just simple catalytic groups. The first example in an artificial enzyme was our compound combining pyridoxamine with cyclodextrin [9]. We saw that this compound could selectively bind ketoacids with hydrophobic sidechains and convert them to amino acids, in particular to phenylalanine and tryptophan. This mimics the transamination by which amino acids are biosynthesized. Subsequently we and others have greatly expanded such examples, adding rigidity to the molecules and additional catalytic groups besides the pyridoxal [3].

Many enzymes use bifunctional catalysis, in which two catalytic groups cooperate in transforming a bound substrate. We constructed an artificial enzyme [10] that mimicked the enzyme ribonuclease A, which uses two histidine sidechains as important catalytic units in the cyclization and then hydrolysis of RNA. Our molecule had two attached imidazoles, the catalytic part of the histidines in ribonuclease, and we saw that their placement was critical in achieving catalysis [11]. We even saw that our catalyst exhibited isotope effects

very much like those of the enzyme [12], and suggested that the enzyme uses a chemical mechanism very much like the one we demonstrated for our artificial enzyme [13].

Artificial enzymes are interesting if they achieve very fast rates of reactions as a result of binding the substrate and catalyzing some process in it, and indeed we have examples in which cyclodextrin-based catalysts can achieve very high hydrolysis rates, for instance [14]. However, in many respects the selectivities of enzyme-catalyzed reactions are more striking, and more important to imitate.

Consider the contrast between selectivity in organic chemistry and selectivity in biochemistry. In organic chemistry, reactions occur at the most reactive parts of the substrate, directed by the intrinsic reactivity of the functional groups. For example, if we want to reduce a ketone there must not be an aldehyde group present, which is more reactive. Similarly, if we want to oxidize a carbon of a substrate it must be the most reactive carbon, for instance part of a double bond. By contrast, in enzymatic reactions the selectivity is directed by the geometry of the enzyme-substrate complex, which frequently overrides the intrinsic reactivity of the substrate.

As an example, in the biosynthesis of cholesterol enzymes oxidize methyl groups attached to saturated carbons of lanosterol, ignoring the much more reactive two double bonds and a secondary alcohol group. This is possible because the oxidizing group in the enzyme--an oxygen atom attached to the iron atom of heme--can reach the methyl groups but not the more reactive double bonds or alcohol carbon. Recently we have succeeded in imitating this geometric control, "liberating organic chemistry from the tyranny of functional groups." The catalysts we have prepared use cyclodextrins to bind the substrate in a well-defined geometry, and attack unactivated C-H bonds with an oxygen atom on a metalloporphyrin, just as in the enzyme group cytochrome P-450.

Our systems use a manganese derivative of a porphyrin, since this is more effective than the iron derivatives that are modeled on the enzymes themselves. In our earliest example [15] we used a tetraphenylporphyrin derivative carrying four cyclodextrin units, and achieved turnover catalysis of the hydroxylation of a steroid at a single unactivated methylene group. Furthermore, the product secondary alcohol was not oxidized to the ketone, for geometric reasons, even though such alcohols are more reactive than are unactivated methyene groups toward random oxidants. The geometry of the substrate-catalyst complex, and of the product alcohol-catalyst, do not permit attach on the C-H bond of the alcohol group, which is necessary for its oxidation to a ketone.

In later work we were able to improve the turnover capacity of our artificial cyctlochrome P-450 by incorporating fluorine atoms to make the catalyst more stable to oxidation [16]. In recent unpublished work we have raised the turnover numbers for a newer version of our catalyst to as much as 3000, again with complete positional and stereochemical product control, dictated by the geometry of the catalyst-substrate complex;.

In further work, we were able to shift the oxidation of the same substrate type to a new position by using three-point binding of the substrate to three cyclodextrin units in the catalyst [17]. Furthermore, in unpublished work we have seen that a carbon-carbon double bond in the substrate is not attacked when the binding geometry puts it out of reach of the manganese-oxygen group. Thus using cyclodextrins we have indeed achieved an important advance in biomimetic chemistry using artificial enzymes--overcoming the intrinsic reactivity of the substrate by imposing geometric control in a complex that mimics the enzyme-substrate complex in biochemistry.

In this most recent work the cyclodextrins were used as binding units to help achieve strong complexing with well-defined geometry. Of course other binding units can also be envisioned, which may ultimately have some advantages. However, the highly available

cyclodextrins have let us make an important start in the field of biomimetic chemistry, in which we hope to utilize the same principal by which enzymes achieve their selectivity. Cyclodextrins have also let us perform these selective transformations in water solution, and thus add an environmental advantage to their other features. We want to change the style of organic chemistry, and to achieve transformations that are otherwise impossible when using previous chemical styles. Cyclodextrins have helped open up this new field of chemistry.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRINS IN INVERSE PHASE TRANSFER CATALYSIS

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Cyclodextrins act as Inverse Phase Transfer catalyst in the hydrolysis of benzyl halides. The reaction is affected by the choice and by the amount of the selected cylodextrin. The reaction proceeds under mild reaction conditions and provides almost exclusively the corrisponding benzyl alcohol as reaction product. No organic solvent are required.

Циклодекстрины действут в качестве обратной фазы трансфер-катализатора в процессе гидролиза бензилгалогенов. Реакция зависит от выбора и количества циклодекстрина. Реакция протекает в умеренных условиях и обеспечивает образование почти исключительно только бензил спирта как продукта реакции. Никаких органических растворителей не требуется.

Ցիկլոդեքստինները գործում են որպես տրանսֆեր-կատալիզատորի հակադարձ ֆազա բենզիլհալոգենների հիդրոլիզի պրոցեսում: Ռեակցիայի ազդեցությունը կախված է ցիկլոդեքստրինի ընտրությունից և քանակից: Ռեակցիան ընթանում է չափավոր պայմաններում և ապահովում է համարյա բացառապես միայն բենզիլ սպիրտի առաջացումը որպես ռեկցիայի արդյունք: Ոչ մի օրգանական լուծիչ չի պահանջվում:

Introduction

Reactions involving reagents soluble in non miscible layers proceed only with low reaction rates or, eventually, they do not occur at all. On the other hand, the reactions between organic compounds and inorganic anions are very common, and to try to overcome this problem is an important goal in organic syntheses.

Phase Transfer Catalysis (PTC) [1] actually represents the better solution to carry out under mild reaction conditions many reaction between molecules soluble in water and different compounds soluble only in a immiscible organic phase. Up to now thousands and thousands of papers and patents have expanded the use of PTC to a wide range of reactions and processes. In any case the PTC catalyst (usually an onium salt or a crown ether) allows to transfer the activated anion in the organic layer according to a mechanism fully proved by Montanari [2].

Recently it was reported that by working under Inverse Phase Transfer Catalysis (IPTC) [3] conditions, the organic molecule is transferred to react in the aqueous phase. In particular it was proved that also cyclodextrins (non reducing, stable and non toxic oligosaccharides) act as IPTC catalyst [4].

By using this synthetic procedure good results were obtained, for instance, in the oxidation of terminal alkenes [5] (Wacker process) and in the carboxylic acid esters hydrolysis reaction [6].

The hydrolysis of the alkyl halides (Figure 1) to form the corresponding alcohol is a well known reaction in organic chemistry, but industrially it is of negligible importance since, generally, alkyl halides are obtained starting from the parent alcohol [7]. The main

exception is represented by the synthesis of benzyl alcohol carried out industrially hydrolysing benzyl chloride being this latter produced by the chlorination of toluene [8].

Because benzyl chloride is insoluble in water, the reaction rate is very low. On the other hand under classical LL-PTC conditions the hydrolysis of benzyl halides is fast, but due to the strong anionic activation, the reaction leads to the formation of the corresponding symmetric ethers [9] following the classical Williamson synthesis for symmetric ethers.

We have found that carrying out the hydrolysis of benzyl chloride or benzyl bromide in the presence of suitable cyclodextrins, the reaction proceeds faster, under milder reaction conditions providing almost exclusively benzyl alcohol as reaction product. No organic solvent was used.

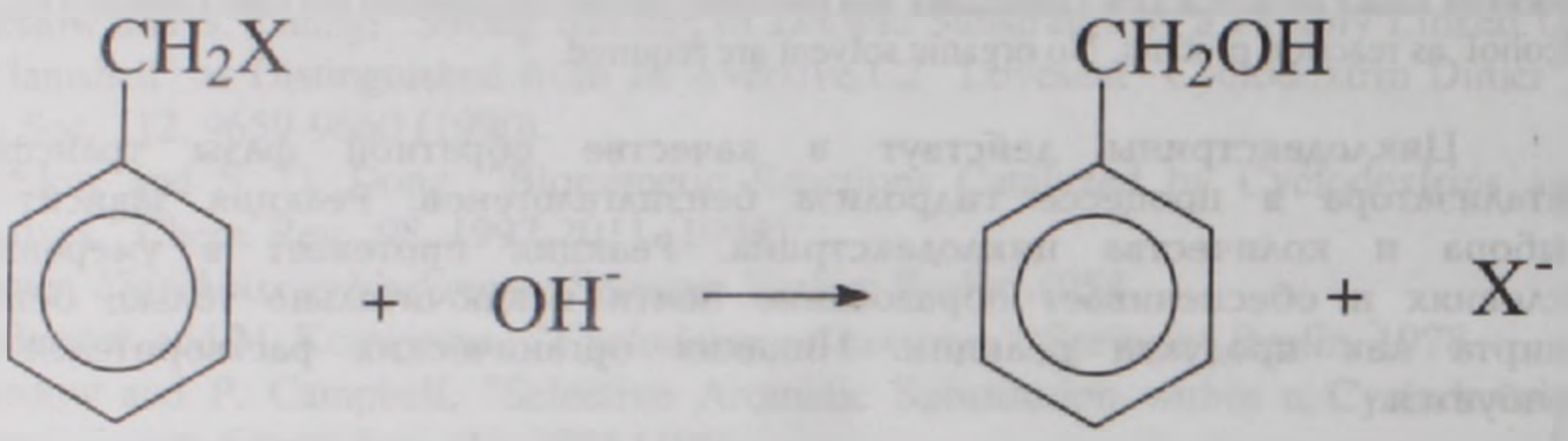

Figure 1. Hydrolysis of alkyl halides.

Material and Methods

β–cyclodextrin was kindly supplied from Roquette-Italia (Cassano Spinola – Italy). Other cyclodextrins were gifted by Wacker-Chemie (Germany). All solvents and reagents employed (ACS grade) were bought from Aldrich (U.S.A.) and used without further purification. GC-MS analysis were performed on a HP 5890 A spectrometer. Magnetic stirring rate was measured with a Cole-Palmer 08199 phototachometer. Reaction environment was thermostated with a Lauda RC6 thermostat.

In a typical experiment to 30 ml of a K_2CO_3 solution (20% w/v) the required amount of catalyst was dissolved. Then 0.1 ml of benzyl chloride was added and the mixture allowed to react at 50°C and 500 rpm. Once the reaction was over, the reaction mixture was extracted twice with diethyl ether and the organic layer analysed to GC-MS. definitive confirmations were obtained by comparison with authentic samples.

Results and Discussion

Since alkyl halides are insoluble in water, their hydrolysis is a reaction extremely low even under drastic stirring of the biphasic system. The use of PTC catalysts increases the reaction rate, but leads to the formation of the corresponding ethers. That is, alcohols could not be obtained by simple hydrolysis of the parent alkyl halide by working under classical PTC conditions.

On the other hand, cyclodextrins greatly affect the solubility in water of the benzyl halides. For example Figure 2 reports the solubilizing effect of different amount of methyl-β-cyclodextrin on benzyl chloride in aqueous solution at room temperature. It is clear that the enhancement in the abosrbance at 258 nm of the solution of benzyl chloride in pure water and in presence of progressive higher amount of methyl-β-cyclodextrin no doubt proves the solubilizing effect of the selected cyclodextrin on benzyl chloride. In particular 10 wt%

solution of methyl-β-cyclodextrin leads to an in crease in the solubility in water more than seven times.

Benzyl halides reaction could be profitably carried out in the presence of cyclodextrins as IPTC catalysts.

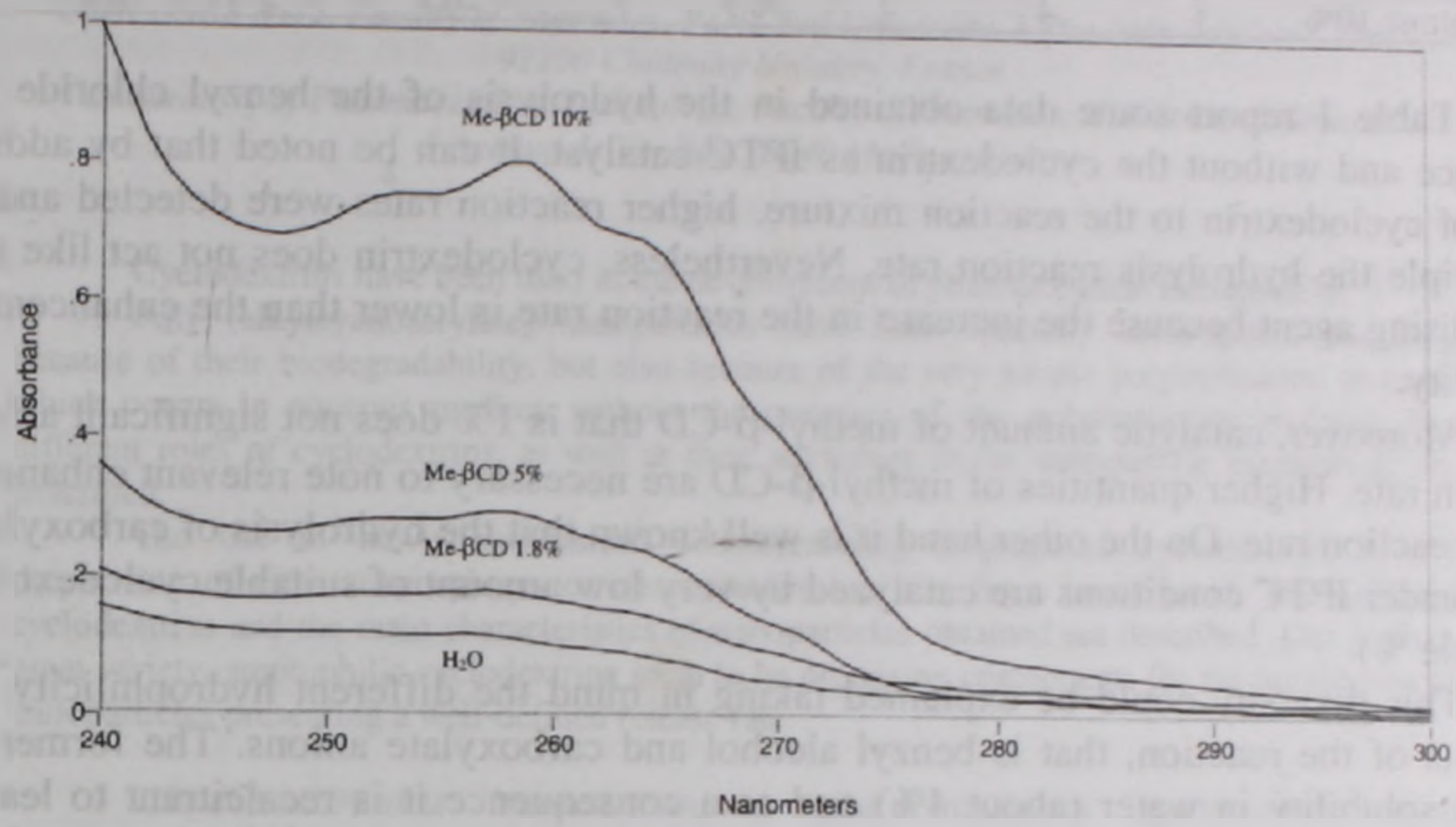

Figure 2. The solubilyzing effect of different amount of methyl-β-cyclodextrin on benzyl chloride in aqueouss solution at room temperature.

An accepted possible reaction mechanism is sketched in Figure 3. The cylodextrins are able to form stable inclusion compounds with the benzyl halide. This latter could be transferred in aqueous phase where the reaction takes place. Actually the cyclodextrin acts as a co-catalyst being the hydroxyl anions the true catalysts in the hydrolysis reaction.

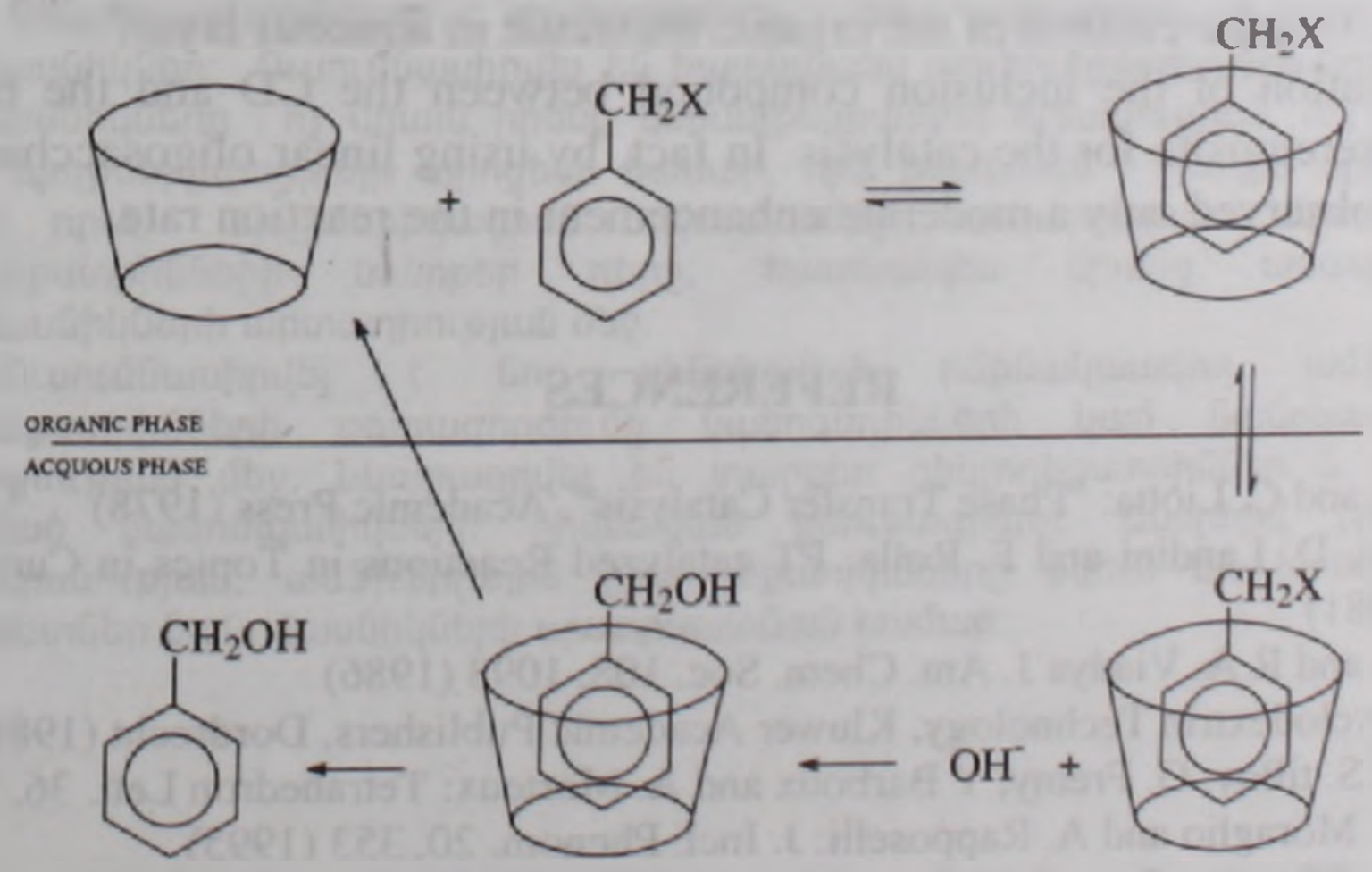

Figure 3. Inverse Phase Transfer Catalysis.

Table 1. Hydrolysis of benzyl halide under IPTC conditions. 50°C 500 rpm.

		Conversion, %		
Catalyst	**Time(h)**	**Catalyzed (a)**	**Uncatalyzed (b)**	**A/b ratio**
Methyl-β-CD 1%	4	38.3	26.1	1.5
Methyl-β-CD10%	4	85.9	26.1	3.3
β-CD 10%	4	42.4	26.1	1.6
Maltose 10%	4	36.9	26.1	1.4

Table 1 report some data obtained in the hydrolysis of the benzyl chloride in the presence and without the cyclodextrin as IPTC catalyst. It can be noted that by adding 10 wt% of cyclodextrin to the reaction mixture, higher reaction rates were detected and more than triple the hydrolysis reaction rate. Nevertheless, cyclodextrin does not act like simple solubilising agent because the increase in the reaction rate is lower than the enhancement of solubility.

Moreover, catalytic amount of methyl-β-CD that is 1% does not significant affect the reaction rate. Higher quantities of methyl-β-CD are necessary to note relevant enhancement in the reaction rate. On the other hand it is well known that the hydrolysis of carboxylic acid esters under IPTC conditions are catalyzed by very low amount of suitable cyclodextrin (i.e. < 1 mole %).

This diversity could be explained taking in mind the different hydrophilicity of the products of the reaction, that is benzyl alcohol and carboxylate anions. The former has a limited solubility in water (about 4%) and as a consequence it is recalcitrant to leave the apolar cavity of the cyclodextrin thus partially blocking the catalysis. As far as carboxylic acid ester hydrolysis is concerned, highly hydrophylic carboxylate anions are obtained that immediately pass in to water solution breaking the inclusion compound and restoring free cyclodextrin cavity to start another catalytic cycle.

Because the hydrolysis of benzyl halide leads to quite lypophilic benzyl alcohol which have a low water solubility, greater amount of the IPTC catalyst, in comparison with other IPTC reactions, is required to show significant enhancement in the reaction rate.

In particular the methyl-β-cyclodextrin acts as better catalyst in comparison with the parent β-cyclodextrin.

This fact could be ascribed to its higher solubility in both phases. As a consequence it is a better phase transfer catalyst of the organic molecule in aqueous layer.

The formation of the inclusion compound between the CD and the benzyl halide seems to be a prerequisite for the catalysis. In fact, by using linear oligosaccharides such as maltose, it was observed only a moderate enhancement in the reaction rate.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRINS IN PHARMACEUTICAL NANOPARTICLES

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Cyclodextrins have been used in the development of pharmaceutical nanoparticles.

Poly (alkylcyanoacrylate) nanoparticles have been specially investigated, not only because of their biodegradability, but also because of the very simple polymerisation process, which occurs in aqueous medium without the presence of any polymerisation initiator. The different roles of cyclodextrins, as well as their advantage in the nanoparticle preparation, are described.

The use of newly synthesized self-assembling amphiphilic cyclodextrins in the preparation of either nanospheres or nanocapsules has also been investigated. The different cyclodextrins and the main characteristics of nanoparticles obtained are described. Due to their great variety, amphiphilic cyclodextrins seem to be promising components for the preparation of nanoparticles presenting a well-defined release rate.

Циклодекстрины использовались для создания фармацевтических наночастиц. Наночастицы поли(акрилцианоакрилат)а особенно изучены не только по их биоразрушаемости, но также из-за очень простого процесса полимеризации, что происходит в водной среде без наличия какого-либо инициатора полимеризации. Описана различная роль циклодекстринов, особенно их преимущества в производстве наночастиц.

Изучено использование новых синтезированных самосвязывающихся амфифилических циклодекстринов в изготовлении наношариков или нанокапсул. Описаны различные циклодекстрины и основные характеристики полученных наночастиц. Благодаря их большому разнообразию амфифилические циклодекстрины представляют собой перспективные компоненты для приготовления наночастиц.

Ցիկլոդեքստրինները օգտագործվել են ստեղծելու դեղագործական նանոմասնիկներ: Ուսումնասիրվել են հատկապես պոլի(ակրիլցիանոակրիլատ)ային նանոմասնիկները ոչ միայն իրենց կենսաքայքայվող ունակության, այլ նաև շատ պարզ պոլիմերիզացիայի պրոցեսի համար, որն ընթանում է ջրային միջավայրում առանց որևէ պոլիմերիզացիոն նախածեռնիչի առկայության: Նկարագրվել է ցիկլոդեքստրինների տարբեր դերը, հատկապես նրանց առավելությունը նանոմասնիկների արտադրության մեջ:

Ուսումնասիրվել է նոր սինթեզված ինքնակապվող ամֆիֆիլային ցիկլոդեքստրինների օգտագործումը նանոգնդիկների կամ նանոկապսուլների արտադրության մեջ: Նկարագրվել են տարբեր ցիկլոդեքստրիններ և տրվել են ստացված նանոմասնիկների հիմնական բնութագրերը: Շնորհիվ իրենց մեծ բազմազանության, ամֆիֆիլային ցիկլոդեքստրինները թվում են խոստումնալից կոմպոնետներ նանոմասնիկների պատրաստման համար:

Introduction

Nanoparticles (nanospheres or nanocapsules) constitute a very interesting target system for drug delivery. Because of their small size they allow a large contact surface with biological membranes and, whatever their administration route, they can significantly improve drug bioavailability. However, some drawbacks can result from the nature of the constituting polymer due to: the polymerisation process, the polymer hydrophily or lipophily, the nanoparticle loading capacity, and the drug release kinetics.

In order to overcome some of these problems, our laboratory developed the use of cyclodextrins in the preparation of nanoparticles. Two main orientations have been investigated [1]: the preparation of combined cyclodextrin/poly(alkyl cyanoacrylate) nanospheres, the preparation of amphiphilic nanoparticles.

I. Combined poly(isoalkyl cyanoacrylate)/cyclodextrin nanospheres.

Nanoparticulate systems can be prepared either by dispersion of preformed polymers or polymerisation.

Dispersion of preformed polymers concerns albumin, gelatine, alginates or synthetic polymers such as poly(lactic acid), poly(lactide-co-glycolide), poly-ε-caprolactone, or cellulose derivatives. The most frequently employed preparation methods, solvent emulsion evaporation or nanoprecipitation, allow the entrapment of highly hydrophobic drugs but require the use of organic solvents more or less toxic which implies a careful purification process.

Polymerisation methods employed in nanosphere preparation depend on the monomer type and polymerisation mechanism. However, the emulsion polymerisation is probably the most frequently employed method. Among the polymers used in such nanosphere preparation are poly(alkyl cyanoacrylates). These are particularly interesting, not only because of their biodegradability, but also because of the very simple polymerisation process which occurs in aqueous medium without the presence of any polymerisation initiator. In fact, the polymerisation process is aroused by a basic compound or more simply by the presence of hydroxyl ions resulting from water dissociation or from the presence of any kind of molecule with hydroxyl groups. One of the major drawbacks of this type of nanosphere is related to the difficulty of entrapping hydrophobic drugs which can hardly be dissolved in the aqueous polymerisation medium. We proposed the use of cyclodextrins in order to overcome this problem [2]

1.1 Preparation and characteristics of combined poly (alkyl cyanoacrylate) / cyclodextrin nanospheres.

The possibility of preparing nanospheres in the presence of cyclodextrins was investigated with poly(isobutyl cyanoacrylate) [2, 3].

Nanospheres were prepared by anionic polymerisation of isobutylcyanoacrylate in 0.01 M hydrochloric acid containing 1% w/v poloxamer 188 in the presence of α-, β-, γ-, hydroxypropyl α-, hydroxypropyl β-, hydroxypropyl γ-, and sulphobutylether β-cyclodextrin. With all the cyclodextrins it was possible to obtain nanospheres (Table 1).

Nanosphere size depends on the cyclodextrin type. The smallest particles being obtained with hydroxypropyl β- or hydroxypropyl γ-cyclodextrin were 103 and 87 nm respectively. It depends also on the cyclodextrin concentration (Table 2), an increase in cyclodextrin concentration leading to a decrease in particle size.

Table 1. Characteristics of poly(isobutyl cyanoacrylate) nanospheres prepared in the presence of cyclodextrins (5 mg/ml) and poloxamer 188 (1%). (Mean of 3 replicates ± SD; ND = not determined).

Cyclodextrin	Size (nm)	Zeta potential (mV)	Cyclodextrin content (ug CD/mg particles)
α-cyclodextrin	228 ± 69	-34.4 ± 4.0	ND
β-cyclodextrin	369 ± 7	-24.7 ± 8.2	360
γ-cyclodextrin	286 ± 9	-22.9 ± 0.6	240
hydroxypropyl α-cyclodextrin	244 ± 25	-27.0 ± 2.2	ND
hydroxypropyl β-cyclodextrin	103 ± 6	-8.6 ± 0.9	247
hydroxypropyl γ-cyclodextrin	87 ± 3	-2.6 ± 2.2	220
sulphobutyl ether β-cyclodextrin	319 ± 10	-45.4 ± 2.4	ND

Table 2. Influence of the cyclodextrin concentration on characteristics of poly(isobutyl cyanoacrylate) nanospheres.

Cyclodextrin	Concentration (mM)	Size (nm)
sulphobutyl ether β-cyclodextrin	5	252
	10	196
	15	171
	20	126
hydroxypropyl γ-cyclodextrin	5	64
	10	53
	15	46

It is interesting to note the role played by cyclodextrins. Not only, their concentration influences the particle size, but the amount of cyclodextrin combined to the nanospheres and measured by the method of Vikmon [4] represents about 1/4 to 1/3 of the nanosphere weight (Table 1). Furthermore, their influence on the ζ potential of the particle, which is about – 40 mV in the absence of cyclodextrin increases to values close to zero in the presence of neutral natural cyclodextrins or their hydroxypropyl derivatives. In the presence of the negatively charged sulphobutyl ether of β-cyclodextrin, it decreases to – 45 mV (Table 1).

These results suggest an influence of cyclodextrins on nanosphere formation, either by initiation of the polymerisation process of alkyl cyanoacrylate or by steric stabilization of the nanospheres.

Normally, the anionic polymerisation starts with the monomer activation by nucleophile groups, such as weak bases or hydroxyl ions from the water dissociation, present in the polymerisation medium. In the present case, the numerous hydroxyl groups of cyclodextrins could behave like polymerisation initiators. However, further studies have shown that nanospheres prepared by nanoprecipitation [5] of either: 1) the product obtained by dissolution in acetonitrile of combined poly(alkyl cyanoacrylate)/hydroxypropyl β-cyclodextrin nanospheres or 2) the poly(alky cyanoacrylate) alone, do not present any difference in their infrared spectra [3, 6], and that infrared spectra of the supernatant of 1) is comparable to that of hydroxypropyl β-cyclodextrin, indicating a high hydroxypropyl β-cyclodextrin concentration in the supernatant. This study leads to the conclusion that there are no covalent bonds between the hydroxypropyl β-cyclodextrin and the polymer chains. The mechanism by which cyclodextrins could initiate the alkyl cyanoacrylate polymerisation is not clearly demonstrated and is disputable.

The alkyl cyanoacrylate polymerisation occurs inside the surfactant (poloxamer 188) micelles and ends with the disappearance of monomers from polymerisation medium, and

with a concomitant release of the surfactant. Normally, the surfactant adsorption at the nanosphere surface results in their steric stabilization. However, in the present case investigations carried out on nanospheres prepared in the presence of hydroxypropyl β-cyclodextrin showed that the presence of poloxamer does not affect the particle size which decreases (from 300 to 50 nm) with an increase in hydroxypropyl β-cyclodextrin concentration (from 0 to 12.5 mg/ml) in the polymerisation medium [3, 6]. Similarly the zeta potential of the particles decreases from a high negative value (– 40 mV) to a value close to 0 mV with an increase in hydroxypropyl β-cyclodextrin concentration, and ζ potential values are not affected by the presence of poloxamer 188 [3]. These results, associated to the high amount of cyclodextrins combined to the nanospheres, leads to the conclusion that cyclodextrins have a steric stabilization effect on poly(alkyl cyanoacrylate) nanospheres.

1.2 Combined poly (alkyl cyanoacrylate) / cyclodextrin nanospheres loaded with active ingredients.

Nanospheres of poly(isobutyl cyanoacrylate) were loaded with a series of steroids (model molecules) [3, 6, 7] and nanospheres of poly(isohexyl cyanoacrylate) were loaded with saquinavir [8]. The loading was carried out by addition of the active ingredient free or included in hydroxypropyl β-cyclodextrin to the polymerisation medium, and the nanospheres were recovered by freeze-drying.

In the case of steroids, except for spironolactone, the nanospheres obtained with the hydroxypropyl β-cyclodextrin inclusions have a smaller diameter (about 100 nm) than the nanospheres obtained with the free steroids (between 200 and 300 nm) (Table 3).

Table 3. Characteristics of steroid-containing poly(isobutyl cyanoacrylate) nanospheres prepared with (+) and without (-) hydroxypropyl β-cyclodextrin. (Mean of 3 replicates ± SD).

Steroid	Size (nm)		Drug loading (μmol/g)		Loading increase (fold number)
(presence of CD)	-	+	-	+	
Hydrocortisone	281 ± 7.5	105 ± 3.5	6.0	2.2	7
Prednisolone	271 ± 3	115 ± 26.5	0.33	43.0	130
Spironolactone	263 ± 7.5	294 ± 4	18.4	127.2	7
Testosterone	216 ± 16	110 ± 6	7.9	67.6	8.5
Megestrol acetate	249 ± 18	93 ± 3.5	0.65	3.6	5.5
Danazol	255 ± 19	100 ± 19	1.0	33.2	33
Progesterone	268 ± 25.5	92 ± 12	2.5	69.6	28

In the presence of hydroxypropyl β-cyclodextrin, a significant increase in loading capacity of nanospheres is observed, varying from 7 times for spironolactone to 130 times for prednisolone.

Physical state of progesterone in combined poly(isobutyl cyanoacrylate)/hydroxypropyl β-cyclodextrin nanospheres was investigated by differential scanning calorimetry and compared with the separate products [3, 6]. In the nanospheres, it is possible to observe the disappearance of the endothermic peak (130 °C) characteristic of progesterone melting, and its replacement by a broad endothermic transition in the region 130-170 °C. This indicates that progesterone is either molecularly dispersed in the nanospheres or, at least, in amorphous state.

The in vitro release of progesterone, studied in alkaline borate buffer of pH = 8.4, occurs with a very fast initial release followed by a much slower release phase reaching a plateau after 30 h. The release rate is faster and higher for small particles than for large ones

(35% in the second phase for particles of 150 nm diameter, and 62% for particles of 70 nm). In the presence of esterases, the progesterone release is almost total after 1 or 2 h depending on the particle size [3, 6]. For its part, hydroxypropyl β-cyclodextrin was totally released in less than 1 h [3].

Loading of nanospheres, which is dramatically increased in the presence of hydroxypropyl β-cyclodextrin, could be explain by three mechanisms. The inclusion compound steroid/hydroxypropyl β-cyclodextrin can be absorbed at the nanosphere surface; this phenomenon corresponds to the steroid fast release observed in the in vitro dissolution tests. On the other hand, the inclusion could be entrapped in the nanosphere core during their formation by polymerisation. However, due to the high hydrophily of the external part of the inclusion compound, this mechanism does not seem to be predominant and may not occur at all. Finally, in the aqueous polymerisation medium, the inclusion dissociates in free species, according to its stability constant:

steroid/ hydroxypropyl β-cyclodextrin ⇔ steroid + hydroxypropyl β-cyclodextrin

The inclusion compound dissociation could be accelerated by capture of the free steroid by the hydrophobic polymer during its formation. In order to verify this hypothesis, a correlation was looked at between the nanosphere loading capacity and the polymer/water partition coefficient in the presence or not of hydroxypropyl β-cyclodextrin and for various steroid concentrations [3, 7]. In fact the value of the partition coefficient polymer/water was replaced by that of the partition coefficient octanol/water, because there is a linear relationship between these two values:

$$\log P(\text{polymer/water}) = a \times \log P(\text{octanol/water}) + b$$

In the presence or not of hydroxypropyl β-cyclodextrin the partition coefficient is not the main factor influencing nanosphere loading. On the other hand, the product of the initial concentration in active ingredient and the partition coefficient is the determining factor of the loading. This result indicates that, in the absence of cyclodextrins, the steroid affinity for the polymer is not sufficient to result in noticeable nanosphere loading.

In conclusion, the active ingredient, free or included in the cyclodextrin, is partly adsorbed at the nanosphere surface; this localisation corresponds to its fast release. The active ingredient is also entrapped in the nanosphere core; this localisation corresponds to the plateau observed in the absence of esterases.

2. Amphiphilic cyclodextrin nanoparticles

Natural cyclodextrins, as well as their hydrophilic derivatives, despite their prominent advantage of being powerful drug solubilizers, have the major inconvenience of their high external hydrophily, which reduces the possibility of interactions with biological membranes. For this reason, several types of amphiphilic cyclodextrin have recently been synthesized [9]. These cyclodextrin derivatives differ not only by the position and length of the substituents, but also by the nature of the chemical bond between the substituent and the cyclodextrin.

The work we carried out was mainly oriented towards the "skirt-shaped" cyclodextrins substituted by esterification (O) on the secondary hydroxyl groups (II) [10, 11]. In a second attempt, we investigated the influence of the chemical bond nature, the chain length and the

substitution localisation. With these amphiphilic cyclodextrins it has been possible to prepare either nanospheres or nanocapsules.

2.1 Preparation and characteristics of skirt-shaped cyclodextrin nanospheres.

Skirt-shaped α- or β-cyclodextrins with hydrocarbon chains from C_6 to C_{14}, which have surfactant characteristics [12], can very easily lead to nanospheres.

Nanospheres of skirt-shaped cyclodextrins can be prepared by different methods: nanoprecipitation [13, 14], the most simple, or emulsion solvent evaporation [15]. The nanoprecipitation method consists in dissolving the amphiphilic cyclodextrin in an organic solvent miscible in water (acetone or absolute ethanol) and injecting this solution in an aqueous phase, with or without surfactant, under stirring. Cyclodextrins precipitate spontaneously and form nanospheres. The organic solvent, and part of the water, are removed by evaporation under vacuum to give the desired concentration in nanosphere suspension.

Nanospheres present very good sphericity and narrow dispersity, even without the presence of surfactant [13] (Table 4). Their size is not affected by stirring rate, but is increased with an increase in ionic surfactant concentration, and is independent of the non-ionic surfactant concentration [13] (Table 5).

Table 4. Characteristics of skirt-shaped β-cyclodextrin nanospheres prepared by nanoprecipitation in the presence or not of surfactant. Influence of the hydrocarbon chain length. (PI: polydispersity index)

Surfactant	βCD- C_6(II,O)		βCD- C_{12}(II,O)		βCD- C_{14}(II,O)	
	Size (nm)	PI	Size (nm)	PI	Size (nm)	PI
(none)	107 ± 17	0.045	103 ± 12	0.032	89 ± 26	0.093
PEF68	103 ± 14	0.096	106 ± 17	0.069	98 ± 16	0.048
Span® 85	120 ± 31	0.072	101 ± 19	0.075	100 ± 18	0.076

Table 5. Characteristics of skirt-shaped β-cyclodextrin nanospheres prepared by nanoprecipitation in the presence or not of surfactant. Influence of nature and concentration in surfactant. (PI: polydispersity index; Pluronic® F68: PEF68; SDS : sodium dodecyl sulphate; BZD^+: benzethonium chloride)

Surfactant (%w/v)	PEF68		SDS		BZD^+	
	Size (nm)	PI	Size (nm)	PI	Size (nm)	PI
-	100 ± 13	0.05	100 ± 13	0.05	100 ± 13	0.05
0.055	103 ± 17	0.03	136 ± 33	0.11	135 ± 54	0.088
0.156	100 ± 23	0.04	140 ± 24	0.032	159 ±34	0.1
0.257	105 ± 14	0.01	139 ± 26	0.018	173 ± 29	0.079

Nanospheres were loaded with either hydrophilic or lipophilic drugs. The encapsulation of doxorubicin hydrochloride in skirt-shaped cyclodextrin (C_6) nanospheres [16, 17] requires the presence of a surfactant in the nanoprecipitation medium. In the presence of poloxamer, the concentration in doxorubicin in nanospheres is high (40% w/w), and the nanospheres are stable in saline buffer medium, however they do not resist aqueous dilution. In the presence of sodium dodecyl sulphate, doxorubicin concentration in the nanospheres is much lower (7% w/w), and the nanospheres, which resist to aqueous dilution are not stable in saline buffer medium. The optimal fixation is obtained by diluting the nanosphere suspension prepared with sodium dodecyl sulphate, by an equivalent amount of poloxamer solution [16, 17].

More interestingly, skirt-shaped γ-cyclodextrin (C_6) nanospheres were loaded with lipophilic drugs: progesterone, testosterone and hydrocortisone, the loading being carried out in the presence of Pluronic F68 either during the nanoprecipitation process or on blank nanospheres. The drug concentration in nanospheres depends on the affinity constant of the drug for the parent cyclodextrin cavity, and depends inversely on the drug water solubility (Table 6).

Table 6. Influence of drug characteristics on the loading capacity of γ-cyclodextrin diester (C_6) nanospheres prepared by nanoprecipitation.

Drug	Loading capacity (μg/mg)	Water solubility (μg/ml)	KγCD
Progesterone	60-80	3.3	24.000
Testosterone	20-30	23.0	16.500
Hydrocortisone	< 15	326.0	2.240

When nanospheres are loaded with progesterone [14], the loading capacity varies with the initial content of drug in the preparation medium, and reaches an optimum corresponding to almost 100% of entrapment efficiency (Table 7).

Table 7. Influence of the initial progesterone content (mg/30 mg amphiphilic cyclodextrin) on the loading capacity and the diameter of skirt-shaped γ-cyclodextrin (C_6) nanospheres prepared by nanoprecipitation in the presence of Pluronic F68

Drug content m g/30 mg γCD-C_6(II,O)	Drug loading		Entrapment	Size
	(%)	(mg/mg γCD-C_6(II,O))	(%)	(nm)
0.5	49.09 ± 10.0	11.5 ± 3.75	71.0 ± 22.09	126 ± 44
1.0	74.91 ± 20.5	24.12 ± 8.65	73.09 ± 26.22	125 ± 44
2.5	91.96 ± 0.92	81.19 ± 15.05	97.81 ± 18.1	118 ± 21
5.0	91.06 ± 0.4	75.07 ± 20.26	45.04 ± 12.13	122 ± 25
10.0	89.15 ± 0.52	80.98 ± 21.82	24.29 ± 6.54	113 ± 28
20.0	97.19 ± 3.09	53.01 ± 1.10	7.50 ± 0.15	121 ± 19

Differential scanning calorimetry studies showed the disappearance of the endothermic melting peak of progesterone at 130 °C in the nanospheres. On X-ray diffraction patterns, peaks characteristic of the amphiphilic γ-cyclodextrin diester are recovered in the unloaded and loaded nanospheres. However, no signal corresponding to progesterone crystalline domains is detected in the loaded nanospheres [14].

The progesterone is very rapidly released in vitro and reaches an equilibrium depending on the nature of the dissolution medium and on the affinity of the drug for this medium. There is no influence of the formulation parameters. Such a fast release profile is of interest when an improvement in the bioavailability of the drug is desired [14]. These results suggest that progesterone is associated molecularly on the surface of nanospheres and not matrix encapsulated. This association probably occurs through hydrophobic interactions at specific sites of the carrier. The possibility of inclusion of the drug in the cyclodextrin cavity is not excluded [14].

2.2 *Preparation and characteristics of skirt-shaped cyclodextrin nanocapsules*

Nanocapsules of cyclodextrin diesters were prepared by a method very similar to the nanoprecipitation of nanospheres [18]. A lipophilic phase, constituted either of Miglyol 812® or benzyl benzoate and the amphiphilic β-cyclodextrin derivative (with either C_6, C_{12}

or C_{14} hydrocarbon chains) is dissolved in acetone and added under mechanical stirring to an aqueous phase with or without surfactant (Pluronic ® PEF68). The nanocapsules are formed immediately, and acetone is removed by evaporation under vacuum together with part of the water to obtain a suspension of the desired concentration. Nanocapsules loading is obtained by adding the drug to the organic phase.

Nanocapsules characterized by transmission electron microscopy and electron microscopy after freeze-fracture present a diameter close to 200 nm and have a low polydispersity index (Table 8) [18].

Table 8. Characteristics of β-cyclodextrin diester nanocapsules containing benzyl benzoate. Influence of the hydrocarbon chain length (PI: polydispersity index)

Surfactant	βCD- C_6(II,O)		βCD- C_{12}(II,O)		βCD- C_{14}(II,O)	
	Size (nm)	PI	Size (nm)	PI	Size (nm)	PI
PEF68	204 ± 49	0.083	183 ± 48	0.057	178 ± 15	0.130
Span® 85	256 ± 66	0.040	223 ± 49	0.001	213 ± 43	0.029

Nanocapsules were loaded either with indomethacin, progesterone or amphotericin B as drug models. The encapsulation yield is 90% or more [19] (Table 9).

Table 9. Encapsulation of various drugs in β-cyclodextrin diester nanocapsules (C_6) containing benzyl benzoate.

Drug	Drug encapsulation yield (%)	Drug content (% w/w)
Indomethacin	90	21.6
Progesterone	>98	7.5
Amphotericin B	90	11

The rat gastric ulcerative effect of indomethacin encapsulated was compared with that of an indomethacin solution (Indocid®). Whatever the administered dose (5 or 10 mg/kg), the ulcerative effect is significantly decreased, without disappearance of the bioavailability of the product [19]. The protection afforded by the encapsulated indomethacin is 82% for 5 mg/kg and 53% for 10 mg/kg administered.

2.3 Preparation and characteristics of amphiphilic cyclodextrin nanocapsules

A series of amphiphilic β-cyclodextrins was prepared, varying by: the substitution localisation: on the primary (I) or the secondary (II) face, the hydrocarbon chain length (C_6 and C_{14}) branched (B) or not, and the bond type (ester: -O, or amide: -N) [20, 21] (Table 10).

Table 10. Physico-chemical properties of amphiphilic β-cyclodextrins (* with decomposition).

Amphiphilic cyclodextrin	Molecular weight (g/mol)	Melting point (°C)	Solubility in ethanol		Solubility in acetone		HLB calculated
			(mM)	(mg/ml)	(mM)	(mg/ml)	
βCD- C_6(II,O)	2506	230	0.6	1.5	1.0	2.5	8.9
βCD- C_6(I,O)	1820	250*	2.0	3.6	2.0	3.6	11.1
βCD- C_6(I,N)	1813	224	1.0	1.8	0.4	0.72	11.2
βCD- C_6B(I,N)	1813	309*	0.2	0.36	0.8	1.44	11.2
βCD- C_{14}(I,N)	2597	198	0.4	1.04	0.54	1.04	7.8

Nanocapsules were prepared using either acetone or ethanol as organic phase [21]. Results obtained with ethanol are presented in Tables 11 and 12.

Table 11. Mean diameter (nm) of amphiphilic cyclodextrin nanocapsules obtained in ethanol as a function of the cyclodextrin molar concentration

Amphiphilic cyclodextrin	Molar CD concentration					
	0.1	0.2	0.4	0.6	0.8	1
βCD- C_6(II,O)	435	763	998			
βCD- C_6(I,O)	399	385	405	430	532	579
βCD- C_6(I,N)	285	279	284	315	307	367
βCD- C_6B(I,N)	305	391	546	583	767	
βCD- C_{14}(I,N)	332	351	883			

Table 12. Polydispersity index of amphiphilic cyclodextrin nanocapsules obtained in ethanol as a function of the cyclodextrin molar concentration

Amphiphilic cyclodextrin	Molar CD concentration					
	0.1	0.2	0.4	0.6	0.8	1
βCD- C_6(II,O)	0.09	0.24	1.20	1.50		
βCD- C_6(I,O)	0.47	0.49	0.53	0.65	0.68	0.70
βCD- C_6(I,N)	0.09	0.21	0.14	0.21	0.15	0.16
βCD- C_6B(I,N)	0.15	0.38	0.79		0.86	
βCD- C_{14}(I,N)	0.24	0.11	0.65			

In the light of this work, it appears that substitution of cyclodextrins on the primary face results in more appropriate nanocapsule forming agents in the sense that monodispersity can be better achieved with these cyclodextrin derivatives. The length of aliphatic chain grafted to the molecule and bond type used in the grafting play important roles in determining the physico-chemical properties of the products. Optimum chain length was linear C_6, with either deoxy or amido bond. Molecules with branched substituents tend to collapse at high concentrations, because of their probably larger surface area, while long aliphatic chain substitution or substitution on the secondary face resulted in instable nanocapsules.

Conclusion. Our laboratory has developed studies on the use of cyclodextrins in modern pharmaceutical dosage forms, among which nanoparticles.

It appears that cyclodextrins and their hydrophilic derivatives can be very useful agents in the preparation and loading of polymer nanoparticles, and specially poly(akyl cycanoacrylate) nanoparticles. Cyclodextrins, not only, act as polymer initiators, steric stabilizing agents but also they significantly increase the nanoparticle loading capacity. It can be assumed that, depending on their nature and concentration, it will be possible to modulate not only the nanoparticle load but also the release kinetic of the active ingredient.

Finally, amphiphilic cyclodextrins represent a new series of surface-active agents capable of self-association to lead either to nanospheres or to nanocapsules. Due to their potential variety, it will be possible to prepare nanoparticles specially tailored to obtain a defined release rate for a given active ingredient.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

COMPLEXATION OF DITOPIC GUESTS BY CYCLODEXTRINS AND DERIVATIVES

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The complexation of ditopic guests by cyclodextrins and their higher degree derivatives is reviewed. Special attention is paid to the complexation of ditopic guests by cyclodextrin dimers, that can lead either to the formation of 1:1 stoichiometry complexes, in which a cooperative effect over the equilibrium constant is observed, or to the generation of supramolecular polymers with n:n stoichiometries.

Рассмотриваются вопросы комплексообразования дитопических "гостей" циклодекстринами и их высокомолекулярными производными. Особое внимание уделяется комплексам дитопических "гостей" димерами циклодекстринов, что может вести либо к образованию 1:1 стехиометрических комплексов, в которых наблюдается совместный эффект над константой равновесия, либо к появлению надмолекулярных полимеров с n:n стехиометрией.

Քննարկվում են ցիկլոդեքստրինների և նրանց բարձրամոլեկուլային ածանցյալների հետ դիտոպիկ «հյուրերի» համալիրագոյացման խնդիրները: Հատուկ ուշադրություն է դարձվում ցիկլոդեքստրինների դիմերների և դիտոպիկ «հյուրերի» միջև համալիրագոյացմանը, որը կարող է տանել կամ 1:1 ստեխիոմետրիկ համալիրների գոյացմանը, որտեղ դիտվում է միացյալ գործոնի վեր լինելը հավասարության հաստատունից, կամ n:n ստեխիոմետրիայով վերնամոլեկուլյար պոլիմերների առաջացմանը:

I. Complexation of ditopic guests by natural cyclodextrins.

Natural cyclodextrins (CD) are cyclic oligomers, with a truncated cone shape, built up from 6, 7 or 8 glucopyranose units, linked by α-(1-4)-glycosidic linkages, named α, β and γ–CD, respectively (Figure 1). They are known to form inclusion complexes in water with a variety of organic molecules, a property used to increase the bioavailability and stability of poorly soluble drugs[1-3] and flavors [4], as photochemical sensors [5-8], enzyme mimics [9-12] or as pollutants removers in environmental processes [1].

Figure 1. Structure and schematic representation of α- β- and γ-cyclodextrin

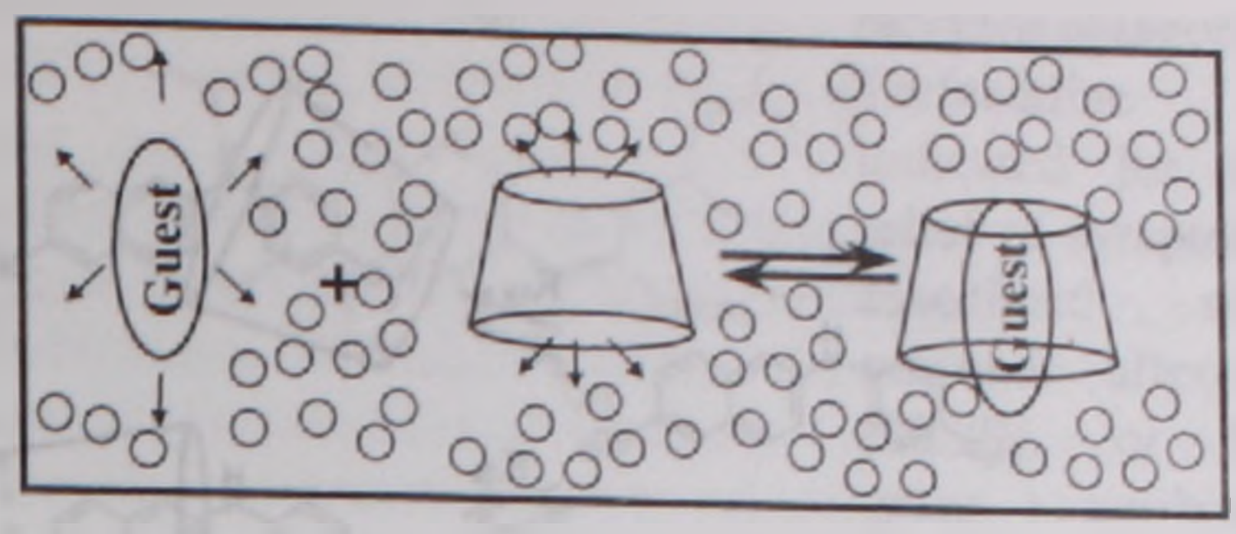
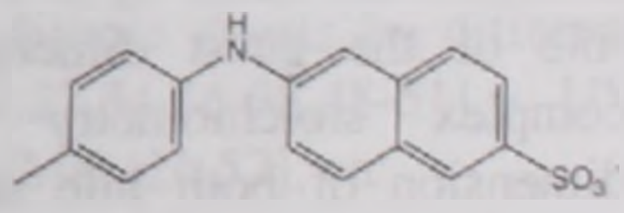


Figure 2. Schematic representation of a 1:1 stoichiometry cyclodextrin inclusion complex.

Due to their α-(1-4)-glycosidic linkages, all primary hydroxyl groups (C-6) are orientated toward one of the edges of the truncate cone, while the secondary hydroxyl groups (C-2 and C-3) are placed on the other edge. Since hydrogens of carbons 3, 5 and 6 and the non-bonding electron pairs of the glycosidic oxygen bridges are orientated toward the inside of the cavity, it has a hydrophobic environment with a high electron density [1,13]. These double characteristic of CDs, in one hand the existence of a hydrophobic cavity and on the other the existence of both hydrophilic hydroxyl rims give them the property to form inclusion complexes in water with a variety of organic molecules. During this process, the less polar guest molecule substitutes the energetically unfavored water molecules that occupy the CD cavity, as shown in figure 2. This process is regulated by non covalent interactions between host and guest: van der Waals forces, hydrogen bonding, electrostatic interactions [1,11].

During formation of the inclusion complex, the guest molecule enters, totally or partially, inside of the slightly apolar cavity of the CD. The simplest, and most frequent complex formed has a 1:1 stoichiometry (CD: guest), in which the guest is totally or partially included inside of a single CD. However, depending on the size of the CD cavity, or the dimensions and apolar sites of the guest molecule, other stoichiometries such as 2:1, 1:2, 2:2 or higher degree complexes do exist [14]. An example of the effect of the dimension of the cavity over the complex stoichiometry is the complexation of sodium deoxycholate (NaDC) by natural CDs. With α-CD, no complex is formed, while with β-CD and γ-CD 2:1 and 1:1 complexes are obtained. [15] In this review we will focus our attention on the complexation of ditopic guests by CDs and their higher degree derivatives (dimers, trimers, tetramers).

The 6-(p-toluidino)naphthalene-2-sulfonate (TNS) **1** has been one of the ditopic guests more wildly used as a model guest in order to compare the binding ability of different β-CD derivatives [16-23] or to determine the stability constant of guests molecules through competitive complexation processes [24,25].



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Recently, from steady-state and time-resolved fluorescence data, ^{13}C NMR titration, ROESY experiments and PCGA analysis, we have proposed that TNS forms two distinct 1:1 inclusion complexes, resulting from the complexation of both toluidin and naphthalensulfonate moieties (with two different microscopic equilibrium constants, K_{1a} and K_{1b}) and one 2:1 complex (with the microscopic equilibrium constants K_{2a} and K_{2b}) with β-CD and its monoamino derivative (β-$CDNH_2$), as shown in Figure 2 [23]. This is in agreement with Schneider *et al* [20]. The TNS molecule is first complexed by one CD (through the secondary face) by either of its two apolar sites (toluidino or naphthalensulfonate) and when the CD concentration increases the 2:1 complex if formed.

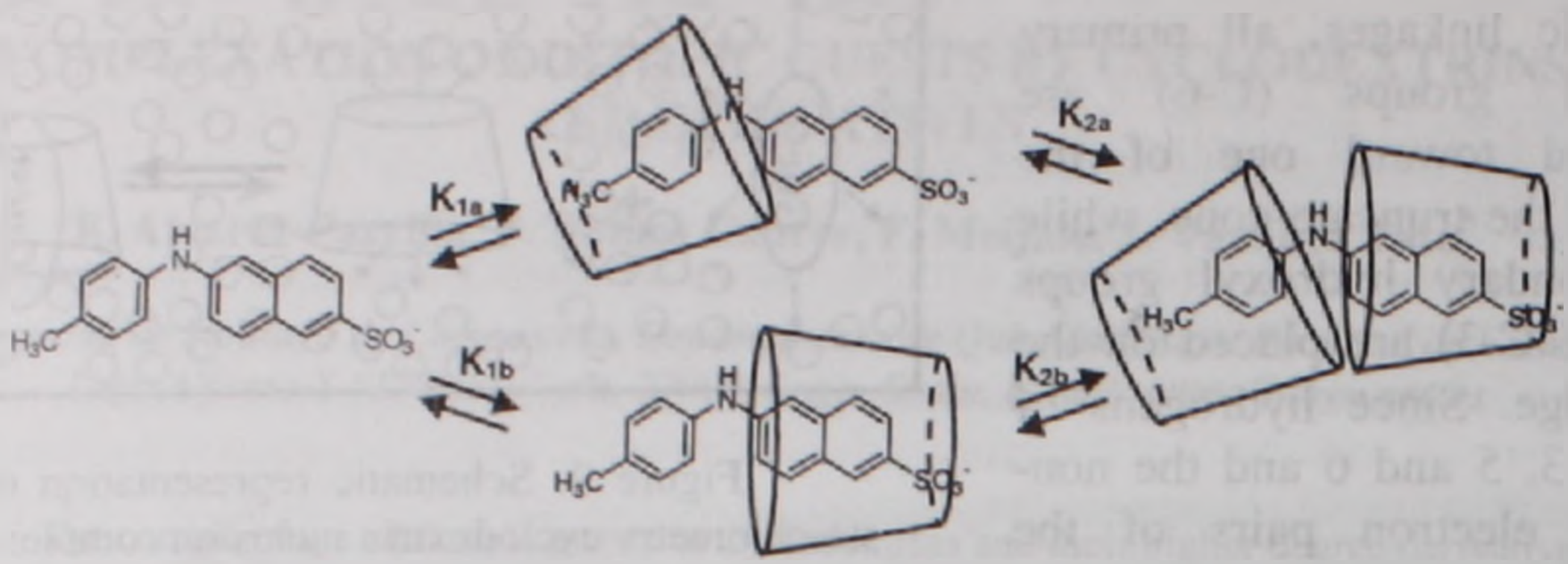
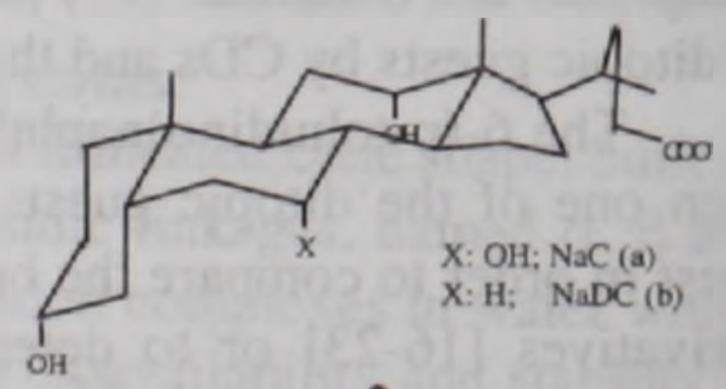


Figure 2. Schematic representation of the complexation of the ditopic guest TNS by β-CD, in which two 1:1 complexes and one 2:1 complex are obtained.

From the microscopic stability constants values (β-CD showed a higher K_{1a} value, while β-$CDNH_2$ showed a higher K_{1b} value) it was possible to conclude that the complexation behavior of TNS with β-CD was regulated by polarity factors, while that for β-$CDNH_2$ was regulated by both polarity and electrostatic interactions between the positively amino group and the negatively charged sulfonate moiety of TNS.

The complexation of the aminonaphthalene sulfonate derivative 6-(p-anilino)naphthalen-2-sulfonate (2,6-ANS) is another example in which electrostatic interactions play an important role on the complexation behavior of a ditopic guest. The structural difference with TNS is that the methyl group in position 6 of the benzene ring is substituted by a hydrogen atom. When ANS is complexed by β-CD it forms only two 1:1 complexes (similar to those of TNS). However, when it is complexed by protonated β-$CDNH_2$, it forms two 1:1 complexes and a 2:1 complex. This different behavior is due to the electrostatic interaction between the protonated amino group and the sulfonate ion [23].

The complexation of the bile salts, sodium cholate **(2a)** and its deoxyderivative sodium deoxycholate **(2b)**, by β-CD helps to understand the role of the guest dimension and polarity on the complex stoichiometry [26]. The longitudinal dimension of both bile salts is identical and large enough (between 12 and 15 Å) to allow the complexation by two β-CDs. From NMR experiments has been demonstrated that NaC forms a stable 1:1 complex, while NaDC forms a 2:1 complex. The only structural difference between both salts is located at C-7 position of the steroid body, NaC having a hydroxyl group and NaDC a hydrogen atom†. This confers a larger hydrophobic region to the NaDC than that of NaC, and consequently it can be complexed by two CDs. The conclusion is that the stoichiometry of the complex formed is regulated by their polarity.



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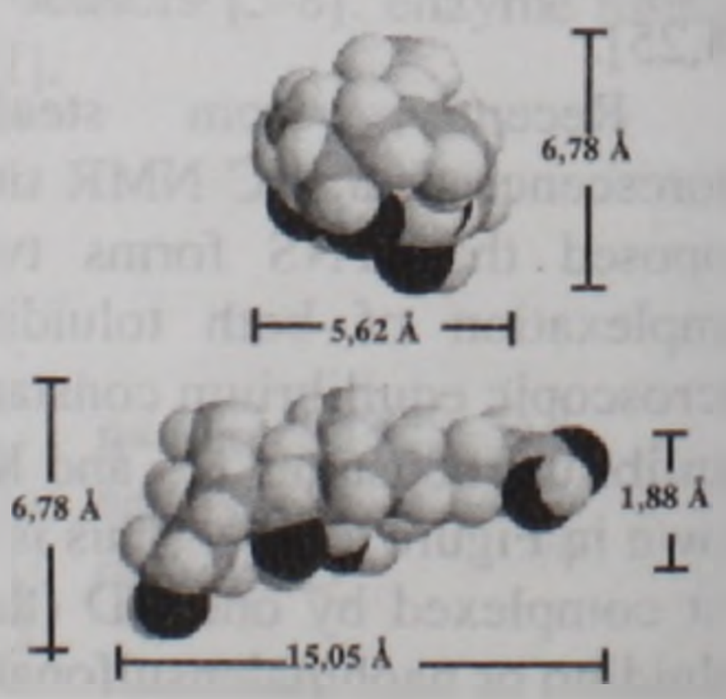
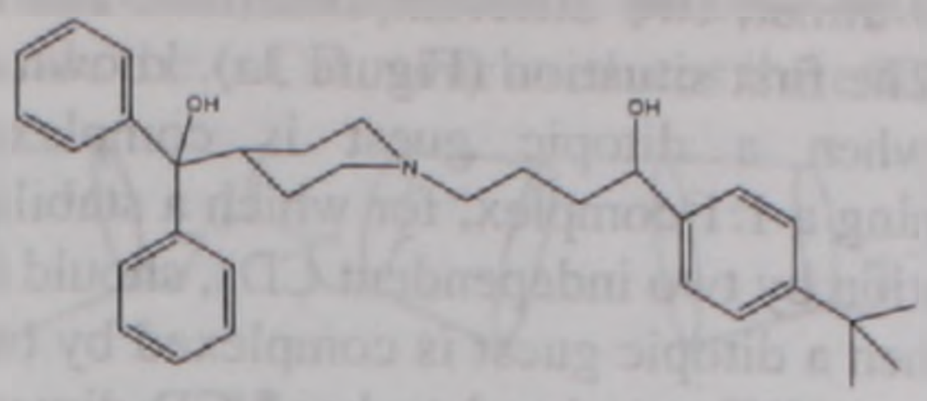


Other ditopic guests such as the histamine H_1-

†This difference has also a strong influence on the aggregation behaviour of both biosurfactants in water as it has been widely demonstrated [27].



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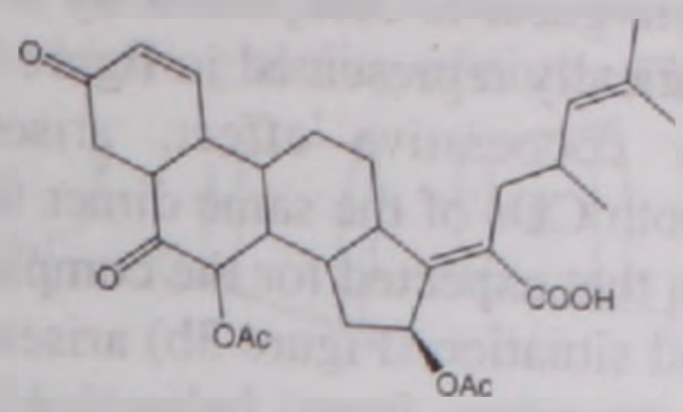

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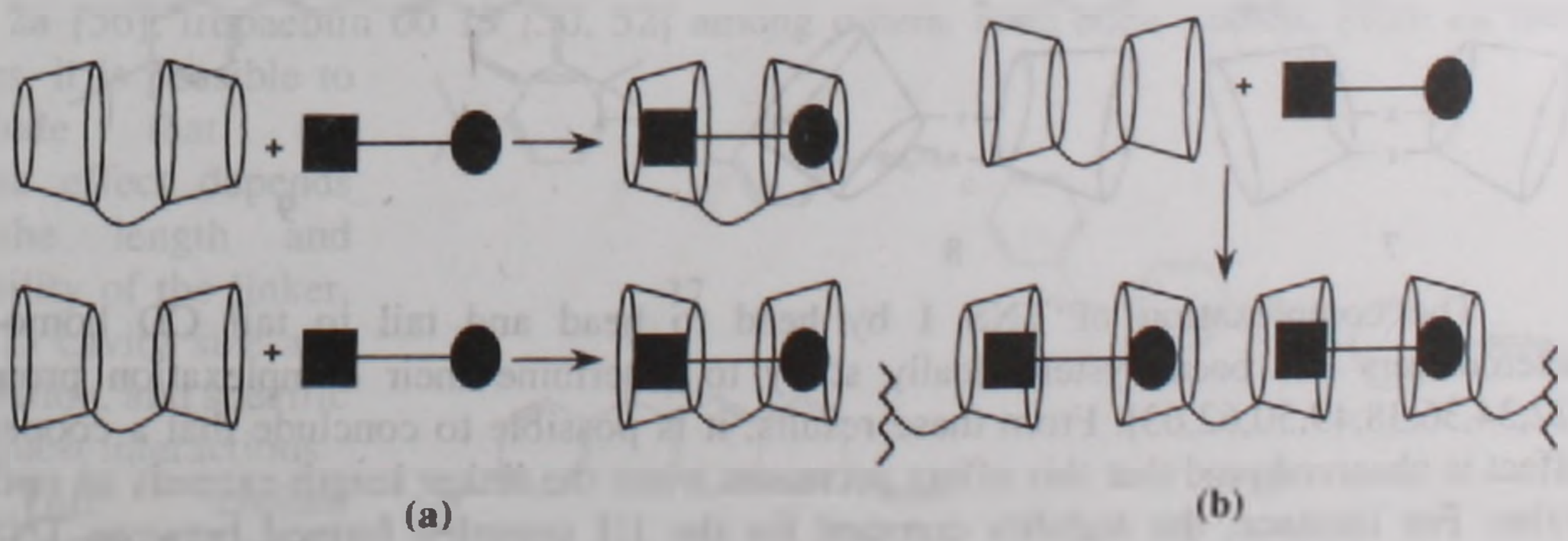
receptor antagonist Terfenadine 3, indicated for the relief of symptoms associated with seasonal allergies [28,29], or the gram + antibiotic helvolic acid 4 (unpublished results) used in dermatological diseases have been complexed by β-CD. Both drugs form 2:1 complexes. It was also evident an increase on their solubilization on water as a result of the complex formation. A study carried out by Wimmer *et al* [30] showed that retinol forms a 2:1 complex with γ-CD, which increases its stability toward UV light and oxygen oxidation and polymerization. Lawrence *et al* [31] observed high stability constants when a tetraaminoporphyrin was complexed by twσ-ĐMβ-CD (heptakis(2,6-di-O-methyl)-β-CD).

II. Complexation of ditopic guests by cyclodextrin dimers.

CD dimers are molecules in which two CD units are linked together through covalent unions. They can be classified in four groups depending on: a) the type of CD present in the structure: homo- [26,32-35] and heterodimer [33,34], when both CDs are or not of the same type; b) the side of the CD were the linking is located: head to head [26,36-39], tail to tail [34,35,38-40] and head to tail [41], where head and tail are the primary and secondary hydroxyl rims of CD; c) the linking bridge: diamine [42], diether [43], diester [44], disulfide [45], imidazolium [33] and diamide [42] are the most common ones; and d) the number of linking groups present in the structure: single and doubly bridged CD dimers [46,47]. Among all CD dimers, the most common ones are the head to head and tail to tail β-CD homodimers.

Over the last 10 years numerous CD dimers have been synthesized in order to study the influence of two adjacent CDs over the complexation of ditopic guests by different instrumental techniques: fluorescence spectroscopy [17,22,34,36-38,48-51], UV spectroscopy [39,52-54], differential calorimetry [35,44,55,56], NMR [26,57], etc.



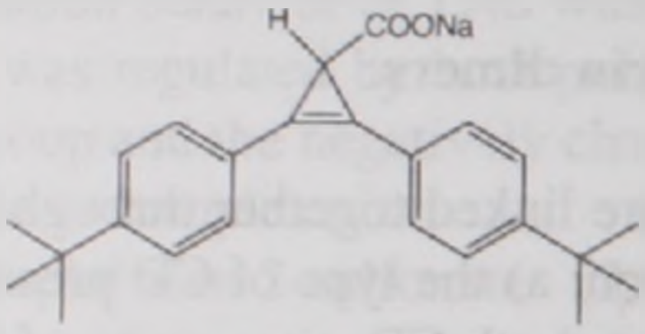
(a) (b)

Figure 3. Schematic representation of the structures that can be formed by the complexation of a ditopic guest by a CD dimer: (a) Chelate binding or cooperative effect; (b) Supramolecular polymer with a n:n stoichiometry.

When a ditopic guest is complexed by a CD dimer, two different situations can be achieved, as schematically represented in figure 3. The first situation (Figure 3a), known as chelate binding or cooperative effect, arises when a ditopic guest is complexed simultaneously by both CDs of the same dimer forming a 1:1 complex, for which a stability constant, higher than that expected for the complexation by two independent CDs, should be expected. The second situation (Figure 3b) arises when a ditopic guest is complexed by two CDs (through their secondary faces) belonging to two different head to head CD dimers, resulting in the formation of a supramolecular polymer with a n:n stoichiometry.

a) Chelate binding.

The chelate binding hypothesis was first suggested by Breslow *et al* [35,37,44, 47,55, 58-61] when proposing CD dimers as mimics for antibodies and enzymes. According to this hypothesis when a ditopic guest is complexed simultaneously by two CDs from the same dimer, the global stability constant should be larger than the square of that for the complexing of each binding site, since the free energy is doubled. As an example, the complexation of the ditopic host **5**, with two tert-butylphenyl groups, by dimer **6** showed a binding constant K_{11} 7 x 10^8 M^{-1}, which is similar to those of medium-affinity antibodies.



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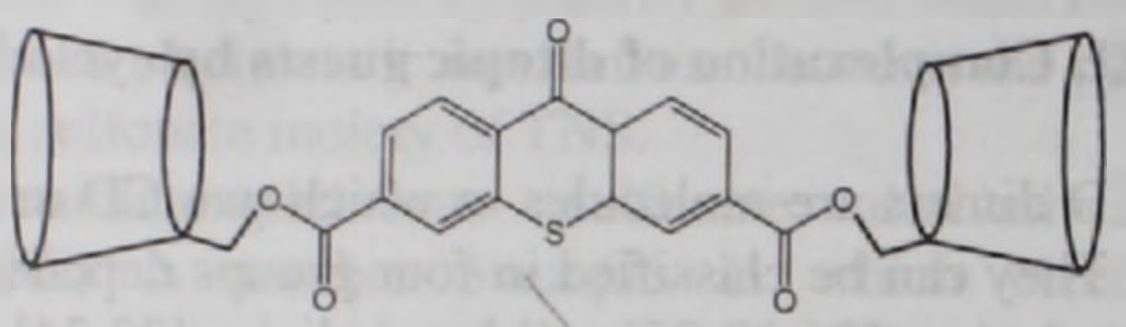
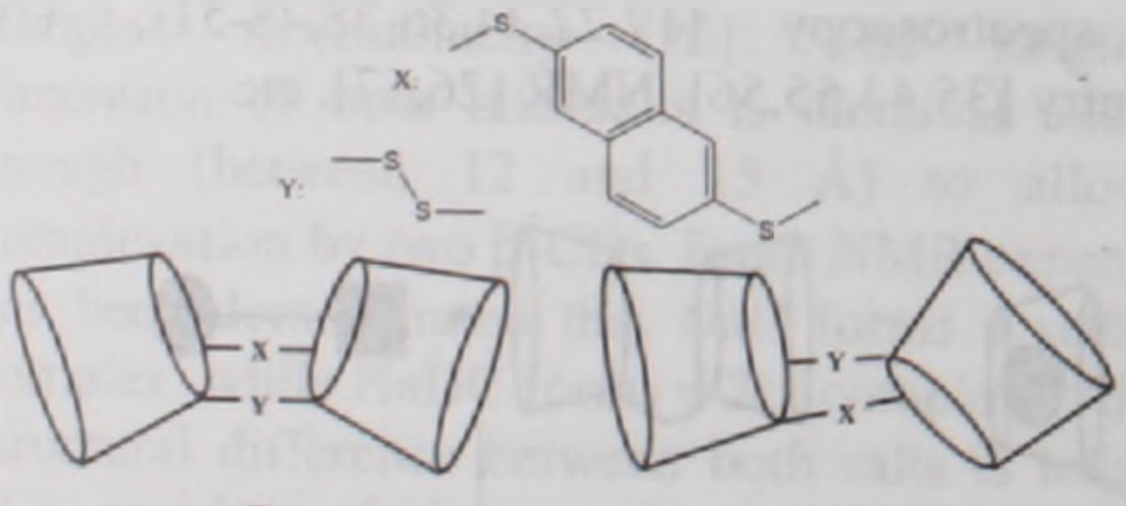
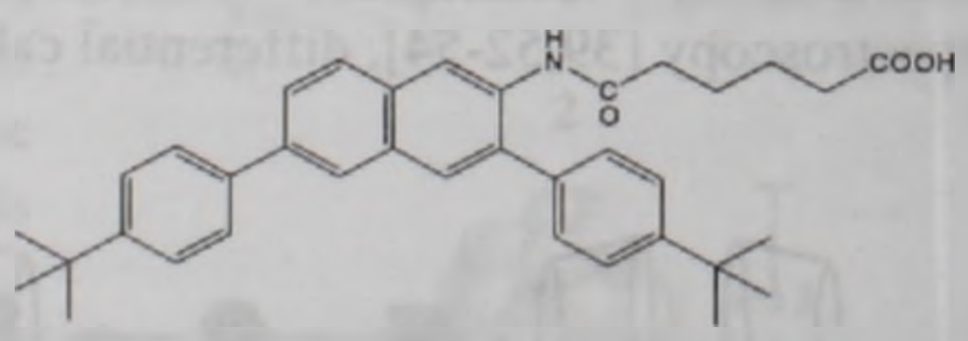


6

In order to study the stereochemical aspects of the complexation process, Breslow *et al* synthesized double linked dimers **7** and **8**, and studied their complexation behavior with the ditopic guest **9**, observing that with the occlusive or clamshell dimer **7** the ditopic guest is cooperatively complexed by both CDs ($K_{11} > 4 \times 10^{11}$ M^{-1}), while with the aversive or loveseat dimer **8**, no cooperative complexation was observed. In further studies these authors found that the binding of ditopic guests by CD dimers in water solution is regulated by enthalpic advantages, instead of the initially considered translational entropy process, that in fact was unfavorable [37,44,61].



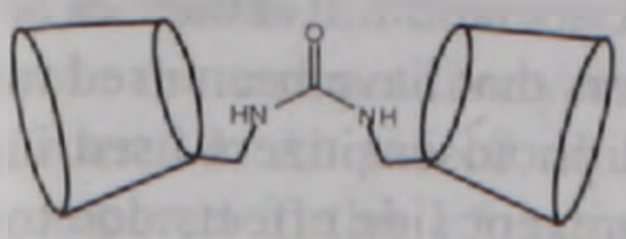
7 **8**



9

The complexation of TNS **1** by head to head and tail to tail CD homo- and heterodimers has been systematically study to determine their complexation properties [22,34,36,38,49,50,62,63]. From these results, it is possible to conclude that a cooperative effect is observed, and that this effect decreases when the linker length exceeds an optimum value. For instance, the stability constant for the 1:1 complex formed between TNS and dimers **10** and **11** reduces from 45700 to 9300 as the number of methylene groups increases, being in all cases higher than that for β-CD (K_{11} 3140 M^{-1} and K_{21} 86 M^{-1}) [22]. When TNS was complexed by the heterodimer **12**, a negative effect was observed, since there is a

competitive complexation process between TNS and the linker that is partially included inside the α-CD, which reduces the effective binding ability of dimer **12** [34].



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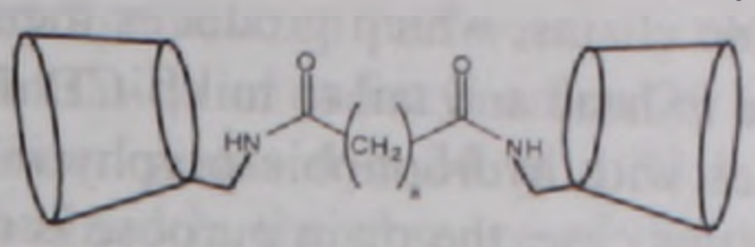

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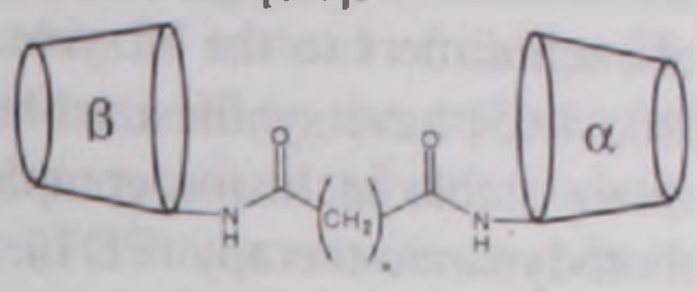

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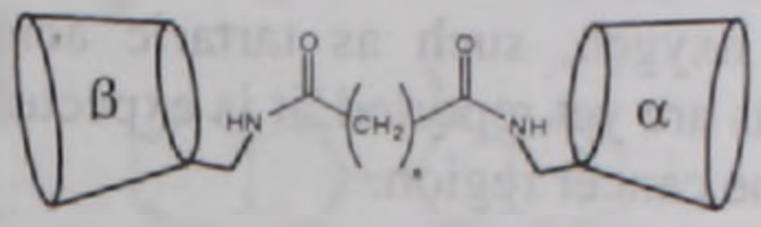

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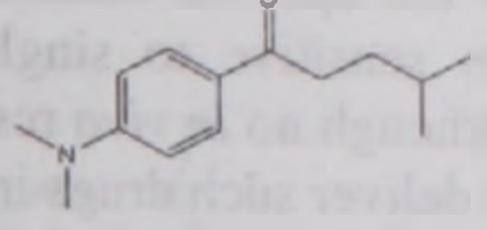
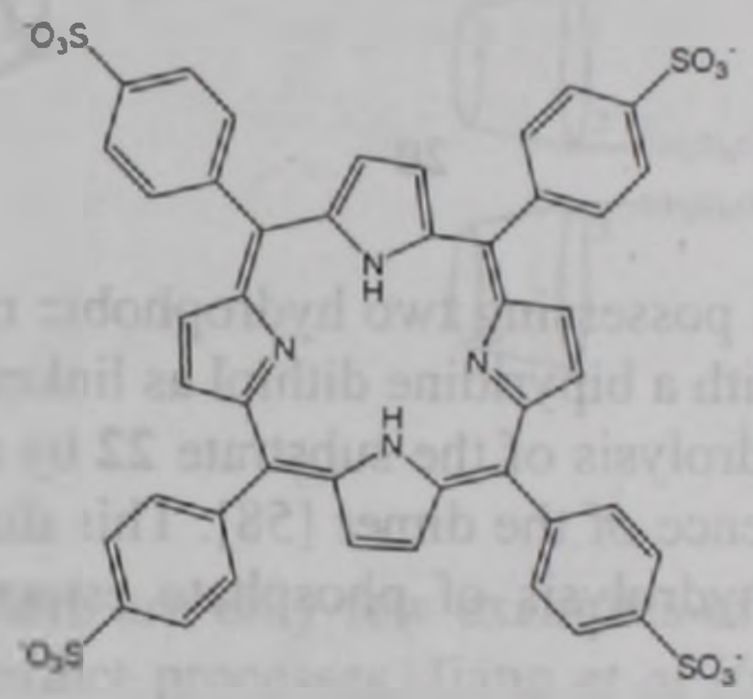


14

The effect of the cavity size of both CDs was studied by Toda *et al* [48]. They observed that the heterodimer **13** showed a cooperative and site-specific binding to DMBA **14**, in which the aromatic moiety is partially included inside of the α-CD cavity, while the alkyl group is included into the β-CD cavity.



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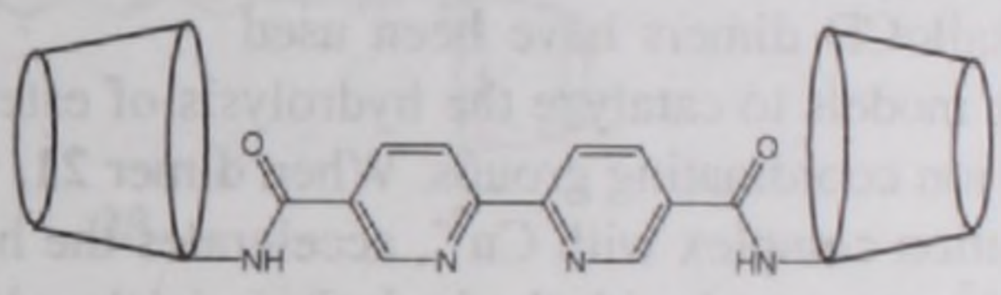
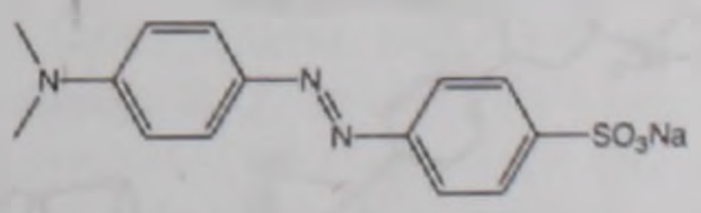


16

When the porphyrin derivative **15** was complexed by dimer **16** a final 2:2 complex was formed, i.e. two porphyrins are complexed by two CD dimers. The process is facilitated by the presence of 0.5 equivalents of Zn^{2+} due to the formation of a tetrahedral metal ion complex with the bipyridine units of both dimers [34].

Other ditopic guests such as methyl orange **17** [36, 49, 50, 52, 53], BNS **18** [35, 50], NaC **2a** [56], tropaeolin (0) **19** [50, 52] among others, have been studied. From all these results, it is possible to conclude that the chelate effect depends on the length and flexibility of the linker, the CD cavity size and orientation, and specific host-guest interactions.



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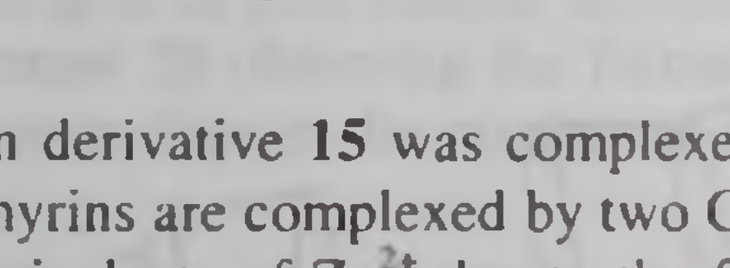

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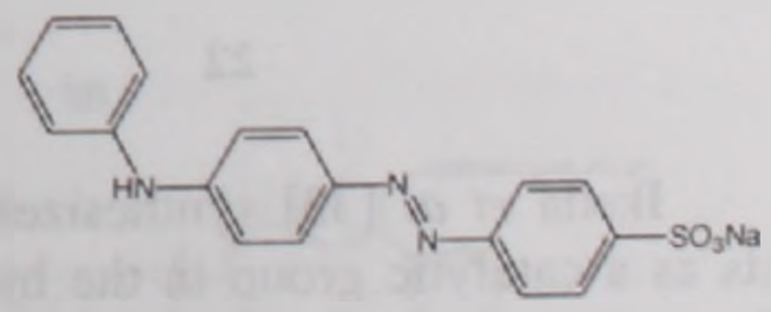
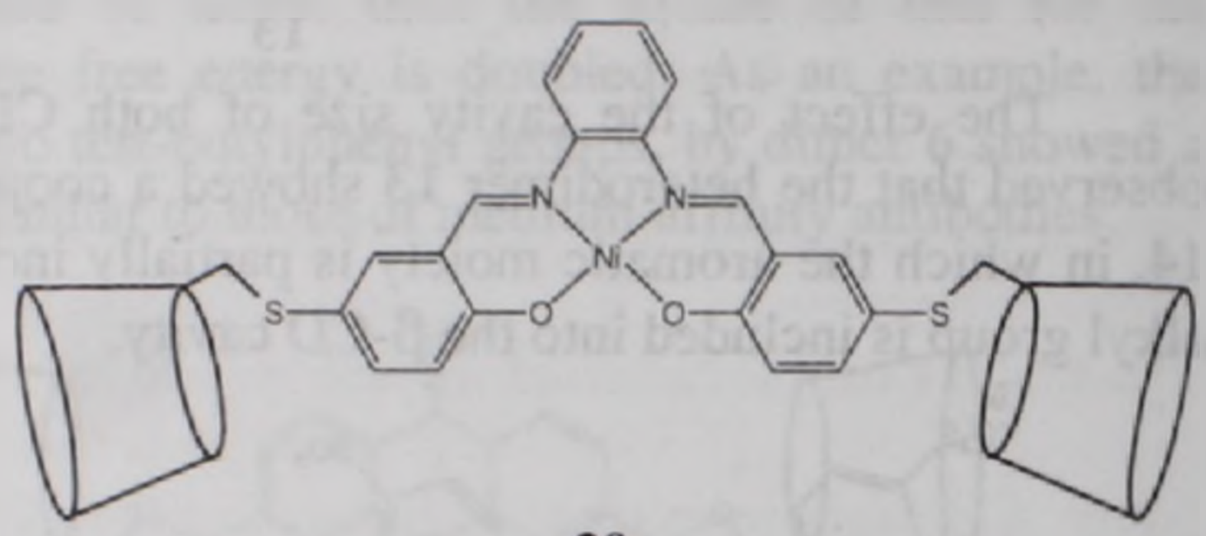


19

This chelate effect of CD dimers can be exploited in order to use them in different chemical and biological

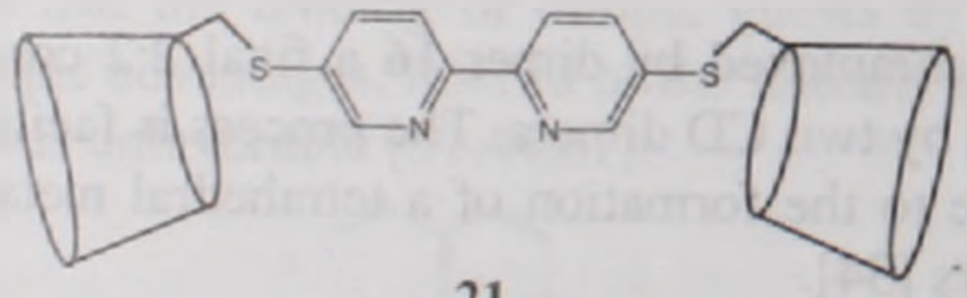
applications. Breslow *et al* [61] found that some CD dimers can selectively inhibit dimeric and tetrameric enzymes as citrate synthase and lactic dehydrogenase, because of the binding of such dimers to the enzyme side chains, which produces their dissociation. Ruebner *et al* [40,64,65] have synthesized head to head and tail to tail β-CD dimers that have been used to obtain stable inclusion complexes with hydrophobic porphyrinoid photosensitizers used in photodynamic therapy (PDT). In this case, the main purpose is to prevent side effects due to unwanted targeting to organs others from the tumor. The optimal spacer length for each porphyrinoid derivative was established, the stability constants values being in the range 10^4 - 10^6 M^{-1}. For the specific release of the included drug at the tumor site, dimers with breaking points sensitive to singlet oxygen, such as tartaric acid moiety, were also synthesized. Although no *in vivo* results are yet reported, it is expected that these CD dimers will selectively deliver such drugs in the cancer region.

Maletic *et al* [66] reported the use of the orange-colored Nickel dimer **20** to selectively binding tripeptids containing either L-Phe-D-Pro or D-Phe-LPro, from a maximum of 24389 different tripeptides. These authors proposed that this selectivity could be exploited for the design of novel sequence-specific peptide-cleaving catalysts.



20

MetalloCD dimers have been used as enzyme models to catalyze the hydrolysis of esters possessing two hydrophobic moieties and metal ion coordinating groups. When dimer **21**, with a bipyridine dithiol as linker, forms a coordination complex with Cu^{2+}, accelerates the hydrolysis of the substrate **22** by a factor of 2.25×10^3, compared with the hydrolysis in the absence of the dimer [58]. This dimer has also shown a really good catalyst effect over the hydrolysis of phosphate esters in the presence of La^{3+} and H_2O_2.



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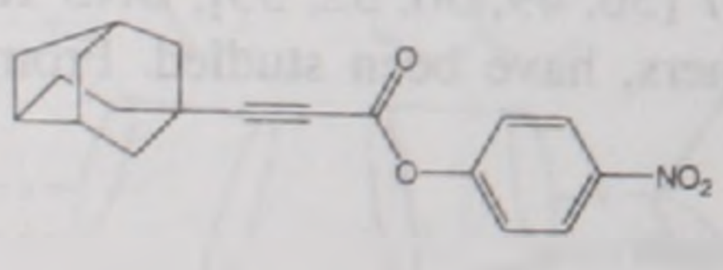

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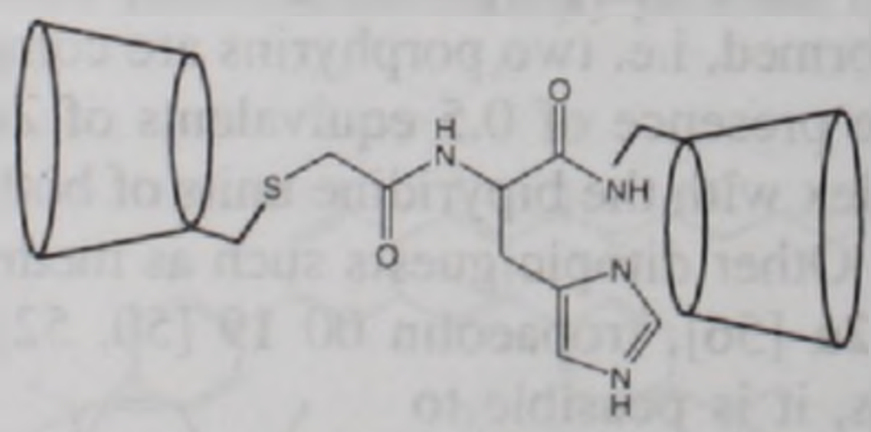
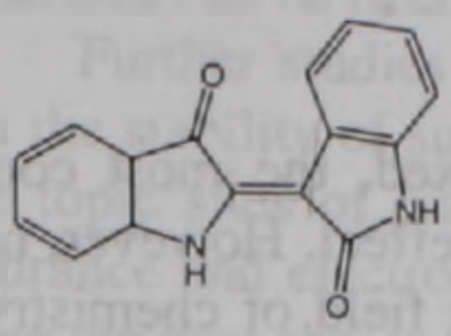


23

Ikeda *et al* [33] synthesized dimer **23** possessing a imidazole-appended group, that acts as a catalytic group in the hydrolysis of nitrophenyl alkanoates, obeying a Michaelis-Menten mechanism. No catalytic effect was observed when one β-CD in the dimer was substituted by α-CD.

An interesting use of CD dimers has been proposed by Easton *et al* [39] for the selectively synthesis of non-linear ditopic molecules from reagents complexed in both CD's of the dimer. In this way, they synthesized preferentially indirubin **24** instead of indigo **25**, from the competitively condensation of indoxyl anion **26** and isatin **27** in the presence of dimer **10**. At pH 10, this dimer yields dye **24** in a 33:1 ratio, which is advantageously compared to the 1:1 ratio obtained in the absence of the dimer. Similar results have been published by Ikeda *et al* [67] for the benzoin condensation reaction from two molecules of benzaldehyde.



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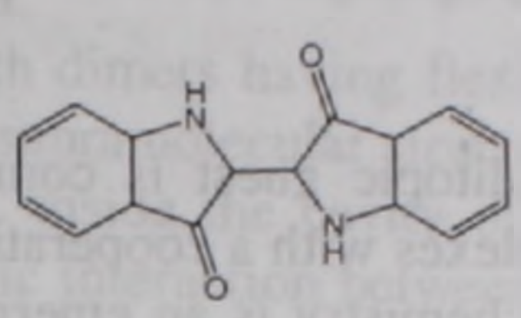

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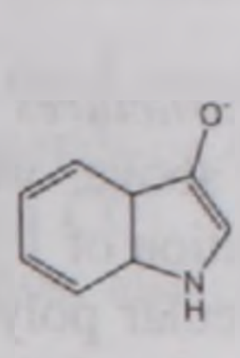

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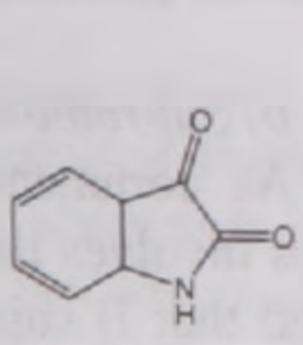

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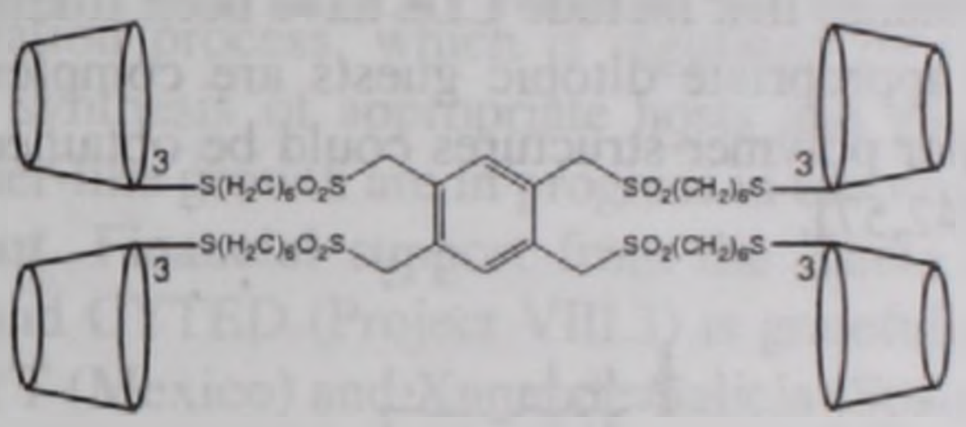
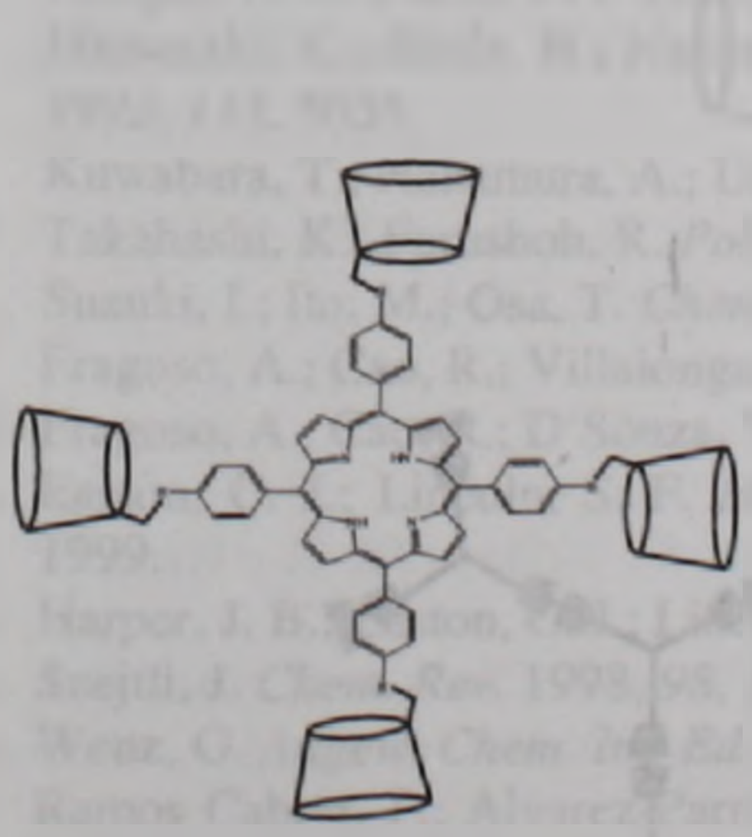


28

There are only few examples of the use of higher degree CD derivatives as hosts in chelate effect processes. Jiang *et al* [68] studied the complexation of tetraporphyrines and metalloporphyrines by tetramer **28** observing the formation of a 1:1 complex, with a K_{11} value as high as 10^8 M^{-1}, even though the number of aryl derivatives complexed is still unknown.



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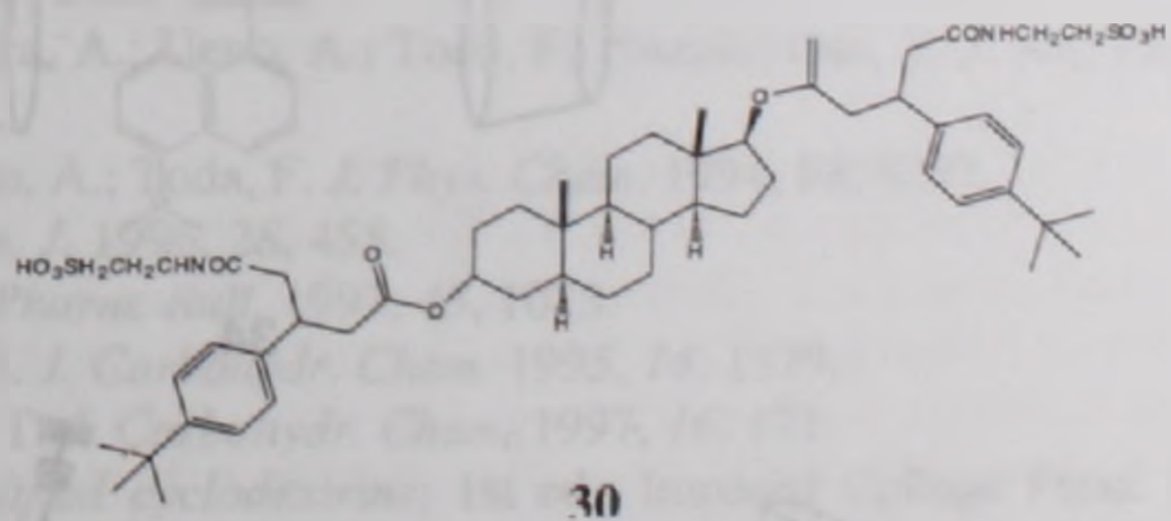

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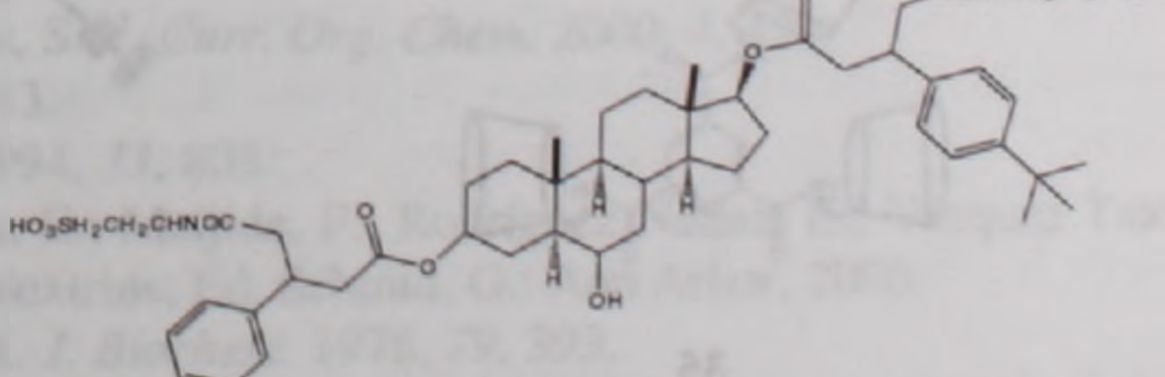
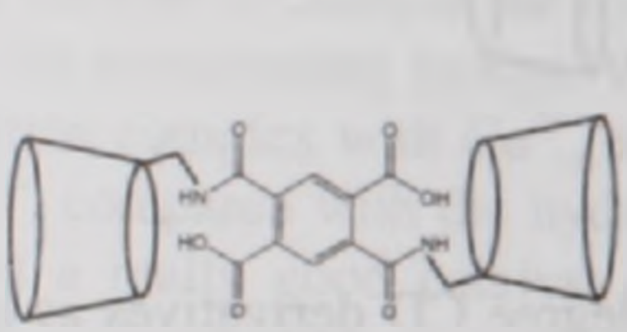


31

Quite interesting was the study of the regioselectivity of artificial enzymes, through chelate binding by CD derivatives carried out by Breslow *et al* [60,61]. The tetraphenylporphyrin tetramer **29** was synthesized, and its Mn(III) complex was used as a mimetic catalyst of cytocrom P-450 for the hydroxylation of saturated carbons. This tetramer complexed steroid **30** by two opposite CDs, placing the steroid body above the Mn(III) complex. When treated with iodosobenzene the hydroxylation of C-6 saturated carbon results in quantitative yield, with stereoselectivity toward the 6α-hydroxysteroid **31**. Further studies confirmed that this regioselectivity permitted to hydroxylate unactivated carbons even in the presence of olefinic and hydroxyl groups, which are more easily oxidized.

b) Supramolecular structures.

As commented on above, when a ditopic guest is complexed, the most common process involves the formation of 1:1 complexes with a cooperative effect. However, having in mind that i) supramolecular polymeric chemistry is an emerging field of chemistry that combines the polymeric chemistry with supramolecular chemistry to design "living" polymers by the self-assembly of complementary monomeric components through molecular interactions and recognition processes [69]; and ii) that no examples others than polyrotaxanes and polycatenanes that include CDs have been studied [70], we have proposed the hypothesis that when appropriate ditopic guests are complexed by head to head CD dimers linear supramolecular polymer structures could be obtained by the way of inclusion complex formation [23,26,42,57].



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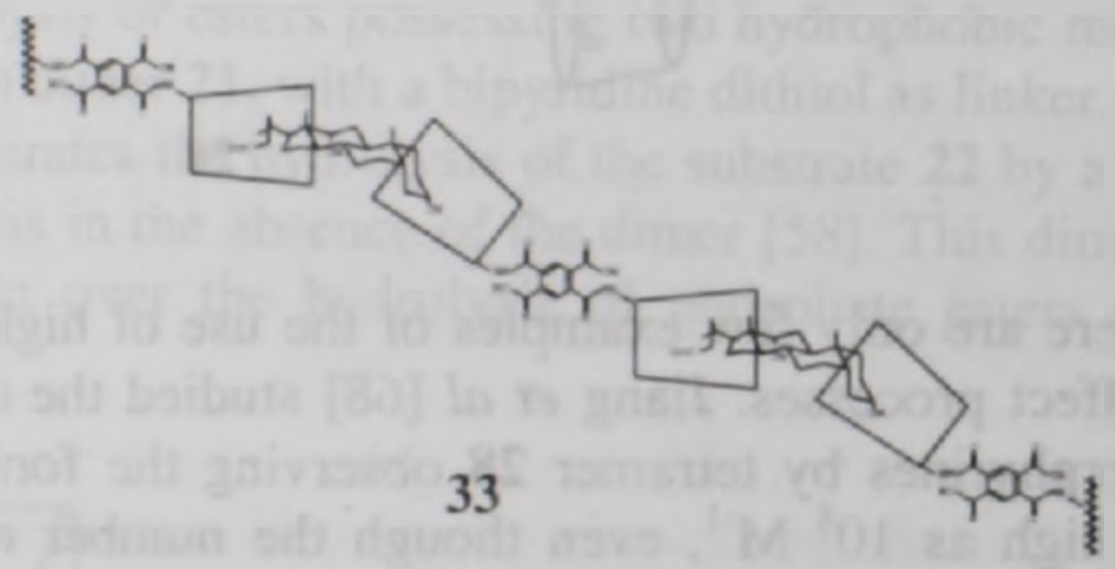

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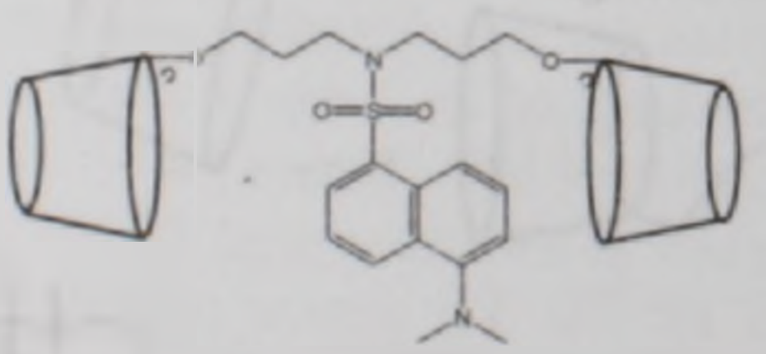

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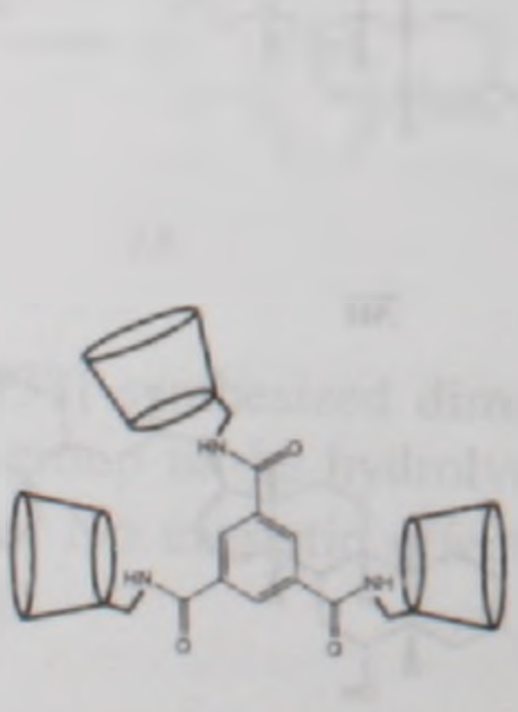

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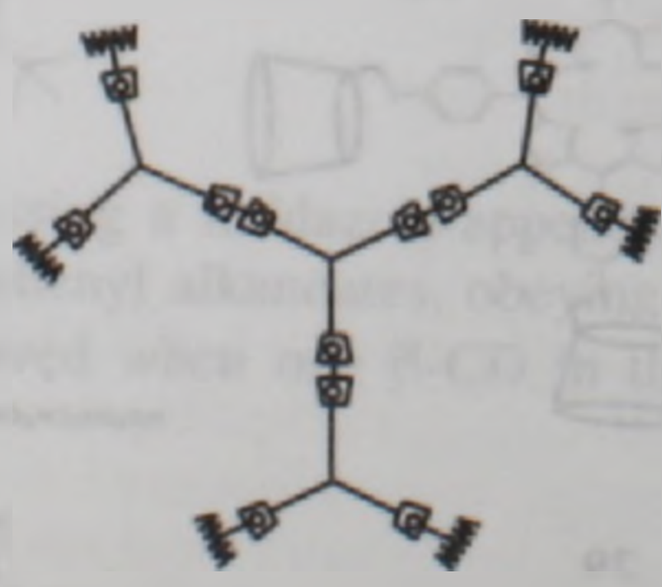

36

In order to prove such a hypothesis, sodium deoxycholate NaDC **2b** was used as ditopic guest, since previous studies showed that this molecule forms 2:1 complexes with β-CD and two amino derivatives through their secondary side [23,26,42,57,71]. As expected, when NaDC was complexed by head to head dimer **32** a linear supramolecular polymer **33** with a n:n stoichiometry was obtained [26]. On the other hand, Huskens, Reinhouldt *et al* [56] observed the formation of a 1:1 complex with chelate effect when NaDC was complexed by tail to tail dimer **34**. From these results, it is possible to conclude that depending on the geometry of both host and guest, either chelate effect or supramolecular structures can be obtained.

Further studies with dimers having flexible or rigid amine or amide linkers, showed that the stability of such supramolecular structures depends on the penetration degree of the two topic sites of NaDC inside the cavity of the two CDs, which is controlled by steric hindrance and electrostatic interaction between both host and guest, a flexible amide linker being the best choice [23,42].

When NaDC was complexed by trimer **35** a dendrimer-like supramolecular polymer **36** was obtained by the way of self-assembly of both structural units. By electron microscopy it was possible to observe different fractal structures that depend on the number of CDs involve in the complexation process, which is regulated by steric hindrance [72]. At the moment studies on the synthesis of appropriate hosts and guests in order to improve the supramolecular dendrimer-like growth are in progress in our research group.

Acknowledgement. Financial support from the Xunta de Galicia (Project XUGA PGIDT99PXI26201B) and CYTED (Project VIII.3) is gratefully acknowledged. E.A.P. and P.R.C. thanks CONACYT (Mexico) and Xunta de Galicia (Spain) for a research scholarship.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRIN INCLUSION COMPLEXES OF THE ANTIHYPERLIPIDEMIC DRUG CLOFIBRIC ACID

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The products of the inclusion of clofibric acid, an antihyperlipidemic, in β-CD, γ-CD, heptakis(2,6-di-O-methyl)-β-CD (DIMEB) and heptakis(2,3,6-tri-O-methyl)-β-CD (TRIMEB), were investigated by thermal analysis and single crystal X-ray diffraction. All complexes have 1:1 host-guest stoichiometry and contain varying amounts of water of crystallization. Thermogravimetric and differential scanning calorimetric traces indicated dehydration as the first thermal event. The TRIMEB complex was the most stable in that it displayed a fusion endotherm while the other complexes decomposed on heating. Single crystal X-ray diffraction revealed that both the β- and γ-cyclodextrin complexes crystallize with the host molecules arranged in channel-mode (space groups C2 and $P42_12$ respectively), but extensive disorder of the guest molecules prevented their modelling. The DIMEB and TRIMEB complexes are orthorhombic (space group $P2_12_12_1$), the former crystallizing in a novel packing arrangement. The clofibric acid molecule is partially disordered when included in DIMEB and is oriented with the chlorine atom at the host primary rim and the carboxylic acid group at the secondary rim. In contrast, the guest is ordered in the TRIMEB complex and it assumes the opposite orientation. Furthermore, inclusion of the guest carboxylic acid group within the TRIMEB cavity is mediated by bridging water molecules.

Методами термального анализа и диффракции рентгеновскими лучами единичных кристаллов изучены продукты включения клофибрилловой кислоты-антигиперлипидемика, в β-ЦД, γ-ЦД, гептакис(2,6-ди-О-метил)-β-ЦД (ДИМЕБ) и гептакис(2,3,6-три-О-метил)-β-ЦД (ТРИМЕБ). Все комплексы имеют 1:1 "гость-хозяин" стехиометрию и содержат разные количества кристаллизационной воды. Термогравиметрические и дифференциально сканированные колориметрические следы указывают, что дегидратация является первым следствием термального воздействия. В этом плане самым стабильным был ТРИМЕБ комплекс, проявляя фузию эндотерм, в то времия как другие комплексы разрушались при нагревании. Методом диффракции рентгеновскими лучами единичных кристаллов выявлено, что как β-, так и γ - циклодекстриновые комплексы кристаллизуются с молекулами "хозяина", располагаясь в виде канала (пространственные группы C2 и $P42_12$ соответственно), однако их моделирование предотвращается выраженным беспорядком молекул "гостя". Комплексы ДИМЕБ и ТРИМЕБ являются орторомбическими (пространственная группа $P2_12_12_1$), предыдущий кристаллизуясь в новом упакованном порядке. Молекула клофибрилловой кислоты частично нарушается, когда включается в ДИМЕБ, и с атомом хлора ориентируется в первичном кольце "хозяина", а группой карбоксиловой кислоты в вторичном кольце. В противоположность этому, в ТРИМЕБ комплексе, "гость" определяет и принимает противоположеную ориентацию. Кроме того, включение группы карбоксиловой кислоты "гостя" в полость ТРИМЕБ опосредовано мостиками молекул воды.

Թերմալ անալիզի և եզակի բյուրեղների ռենտգենյան ճառագայթների դեֆրակցիայի մեթոդներով ուսումնասիրվել են կլոֆիբրիլաթթվի հակահիպերլիպիդեմիկի, ներառման նյութերը β-ՑԴ-ի, γ-ՑԴ-ի, հեպտակիս (2,6-դի-Օ-մեթիլ)-β-ՑԴ (ԴԻՄԵԲ)-ի և հեպտակիս (2,3,6-տրի-Օ-մեթիլ)-β-ՑԴ (ՏՐԻՄԵԲ)-ի մեջ : Բոլոր համալիրները ունեն 1:1 «տեր-հյուր» ստեխիոմետրիա և պարունակում են բյուրեղացած ջրի տարբեր քանակներ: Թերմոգրավիմետրիկ և դիֆերենցիալ սկանող կոլորիմետրիկ հետքերը ցույց են տալիս, որ դեհիդրատացիան համարվում է թերմալ

ազդեցության հետևանքը: Այս դեպքում ամենակայունը եղել է ՏՐԻՄԵԲ համալիրը, ցուցաբերելով էնդոթերմի ֆուգիա, այն դեպքում երբ մյուս համալիրները քայքայվել են տաքացնելիս: Եզակի բյուրեղների ռենտգենյան ճառագայթների դեֆրակցիայի մեթոդով բացահայտվել է, որ ինչպես β-, այնպես էլ γ-ցիկլոդեքստրինային համալիրները բյուրեղանում են «տիրոջ» մոլեկուլների հետ, տեղավորվելով նեղուցի տեսքով (համապատասխանաբար C2 և P 42₁2 տարածական խմբերը), իսկ նրանց մոդելավորումը կանխվում է «հյուրի» մոլեկուլների լայնատարած անկարգավորվածությամբ: ԴԻՄԵԲ և ՏՐԻՄԵԲ համալիրները օրթոռոմբաձև են ($P2_12_12_1$ տարածական խումբը), նախապես բյուրեղանալով նոր փաթեթավորված տեսքով: Կլոֆիբրիլաթթուն մասնակիորեն քայքայվում է, երբ ներառվում է ԴԻՄԵԲ-ի մեջ և բոլոր ատոմով կողմնորոշվում է «տիրոջ» առաջնային օղակի, իսկ կարբօքսիլաթթվի խմբով երկրորդային օղակի նկատմամբ: Հակառակ դեպքում, ՏՐԻՄԵԲ համալիրում, «հյուրն» է որոշում և ընդունում է հակառակ կողմնորոշում: Բացի այդ «հյուրի» կարբօքսիլաթթվի խմբի ներառումը ՏՐԻՄԵԲ-ի խոռոչի մեջ կատարվում է ջրի մոլեկուլների կամրջակների միջոցով:

Introduction

The manifold advantages of inclusion of poorly soluble drug molecules in cyclodextrins (CDs) is well documented [1,2]. Our studies in this area have focused on the preparation of crystalline CD-drug complexes and their physicochemical characterization by thermal and X-ray diffraction methods. Together, these techniques provide unequivocal proof of the authenticity of CD inclusion complexes and yield detailed information on the mode of drug inclusion in the CD cavity [3]. Representative drugs whose CD complexes have been characterised in this laboratory include ibuprofen [4], naproxen [5], diclofenac sodium [6], acetaminophen [7] and (L)-menthol [8].

Here we report results obtained by thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), UV and infrared spectroscopy, and single crystal X-ray diffraction for the inclusion complexes of the antihyperlipidemic clofibric acid with β-CD, γ-CD, heptakis(2,6-di-O-methyl)-β-CD (DIMEB) and heptakis(2,3,6-tri-O-methyl)-β-CD (TRIMEB). The guest clofibric acid [2-(4-chlorophenoxy)-2-methyl propionic acid], shown in Figure 1, is known to reduce triglyceride and cholesterol concentration in the serum [9]. It is poorly soluble in aqueous media, rendering it a good candidate for solubilization by CDs. The selection of clofibric acid as a target for CD inclusion was also prompted by the fact that it is the active metabolite resulting from *in vivo* hydrolysis of its ethyl ester, clofibrate, for which studies of CD-inclusion have already been reported [10,11].

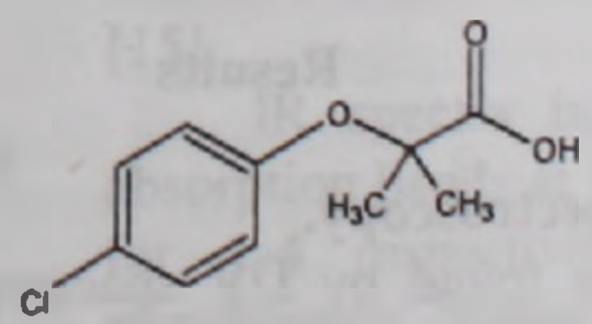

Figure 1. Chemical structure of clofibric acid.

A novel feature of the present study is the X-ray structural elucidation of four distinct crystalline CD complexes of the same drug molecule, revealing a variety of modes of inclusion ranging over extensive guest disorder in β-CD and γ-CD, partial order in DIMEB

and an ordered arrangement in TRIMEB. In the latter case, water molecules are shown to play a definitive role in the inclusion process.

Materials and Methods

Clofibric acid was purchased from Sigma Chemical Co. (Missouri, USA). The cyclodextrin host compounds β-CD, γ-CD, DIMEB and TRIMEB were supplied by Cyclolab (Budapest , Hungary).

The complexes of clofibric acid with β-CD and γ-CD were prepared by addition of the solid guest to hot saturated aqueous solutions of the CDs (host-guest ratio 1:1) with stirring for 12h at 65°C. Slow evaporation of filtered solutions yielded the crystalline complexes. Complexes with the methylated CDs were prepared by addition of clofibric acid to saturated aqueous solutions of the CDs (host-guest ratio 1:1) at 20°C, followed by incubation of the solutions at 50°C.

Thermal analysis was performed on a Perkin Elmer PC7 Series system with sample masses in the range 3-5 mg and a heating rate of 10°Cmin^{-1} under a nitrogen gas purge of flow rate 30cm^3 min^{-1}. For TGA, samples were placed in open aluminium pans while vented pans were used for DSC runs.

Complex stoichiometries were ascertained from a combination of TGA (yielding the water content) and UV spectrophotometry (host-guest ratio). For the latter, absorbance measurements were recorded on a Philips PU8700 UV/vis spectrophotometer in the range 200-400nm.

IR spectra were recorded on a Perkin Elmer 983 IR spectrometer with samples in the form of Nujol mulls.

To confirm phase transitions on heating, powder X-ray diffraction (PXRD) traces of the methylated CD complexes at different stages of heating were recorded photographically with samples in capillary tubes. The specimens were irradiated with Ni-filtered CuKα-radiation (λ = 1.5418Å).

Single crystals were examined for diffraction quality by X-ray photographic methods which also yielded unit cell and space group data. Intensity measurements were performed on a Nonius Kappa CCD diffractometer at 293(2)K with graphite-monochromated MoKα-radiation (λ = 0.71069Å) using a combination of φ- and ω-scans (0.5° for the DIMEB complex and 1° for each of the others). Detector to crystal distances were in the range 45-50mm and exposure times in the range 160-243s. Data-reduction was carried out with the program DENZO-SMN [12]. The structures of the β-CD, γ-CD and TRIMEB complexes were solved by isomorphous replacement using atomic coordinates of the rigid fragments of CD hosts from previous structure determinations [6,8]. The structure of the DIMEB complex was solved by the Patterson search method using program PATSEE [13]. Guest and water molecules were located from difference electron density maps. Full-matrix least-squares refinements based on F^2 with program SHELXL-93 [14] included mixed modes of isotropic and anisotropic thermal treatment and inclusion of H atoms in idealized positions. Least-squares weights of the form $w = 1/[\sigma^2(F_o)^2 + (aP)^2 + bP]$ were employed, with $P = [\max(F_o^2, 0) + 2F_c^2]/3$.

Results

Thermal analysis and spectroscopy.

All four complexes were found by UV spectrophotometry to have 1:1 host-guest stoichiometry. In Table 1, TG-DSC data are listed for the complexes whose compositions are as follows:

1 (host β-CD): $C_{42}H_{70}O_{35}\cdot C_{10}H_{11}O_3Cl\cdot 8.4H_2O$,
2 (host γ-CD): $C_{48}H_{80}O_{40}\cdot C_{10}H_{11}O_3Cl\cdot 15.5H_2O$,
3 (host DIMEB): $C_{56}H_{98}O_{35}\cdot C_{10}H_{11}O_3Cl\cdot 5.4H_2O$,
4 (host TRIMEB): $C_{63}H_{112}O_{35}\cdot C_{10}H_{11}O_3Cl\cdot 1.4H_2O$.

Table 1. TG-DSC data for the CD inclusion complexes of clofibric acid.

Complex	Event (DSC)	Onset (t/°C)	Peak (t/°C)	% H_2O (TGA)
1	Endo A	48	66	10.1
	Endo B	104	110	
	Exo C	302	309	
2	Endo A	50	75	15.6
	Exo B	301	309	
3	Endo A	54	76	5.9
	Endo B	149	151	
	Endo C	201	207	
4	Endo A	30	45	1.5
	Endo B	129	132	
	Endo C	145	147	

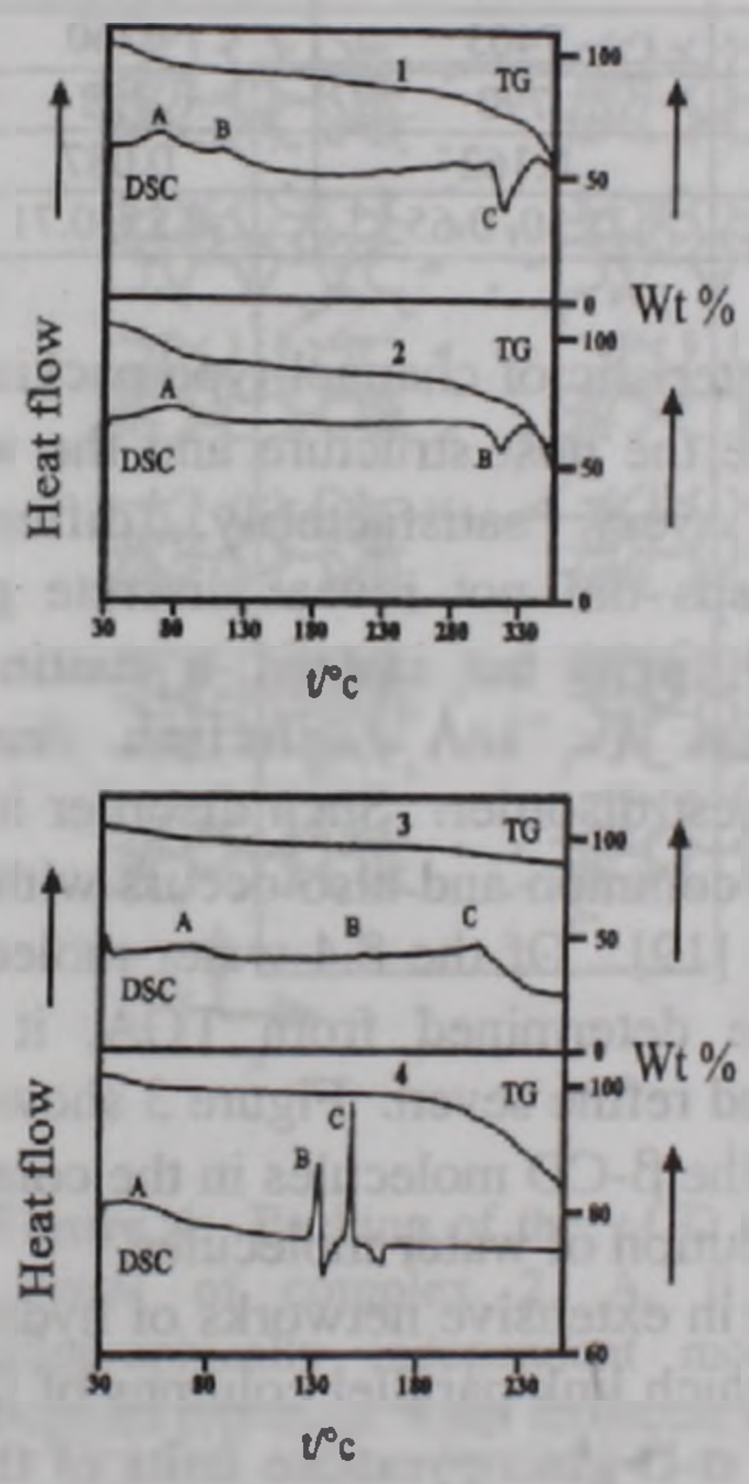

Figure 2. TG and DSC traces for CD complexes of clofibric acid.

Figure 2 shows the combined TG/DSC traces for the complexes. Those for complexes **1**, **2** and **4** were discussed elsewhere recently with an emphasis on dehydration aspects [15] and only a brief summary is therefore given here. For completeness, the traces for complex **3** are included in this report. Dehydration is a two-step process for the β-CD complex (endo A, endo B, Table 1) and one-step for the other complexes. The TG traces for **1** and **2** indicate gradual weight loss after dehydration and major decomposition (exothermic from DSC) following at around 300°C. In contrast, complexes of the methylated CDs, **3** and **4**, are thermally more stable, as indicated by the relatively small weight loss in TG following dehydration (endo A). Complex **3** undergoes a phase transition (endo B) followed by decomposition (endo C). The TRIMEB complex undergoes an endothermic phase transition (endo B) followed by exothermic recrystallization, and finally melting (endo C). The presence of a fusion endotherm with zero weight loss in TG is characteristic of TRIMEB complexes [15].

IR spectra in the carbonyl region yielded absorption bands at 1730, 1729, 1734 and 1726cm^{-1} for **1**-**4** respectively. These frequencies are significantly higher than the value of 1706cm^{-1} reported for pure clofibric acid, indicating stronger C=O bonds in the complexed materials. The magnitudes of the shifts in ν_{CO} are similar to those reported by Wei *et al.* for CD-tolbutamide complexes [16].

Single crystal X-ray analyses.

Crystallographic data for the complexes and details of structure refinements are listed in Table 2.

Table 2. Crystal Data and Structural Refinement Details for CD complexes of Clofibric Acid.

Parameter	Complex 1	Complex 2	Complex 3	Complex 4
Host	β-CD	γ-CD	DIMEB	TRIMEB
Complex M/gmol^{-1}	1501.0	1791.0	1643.3	1669.4
Crystal system	Monoclinic	Tetragonal	Orthorhombic	Orthorhombic
Space group	C2	$P4_12$	$P2_12_12_1$	$P2_12_12_1$
a/Å	18.818(6)	23.647(9)	10.783(5)	11.601(3)
b/Å	24.476(8)	23.647(9)	15.338(6)	26.284(7)
c/Å	15.761(5)	23.064(9)	49.39(1)	28.882(6)
α/°	90	90	90	90
β/°	110.43(1)	90	90	90
γ/°	90	90	90	90
V/ Å^3	6803	12897	8167	8807
Z	4	6	4	4
Crystal size/mm	0.2 x 0.3 x 0.3	0.3 x 0.4 x 0.3	0.3 x 0.4 x 0.3	0.2 x 0.3 x 0.3
Refinement data	12828	11207	7492	41775
Unique data	12631	10960	7477	21770
Data with $I>2\sigma(I)$	9347	6590	7463	6760
L.S. parameters	546	634	790	758
R (on F)	0.121	0.101	0.162	0.087
Δρ min, max. /e Å^{-3}	-0.52, 1.05	-0.58, 0.64	-1.30, 0.65	-0.85, 0.71

The unit cell and space group data for **1** are characteristic of channel-type packing of dimeric units in β-CD complex crystals [17,18]. While the host structure and the water molecules refined very satisfactorily, difference electron density maps did not reveal discrete guest atoms in the β-CD cavity but instead, a continuous distribution of weak (< 1eÅ^{-3}) electron density, indicating severe guest disorder. Such disorder in the channel of β-CD is common and also occurs with e.g. the guest ibuprofen [19]. Of the 8.4 water molecules per β-CD molecule determined from TGA, it was possible to locate and refine seven. Figure 3 shows the channel packing of the β-CD molecules in the complex as well as the distribution of water molecules.

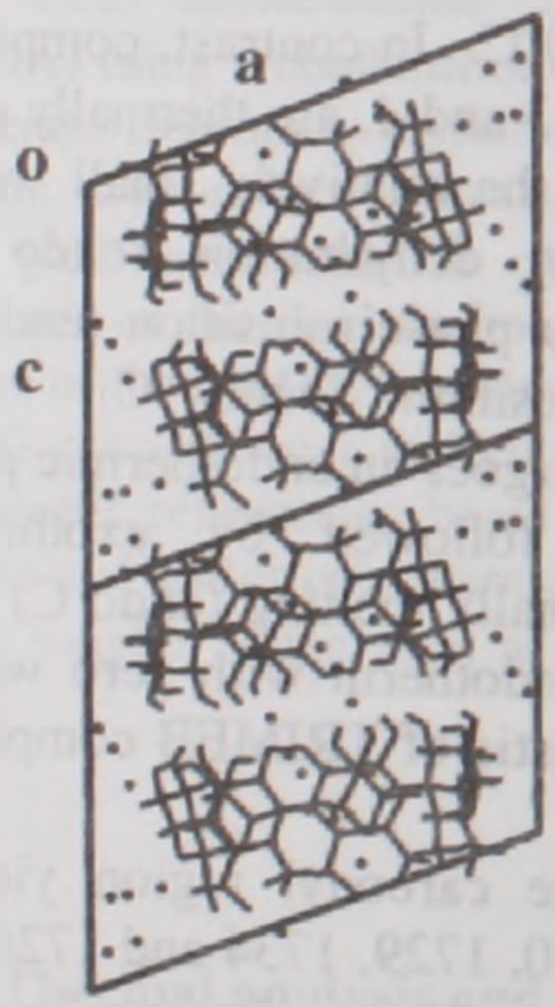

Figure 3. Packing of the β-CD molecules in the crystal of complex **1**. Small circles represent water molecule sites.

These engage in extensive networks of hydrogen bond interactions which link parallel columns of β-CD dimeric units. The α-D-glucopyranose units of the β-CD molecule adopt the usual $^4C_1$ chair conformation. One primary hydroxyl group is in a (-)-*gauche* conformation and the others are in a (+)-*gauche* conformation. The 'round' shape of the macrocycle is maintained by intramolecular $O3G_n\cdots O2G_{n+1}$ hydrogen bonds [17] with O···O distances in the range 2.75(1)-2.91(1)Å. Other standard geometrical data, with their observed ranges, include the glycosidic oxygen angles $C4G_n$-$O4G_n$-$C1G_{n+1}$ (117.9(4)-119.0(4)°), torsion angle index (113.5-125.5°), tilt angles (0.6(2)-8.0(2)°), heptagon radii (4.94(1)-5.22(1)Å) and $O4Gn\cdots O4G_{n+1}$ distances (4.32(1)-4.47(1)Å). The values of these parameters indicate that there are no unusual host distortions as a result of complexation with

clofibric acid. In this structural arrangement, the β-CD molecules form dimers with twofold crystallographic symmetry through multiple O-H···O hydrogen bonds across their secondary faces ($O3G_n$···$O3G_{8-n}$, 2.78(1)-2.92(1)Å). Each unit cell in Figure 3 shows such a dimer. On average each β-CD dimer accommodates two molecules of clofibric acid but guest disorder prevents description of the mode of inclusion. From the IR data, however, we conclude that the included clofibric acid molecules are unlikely to form carboxylic acid dimers through hydrogen bonding as they do in the pure solid drug.

Crystals of complex **2** have the tetragonal symmetry (Table 2) common to all γ-CD complexes [18]. The crystal packing is of the channel-type based on stacking of three γ-CD molecules in head-to-head, tail-to-tail and head-to-tail arrangements along the *c*-axis (Figure 4).

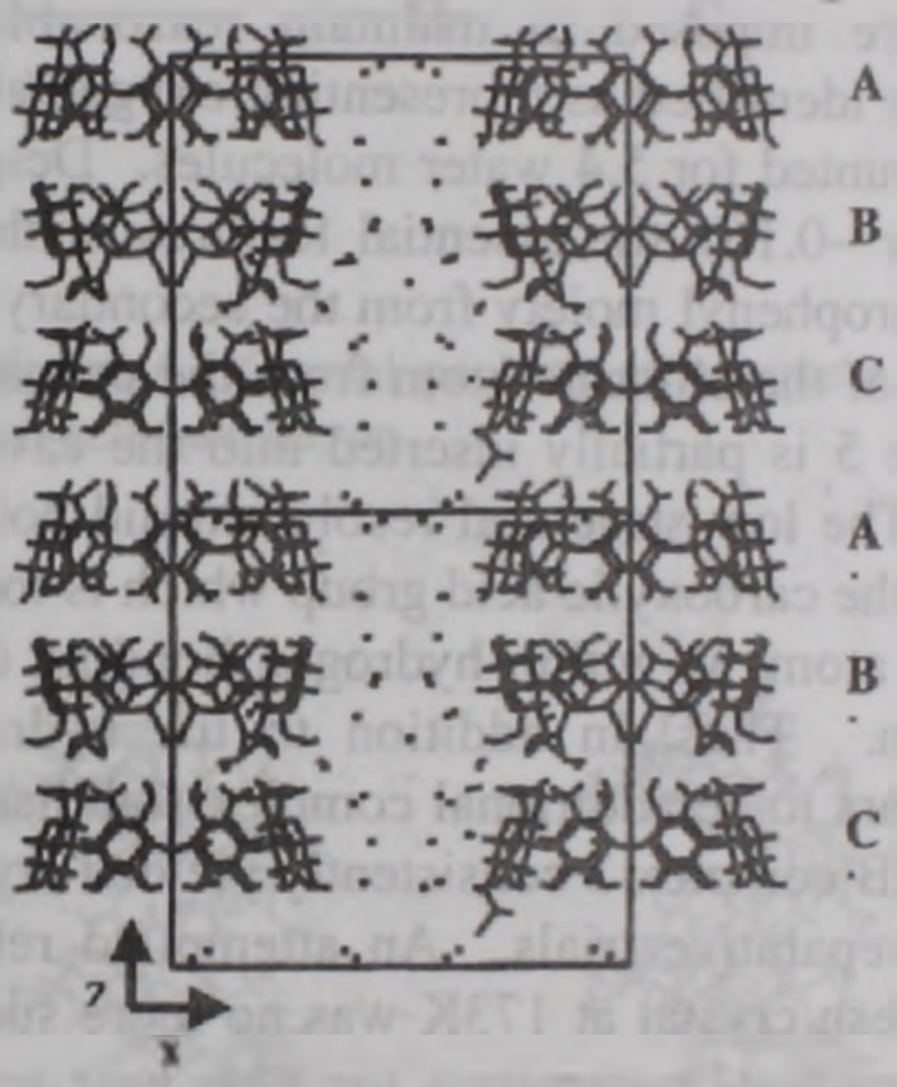

Figure 4. Packing of the γ-CD molecules in the crystal of complex **2**. A, B, C denote crystallographically independent molecules and small circles represent water molecule sites.

A fourfold crystallographic axis runs through the centre of the γ-CD channel. Consequently, unless the included guest molecule has inherent fourfold symmetry, it will be disordered within the channel. While refinement of the host molecule and several water molecules of complex **2** proceeded satisfactorily, the guest clofibric acid molecule was only evident as a distribution of very weak electron density, analogous to the situation for the β-CD complex **1**. Of the 15.5 water molecules per γ-CD molecule expected from TGA measurements, only 6.3 discrete water molecules were accounted for in the final model, the remainder being disordered over a large number of sites. Most of the molecules located were outside the γ-CD cavity. The α-D-glucopyranose rings of the three independent γ-CD molecules are in the $^4C_1$ chair conformation. As for β-CD, the 'round' shape of the γ-CD macrocycle is maintained by intramolecular $O3G_n$···$O2G_{n+1}$ hydrogen bonds (range 2.77(1)-2.89(1)Å). Other parameters and their ranges include the glycosidic bond angles (115.0(4)-116.7(4)°), tilt angles (5.8(2)-15.6(3)°), the octagon radii (5.82(1)-5.90(1)Å) and $O4G_n$···$O4G_{n+1}$ distances (4.49(1)-4.50(1)Å). As for the β-CD complex **1**, the crystallographic and UV data prove that an inclusion complex forms between clofibric acid and γ-CD. The guest disorder in the case of this host results primarily from the high symmetry requirements of the space group and is exacerbated by the larger diameter of the γ-CD channel. We recently reported a study of the kinetics of dehydration of complexes **1** and **2** [15]. This showed that the process is diffusion-controlled, with activation energies significantly lower than those for the respective parent CD hydrates.

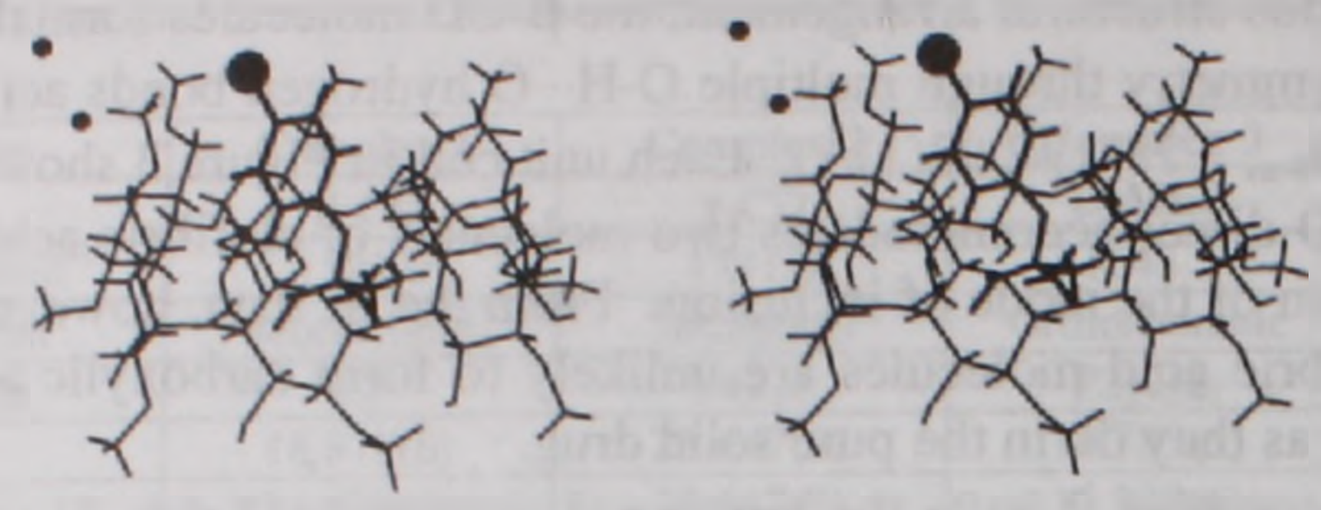

Figure 5. Stereoview of complex **3**. Water molecules are represented by small circles and the guest chlorine atom by a large circle.

Figure 5 is a stereoview of the complex **3**. Considerable difficulty was encountered during the refinement of this structure due to generally low electron densities observed for the guest atoms in the DIMEB cavity. In particular, the carboxylic acid group was poorly defined and extensive distance restraints were imposed to maintain reasonable guest geometry. In addition only two peaks could be identified as representing oxygen atoms of water molecules, whereas the TG analysis accounted for 5.4 water molecules. Despite the relatively poor quality of the analysis (R-factor ~0.16), the essential features of the guest inclusion are clear, namely insertion of the chlorophenyl moiety from the secondary side of the host DIMEB and the significant protrusion of the chlorine atom from the primary side. As detailed below, the chlorine atom in Figure 5 is partially inserted into the cavity of a DIMEB molecule directly above that shown. The low structural resolution did not permit distinction between the C=O and C-O bonds of the carboxylic acid group which is located at the host secondary rim, but one of the oxygen atoms is within hydrogen bonding distance (2.84(2)Å) of a host glycosidic oxygen atom. Thus, in addition to the hydrophobic interactions between host and guest, there appears to be additional complex stabilisation by direct host-guest hydrogen bonding. The DIMEB complex **3** consistently yielded crystals of mediocre diffraction quality despite several preparative trials. An attempt to refine the structure with intensity data collected from a fresh crystal at 173K was no more successful than the present one. The low precision of structural parameters for both host and guest preclude detailed discussion of molecular parameters.

An important feature of complex **3** is that the crystal packing arrangement (Figure 6) is a novel one for DIMEB complexes. Generally, DIMEB complexes crystallize in the space group $P2_12_12_1$ with typical unit cell dimensions a ~ 14.8, b ~18.9, c ~ 28.8Å and the representative X-ray powder pattern for this isostructural series has been reported [18]. Complex **3** crystallizes in the same space group but with different cell constants, one of them being ~10.8Å (Table 2), which is the approximate height of the complex molecule in the orientation shown in Figure 5. The result is that the DIMEB cavities are strictly aligned along the crystal x-direction, as shown in Figure 7 for three translated molecules. It is evident that successive guest molecules are virtually in contact in head-to-tail mode (e.g. the shortest intermolecular guest Cl···C(methyl) distance is only 3.51Å). This was confirmed by inspection of space-filling diagrams which show the head-to-tail contact of the molecular surfaces. In this novel packing arrangement, the guests thus form a 'continuum' along the channel axis. In contrast, in the other known packing arrangement of DIMEB complexes, the axes of the macrocycles are offset from one another, producing a decidedly non-linear 'channel'.

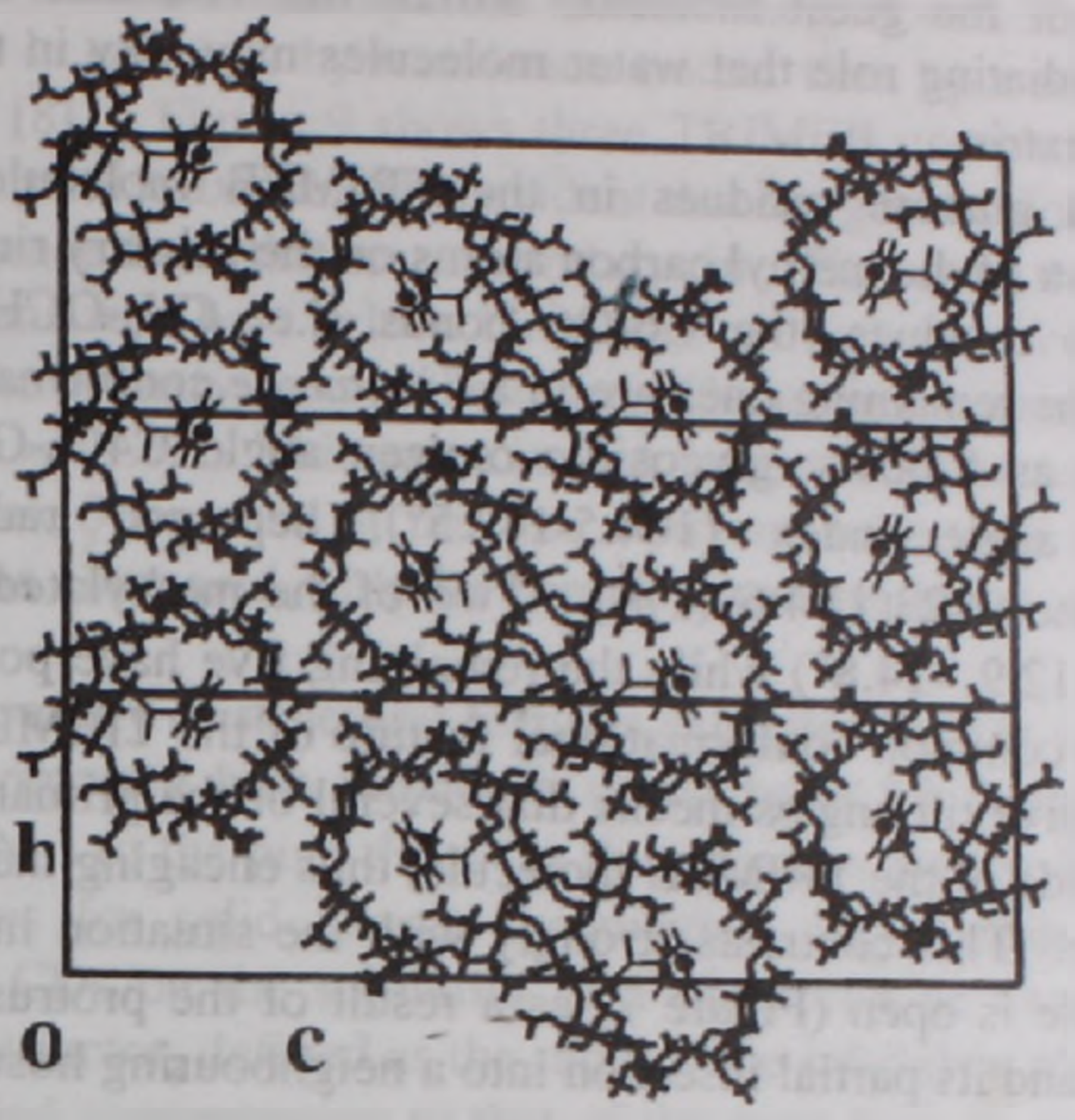

Figure 6. Crystal packing arrangement in complex **3** shown in projection down the *a*-axis. Three unit cells are shown.

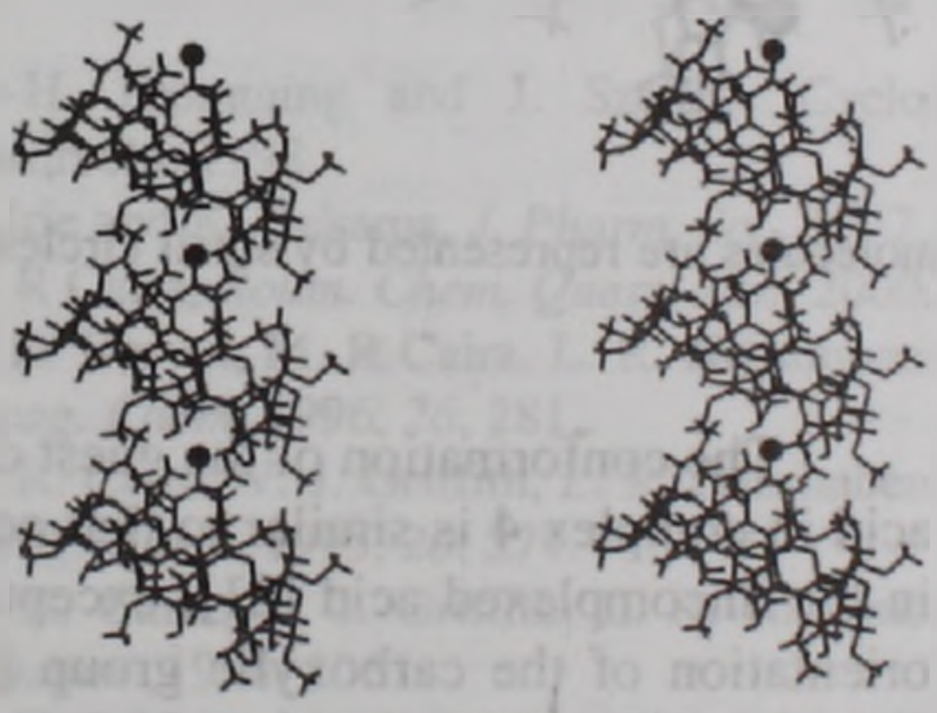

Figure 7. Stereoview showing three molecules of complex **3** related by translation along the *a*-axis (vertical).

The structure of **4**, the TRIMEB complex of clofibric acid. is illustrated in the stereoview of Figure 8. Of the four complexes analysed. this one yielded the best resolution of the guest molecule as well as the water molecules of crystallization. The orientation of the guest in the TRIMEB cavity is opposite to that observed in the DIMEB complex **3**. The chlorophenyl residue partially protrudes from the secondary side of the TRIMEB cavity while the carboxylic acid moiety is located inside the cavity and is linked to the host primary side via hydrogen bonding through bridging water molecules. Refinement of the water molecule site-occupancy factors yielded values of 0.89 and 0.51, the total corresponding to the stoichiometric value of $1.4H_2O$ observed in TGA. Figure 8 shows that one water molecule is hydrogen bonded to the carbonyl O atom of the guest while the other is hydrogen bonded to the guest hydroxyl group. Each water molecule is in turn hydrogen bonded to a member of a disordered pair comprising a DIMEB primary oxygen atom. The four O···O distances for these hydrogen bonds are in the range 2.53(1)-2.76(1)Å, indicating that strong attractive interactions are responsible for anchoring the

hydrophilic portion of the guest molecule within the TRIMEB cavity. This is a rare illustration of the mediating role that water molecules may play in the inclusion of a guest molecule in a cyclodextrin.

All methylated glucose residues in the TRIMEB molecule adopt the usual $^4C_1$ conformation with four of the methyl carbon atoms on the primary rim being disordered over two sites. For two residues, the C6-O6 bonds (i.e. CH_2-OCH_3) have a (+)-*gauche* conformation while the remaining ones are in a (-)-*gauche* conformation. Other parameters and their ranges are as follows: glycosidic oxygen angle $C4G_n$-$O4G_n$-$C1G_{n+1}$ (114.8(5)-118.8(3)°), torsion angle index (104.5-142.5°), heptagon radii (4.60(1)-5.41(1)Å), $O4G_n\cdots O4G_{n+1}$ distance (4.23(1)-4.62(1)Å). Two of the methylated glucose residues have negative tilt angles (-12.9, -14.8°) while the remaining five have positive tilt angles (range 9.9-42.4°) which is a common conformational feature of the TRIMEB molecule [20]. The predominance of positive tilt angles means that several of the primary methoxy groups tend to close the primary side of the TRIMEB molecule, thus encaging the guest within the bowl-like TRIMEB surface. This contrasts strongly with the situation in complex **3**, where the 'lid' of the macrocycle is open (Figure 7) as a result of the protrusion of the bulky guest chlorophenyl residue and its partial insertion into a neighbouring host cavity.

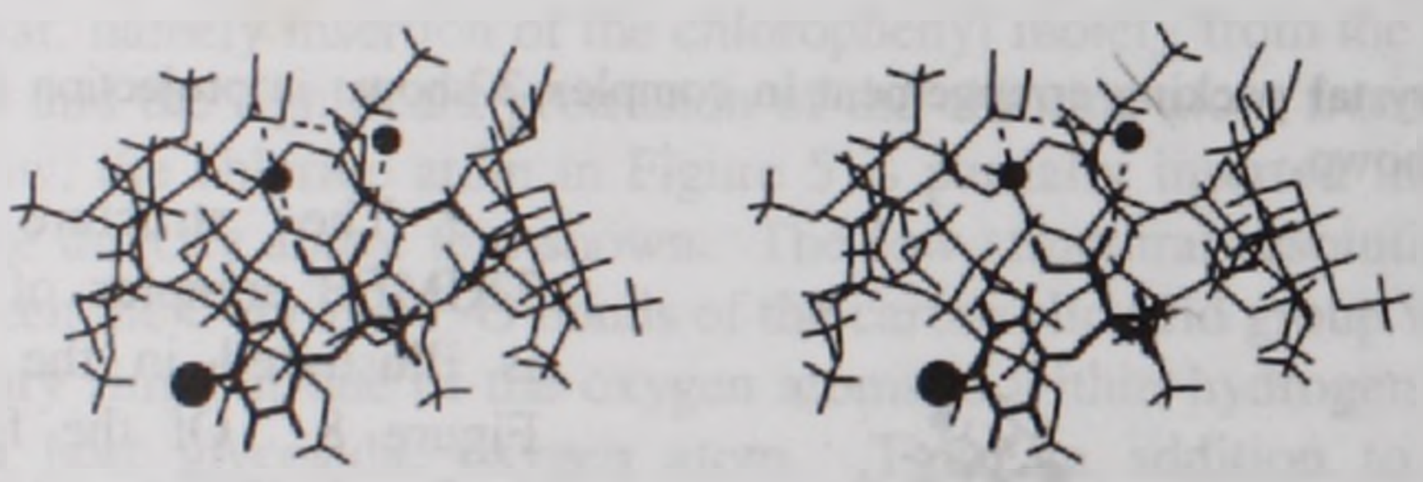

Figure 8. Stereoview of complex **4**. Water molecules are represented by small circles and the guest chlorine atom by a large circle.

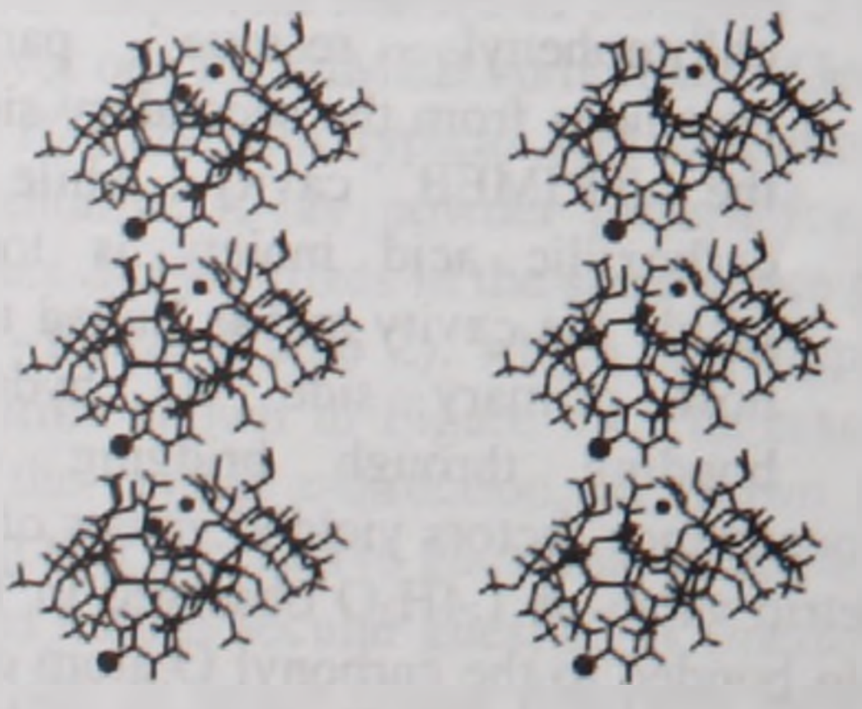

Figure 9. Stereoview showing three molecules of complex **4** related by translation along the *a*-axis (vertical).

The conformation of the guest clofibric acid in complex **4** is similar to that occurring in the uncomplexed acid [21], except for the orientation of the carboxylic group [O-C-C-O(hydroxyl) torsion angles 28.5(9) and -35.9(2)° respectively]. The high precision of the analysis permitted a distinction between the carboxylic C-O and C=O bonds (1.30(1), 1.17(1)Å respectively). The C=O distance is significantly shorter than the value of 1.231(6)Å reported for this bond in the uncomplexed clofibric acid molecule, a result consistent with the IR data which showed that complexation is accompanied by an increase in ν_{CO}. This complex is isostructural with the (L)-menthol complex of TRIMEB [8] whose packing arrangement is uncommon for

TRIMEB complexes. Details were reported earlier where it was shown that molecules in this arrangement pack in columns by translation (rather than by a screw-axis, which is the more common motif) [8]. Figure 9 shows three TRIMEB complex molecules related by translation along the crystal *a*-axis and it is evident that the guest chlorine atom and a portion of the phenyl ring protrude from the host secondary rim. However, they do not penetrate the primary side of the translated host below, but rather act as a spacer between the secondary face of one host and the primary face of the other. The complex units thus pack head-to-tail with a periodicity of a = 11.6Å. This value is ~1Å longer than the corresponding parameter of the DIMEB complex **3** because of the less efficient 'cage-type' inclusion by TRIMEB molecules (Figure 9) as compared with the complete encapsulation of the guest by DIMEB molecules (Figure 7).

Apart from the variety of structural features exhibited by this series of complexes, the characterization data reported here provide a sound basis for further development of the complexes as alternatives to the pure drug. In this regard, only the β- and γ-CD complexes are realistic candidates for solid dosage formulation, for reasons of safety and cost.[22] Finally, the effect of CDs on the solubility of a guest can be expressed in terms of the solubility enhancement factor, defined as the ratio of the solubility of the drug in an aqueous CD solution of specified concentration to that of the pure drug in water [1]. In the case of clofibric acid, the solubility enhancement factors at 25°C were found to be 1.4 and 2.1 for β- and γ-CD respectively [23].

Acknowledgements. We express our thanks to the NRF (Pretoria) and the University of Cape Town for financial assistance.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

INTERACTION OF IBUPROXAM WITH γ-CYCLODEXTRIN IN SOLUTION AND IN SOLID STATE

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Interaction between Ibuproxam (Ib) and γ-cyclodextrin (γ-CD) was studied in liquid and solid states. In solution, complexation was evaluated by solubility studies. Also, we studied the effect of temperature on the complexation, to propose a model of the complexation mechanism in solution. The results suggested that complexation is a spontaneous and exothermic process. In the solid state, binary systems were prepared by kneading, heating in a sealed container, and spray-drying methods. The products obtained were studied using Differential Scanning Calorimetry (DSC), Hot Stage Microscopy (HSM), Fourier Transformed Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and compared by their dissolution rate performance. SEM, DSC and HSM indicated that Ib is partially found as crystalline non-complexed form in the kneaded sample, while in the sealed-heated and spray-dried products Ib is totally dispersed in the carrier. Finally, this inclusion process in the γ-CD significantly increases the dissolution rate of Ib, especially in the spray-dried system, where the amorphous nature of this one plays a significant role.

Изучена реакция между ибупроксамом (Ib) и γ-циклодекстрином (γ-ЦД) в жидком и твердом состоянии. В растворе комплексообразование оценивалось по результатам растворимости. Изучали также влияние температуры на комплексообразование, чтобы предложить модель механизма комплексообразования в растворе. Результаты показали, что комплексообразование - спонтанный и экзотермический процесс. В твердом состоянии бинарные системы изготовлялись смешиванием, нагреванием в изолированном контейнере, а также методом распылительной сушки Полученные продукты изучались дифференциальной сканирующей колориметрией (ДСК), жарофазной микроскопией (ЖФМ), трансформационной инфракрасной спектроскопией Фурие (ТИКФ), сканнирующей электронной микроскопией (СЭМ) и сравнивались по их скорости растворимости. Результаты СЭМ, ДСК и ЖФМ показали, что Ib частично обнаруживается в виде кристаллической некомплексной формы в смешанном образце, тогда как в герметически нагретых и в препаратах, высушенных распылительной сушке Ib полностью диспергирован в носителе. В конечном счете, этот процесс включения в γ-ЦД значительно увеличивает скорость растворимости Ib, особенно при распылительной сушке, где его аморфная природа играет важную роль.

Ուսումնասիվել է իբուպրոքսամի (ԻԲ) և γ-ցիկլոդեքստրինի (γ-ՑԴ) միջև ռեակցիան հեղուկ և պինդ վիճակում: Լուծույթում համալիրագոյացումը գնահատվել է ըստ լուծելիության: Ուսումնասիրվել է նաև ջերմաստիճանի ազդեցությունը համալիրագոյացման վրա, առաջարկելու համար լուծույթում համալիրագոյացման մեխանիզմի մոդել: Արդյունքները ցույց են տվել, որ համալիրագոյացումը ինքնածին և էկզոթերմիկ պրոցես է: Պինդ վիճակում բինար սիստեմները պատրաստվել են խառնելով, տաքացնելով մեկուսացված կոնտեյների մեջ, ինչպես նաև փոշիացման-չորացման մեթոդներով: Ստացված նյութերը ուսումնասիրվել են դիֆերենցիալ սկանող կոլորիմետրիայի (ԴՍԿ), շոգե-ֆազային միկրոսկոպիայի (ՇՖՄ), Ֆուրիեի

տրանսֆորմացիոն ինֆրակարմիր սպեկտրոսկոպիայի (ՖՏԻԿ), սկանող էլեկտրոնային միկրոսկոպիայի (ՍԷՄ) մեթոդներով և համեմատվել են ըստ նրանց լուծելիության արագությունների: ՍԷՄ, ԴՍԿ և ՇՖՄ արդյունքները ցույց են տվել, որ ԻԲ-ն մասնակիորեն գտնվում է բյուրեղային ոչ համալիր ձևով խառնված նմուշում, այն դեպքում որ հերմետիկորեն տաքացված և փոշիացմամբ չորացված պատրաստուկներում ԻԲ-ն ամբողջապես ցրված է կրիչի մեջ: Վերջապես γ-ՑԴ -ում ներառման պրոցեսը բարձրացնում է ԻԲ-ի լուծելիության արագությունը, հատկապես փոշիացնող չորացնող համակարգում, որում նրա ամորֆ բնույթը մեծ դեր է խաղում:

Introduction

Cyclodextrins (CDs) are well-known complexing agents widely used in the pharmaceutical field [1]. The CD complexation has been very employed to improve the stability, dissolution rate and bioavailability in oral, rectal, dermal and parenteral administration [2, 3].

Ibuproxam (Ib) is a non-steroid anti-inflammatory agent with good analgesic and antipyretic properties [4]. It is characterised by a poor aqueous solubility, which limits its dissolution rate and, consequently, its bioavailability.

Earlier investigations showed that Ib complexation with γ-CD increases its solubility [5-7]. However, some aspects, such as thermodynamics of the complexation process and the isolation of solid complexes have not been treated extensively. For this reason, the primary objective of the present study was to investigate the potentiality of interaction of Ib with γ-CD with the aim to reach solid inclusion complexes able to yield further improvement of drug dissolution characteristics. Moreover, the thermodynamic parameters of the complexation were determined, in order to explain the mechanism of this one at the liquid phase.

Materials and Methods

Ib was supplied by Manetti and Roberts (I-Firenze) and γ-CD by Cyclolab (H-Budapest).

Study of the complexation in aqueous solution.

The complexation of Ib with γ-CD in aqueous solution at different temperatures (25, 37 and 50 °C) has been studied using the solubility method described by Higuchi and Connors [8]. 50 mg of Ib were added to 10 ml solutions containing various concentrations of γ-CD (10- 100 mM). The Erlenmeyer flasks were sealed and stirred for one week, until the solubility equilibrium was reached. Then, their content was filtered through 0.22 μm cellulose nitrate membrane filters. The filtrates, properly diluted with distilled water, were analysed spectrophotometrically at 262.5 nm. The apparent 1:1 stability constant (Kc) was calculated from the slope of the phase solubility diagram following the Higuchi and Connors equation.

Preparation of the solid complexes.

The samples, according to the solubility curve results, were prepared in 1:1 molar ratio by the following methods:

Kneading: γ-CD and Ib were kneaded in a mortar with the aid of few drops of ethanol, until a homogenous paste was obtained. The process continued for 45 min and the final paste was dried in an oven at 35 °C for 24 hours.

Spray-drying: The required amounts of Ib and γ-CD were dissolved, respectively, in 400 ml of 96 % ethanol and 400 ml of purified water. Both solutions were mixed by sonication (20 min), to produce a clear solution, which was then spray-dried (Büchi 190M miniSpray-Dryer, Switzerland). The drying conditions were: flow rate: 400 ml· h^{-1}; inlet temperature: 115 °C; outlet temperature: 70 °C; air flow rate: 400 Nl· h^{-1}.

Heating in a Sealed Container: 400 mg of a 1:1 mol:mol Ib-γ-CD physical mixture was sealed in a 2 ml glass ampoule containing 125 µL of distilled water. The ampoule was vigorously shaken and then heated at 100 °C for 2 h. The final product was dried under the same conditions that the kneaded system.

Physical Mixture: drug and cyclodextrin in equimolar proportion and previously sieved, were mixed during 15 min in a flask under manual agitation.

All the obtained products were finally ground and sieved (50- 200 µm).

Study of the complexation in solid state.

DSC measurements were carried out using a DSC equipment (Mettler TA4000) equipped with a DSC 25 cell. Samples of 5-8 mg were put into aluminium crucibles, which firm was pierced to allow the leaving of gases evolved during the heating process. The conditions were: static air atmosphere, temperature range from 30 to 320 °C and 10 °C· min^{-1} heating rate.

Different observations were made during heating using a HSM (Mettler model FP82HT) attached to an Olympus BH-2 microscope. Approximately 0.1 mg of samples was placed on glass slides with coverglass and heated at 5 °C· min^{-1}.

The infrared spectra of the different samples were obtained by means of a Bomem M-120 IR equipment. The samples were mixed with KBr and compressed as disks. The selected wavenumber ranged between 600 and 4000 cm^{-1}, being the speetra resolution of 4 cm^{-1} and 20 the number of scans.

The microscopic features of the single raw materials were compared with those of the products obtained by kneading, spray-drying and sealed heating by examination under the SEM (Philips XL30). Samples were previously coated with Au, in order to make them conductor.

Dissolution rate studies.

The dissolution rate studies were performed according to the USP 23 paddle method. The samples, equivalent to 40 mg of Ib, were previously placed into hard gelatine capsules. Dissolution medium was 1000 mL of distilled water. The stirring speed was 50 rpm and the temperature 37 ± 0.5 °C. Aliquots of 3 ml were withdrawn at settled time intervals using a filter syringe, and analysed spectrophotometrically at 262.5 nm. All tests were performed in triplicate.

Results and Discussion

Complexation in aqueous solution.

Figure 1 shows the obtained phase solubility diagrams. The solubility of Ib increased in a linear fashion as function of CD concentration, being classified as A_L Higuchi type curves. In this system a water soluble complex may exist in the solution since no precipitation was observed even at concentration of CD as high as 100 mM.

The variation of stability constant as a function of temperature was used to determine the thermodynamic parameters for Ib inclusion complex with γ-CD. The stability constants calculated at different temperatures and the thermodynamic parameters for the complexes are shown in Table 1. For 1:1 complex formation, the equilibrium constant Kc, was determined according to $Kc = m / S_0 (1-m)$, where m is the slope of solubility diagram shown in Figure 1, and S_0 is the intrinsic solubility of Ib. As shown in Figure 1, temperature significantly affected the interaction. So, the Kc values decrease with increase in temperature. For calculate the thermodynamic parameters of the inclusion phenomenon, we have previously calculated the association constants at three different temperatures.

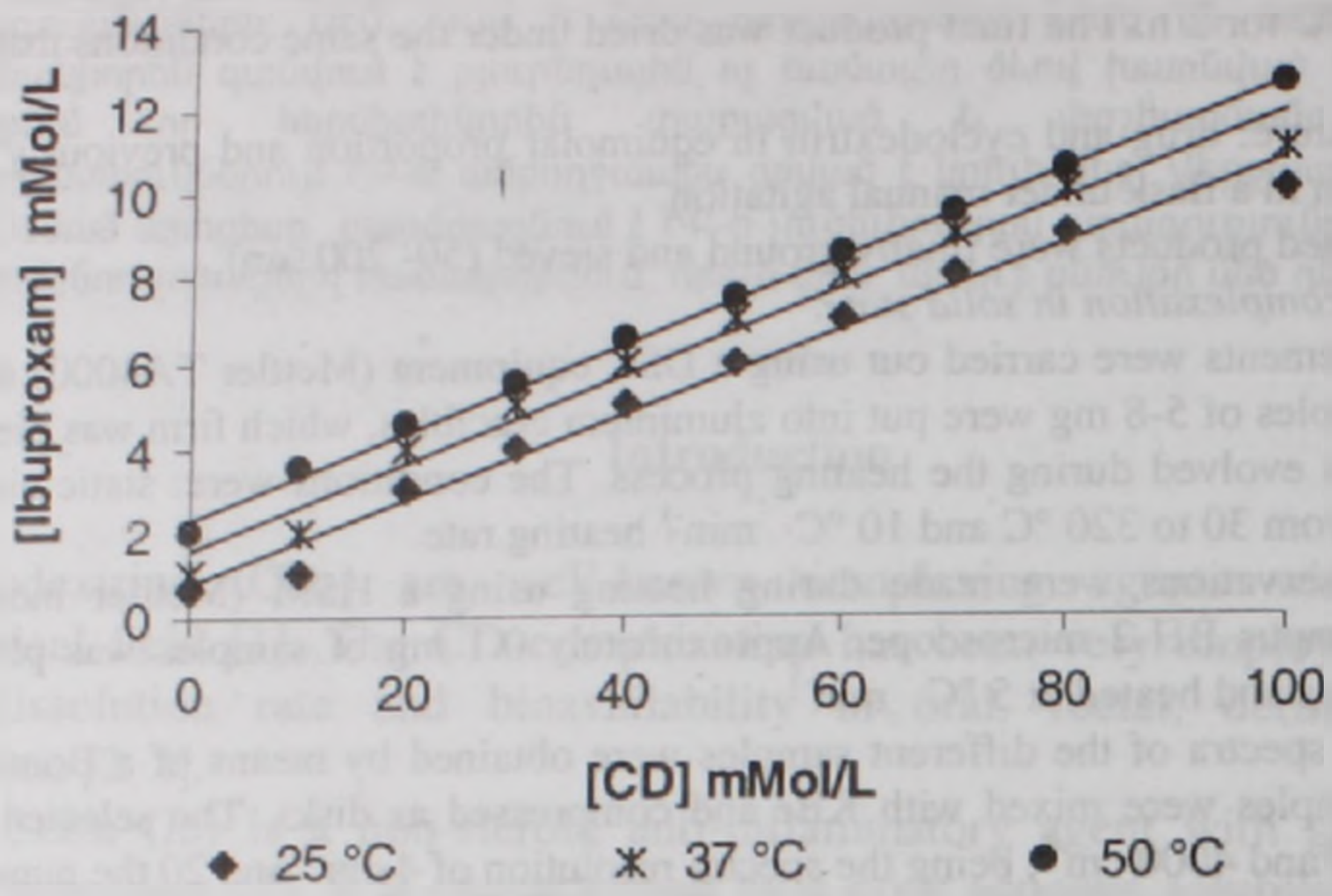


Figure 1. Phase-solubility diagram of Ib-γ-CD system at 25, 37 and 50°C. The calculation of the thermodynamic parameters was carried out using the following equations:

$$\ln \frac{K_2}{K_1} = \frac{\Delta H}{R}\left[\frac{T_2 - T_1}{T_2 \cdot T_1}\right] \qquad \text{Eq. (1)}$$

$$\Delta Gi = -RTi \ln Ki \qquad \text{Eq. (2)}$$

$$\Delta Si = \frac{\Delta Hi - \Delta Gi}{Ti} \qquad \text{Eq. (3)}$$

Where K_1 and K_2 are the stability constants at 25 at 37 °C respectively, R is the gas constant (8.314 J· mol^{-1}· K^{-1}), being T_1 and T_2 the temperatures in Kelvin degrees.

Table 1. Thermodynamic parameters calculated from solubility studies at different temperatures.

Temperature (K)	Slope	*R*	K_c (M^{-1})	ΔG (KJ· mol^{-1})	ΔH (KJ· mol^{-1})	ΔS (J· mol^{-1}· K^{-1})
298	0.09979	0.9915	142.0	-12.278	-34.25	-73.73
310	0.10303	0.9898	73.6	-11.079	-34.25	-74.74
323	0.10248	0.9982	48.7	-10.434	-34.25	-73.73

The large negative magnitude of enthalpies (ΔH) obtained for all the interactions, suggesting that strong intermolecular forces are involved in the complex formation, as hydrogen bonding, which energy is between 12 and 20 KJ· mol^{-1} [9].

It is worth noting that the complexes gave negative entropy changes (ΔS), which were unfavourable for the complex formation. These results may indicate a more ordered state upon complexation. The overall entropy change is the sum of two opposite effects. First, a negative contribution, due to the reduction of traslational and rotational degrees of freedom of the host and guest molecules by complexation. Also, a positive effect, related with the solvent ordering, which is due to the displacement of the highly ordered water molecules of the cavity of the CD and those surrounding the hydrophobic portions of the guest molecule

when it interact with the inner of the CD. In our case, the first factor seems to be the prevalent.

On the other hand, negative free energy values (ΔG) were obtained for all the temperatures, indicating that the inclusion process is spontaneous. This is due to the negative ΔH change that compensates the unfavourable ΔS, indicating also the exothermic nature of the complexation process under study. This contribution of ΔH to the ΔG value confirm that this type of interaction is enthalpy driven.

The Kc values decrease whit increase in temperature, as found for a large variety of drugs [10], can be interpreted as a result of displacement of cavity water molecules by means of the entering guest, enabling them to form full hydrogen bonds with adjacent water molecules.

Complexation in solid state.

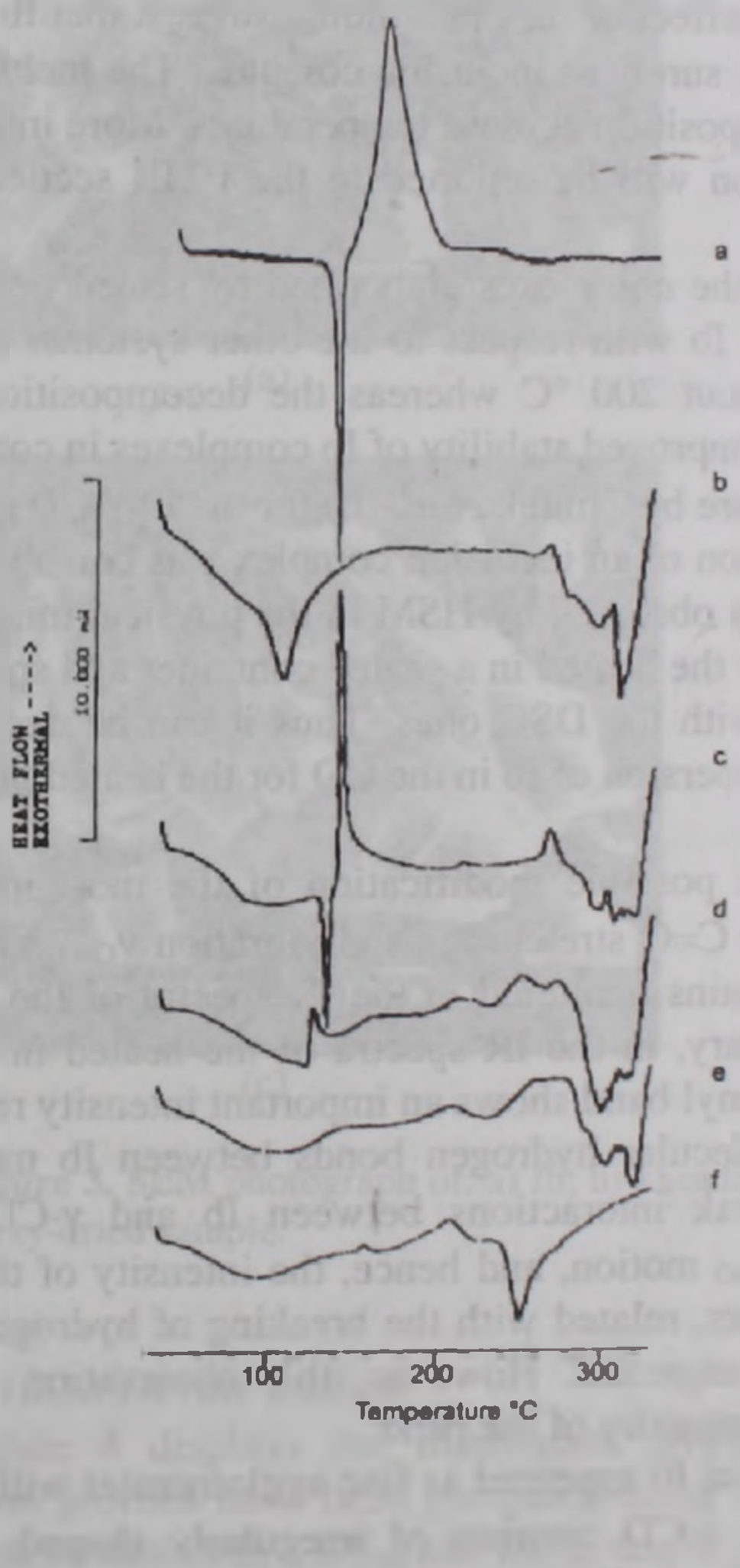


Figure 2. DSC curves of: a) Ib; b) γ-CD; c) physical mixture; d) kneaded; e) heated in a sealed container and f) spray-dried.

The DSC traces of the systems under study are represented in Figure 2. The DSC curve of Ib (Figure 2a) is characterised by the presence of typical endo- and exo-thermal peaks (endo: Tonset = 126.1 (± 0.6)°C, Tpeak = 130.2 (± 0.7)°C, ΔHf = 129 (± 9) J· g^{-1} and exo: Tonset = 147.2 (± 1.8)°C, Tpeak = 165.3 (± 0.6)°C, ΔHf = 573 (± 63) J· g^{-1}) corresponding to the melting and oxidative degradation of the drug, respectively. These results are in accordance with the literature data [6]. On the other hand, the γ-CD (Figure 2b) shows a broad endothermic effect around 100 °C related to the CD dehydration.

The thermogram corresponding to the physical mixture (Figure 2c) displays the endo-exothermic effect of Ib (endo: Tonset = 122.9 (± 0.7)°C, Tpeak = 131.5 (± 0.5)°C, ΔHf = 115 (± 10) J· g^{-1} and exo: Tonset = 138.3 (± 1.9)°C, Tpeak = 142.6 (± 0.6)°C, ΔHf = 290 (± 30) J· g^{-1}). It is important to note that the exothermic effect shows a significant difference in the temperature and shape of the peak, which may be attributed to

the degradation of the drug itself. If we take into account that this is an oxidation process, in the pure drug, when melts, the contact with the oxygen is limited to the outer layer in the crucible. The rest of the drug is protected against oxygen by its own liquid, which delays the oxidation process. On the contrary, in the physical mixture, the drug, in a relatively low w/w percentage is molten beneath the carrier particles, allowing its simultaneous contact with the atmospheric oxygen.

The thermogram corresponding to the kneaded system (Figure 2d) reveals that the endo-exothermic effect of Ib does not disappear (endo: Tonset = not determinable, Tpeak = 120.4 (± 0.7)°C, ΔHf = not determinable and exo: Tonset = 122.9 (± 1.4)°C, Tpeak = 124.9 (± 0.8)°C, ΔHf = 28 (± 4) J· g^{-1}). In this product, a clear size reduction and broadening of both peaks is observed, with a concomitant shift to lower temperatures. This result may be interpreted in terms of incomplete inclusion formation between Ib and γ-CD [13-14].

The sealed heating (Figure 2e) and spray-drying (Figure 2f) treatments leaded to the total disappearance of the endo-exothermic effect of Ib. This finding suggest that Ib exists in a molecularly dispersed state into the γ-CD, surely as inclusion complex. The included drug should not suffer neither melting not decomposition at these temperatures. More information about the nature of the drug-CD interaction will be reported in the FTIR section of this paper.

The thermograms also revealed that the complexes elaborated by sealed heating and spray drying have an improved stability of Ib with respect to the other systems. Thus, the complexes start their decomposition at about 200 °C whereas the decomposition of the commercial Ib arises at about 165 °C. This improved stability of Ib complexes in comparison with pure Ib appears described in the literature by Zmitek *et al.* (6) for the Ib-γ-CD complex.

The feasibility of the possible formation of an inclusion complex was corroborated by HSM. The melting process of Ib was clearly observed by HSM in the physical mixture and, partially, in the kneaded product, but not for the heated in a sealed container and spray-dried samples. These results are in accordance with the DSC ones. Thus it can be deduced the reality of the formation, at least, of a true dispersion of Ib in the CD for the heated in a sealed container and spray-dried products.

The IR studies were focused on the possible modification of the most interesting absorption band of Ib, corresponding to the C=O stretching band vibration $\nu_{C=O}$, situated at 1634 cm^{-1} in the pure drug. This band remains unaltered in the IR spectra of the physical mixture and kneaded system. On the contrary, in the IR spectra of the heated in a sealed container and spray-dried systems, the carbonyl band shows an important intensity reduction. It is related with the breaking of intermolecular hydrogen bonds between Ib molecules, accompanied by the establishment of weak interactions between Ib and γ-CD in the complexed state [13], which reduce the $\nu_{C=O}$ motion, and hence, the intensity of this band. Also, a possible shift to higher wavenumbers, related with the breaking of hydrogen bonds between drug molecules [14] would be expected. However, this observation was not possible, due to the strong reduction of the intensity of the band.

The SEM analysis revealed as the commercial Ib appeared as fine agglomerates with smooth surfaces (Figure 3a). On the other hand, γ-CD consists of irregularly shaped crystals.

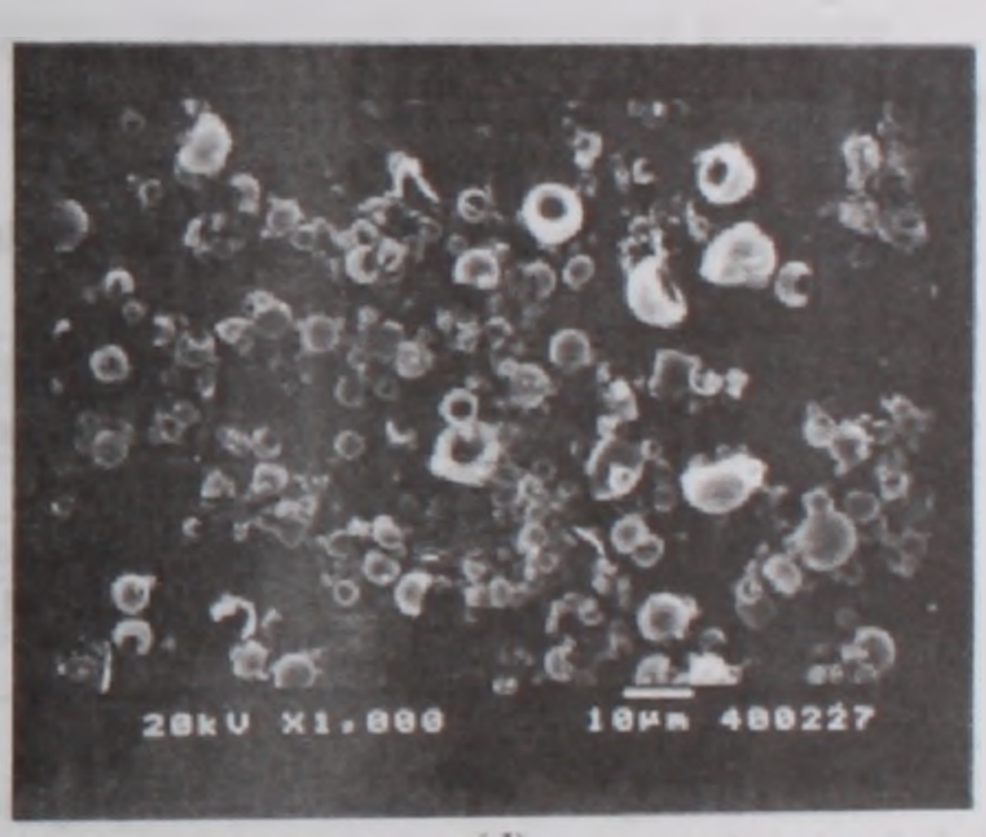
The kneaded mixture photograph (Figure 3b) is characterised by the influence of the preparing technique in the sample morphology. Thus, mixed crystals constitute the kneaded product, which present adhered crystals of original Ib. This observation is in agreement with the results obtained from the former analytical techniques, that reveal the kneaded system does not constitutes a true inclusion complex. Figure 3c shows the heated in a sealed

container product, where the original morphology of both Ib and γ-CD has disappeared, being impossible the differentiation of the components. The spray-drying technique, in contrast, yields products of amorphous appearance (Figure 3d), with the presence of spherical homogeneous particles, which display a quite significant reduction in the particle size. These particles are distinguished by the absence of agglomeration. These observation help to contemplate the presence of a single component in the preparations obtained by sealed heating and spray-drying methods.

(a)

(b)

(c)



(d)

Figure 3. SEM photograph of: a) Ib; b) kneaded sample; c) heated in a sealed container sample and d) spray-dried sample.

Dissolution rate studies.

Figure 4 displays the dissolution profiles of Ib and the Ib-γ-CD systems. The dissolution profiles have been compared using the dissolution efficiency parameter [15] at 15, 30 and 60 min (DE_{15}, DE_{30} and DE_{60}, respectively) (Table 2).

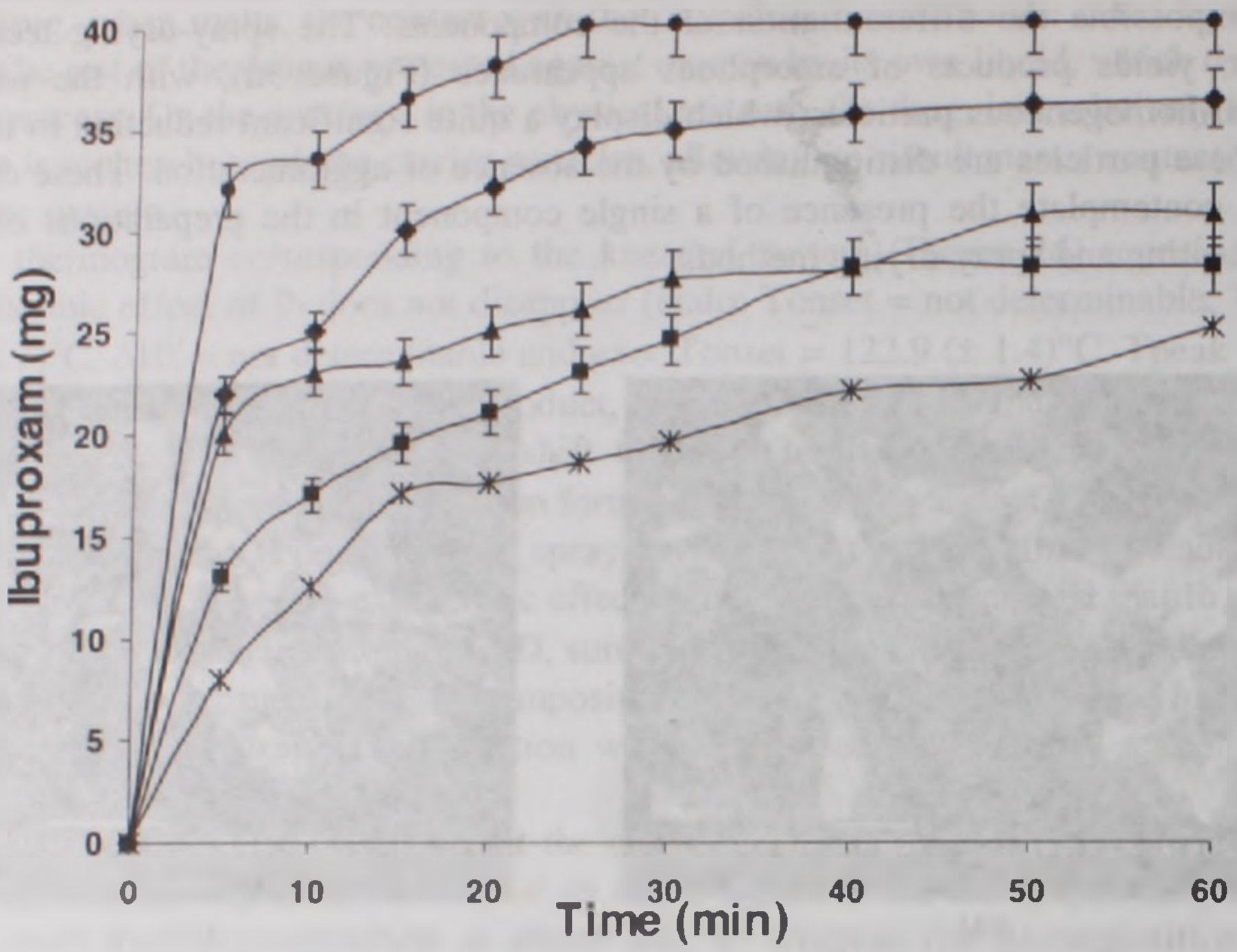


Figure 4. Dissolution curves of Ib and Ib-γ-CD binary systems: ✱: Ib; ■: physical mixture; ▲: kneaded; ●: heated in a sealed container and ●: spray-dried.

Table 2. Dissolution efficiency values at 15, 30 and 60 min for the Ib and Ib-γ-CD binary systems.

	Elaboration Method	DE15	DE30	DE60
Binary Systems	Physical Mixture	0.331	0.441	0.563
	Kneaded	0.456	0.547	0.638
	Heated in a sealed container	0.510	0.668	0.780
	Spray-Dried	0.698	0.833	0.917
Ibuproxam		0.242	0.347	0.451

It is clearly observed that the physical mixture and kneaded sample improve only slightly the dissolution rate of Ib. The low dissolution rate from the kneaded system may be attributed to the fact that the isolated product has a low percentage of complexed drug.

For the heated in a sealed container and spray-dried samples, we can appreciate a notable increase in the release rate of the drug. These results are due to the major interaction between drug and CD in these systems in contrast with the physical mixture and kneaded sample, which is translated in a more effective inclusion complexation of Ib into the CD cavity. In the spray-dried sample, the special increase in the dissolution rate may be also explained by the higher specific surface of the product produced from the molecular dispersion and, also, by the amorphous structure of the product.

Acknowledgements. The authors wish to thank Dr. Mª del Carmen Ortiz Mellet (Department of Organic Chemistry, Faculty of Chemistry, University of Seville) for her kind help in carrying out and interpreting the IR spectra.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

THE ENHANCED D-GLUCOSE UPTAKE IN BRUSH BORDER MEMBRANE VESICLES BY 2,6-DI-O-METHYL-β-CYCLODEXTRIN

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D-glucose uptake in jejunal brush-border membrane vesicles (BBMV) isolated from rats, investigated by a filtration method was found to be significantly elevated in 2 mM 2,6-di-O-methyl-β-cyclodextrin (DM-CD) treated BBMV compared to control BBMV. In the presence of an inwardly directed NaCl gradient, D-glucose uptake in control vesicles at 15s was seven-fold over that of the equilibrium level at 30 min. As for the DM-CD-treated BBMV in the presence of the NaCl gradient, the overshoot magnitude of the D-glucose uptake was much higher than that in the control BBMV. The D-glucose uptake in control and DM-CD-treated BBMV could be described by an equation giving sum of a saturated term displaying Michaelis-Menten kinetics and a passive diffusion term. By using the equation, the calculation of stereospecific Na^+-dependent D-glucose uptake in control BBMV gave an apparent Km and Vmax values of 0.63 (mM) and 0.87 (*nmole/mg protein/15s*), and the DM-CD-treated BBMV showed 0.44 (mM) and 1.79 (*nmole/mg protein/15s*), respectively. There was a significant increase in Vmax value in the DM-CD-treated BBMV compared to the control, but the Km and passive diffusion term showed no significant differences. The membrane fluidity of the lipid layer and cholesterol content of BBMV were remarkably changed by DM-CD-treatment, indicating that the microenviromental conditions around the glucose carrier in the BBMV may be altered by DM-CD-treatment.

Методом фильтрации выявлено, что поглощение D-глюкозы в мембранах крайных ворсинок у везикул (МКВВ) тощей кишки, выделенных из крыс, значительно повышается при их обработке 2мМ раствором 2,6-ди-О-метил-β -циклодекстрина (ДМ-ЦиД), чем в контрольных необработанных вариантах. При наличии внутренне контролируемого градиента NaCl поглощение D-глюкозы в контрольных везикуллах было 7-раз больше за 15 сек, чем это показание при уровне равновесия за 30 мин. При градиенте NaCl в ДМ-ЦиД обработанных МКВВ вариантах величина связывания D-глюкозы намного выше, чем в контрольных МКВВ вариантах. Поглощение D-глюкозы в контрольных и ДМ-ЦиД обработанных МКВВ вариантах можно описать уравнением, показывающим сумму насыщенного периода по кинетике Михаэлиса-Ментена и пассивного диффузионного периода. С помощью уравнения рассчитаны значения Km и Vmax для Na^+-зависимого связывания D-глюкозы, что составляет 0,63(мМ) и 0,87 (нмоль/мг белка/15сек) в контрольных и 0,44(мМ) и 1,79 (нмоль/мг белка/15сек) в ДМ-ЦиД обработанных МКВВ вариантах, соответственно. Наблюдается значительное повышение значения Vmax в ДМ-ЦиД в обработанных МКВВ вариантах по сравнению с контрольными вариантами, но значения Km и периода пассивной диффузии отличаются незначительными разницами. Текучесть липидного слоя и содержание холестерола в МКВВ заметно менялись при ДМ-ЦиД обработке, указывая, что условия микросреды вокруг носителя глюкозы в МКВВ могут быть изменены при обработке ДМ-ЦиД.

Ֆիլտրացիայի մեթոդով բացահայտվել է, որ կապումը D-գլյուկոզի կլանումը առնետից անջատված բարակ աղիքի վեզիկուլների սահմանային թավիկների մեմբրաններում (ՎՍԹՄ), բավականին բարձրանում է դրանց 2,6-դի-O-մեթիլ- β -ցիկլոդեքստրինի (ԴՄ-ՑիԴ) 2մՄ լուծույթով մշակելիս, քան ստուգիչ չմշակված նմուշներում: NaCl-ի ներքին կարգավորող գրադիենտի առկայությամբ D-գլյուկոզի կլանումը ստուգիչ վեզիկուլներում եղել է 7 անգամ ավելի 15 վրկ ընթացքում, քան այդ

ցուցանիշը հավասարության մակարդակի դեպքում 30 րոպեի ընթացքում: NaCl-ի գրադիենտի դեպքում ԴՄ-ՑիԴ-ով մշակված ՎՍՄԹ-ի նմուշներում D-գլյուկոզի կլանման արժեքը բավականին բարձր է, քան ՎՍՄԹ-ի ստուգիչ նմուշներում: D-Գլյուկոզի կլանումը ստուգիչ և ԴՄ-ՑիԴ-ով մշակված ՎՍՄԹ-ի նմուշներում կարելի է նկարագրել հավասարմամբ, որը ցույց է տալիս Միխաելիս-Մենտենի կինետիկայի հագեցման ժամանակահատվածի և պասսիվ դիֆուզիայի ժամանակահատվածի գումարը: Հավասարման միջոցով հաշվարկել են Na+-ից կախված D-գլյուկոզի կլանման Km և Vmax արժեքները, որը կազմում է 0,63 (մՄ) և 0,87 (նմոլ/մգ սպիտակուց/15վրկ) և 0.44(մՄ) և 1,79 (նմոլ/ մգ սպիտակուց/15վրկ) ՎՍԹՄ-ի ստուգիչ և ԴՄ-ՑիԴ-ով մշակված նմուշներում համապատասխանաբար: Դիտվում է Vmax արժեքի բավականին բարձրացում ԴՄ-ՑիԴ-ով մշակված ՎՍԹՄ-ի նմուշներում, համեմատած ստուգիչ նմուշների հետ, բայց Km-ի և պասսիվ դիֆուզիայի ժամանակահատվածի արժեքները զանազանվում են չնչին տարբերությամբ: Լիպիդային շերտի հոսունությունը և խոլեստերոլի քանակը ՎՍԹՄ-ում նկատելիորեն փոփոխվում են ԴՄ-ՑիԴ-ով մշակելիս, որը ցույց է տալիս, որ միկրոմիջավայրի պայմանները ՎՍԹՄ-ում գլյուկոզի կրիչի շուրջը կարող են փոփոխվել ԴՄ-ՑիԴ-ով մշակելիս:

Introduction

D-glucose is actively cotransported with sodium across the intestinal brush-border membranes (BBM) via carrier- mediated process. It has been demonstrated that alterations in the lipid composition and /or physical state of the plasma membranes of intestinal cells can influence the activity of several important enzymes and transport systems located in these membranes [1]. The intestinal uptake of D-glucose was shown to be increased by the manipulation of dietary fatty acids [2] and by enzyme-inducing agents, [3] whereas it was decreased by drugs [4,5] and by some kind of disease [6,7]. Further, the increased D-glucose uptake was modified by manipulation of dietary macronutrients [8]. Thus, there seems to be association between alterations in membrane fluidity and changes in transport functions, although this association appears to vary according to membrane components [9-11].

Cyclodextrins (CD), a cyclic oligosaccharide consisting of 1,4-linked glucose units, form inclusion complexes with hydrophobic drugs. α, β and γ-CD have different internal cavity diameters, each showing a different formation constant with different-sized guest molecules. CD-membrane interactions have been reported: CD induced morphological changes and hemolytic activity in human erythrocytes [12] and enhanced the leakage of calcein from liposomes [13]. Although, the effects of CD on artificial membranes or red cell membranes are thought to be due to an interaction with membrane lipid components, the direct action of CD on biological membranes at the absorption site of drugs has not yet been completely clarified. We have reported that the membrane permeability for drug penetrated by a passive diffusion across the intestinal membrane was remarkably enhanced by β-CD [14, 15] That was due to the interaction between the membrane lipid components and CD.

The present study was undertaken to investigate whether 2,6-di-O-methyl-β-cyclodextrin (DM-CD) enhances the carrier mediated D-glucose uptake by BBMV DM-CD, the methylated form of natural β-CD, is more soluble in water than the parent compound. We found that the uptake of D-glucose in BBMV treated with DM-CD was significantly increased compared with uptake in control BBMV. Therefore, the perturbation of lipid layer of BBMV induced by DM-CD caused the enhancement of D-glucose uptake via carrier mediated process.

Experimental

Materials. All reagents were of the highest purity available. 2,6-di-O-methyl-β-cyclodextrin (DM-CD), tris(hydroxymethyl)-aminomethane (Tris) and N-2-hydroxyethylpiperadine-N'-2 - ethanesulfonic acid (Hepes) were purchased from Wako Pure chem. Ind. ^{3}H labelled D-glucose was obtained from the Radiochemical Center, Amersham.

Preparation of BBMV from Rat Small Intestine. BBMV were prepared from Wistar rats (230-250g) according to the method of Kesseler et al. with some modifications [16]. Segments of small intestine (50cm), except for the ileum, were used for the preparation of the vesicles. The activity of alkaline phosphatase [17], sucrase [18] used as marker enzymes of the BBMV and Na-K ATPase [19] as a marker enzyme of the basolateral membrane were determined. Alkaline phosphatase and sucrase were enriched during purification process. Na-K ATPase activity was not detected in the BBMV preparation. The protein concentration was determined by the method of Lowry et. al [20] using bovine serum albumin as a standard.

DM-CD Treatment of BBMV. The BBMV suspension was incubated with 2 mM DM-CD in pH 7.5 100 mM mannitol and 10mM Hepes-Tris buffer at 37°Cfor 10 min and then centrifuged at 27,000 g for 20 min. The pellet was resuspended in the same buffer and the protein content was determined. Control BBMV suspension was incubated in pH 7.5 100 mM mannitol and 10 mM Hepes-Tris buffer at 37°C for 10 min and then treated in the same manner as the DM-CD treatment.

Glucose Uptake Experiment. BBMV suspension (50 ml) in 100 mM mannitol and 10 mM Hepes-Tris buffer (pH 7.5) was added to a 50 ml of transport buffer (100 mM NaCl, 100 mM mannitol, and 10 mM Hepes-Tris, at pH 7.5) with 0.1-10 mM D-glucose tracer labelled with ^{3}H. The mixture was incubated at 25°C and the reaction was stopped with 2ml ice-cold 200mM NaCl and 10mM Hepes-Tris buffer (pH 7.5). We calculated the uptake from radioactivity trapped on the filter by dissolving the filter overnight in Creosol (Nacalai Tesque Ind.,) and determining the radioactivity with a liquid scintillation counter (LSC 3500, Aloka).

Measurement of Fluidity. The lipid fluidity of the BBMV was determined by the measurement of fluorescence polarization, using 2-(9-anthroyloxy)stearic acid (2-As; excitation, 390 nm; emission, 452 nm), 1,6-diphenyl-hexatriene (DPH; excitation, 380 nm; emission, 455 nm) and N-(1-anilinonaphtyl-4)maleimide (ANM; excitation, 355 nm; emission, 448 nm) as fluorescent probes. The 2-AS and DPH were dissolved in tetrahydrofuran, each to a concentration of 1 mM; ANM was dissolved in the same buffer solution. An aliquot of fluorescent probe solution was added to the BBMV suspensions, followed by incubation for 30 min at 37°C. The BBMV suspensions were separated from unlabelled fluorescent probes by washing with buffer and centrifuging. This procedure was repeated 3 time at 4°C. Fluorescence polarization was determined with fluorescence spectrophotometer (Hitachi, F3010) at 25°C. The fluorescence polarization (P) was calculated with the following equation:

$$P = \frac{Ivv - G * Ivh}{Ivv + G * Ivh} \qquad G = \frac{Ihv}{Ihh}$$

where Ivv, Ivh, Ihv and Ihh were the fluorescence intensities oriented in a parallel manner (Ivv) and in a perpendicular manner(Ihh).

Measurement of Cholesterol and Phospholipid in BBMV. Total phospholipid and cholesterol in the BBMV were extracted according to the method of Folch et al [21]. The lipids extracted with mixed solvent (chloroform-methanol/2:1) were washed with 0.1mM KCl, and concentrated by evaporating under N_2 stream. Cholesterol and total phospholipid in the BBMV were determined by the method of Zlatkis and Zak [22] and Bartlett [23], respectively.

Results

The D-glucose uptake of BBMV was determined in the presence of an initial 100 mM NaCl gradient (inside<outside). As shown in Figure 1, the values for initial rate and overshoot magnitude of D-glucose uptake in the vesicles was increased significantly by the treatment with DM-CD compared with values of control vesicles. The equilibrium values of D-glucose uptake by both control and treated membrane vesicles were similar, suggesting that the intravascular volume of BBMV was not changed by the DM-CD treatment. Further the initial uptake rates of D-glucose in the BBMV in the presence of an initial 100 mM KCl gradient showed no significant difference between BBMV treated with DM-CD and the controls.

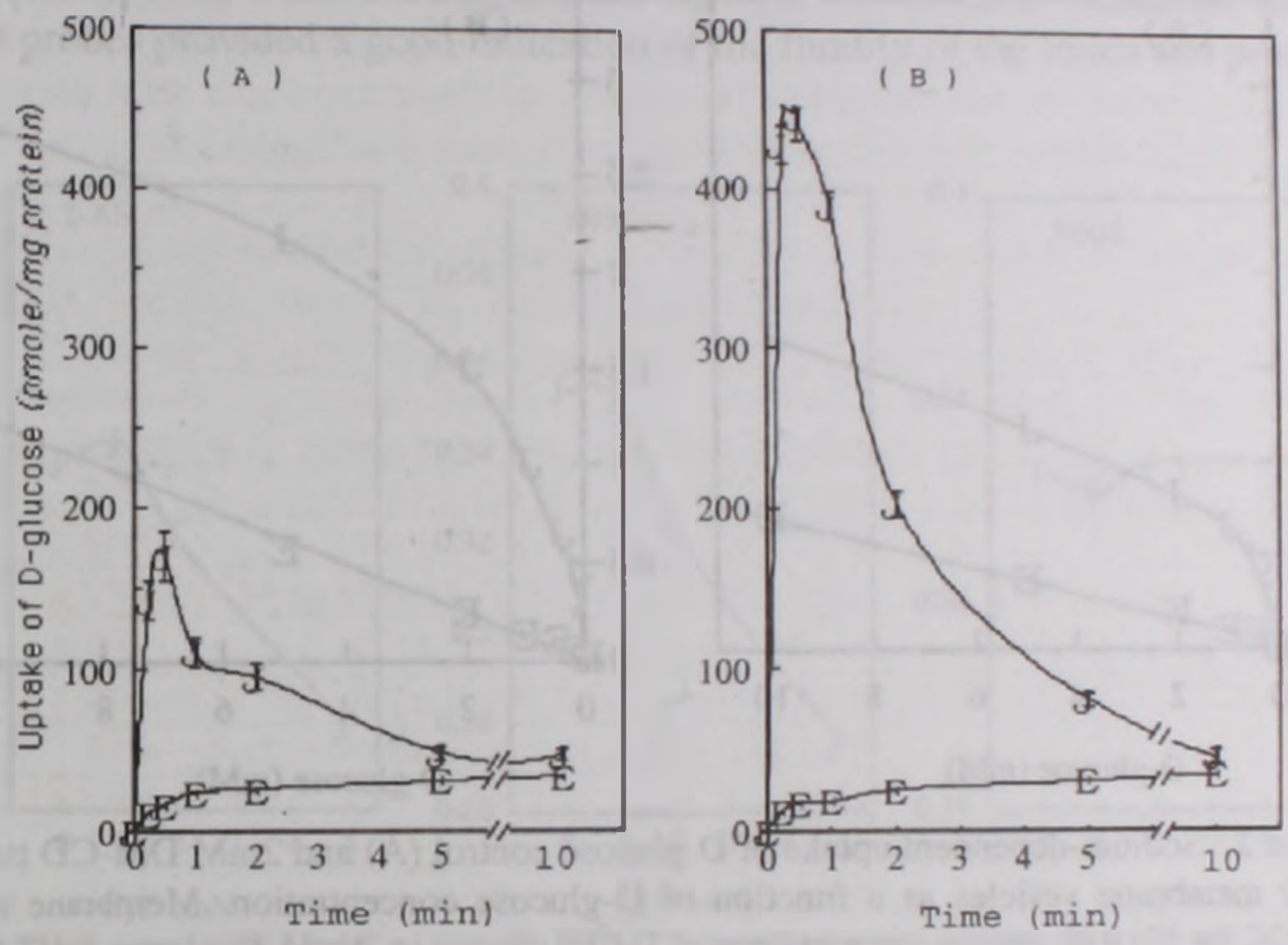

Figure 1. Time course of D-glucoseuptake by brush border menbrane vesicles; control (A) and 2mM DM-CD treatment (B). Control and 2mM DM-CD-treated membrane vesicles were incubated at 25C in 20mM Tris-hepes (pH7.4) containing 100mM mannitol in thepresence of either 100mM NaCl (J) or 100mM KCl (E). Each point represents the mean of five experiments performed in duplicate determinations.

Figure 2 shows the initial uptake of D-glucose as a function of the initial concentration in the presence of NaCl or KCl gradient. The relationship between the concentration and the rate of uptake was non-linear in the presence of NaCl gradient and linear in the presence of KCl gradient. The uptake of D-glucose in the presence of NaCl gradient was assumed to be described by the sum of a saturated term displaying Michaelis-Menten kinetics plus a second non-saturable term, since the D-glucose uptake rate (V) can be expressed by the following equation:

$$V = \frac{V_{max} * (D-glucose)}{K_m + (D-glucose)} + P * (D-glucose)$$

where Vmax is the maximum velocity of Na^+-dependent uptake, Km is the D-glucose concentration at which the Na^+-dependent uptake is half maximal, and P is the permeability coefficient. The non-linear least squares fitting of the experimental data gave the parameters listed in Table 1. In the control experiment, the apparent Km and Vmax were 0.63 (mM) and 0.87 (nmoles/mg protein/15s), whereas the Km of the 2mM DM-CD treated BBMV was not significantly different from that of the control. However, the Vmax of the DM-CD-treated BBMV was two-fold that of the control. The passive permeability of the DM-CD-treated BBMV was slightly, but not significantly increased compared with that of the control.

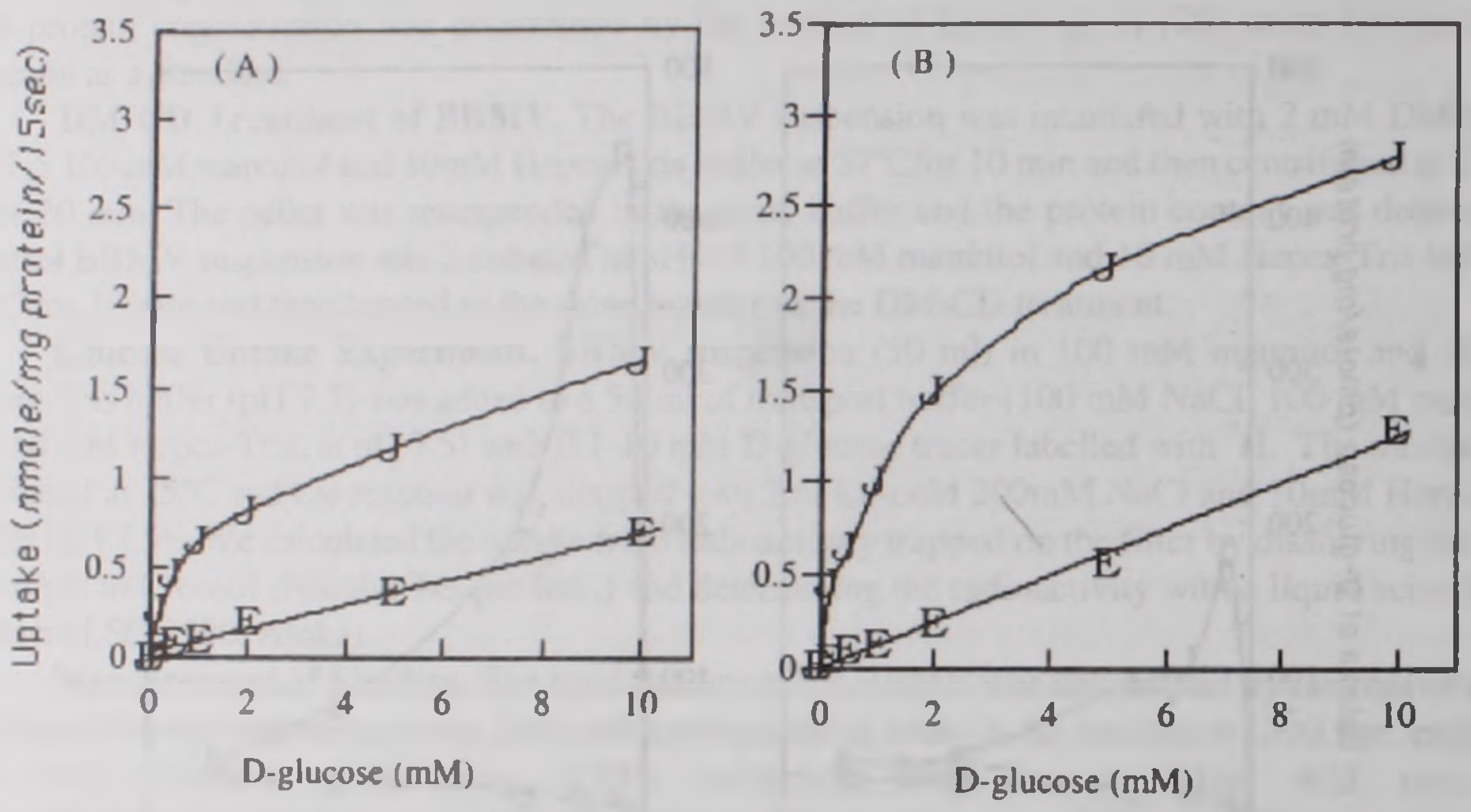

Figure 2. Sodium-dependent uptake of D-glucose control (A) and 2mM DM-CD treatment (B) brush border membrane vesicles as a function of D-glucose concentration. Membrane vesicles were incubated at 25C for 15s with various concentrations of D-[^{3}H] glucose in 20mM Tris-hepes (pH7.4) containing 100mM mannitol in the presence of either 100mM NaCl (J) or 100mM KCl (E). Each point represents the mean of five experiments performed in duplicate determinations.

Table 1. Kinetic Parameters for the Uptake of D-Glucose in Brush Border Membrane Vesicles.

	Michalis-Menten kinetic parameters		First order rate constant
	Km (mM)	Vmax (*nmole/mg protein/15s*)	(*μl / mg protein/15s*)
control	0.631 ± 0.071	0.872 ± 0.045	0.080 ± 0.005
DM-CD	0.431 ± 0.066	1.786 ± 0.110*	0.103 ± 0.013

Each point represents the mean ± S.E. of five experiments performed in threee determinations.
* Significantly different from control, $p < 0.001$

To obtain information on the mechanisms of the enhancing effect of DM-CD on the D-glucose uptake was measured membrane fluidity of BBMV fluorescence spectrophotometrically. The fluorescence polarization of 2-AS, DPH and ANM measured in control and DM-CD-treated BBMV is shown in Fig.3. 2-AS and DPH were used as probes for monitoring the degree of fluidity of the lipid bilayer. 2-AS probably localizes in the bilayer closer to the aqueous interface [24], and DPH has been found to localize in the hydrophobic interior of the BBMV [25]. ANM was used for monitoring the fluidity of the protein. The fluorescence polarization measurements of the BBMV were carried out at 25°C after pretreatment with DM-CD. 2 mM DM-CD enhanced the uptake of D-glucose in the BBMV, and also remarkably altered the fluorescence polarization of 2-AS and DPH. The fluorescence polarization of ANM, which binds covalently and preferentially to sulfhydryl groups of proteins [26], showed no significant changes with DM-CD treatment. Thus, use of fluorescent probes provided a good indication of the fluidity of the lipids and proteins in the BBMV.

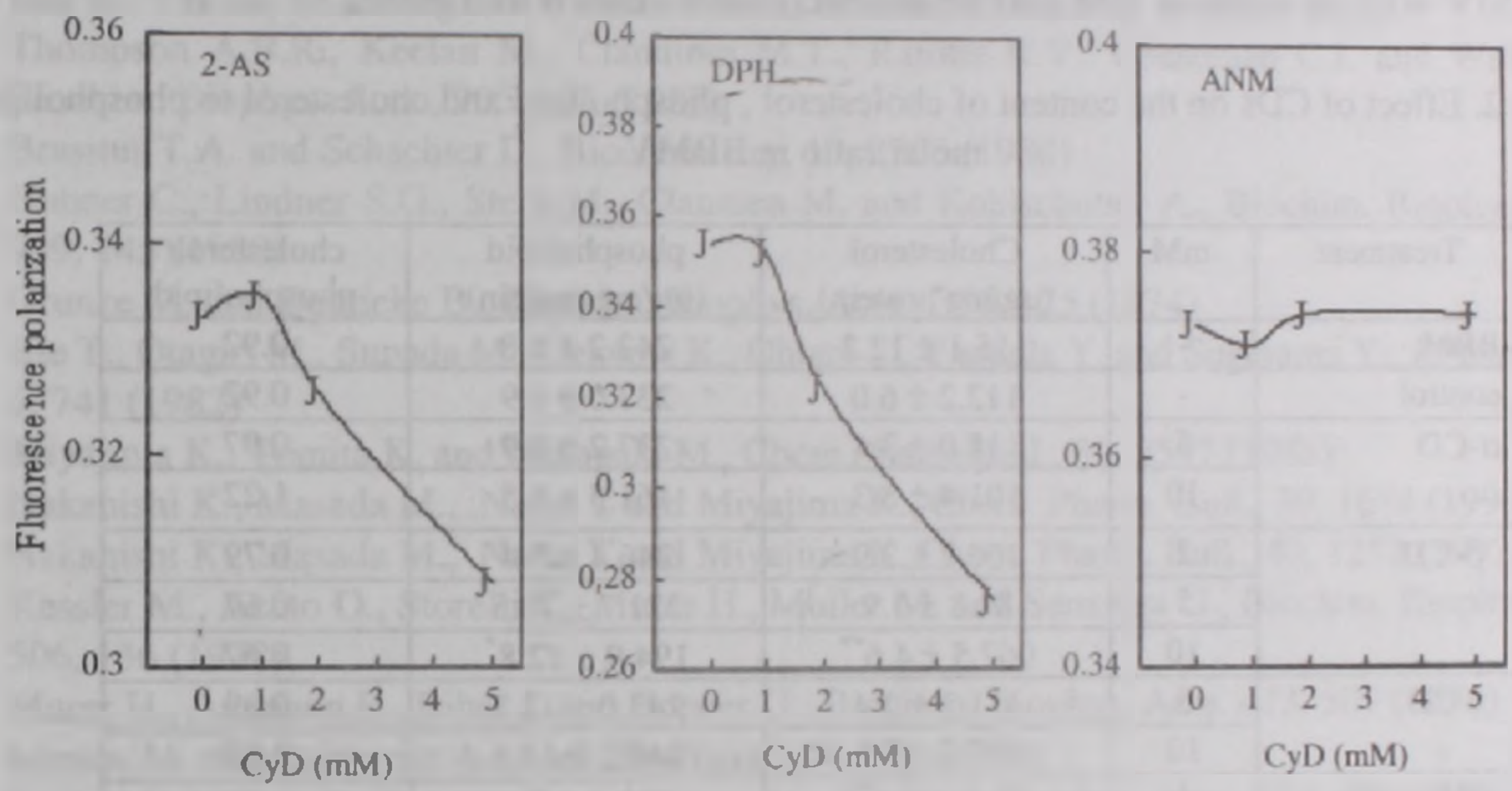

Figure 3. Effect of DM-CD on the fluoresecence polarization of 2-AS-, DPH-, and ANM-labeled bruch border membrane vehicles. Each point represents the mean of four experiments performed in duplicate determinations.

Discussion

Values for osmotically active space and the non-specific binding for D-glucose in the DM-CD-treated BBMV were similar to those values in control BBMV (Fig. 1). There was no significant difference in the passive transport properties of control and DM-CD-treated BBMV, in terms of the time-dependence of D-glucose influx in KCl solution. Thus, we regarded the integrity and membrane barrier function of DM-CD-treated BBMV as not so much altered.

The Vmax of the 2 mM DM-CD-treated BBMV differed significantly from that of control membranes ($p<0.001$); however the Km showed no difference in control and DM-CD-treated membranes. The increased absorption of D-glucose from the intestine in diabetes mellitus is well known. Tuji et al. reported an increased Vmax for glucose uptake in streptozotocin-induced diabetic rats, without any effect on Km value, suggesting that number

of glucose transporters was increased [27]. Tompson also reported similar phenomena in intact intestinal tissue and diabetic rats [28]. Thus, the increased Vmax of DM-CD-treated BBMV would appear to reflect changes in the number of D-glucose transporters. However, the enhanced D-glucose uptake in BBMV treated with DM-CD in this study can not be explained by an increase in the number of carriers, as we show below.

In this study, fluorescence polarization measurements with 2-AS and DPH as probes indicated a marked increase in the membrane fluidity of the aqueous interface and the hydrophobic interior of the BBMV. However, fluorescence polarization of ANM-labelled BBMV did not induce by DM-CD treatment, indicating that DM-CD may not interact with protein of the BBMV.

An increase in membrane cholesterol content reduced membrane fluidity [30], whereas conversely, a decrease in cholesterol content increased membrane fluidity [31]. The activity of some enzymes in the membrane was affected by alterations in the cholesterol content of the membrane [1]. Therefore, we measured the content of cholesterol and total phospholipid in the BBMV with or without DM-CD treatment, that is shown in Table 2.

Table 2. Effect of CDs on the content of cholesterol , phospholipid and cholesterol to phospholipid molar ratio in BBMV

Treatment	mM	Cholesterol (μg/mg protein)	phospholipid (μg/mg protein)	cholesterol/ phospholipid
BBM	-	115.1 ± 12.2	242.2 ± 6.8	0.92
control	-	112.2 ± 6.0	237.0 ± 6.9	0.92
α-CD	5	118.0 ± 3.4	237.2 ± 6.9	0.97
	10	101.4 ± 8.3	161.5 ± 5.5	1.22
β-CD	2	100.2 ± 2.9	246.4 ± 5.4	0.79
	5	81.8 ± 3.9	221.7 ± 10.5	0.67
	10	067.5 ± 4.6**	194.9 ± 12.8*	0.67
γ-CD	5	111.2 ± 2.4	243.0 ± 12.3	0.89
	10	107.2 ± 5.6	240.2 ± 13.6	0.87
DM-β-CD	1	86.8 ± 3.3**	240.6 ± 5.1	0.70
	2	74.9±3.7**	206.9 ± 21.6	0.70
	5	26.9 ±2.5**	34.6±10.4**	0.40

Each point represents the mean ± S.E. of five experiments performed in threee determinations. Significant difference from control, * $p < 0.05$, ** $p < 0.01$

DM-CD caused the liberation of cholesterol depending on the concentration, whereas did not cause phospholipid from BBMV (2mM DM-CD). The lowering of the cholesterol content of the BBMV by DM-CD induced the increase of membrane lipid fluidity, leading to an increased membrane permeability. We have reported that β-CD interacted with the cholesterol of intestinal membrane, resulting in withdrawal the lipid molecules by forming inclusion complexes [15]. We found here that D-glucose uptake in the BBMV was remarkably increased by the short-term treatment with DM-CD. DM-CD, as well as β -CD, has also been shown to interact with lipid components of the BBMV and to induce the membrane fluidity; this action may influence the transport properties of BBMV by altering membrane cholesterol content. The enhanced mobility of membrane lipids may result in perturbation of boundary lipids near protein molecules in the membrane. Thus, the enhanced D-glucose uptake by the BBMV treated with DM-CD may be due to alterations of membrane

components of the BBMV, resulting in increased membrane fluidity. The complexity of the situation in the treated BBMV requires additional studies before a definitive conclusion can be reached. Further investigations are currently underway to clarify the mechanism underlying the enhanced D-glucose uptake of DM-CD-treated BBMV.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CURRENT STATUS OF ATP-ase PROTON PUMP INHIBITOR COMPLEXATION WITH CYCLODEXTRINS

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The inclusion complexes of cyclodextrins with drugs where the changes brought to the physical, chemical and biological properties of the guest molecules endow the final product with considerable pharmaceutical potential. The complexation properties of β- and γ-cyclodextrins with a variety of drugs widely used in clinical practice such as omeprazole and gliclazide have been studied. The researches on the interactions between cyclodextrins and a particular class of drugs known as the proton pump inhibitors have been carried out.

Инклюзионные комплексы циклодекстринов с лекарствами, которые приводят к изменениям физических, химических и биологических особенностей молекул "гостя", наделяют конечный продукт определенным фармацевтическим потенциалом. Изучены свойства комлесообразования β- и γ-циклодекстринов с разными лекарствами, широко используемыми в клинической практике, как омепразол и гликлазид. Проведены исследования по изучению взаимолействия между циклодекстринами и особым классом лекарств, известных как ингибиторы протонового насоса.

Ցիկլոդեքստրինների և դեղերի ինկլյուզիոն համալիրները, որոնք բերում են «հյուրի» մոլեկուլների ֆիզիկական, քիմիական և կենսաբանական առանձնահատկությունների փոփոխություններին, օժտում են վերջնական նյութը որոշակի դեղագործական պոտենցիալով: Ուսումնասիրվել են β և γ-ցիկլոդեքստրինների հետ տարբեր դեղերի, ինչպես կլինիկայում լայնորեն կիրառվող օմեպրազոլի և գլիկլազիդի, համալիրագոյացման հատկությունները: Ուսումնասիրվել են դեղերի հատուկ խմբի, որոնք հայտնի են որպես պրոտոնային մխոցի արգելակիչներ, և ցիկլոդեքստրինների միջև փոխազդեցությունները:

Introduction

Cyclodextrins have come a long way since they were first isolated by Villiers in 1891 from the degradation products of starch in butyric fermentation [1] and since their preparation and complete characterization by Schardinger in the early 1900s [2]. Their ability to form inclusion compounds as a result of host-guest complexation, both in the solid state and in solution, is widely appreciated in various sectors [3] and particularly, of direct interest to us, in the field of Pharmaceutical Development [4].

Cyclodextrins are cyclic (α–1,4)–linked oligosaccharides of α–D–glucopyranose which, owing to the impossibility of free rotation about the connecting glycosidic bonds, assume the shape of a torus or a truncated cone rather than that of a perfectly cylindrical molecule (Figure 1). The carbon skeleton of each glucose monomer, present in a $^1C_4$ chair conformation, together with the interconnecting ether bridges delimit an interior, hydrophobic cavity whereas all the hydroxyl groups are oriented outwards to form a hydrophilic external surface. The primary hydroxyl groups are orientated in such a way as to

form the narrow rim of the torus while the secondary hydroxyl groups define the wider rim [5]. The most common cyclodextrins, α-, β- and γ-, are made up of six, seven and eight glucopyranose units respectively. One can easily imagine the central cavity housing hydrophobic/lipophilic, and hence poorly hydrosoluble substances, engulfing them in a hydrophilic shield. Indeed, this is what happens during the formation of inclusion compounds where the changes brought to the physical, chemical and biological properties of the guest molecules endow the resulting complex with considerable pharmaceutical potential [6].

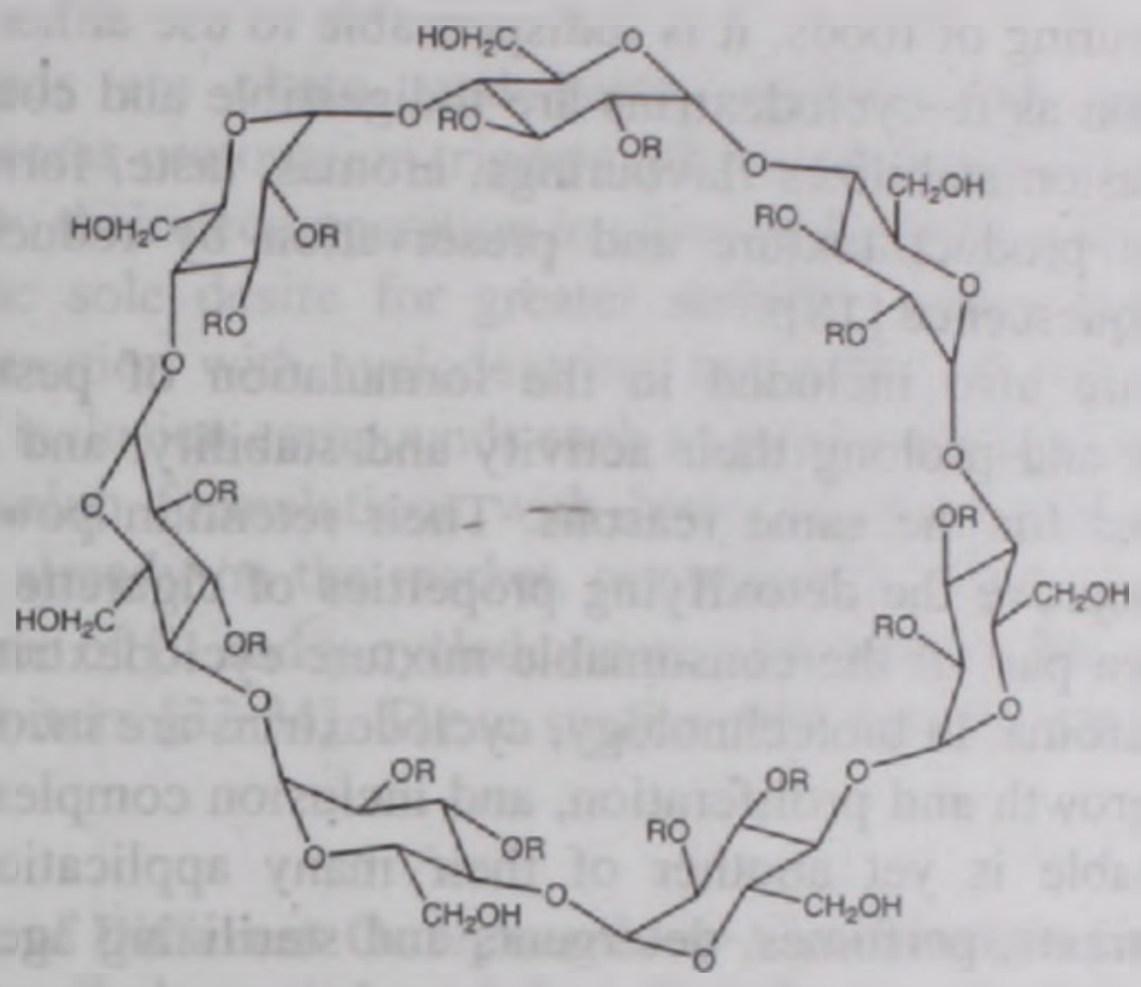


Figure 1. Molecular Structure of β-CD (R=H).

The formation of such inclusion complexes does not involve the formation of covalent bonds and, in aqueous solution, drug molecules are in dynamic equilibrium with those held within the cyclodextrin cavity. The main driving force of complex formation is probably the release of enthalpy-rich water molecules located inside the cavity that cannot satisfy their hydrogen bond potential and are of a higher enthalpy than the water molecules in solution [7, 8]. During complexation, the binding of non-polar drug molecules releases enthalpy-rich water molecules from the cavity into solution and the energy of the system is lowered [9].

Cyclodextrins occupy an important and ever-expanding pharmaceutical niche [10]. They are put to use essentially to increase molecular stability, enhance drug solubility in aqueous solutions, improve dissolution rate and bioavailability [11-13]. Other uses include that of converting liquid drugs into microcrystalline powders, preventing drug-drug or drug-excipient interactions, reducing drug irritation after oral or topical administration and improving the organoleptic characteristics of the formulation. Stability improvement aims at decreasing volatility and bettering heat stability, resistance to oxidation and hydrolysis whereas, improved drug-bioavailability from a cyclodextrin-containing formulation arises predominantly from an increase in the apparent solubility of the hydrophobic drug which would normally be prone to a dissolution rate-limited absorption.

Indeed, such interactions have been studied extensively and several inclusion complexes are now on the market in Japan and Europe [14]. In the U.S.A., cyclodextrin-containing products have not yet met with F.D.A. approval which is unfortunate because such an approval conditions worldwide opinion and would be of paramount importance to pharmaceutical use and commercial viability of these valuable materials [15].

Furthermore, cyclodextrins and their derivatives have found their way into countless products of the cosmetic, chemical and alimentary industries where inclusion compounds continue to be appreciated for their enhanced solubility of poorly hydrosoluble compounds and their molecular stabilization to heat, oxidation, light and hydrolysis [16].

In cosmetics, β–cyclodextrin inclusion compounds are used as an alternative method to the micellar solubilization of liposoluble ingredients which usually employs potentially irritating surfactants, for their slow and prolonged release of colorants and perfumes, for stabilizing essential oils and protecting them from oxidation and volatilization and for their ability to include malodorous compounds [17].

In the manufacturing of foods, it is indispensable to use either β– or γ–cyclodextrins for human consumption as α–cyclodextrins are indigestible and could lead to problems of chronic toxicity. Inclusion stabilizes flavourings, aromas, taste, form and colour, and may also help to improve product texture and preservation by reducing the end product's hygroscopicity or deliquescence [18].

Cyclodextrins are also included in the formulation of pesticides, herbicides and fungicides to enhance and prolong their activity and stability, and in products containing plant growth hormones for the same reasons. Their retention power is exploited by the tobacco industry to improve the detoxifying properties of cigarette filters against nicotine and tars whereas, when part of the consumable mixture cyclodextrins are used to stabilize and enhance tobacco aroma. In biotechnology, cyclodextrins are incorporated in cell culture media to favour cell growth and proliferation, and inclusion complexation with enzymes to render them more stable is yet another of their many applications. Anti-foam agents, industrial dyes, deodorants, perfumes, detergents and sterilizing agents are only but a few other examples from the never ending list of countless cyclodextrin-containing products [19].

Of equal importance is the application of natural and modified cyclodextrins in the field of analytical chemistry. Here, they have been successfully employed in chiral capillary electrophoresis [20] as the chiral selector component of the background electrolytes [21], as chiral stationary phases in both liquid and gas chromatography and as selective components of the mobile phase in thin layer and high performance liquid chromatography [22]. The basis of this use lies in the ability of cyclodextrins to select guest molecules in view of their size and geometry.

Our research group has been working with cyclodextrins for some time now and in particular we have studied the complexation properties of β- and γ-cyclodextrins with a variety of drugs widely used in clinical practice such as omeprazole and gliclazide [23-26]. Here, we present a brief over-view of the research carried out on the interactions between cyclodextrins and a particular class of drugs known as the proton pump inhibitors.

H^+/K^+ ATP-ase Proton Pump Inhibitors.

The ultimate mediator of acid secretion in the stomach is the H^+/K^+ ATP-ase transmembranal protein uniquely present in the apical membrane of the parietal cell and commonly referred to as the proton pump. By covalently binding to certain critical cystein residues present in the extracellular luminal domain of this protein, the proton pump inhibitors block the conformational reassessments that are responsible for this protein's pumping action, and thus inhibit the extrusion of protons into the lumen of the stomach. In doing so, these drugs block both basal and stimulated secretion of gastric acid [27-29].

Omeprazole, Pantoprazole, Lansoprazole, Rabeprazole and now also Esomeprazole, which constitute this class of highly specific proton pump inhibitors, incorporate both a

benzimidazole moiety and a pyridine ring bridged together by a methylsulfinyl link (Figure 2). Theoretically, both of these aromatic portions are able to interact with the cyclodextrin cavity. At neutral pH these compounds are devoid of inhibitory activity and require an acidic environment for their activation and as such, these drugs are, in fact, prodrugs. On reaching the parietal cells from the blood, these neutral amphiphilic compounds diffuse into the secretory canaliculi where they become protonated and trapped, unable to cross back over the cellular membrane into the parietal cell. The protonated form rearranges to form a sulfenic acid and a sulfenamide and it is as a sulfenamide that these compounds form disulphides with the sulphydryl groups of the cysteinic amino acids to block the pump [30,31].

These compounds are photo- and heat- sensitive and cannot withstand acidic conditions. Infact, whereas protonation triggers off their biological activity *in vivo*, the same series of events leads to their decomposition *in vitro*, making them difficult to manage during drug formulation. The sole desire for greater stability makes these compounds perfect candidates for complexation with cyclodextrins, but other advantages may also be drawn from the formation of inclusion compounds such as greater aqueous solubility.

In order to develop formulations with better technological and biopharmaceutical properties than those already on the market, our research group carried out studies on the complexation behaviour of β- and γ-cyclodextrins with omeprazole, as the representative of the proton pump inhibitors [32-34]. These studies will now be dealt with in the following sections.

Thermal Study of Different Omeprazole–γ–Cyclodextrin Co-Ground Systems.

This first line of research employed techniques of thermal analysis, such as Differential Scanning Calorimetry (DSC), Thermal Gravimetric/Differential Thermal Gravimetric Analysis (TG/DTG), Evolved Gas Detection (EGD) and Hot Stage Microscopy (HSM) to study and characterize both the inclusion complexes formed and the state of dispersion obtained after co-grinding equimolar mixtures of omeprazole and γ-cyclodextrins under various conditions [32]. These techniques prove ideal for following inclusion complex formation, as they provide a precise insight into the binding events which occur between the cyclodextrin host and the guest molecules.

The samples were prepared by milling a 1:1 physical mixture of omeprazole (OME) and γ-cyclodextrin (γ-CD) in a semi industrial mill (Fritsch Pulverisette, type 02102) for 4 h following four different methods:

1. dry co-grinding;
2. wetting of physical mixture with absolute alcohol;
3. wetting of physical mixture with a 1:1 ethanol/phosphate buffer solution;
4. wetting of physical mixture with a phosphate buffer.

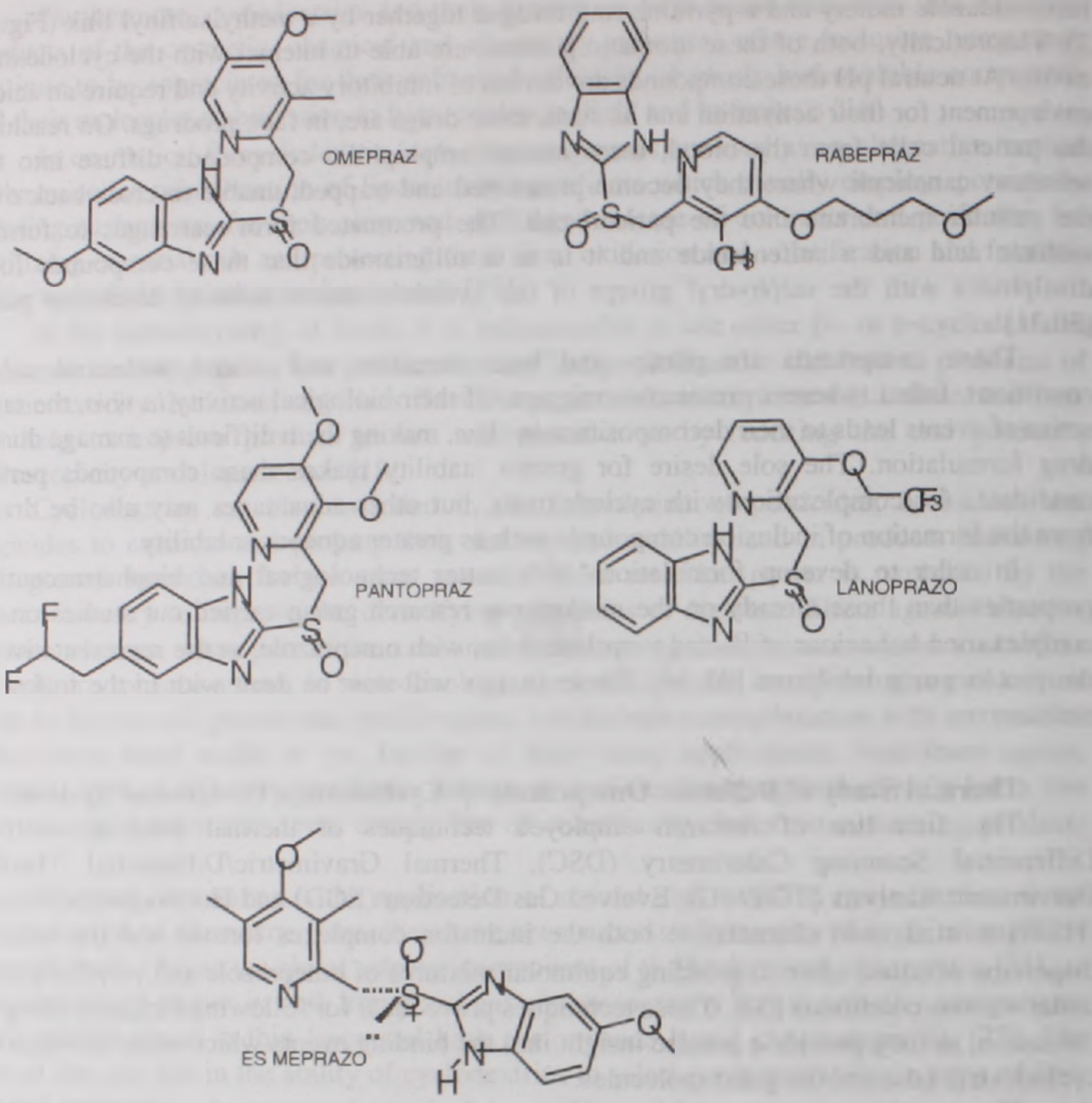


Figure 2. Molecular Structure of the Proton Pump Inhibitors.

The phosphate buffer was used instead of water to wet the systems in order to preserve the drug, under examination, from degradation.

The DSC curves of the co-ground systems were compared with those of the pure components, the dry-ground OME and the physical mixture (Figure 3). Curves 3(a) and 3(b) belong to OME and γ-CD respectively. Curve 3(a) is characterized by the presence of a sharp endothermic effect at 148°C due to fusion, followed by a broad exothermic peak at about 160°C caused by decomposition. In curve 3(b), two endothermic effects (a broad peak at 50°C and a less pronounced one at about 100°C) can be observed as a result of the gradual loss of two types of differently retained water molecules from pure γ-CD. Another endothermic peak at around 275°C indicates the onset of the solid's decomposition which is extended to the liquid phase after all the γ-CD has melted. Curve 3(c), arising from the physical mixture OME/γ-CD, is a mere superposition of the curves given by the pure components of the mixture, apart from dehydration occurring at a lower temperature.

indicating a tendency of the drug to favour CD dehydration [35]. From the DSC curve relative to dry-ground OME (curve 3(d)), it can be noted that the melting endotherm at about 150°C is less pronounced than that in curve 3(a), evidencing only partial amorphization of the drug during dry-grinding.

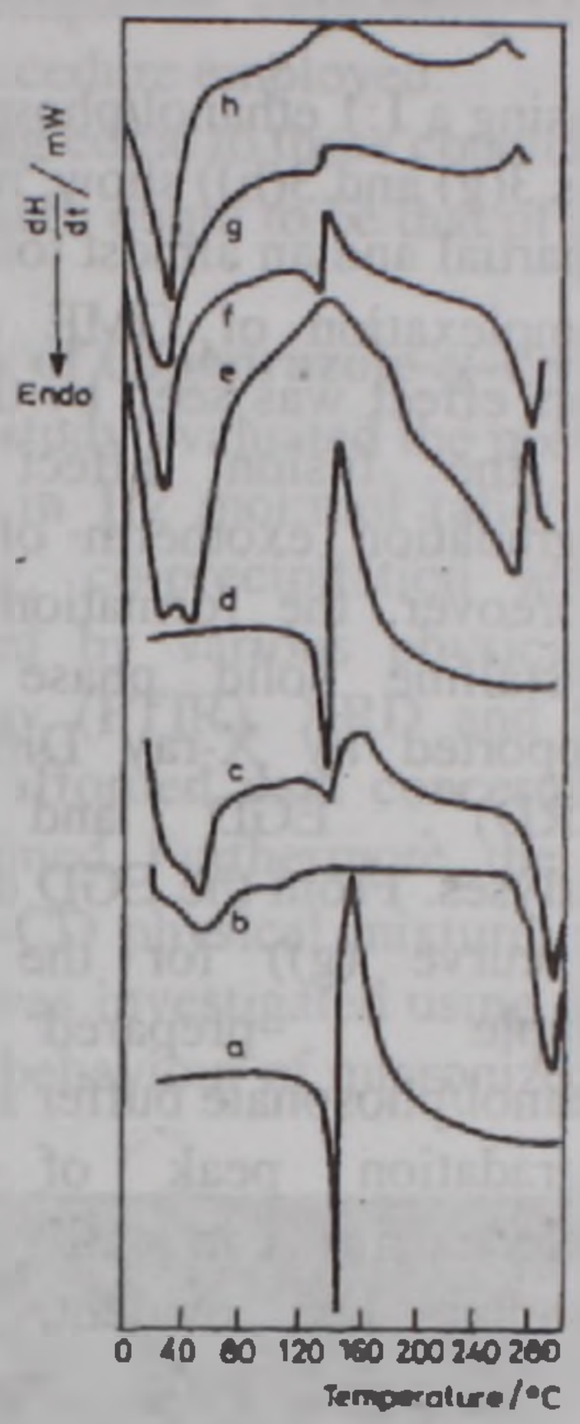


Figure 3. DSC curves corresponding to OME/γ-CD binary systems treated by different co-grinding procedures: (a) micronized OME, (b) γ-CD, (c) physical mixture, (d) dry-ground OME, (e) dry co-ground mixture, and samples obtained by wetting with absolute ethanol (f), 1:1 ethanol/phosphate buffer solution (g) and phosphate buffer (h). (*with kind permission from Kluwer Academic Publishers*)

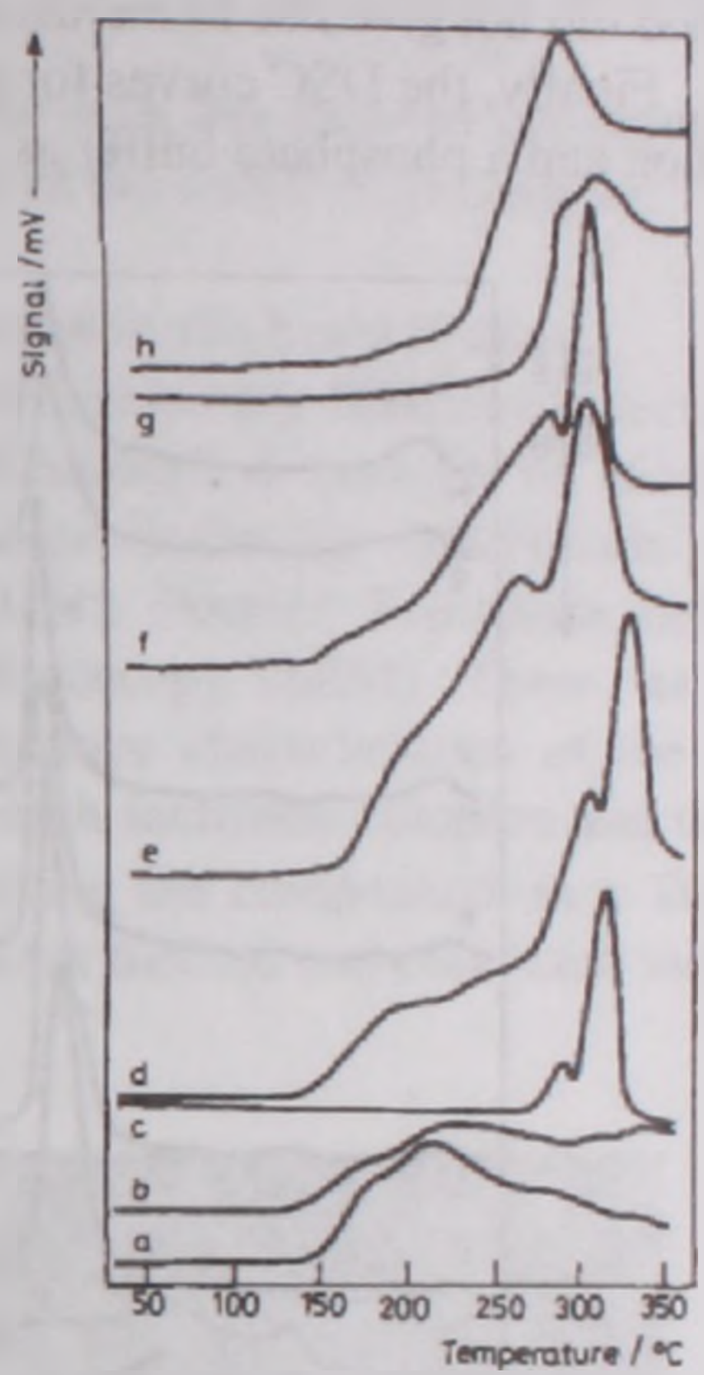


Figure 4. EGD curves corresponding to OME/γ-CD binary systems treated by different co-grinding procedures: (a) micronized OME, (b) γ-CD, (c) physical mixture, (d) dry-ground OME, (e) dry co-ground mixture, and samples obtained by wetting with absolute ethanol (f), 1:1 ethanol/phosphate buffer solution (g) and phosphate buffer (h). (*with kind permission from Kluwer Academic Publishers*)

A drastic change is observed in the thermal profile of the dry co-ground mixture (curve 3(e)). The absence of the characteristic melting peak for OME indicates the presence of an amorphous or poorly crystalline solid state. Although this behaviour has often been interpreted as a consequence of inclusion complex formation, in this case it is probably due to the formation of an amorphous solid dispersion[36]. This assumption, corroborated by the EGD results (Figure 4, curve (e)), agrees with the idea that, the self-absorbed water content of the cyclodextrin host is not normally sufficient to form a real inclusion complex during co-grinding. Moreover, the fact that this inclusion compound was not formed is sustained by the TG/DTG curve (Figure 5, curve (e)), where the mass loss at about 130-140°C has been attributed to degradation of the free drug. As a matter of fact, the TG/DTG profiles, relative to micronized and dry-ground OME and to the OME/γ-CD physical mixture (Figure 5, curves (a), (b) and (d), respectively), display a mass loss over the latter temperature range.

Furthermore the sample obtained by wetting the physical mixture with absolute ethanol (Figure 3, curve (f)), presents the melting endotherm of OME, thus proving that an inclusion compound is not formed when absolute ethanol is used as the binding liquid, suggesting that water is essential for complex formation. In fact, EGD analysis (Figure 4, curve (f)) shows that the sample started to decompose at around 150°C, indicating that this method did not give rise to inclusion complexation.

Finally, the DSC curves for the samples prepared using a 1:1 ethanol/phosphate buffer solution and a phosphate buffer as binding liquids (curves 3(g) and 3(h)) show, respectively, a partial and an almost total inclusion complexation of OME with γ-CD. This effect was seen by the decrease in the fusion effect and the degradation exotherm of the drug. Moreover, the formation of a new crystalline solid phase was also supported by X-ray Diffractometry (XRD)[37], EGD and TG/DTG analyses. From the EGD data (Figure 5, curve (g)) for the co-ground sample prepared with ethanol/phosphate buffer solution, the degradation peak of the free cyclodextrin can still be seen, although less evident, at around 150°C, suggesting only partial complexation. On the other hand, in the case of the sample obtained by using phosphate buffer as the wetting liquid, this thermal event caused by drug decomposition is absent (Figure 5, curve (h)), the only gas evolution effect visible being that at 270°C assigned to complex degradation.

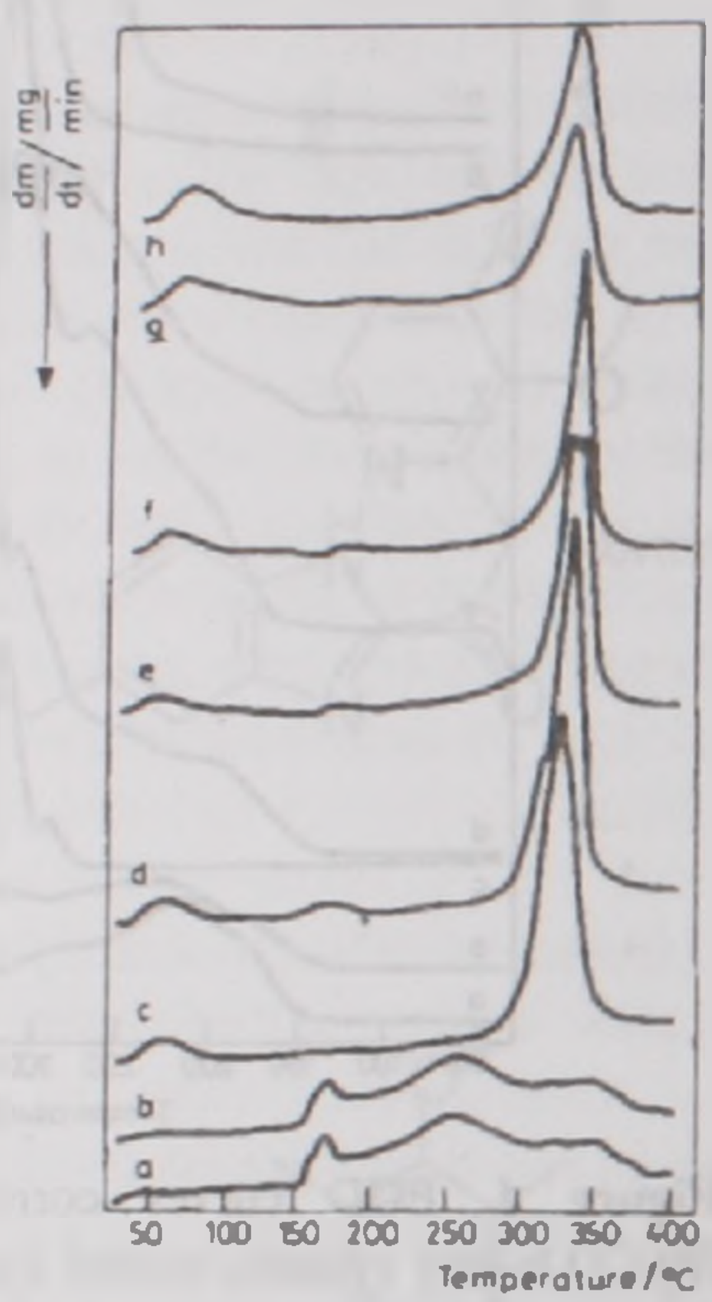


Figure 5. TG/DTG curves corresponding to OME/γ-CD binary systems treated by different co-grinding procedures: (a) micronized OME, (b) γ-CD, (c) physical mixture, (d) dry-ground OME, (e) dry co-ground mixture, and samples obtained by wetting with absolute ethanol (f), 1:1 ethanol/phosphate buffer solution (g) and phosphate buffer (h). (*with kind permission from Kluwer Academic Publishers*)

The feasibility of the formation of an inclusion complex in the co-ground sample prepared with phosphate buffer was corroborated by the HSM data. Crystals of pure OME underwent a melting process at about 159°C, followed by immediate degradation at 162°C. The physical mixture underwent γ-CD dehydration, melting of the drug, partial adsorption of the melt onto the cyclodextrin mass, degradation of the molten drug and finally melting of the γ-CD with decomposition. The same behaviour, although not as pronounced, was observed for the "1:1 ethanol/phosphate buffer solution" sample, whereas melting of the drug was not registered for those samples prepared via dry-grinding and through sole use of phosphate buffer.

In conclusion, all these thermal techniques together revealed that the changed crystalline properties of the composites obtained by co-grinding were due to distinct types of interactions between the components, as a consequence of the variations in the grinding procedure. The experimental data clearly revealed that a true solid complex was obtained

only by wetting the mixtures with a phosphate buffer during co-grinding. Other co-grinding procedures yielded only amorphous drug/cyclodextrin mixtures or their combination with partial complexation. Indeed, since grinding also affects the crystalline properties of a solid, it should be possible to obtain either amorphous mixtures of the co-ground components or inclusion complexes, depending on the characteristics of the starting materials and on the grinding procedure employed.

Having come to these conclusions, it seemed obvious to us that the next step along this line of research ought to be that of studying the solid state of the OME/γ-CD complex.

Study of Omeprazole–γ–Cyclodextrin Complexation in the Solid State

This study evaluated the possibility of obtaining and comparing OME/γ-CD inclusion complexes, in 1:2 mol:mol ratio, using the different technological methods of kneading, spray-drying, co-precipitation and freeze-drying[33]. These inclusion compounds were characterized by various physical methods, such as DSC, Fourier Transform Infrared Spectroscopy (FTIR), XRD and Scanning Electron Microscopy (SEM). These last two techniques afforded data concerning crystallinity and surface characteristics of the solid phases obtained. Furthermore, the dissolution profile of each inclusion complex and that of 1:2 OME/γ-CD physical mixture, prepared by gently mixing the components in a Turbula apparatus, was investigated using the USP 23 rotating basket method and compared with the dissolution behaviour of micronized OME.

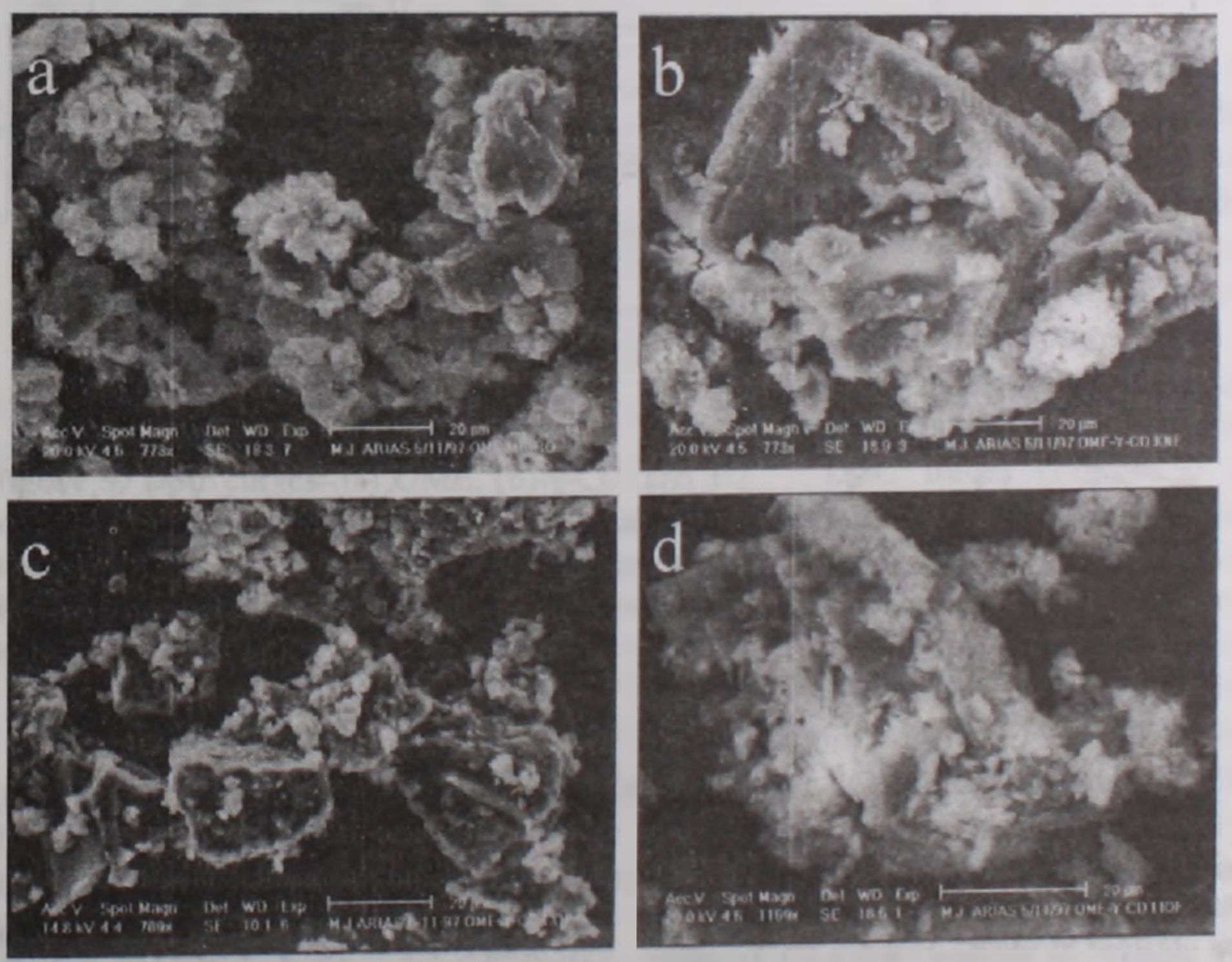


Figure 6. SEM photomicrographs: (a) micronized OME, and 1:2 mol:mol OME/γ-CD systems obtained by kneading (b), co-precipitation (c), and freeze-drying (d). (*with kind permission from Marcel Dekker Inc.*)

Figure 6 shows several SEM photomicrographs of the samples under study. Although this technique is not conclusive for assessing the existence of a true inclusion compound in the solid state, it can be useful to prove the homogeneity of the solid phases. Micronized OME is characterized by the presence of crystalline particles of regular size, while γ-CD appears as crystalline particles without a definite shape (Figure 6, curve (a)). The kneaded system (Figure 6, curve (b)) showed crystalline particles of a single component without any visible drug crystals. A similar morphology was presented by samples prepared via coprecipitation (Figure 6, curve (c)), although in this case the crystals were smaller. The solid phases obtained by freeze-drying (Figure 6, curve (d)) showed a remarkable decrease in crystallinity probably due to the formation of an amorphous inclusion compound. The freeze-dried product appeared to be of a lesser crystalline structure with a soft, fluffy appearance and again, the crystals of the single components were still not distinguishable. The spray-dried sample presented a morphology typical of this preparation i.e. very small, spherical particles with a high tendency to aggregate [24].

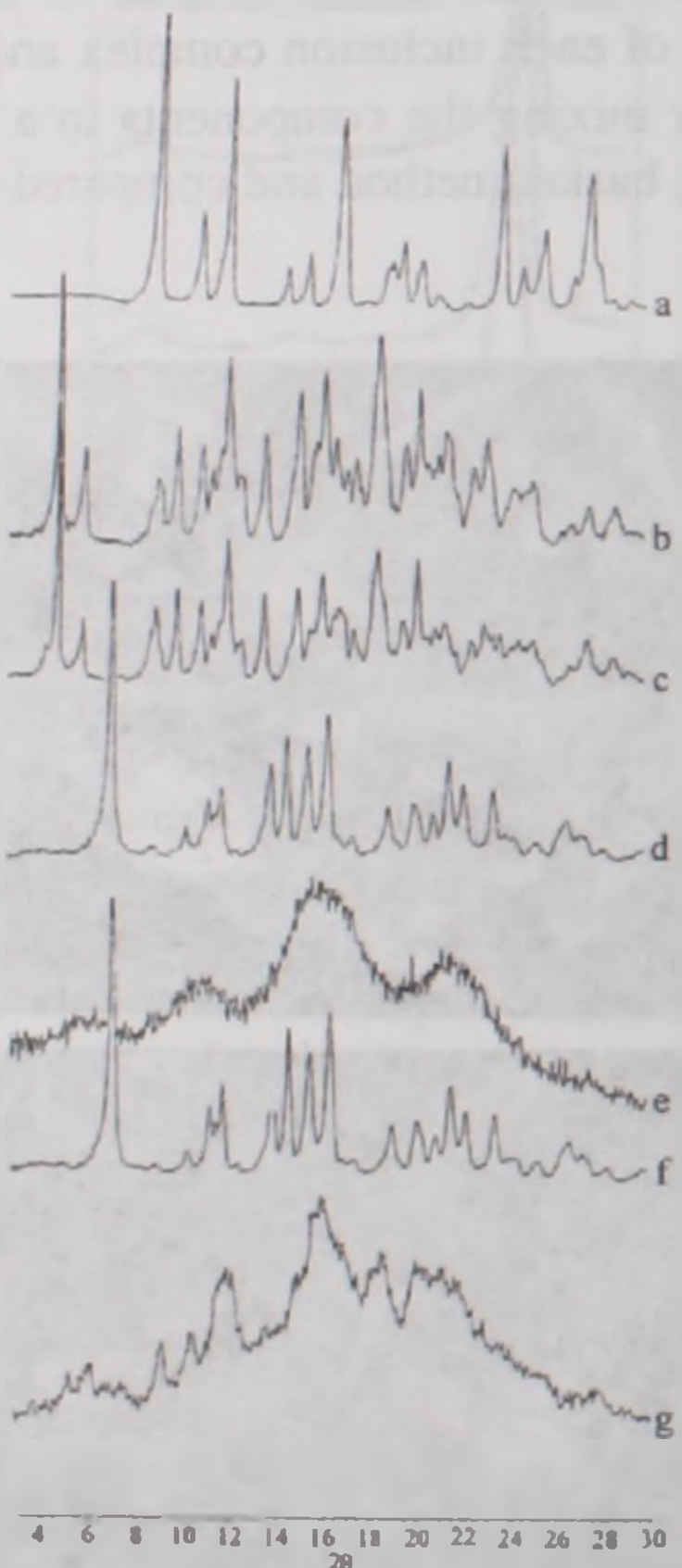


Figure 7. X-ray diffraction patterns on powder: (a) OME, (b) γ-CD, and 1:2 mol:mol OME/γ-CD systems obtained by physical mixing (c), kneading (d), spray-drying (e), co-precipitation (f), and freeze-drying (g). (*with kind permission from Marcel Dekker Inc.*)

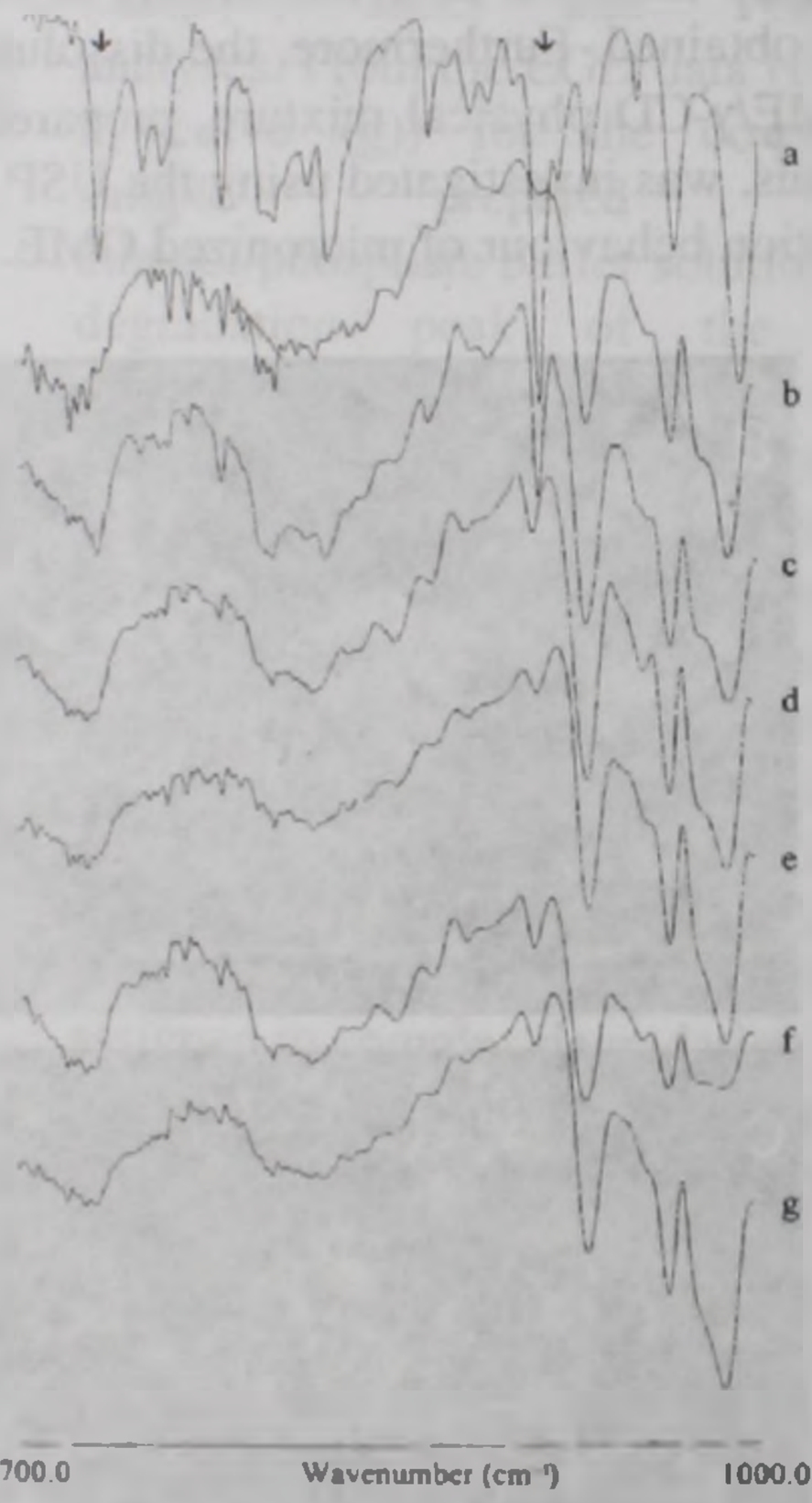


Figure 8. FTIR spectra: (a) OME, (b) γ-CD, and 1:2 mol:mol OME/γ-CD systems obtained by physical mixing (c), kneading (d), spray-drying (e), co-precipitation (f), and freeze-drying (g). (*with kind permission from Marcel Dekker Inc.*)

The X-ray diffraction patterns are depicted in Figure 7. As expected, the physical mixture (curve (c)) corresponds to the simple superposition of the X-ray patterns of OME (curve (a)) and γ-CD (curve (b)). The extremely characteristic diffractograms of the products prepared by kneading and co-precipitation (curves (d) and (f), respectively) probably arose from the formation of a new crystalline phase. The solid phases obtained via freeze-drying (curve (g)) and in particular via spray-drying (curve (e)) showed a remarkable decrease in crystallinity, probably due to the formation of an amorphous inclusion compound. These findings were in-line with those of the FTIR spectral analysis, shown here in Figure 8, where two characteristic bands at 1625.7 cm^{-1} (C=C-N and S-C=N link vibration stretching) [38] and 1204 cm^{-1} (Ar-C-O-CH_3 bending vibration) were chosen from OME's FTIR spectrum to indicate the solid state interactions during complexation. In the spectrum for the physical mixture (curve 8(c)), these two bands remain practically unchanged for position and intensity with respect to the FTIR spectrum of OME alone, indicating that simple mixing did not result in any interaction involving these chromophores. In the FTIR spectra of all the other binary systems, the absorption band at 1625 cm^{-1} disappeared and the intensity of the band at 1204cm^{-1} also markedly decreased probably due to a restriction arising from complexation.

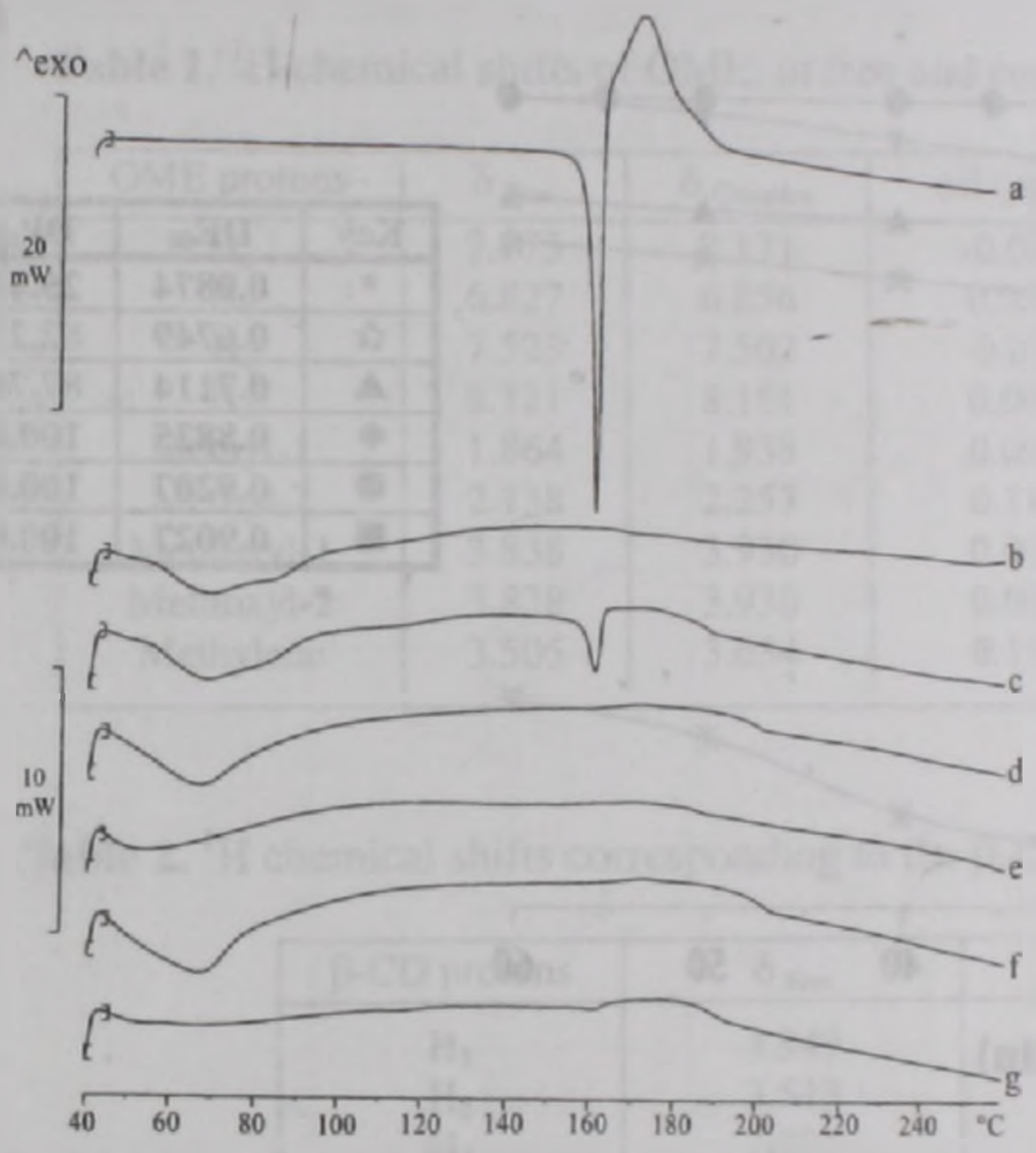
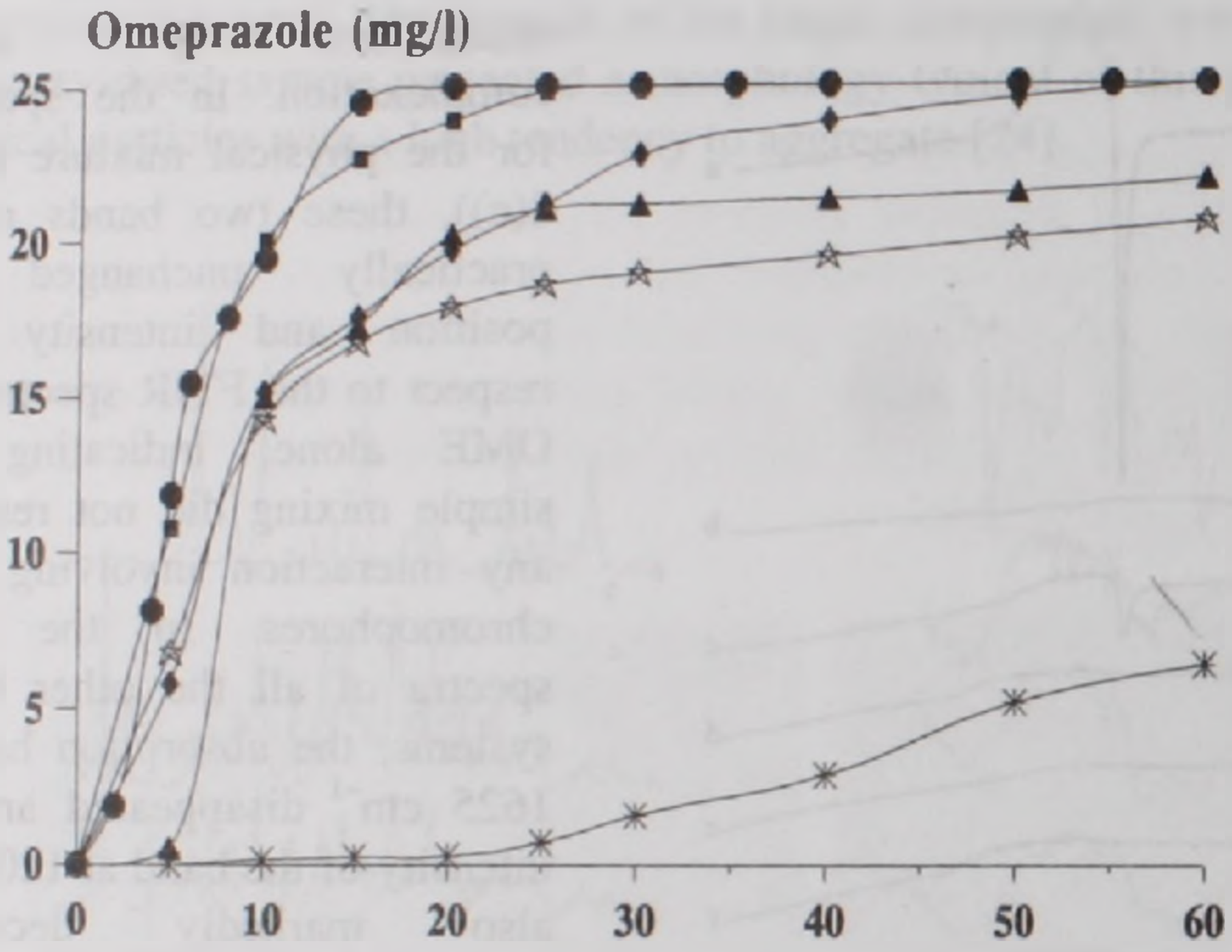


Figure 9. DSC curves: (a) OME, (b) γ-CD, and 1:2 mol:mol OME/γ-CD systems obtained by physical mixing (c), kneading (d), spray-drying (e), co-precipitation (f), and freeze-drying (g). (*with kind permission from Marcel Dekker Inc.*)

The formation of inclusion compounds was confirmed by DSC traces (Figure 9) of the kneaded, spray-dried and co-precipitated systems. Curves (d)-(g) show that the melting endotherm of OME is absent, while it is present in the micronized OME (curve (a)) and in the physical mixture (curve (c)), as expected. However, a residual endothermic effect at around 161°C is observed in the run of the freeze-dried sample, indicating that a small fraction of crystalline free OME is still present, corresponding to approximately 3.5% of the sample.

The dissolution profiles for the systems under study are depicted in Figure 10. For their evaluation two parameters, dissolution efficiency[39] and the percentage of drug dissolved after 60 min (DE_{60} and DP_{60}, respectively), were measured. Among the solid phases obtained, the co-precipitated and the freeze-dried products presented the highest dissolution rates with complete dissolution being achieved within approximately 20 minutes, while the product of spray-drying had a lower dissolution rate. This may be attributed to the high tendency of the

spherical particles to agglomerate in the spray-dried form, probably due to the presence of electrostatic forces. The low DE_{60} and DP_{60} values for the kneaded product could, on the other hand, be traced to the self-aggregation of particles which reduces the surface area available for dissolution. Finally, the physical mixture also shows a significant enhancement in OME dissolution rate with respect to OME alone. This may be ascribed to two concomitant effects, the improved wettability of the physical mixture brought about by the presence of γ-CD, a highly hydrophilic component, and to the possible formation *in situ* of the relevant inclusion compound when the physical mixture comes into contact with the dissolution medium.



Key	DE_{60}	DP_{60}
*	0.0874	25.44
☆	0.6749	82.25
▲	0.7114	87.74
◆	0.8825	100.0
●	0.9207	100.0
■	0.9027	100.0

Figure 10. Dissolution curves and dissolution parameters of micronized OME and 1:2 mol:mol OME/γ-CD systems (* OME, ☆ physical mixture, ▲ kneaded, ◆ spray-dried, ● co-precipitated and ■ freeze-dried). (*with kind permission from Marcel Dekker Inc.*)

Having successfully prepared and thoroughly characterized OME/γ-CD inclusion compounds, we were eager and ready to proceed with our research and to study the complexation properties of β-CD with OME in aqueous media.

Investigation of the Inclusion Complex Formation between Omeprazole and β–Cyclodextrin by Phase-solubility Analysis, ^{1}H and ^{13}C NMR Spectroscopies and Molecular Modelling.

This third section reports the investigation of the complexation mechanism and the driving forces involved in the interaction between OME and β-CD in aqueous media[26,34,40]. The effect of host concentration on apparent guest solubility, the possible separation of solid complexes, their stoichiometric ratios in solution and in the solid state, the calculation of apparent stability constants and the molecular geometry of the relative inclusion compounds were all aspects of complexation that were thoroughly examined in this study using techniques of Phase Solubility Analysis, ^{1}H and ^{13}C NMR Spectroscopy and Molecular Modelling.

In the outcome of the guest molecule being included within the host molecule, the NMR spectra of the inclusion compound will differ, in terms of chemical shift values, from both that of the sole guest molecule and that of the cyclodextrin itself.

The ^{1}H NMR spectral data of the drug and β-CD both in the free and complexed states are reported in Tables 1 and 2, respectively. The peak assignments for OME are shown in Figure 11 and those for β-CD are shown in Figure 12. In the case of the cyclodextrin host, the greatest differences in chemical shift ought to be encountered for those protons situated within the hydrophobic cavity as they will experience this shielding effect the most once the guest molecule has been included whereas, any possible external guest/host interaction will not alter these chemical shifts. This was found to be true for host protons H5 and H6, situated towards the narrower rim of the internal surface.

Table 1. ^{1}H chemical shifts of OME, in free and complexed states in $NaOD/D_2O$ solution.

OME protons	δ_{Free}	$\delta_{Complex}$	$\Delta\delta$ (ppm)	Peak multiplicity
H_a	7.173	7.171	-0.002	doublet (coupled with H_b)
H_b	6.827	6.856	0.029	doublet (coupled with H_a)
H_c	7.523	7.502	-0.021	doublet
H_d	8.121	8.181	0.060	singlet
Methyl-1	1.864	1.958	0.094	singlet
Methyl-2	2.138	2.253	0.115	singlet
Methoxyl-1	3.838	3.930	0.092	singlet
Methoxyl-2	3.838	3.930	0.092	singlet
Methylene	3.505	3.634	0.129	singlet

Table 2. ^{1}H chemical shifts corresponding to the β-CD, in the presence and absence of OME.

β-CD protons	δ_{Free}	$\delta_{Complex}$	$\Delta\delta$ (ppm)
H_1	4.949	4.969	0.020
H_2	3.513	3.531	0.018
H_3	3.875	3.878	0.003
H_4	3.427	3.448	0.021
H_5	3.802	3.765	-0.037
H_6	3.870	3.803	-0.067

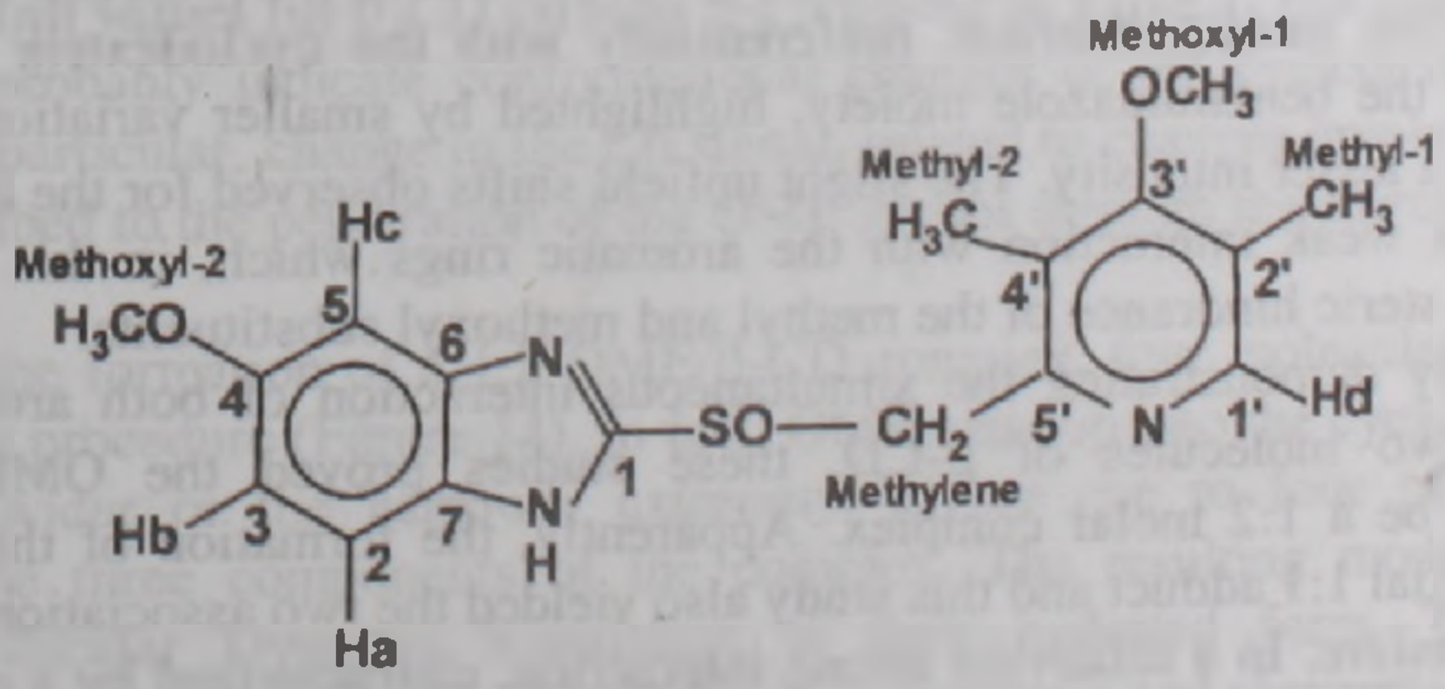


Figure 11. Structure and labelling scheme of OME.

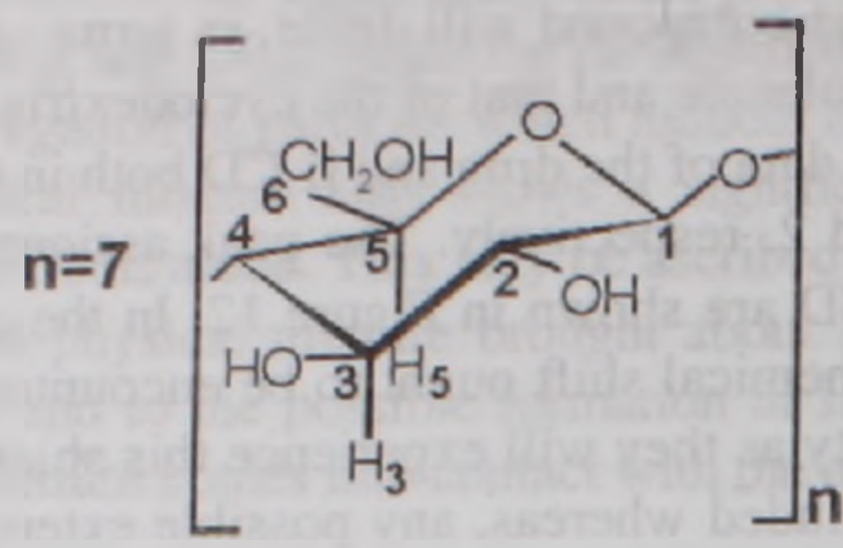


Figure 12. Labelling scheme of β-CD.

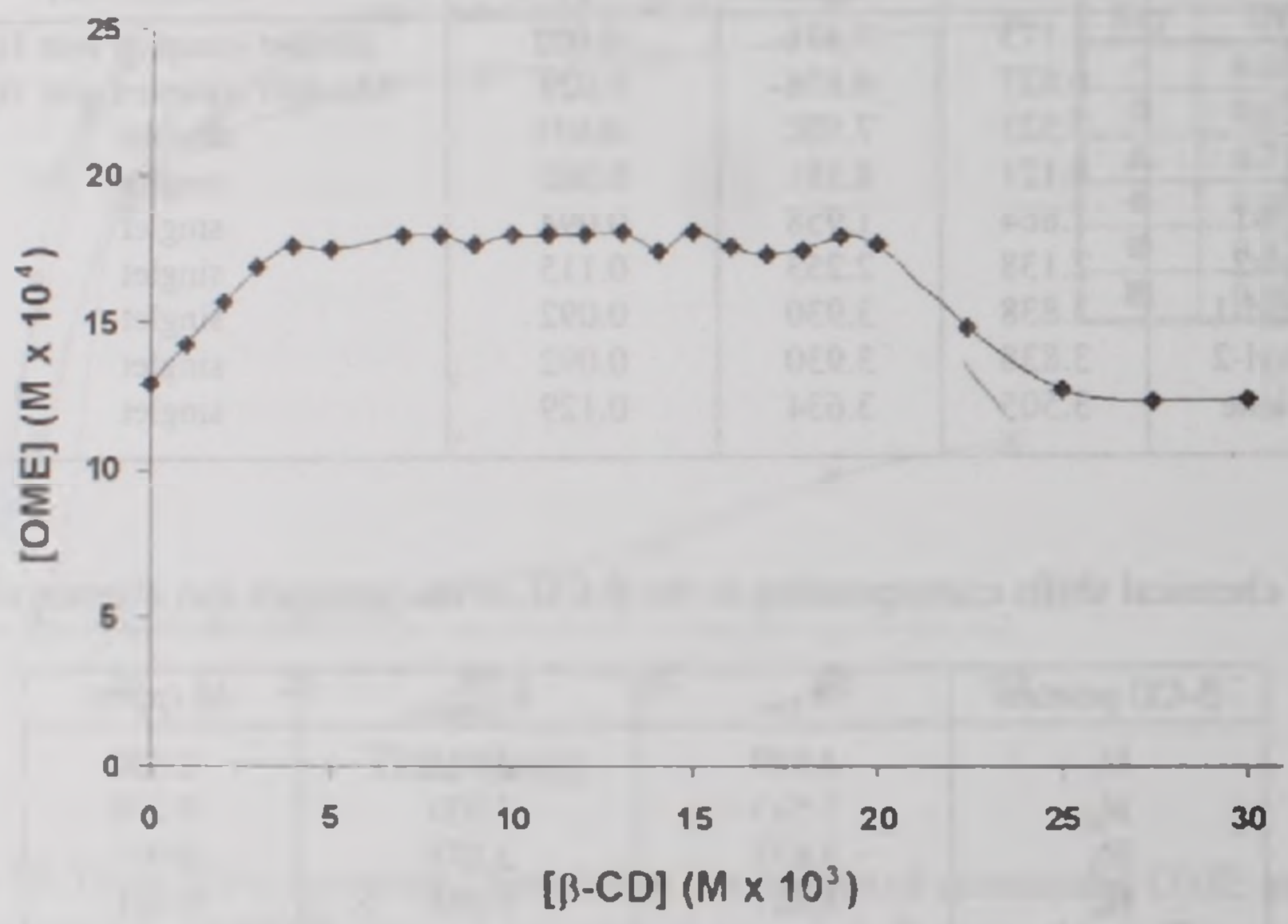


Figure 13. Phase-solubility diagram of OME in presence of β-CD at 25 °C.

The observed downfield shifts of the guest protons in the presence of β-CD, indicated that the pyridine moiety interacts preferentially with the cyclodextrin cavity, while the interaction of the benzimidazole moiety, highlighted by smaller variations in its chemical shifts, was of a lesser intensity. The slight upfield shifts observed for the inner protons of β-CD indicate a weak interaction with the aromatic rings which, in this case, is probably caused by the steric hindrance of the methyl and methoxyl substituents.

Thus, by demonstrating the simultaneous interaction of both aromatic portions of OME with two molecules of β-CD, these studies proved the OME/β-CD inclusion compound to be a 1:2 molar complex. Apparently, the formation of this complex passes through an initial 1:1 adduct and this study also yielded the two association constants for this stepwise procedure. In a relatively strong interaction, characterized by a constant $K_1 = 92.6$ M^{-1}, the pyridine moiety of the guest molecule becomes entrapped by a first host molecule, and the benzimidazole moiety of the guest then accommodates itself within a second host molecule to give the 1:2 complex. However, a second relatively low association constant (K_2

= 4.4 M^{-2}) suggests a rather weak interaction. Initial formation of a 1:1 complex is further supported by the phase-solubility curve (Figure 13). The ascending linear portion has a slope < 1, indicating that the increase in the apparent solubility of OME is most probably due to the formation of an equimolar complex.

The hypothesis that the pyridine portion has a higher affinity for the cyclodextrin cavity than the benzimidazole moiety does, is sustained by ^{13}C NMR data. The ^{13}C NMR chemical shifts for omeprazole carbons, both free and complexed, are shown in Table 3. As a rule, the guest carbons lining the cavity are shielded, while those situated near the rim are deshielded. The observed upfield chemical shifts of several pyridine carbon atoms indicate its inclusion by the cyclodextrin. On the other hand, the downfield shifts observed for C3' and C5' atoms suggest that these carbons are positioned towards the outside of the cavity, and indicate the degree of penetration into the host. Furthermore, upfield shifts were observed for C3 and C5 of the benzimidazole moiety, whereas the signal of C1 was shifted downfield, indicating only partial penetration.

Table 3. ^{13}C chemical shifts of OME in free and complexed states in $NaOD/D_2O$ solution.

OME carbons	δ_{Free}	$\delta_{Complex}$	$\Delta\delta$ (ppm)
C_1	155.344	155 621	0.277
C_2	100.318	100.530	0.212
C_3	112.340	111.954	-0.386
C_4	148.783	n.a.	n.a
C_5	118.743	118.510	-0.233
C_6	141.118	141.135	0.017
C_7	146.453	146.242	-0.211
C_1'	149 538	149 490	-0 048
C_2'	127.650	126.891	-0.759
C_3'	164.772	165.011	0 239
C_4'	128.582	128.189	-0.393
C_5'	158.733	159.171	0.438
Methyl-1	11.292	11.551	0 259
Methyl-2	13.134	13.787	0.653
Methoxyl-1	60.694	n.a.	n.a
Methoxyl-2	60.694	n.a.	n.a
Methylene	56.614	57.472	0.858

Chemical shift values for β-CD carbons are reported in Table 4. The observed shifts of C1, C4 and C6 probably indicate conformational changes of the cyclodextrin ring upon complexation. In particular, change in the C6 signal, related to changes involving the C5-C6 bond, can be ascribed to the penetration of the OME groups through the narrow extremity of the β-CD.

Assuming the formation of a 1:2 OME/β-CD complex, four molecular models were built by a docking procedure (Figure 14). In fact, OME can slip into the cyclodextrin cavity either from the wider or the narrower extremity, giving rise to four putative mutual orientations of the three components of the complex. The resulting models were then submitted to Molecular Dynamic Simulation in their hydrated form. The following parameters were calculated and reported in Table 5: i) the steric and electrostatic interaction energy between OME and the two host molecules; ii) the distances between OME's centre of mass and those of the two β-CD molecules. Figure 15 depicts the average coordinates of the

four models. From these data, host molecule CD1 seems to bind more firmly to the pyridine ring than CD2 does to the benzimidazole group. This is explained by molecular dynamic studies as being due to the wider dimensions of the substituted pyridine group, which stops the β-CD molecule from "slipping off". In models (b) and (c), strong electrostatic interactions occur between the polar core of OME and the secondary hydroxyl groups of β-CD which could explain why the association constant for the 1:1 complex is greater than that of the 1:2 complex. These observations are consistent with ^{1}H NMR and ^{13}C NMR data. As for the mutual arrangement of the complex constituents, the computational results do not provide indications about a strong preference between the two possible orientations of CD1. Nevertheless, when oriented as in models (b) and (c), CD1 shifted towards OME's centre, thus generating repulsive forces with the second β-CD molecule. On its own, CD2 interacts with OME to a lesser extent when inserted from the wider side as depicted in models (b) and (d). In model (b), where these two conditions are combined, CD2 is completely lost at the end of the simulation.

Table 4. ^{13}C chemical shifts corresponding to the β-CD, in the presence and absence of OME.

β-CD carbons	δ_{Free} (ppm)	$\delta_{Complex}$ (ppm)	$\Delta\delta$ (ppm)
C_1	103.576	103.501	-0.075
C_2	73.828	73.760	-0.068
C_3	74.662	74.722	0.060
C_4	82.556	82.293	-0.263
C_5	72.630	72.676	0.046
C_6	61.238	60.901	-0.337

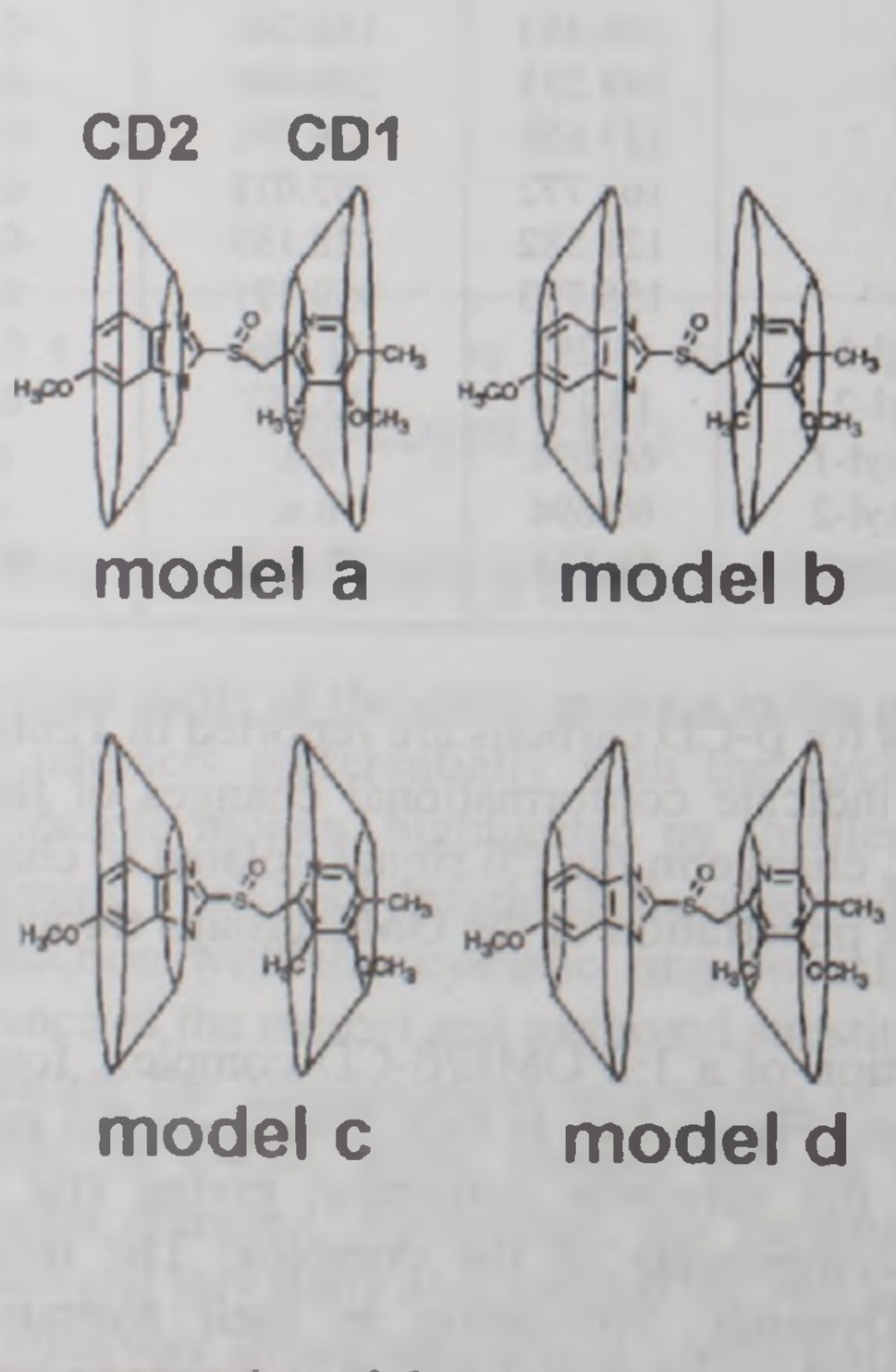


Figure 14. Schematic representation of four molecular models of the OME/β-CD inclusion compound.

Table 5. Molecular dynamics results. Mean values (s.d.) of the distances between OME and β-CD centers of mass, and of calculated interaction energies (CD1 from the pyridine side, CD2 from the benzimidazole side), measured over the last 30 picoseconds of the analysis

Model	Dist. (Å)	CD1 Electrostatic (kcal/mol)	Steric (kcal/mol)	Dist (Å)	CD2 Electrostatic (kcal/mol)	Steric (kcal/mol)
A	3.89 (0.25)	−7.41 (1.47)	−19.43 (2.56)	7.01 (0.38)	−1.43 (2.88)	−16.15 (1.34)
B	1.22 (0.33)	−18.22 (3.94)	−23.82 (1.53)	19.93 (0.57)	0.17 (0.37)	−0.07 (0.05)
C	1.33 (0.30)	−20.45 (2.41)	−21.69 (1.69)	10.21 (0.37)	−6.27 (2.96)	−7.49 (1.47)
D	3.08 (0.22)	−12.02 (1.95)	−23.22 (2.03)	11.43 (0.47)	−0.61 (1.14)	−2.88 (1.17)

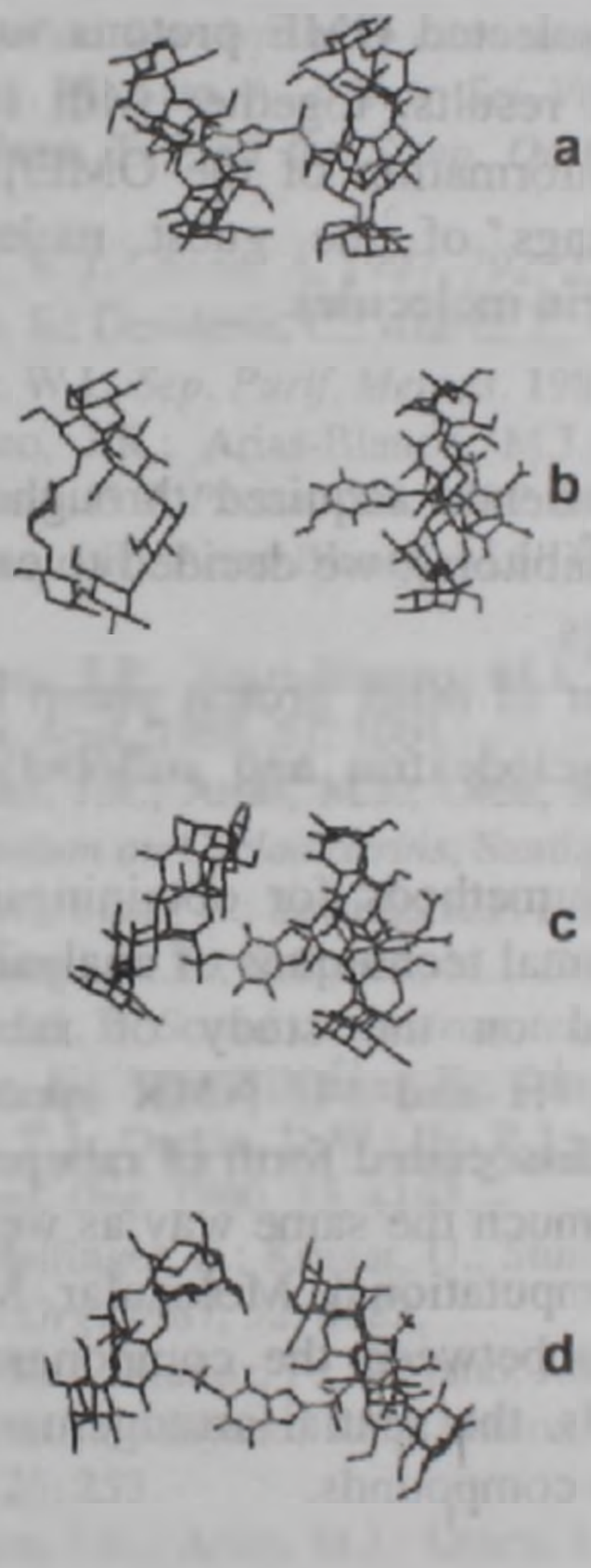


Figure 15. Molecular dynamics average structures of models (a)-(d).

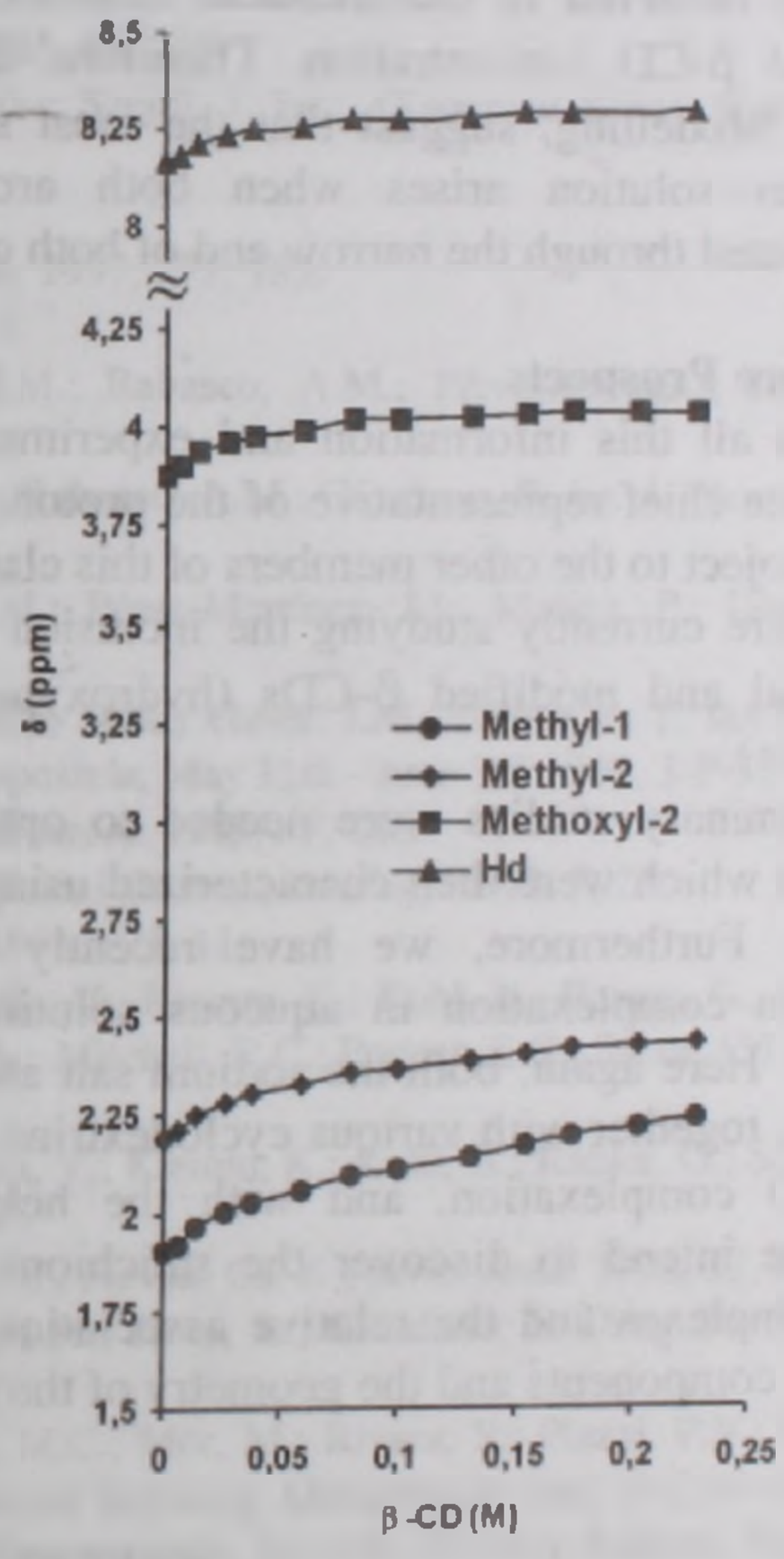


Figure 16. Plot of chemical shift changes of selected protons of OME *vs* the CD concentration.

Therefore, more than one mutual arrangement could be present in solution, and the NMR behaviour could be the result of different situations. Moreover, if CD1 is oriented as in models (a) and (d), the complexation of CD2 around the benzimidazole seems to be favoured, particularly if this is inserted from the narrower rim. Model (a) could therefore explain the chemical shift displacements for OME: the largest ^{1}H and ^{13}C chemical shift variations, observed for the methylene and methyl signals, could be due to the fact that these nuclei are surrounded by the narrower and the wider rim of CD1, respectively (Figure 15). This could also explain the downfield shifts of the corresponding ^{13}C signals, due to interactions with the β-CD rims, and the upfield shifts of the pyridine C1', C2' and C4' signals, considering that these atoms lie within the β-CD cavity. On the contrary, when CD1 is oriented as in models (b) and (c), the methylene group is positioned inside the β-CD hydrophobic cavity and its ^{13}C signal should experience an upfield shift. For the β-CD chemical shift displacements, the highest values were observed for H5, H6 and C6, indicating stronger interactions with the narrower rim, as in model (a). Figure 16 shows how the change observed in the chemical shift values for selected OME protons varies as a function of β-CD concentration. Therefore, the NMR results, together with studies of Molecular Modelling, suggest that the most stable conformation of the OME/β-CD 1:2 complex in solution arises when both aromatic rings of the guest molecule are accommodated through the narrow end of both cyclodextrin molecules.

Future Prospects.

With all this information and experimental experience acquired through studying OME (as the chief representative of the proton pump inhibitors), we decided to extend this research project to the other members of this class of drugs.

We are currently studying the inclusion behaviour of other proton pump inhibitors with natural and modified β-CDs (hydroxypropyl-β-cyclodextrin and sulfobutylether-β-cyclodextrin).

Preliminary studies were needed to optimize the methods for obtaining inclusion compounds which were then characterized using the thermal techniques of analysis already mentioned. Furthermore, we have recently embarked on the study of rabeprazole-cyclodextrin complexation in aqueous solution, using ^{1}H and ^{13}C NMR spectroscopic techniques. Here again, both the sodium salt and the undissociated form of rabeprazole are considered, together with various cyclodextrins. In very much the same way as we did with OME/β-CD complexation, and with the help of Computational Molecular Modelling Studies, we intend to discover the stoichiometric ratio between the components of the various complexes and the relative association constants, the spatial arrangements of the interacting components and the geometry of the inclusion compounds.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRIN STABILISATION OF STEROID AGAINST MICROBIAL DEGRADATION

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Microbial degradation of hydroxy androstenedione in solutions of methylated cyclodextrin was investigated. Stability constant of CD-steroid complex was determined independently by competitive spectrometric method. Concentrations of free and bound steroid forms have been estimated. By analysis of kinetic parameters of microbial degradation the role of free and complex steroid forms has been evaluated. It is concluded that CD-steroid complex is not available for the key microbial degradation enzymes - steroid-1-dehydrogenases.

Изучена микробная деградация гидроксиандростенедиона в растворах метилированного циклодекстрина. Константа стабильности ЦД-стероидного комплекса определялась отдельно спектрометрическим методом. Измерялись концентрации свободных и связанных стероидных форм. Анализом кинетических параметров микробной деградации оценивалась роль свободных и комплексных стероидных форм. Выявлено, что ЦД-стероидный комплекс не подвержен микробной деградации ферментами - стероид-1-дегидрогеназами.

Ուսումնասիրվել է հիդրօքսիանդրոստենեդիոնի մանրէային քայքայումը մեթիլացված ցիկլոդեքստրինի լուծույթներում: ՑԴ-ստերոիդային համալիրի կայունության կոնստանտը որոշվել է առանձին սպեկտրոմետրիկ մեթոդով: Որոշվել են ազատ և կապված ստերոիդային ձևերի քանակները: Մանրէային քայքայման կինետիկ պարամետրերի անալիզի միջոցով գնահատվել է ազատ և համալիր ստերոիդային ձևերի դերը: Բացահայտվել է, որ ՑԴ-ստերոիդային համալիրը չի ենթարկվում քայքայման մանրէային ֆերմենտների ստերոիդ-1-հիդրոգենազների կողմից:

Introduction

Among the key precursors for the synthesis of pharmaceutical steroid drugs of the pregnane group is 9α-hydroxy-androsta-4-ene-3,17-dione (9α-OH-AD). It can be obtained by regiospecific microbial introduction of hydroxy function at C9 4-androstene-3,17-dione or by microbial cleavage of the side chain of the sterols. The necessary condition for accumulation of 9α-OH-AD is blocking of steroid-1- dehydrogenase activity of mutant bacterial strains [1,2]. Residual activity of steroid-1-dehydrogenase causes the transformation of 9α-OH-AD into unstable 9α-hydroxy-androsta-1,4-diene-3,17-dione. The latter changes with the destruction of B ring of steroid structure into respective seco-form, which is further oxidized.

For prevention of the destruction of the labile structures the cyclodextrins (CD) are actively used owing to their ability to form a stabile inclusion complex of the guest-host type. So the rate of photochemical oxidation of the photolabile riboflavin [3] and sodium ascorbate [4] distinctly decreases in presence of CD, because the rate constant for the decomposition of CD-bound form is considerably lower than the respective constant for the free form. The effect of inhibition of enzyme reaction is also connected with the formation of inclusion complex, which blocks the availability of the substrate [5]. We suppose a presence

of such effect for the case of microbial destruction 9α-OH-AD. taking into account the phenomenon of permeability of cell walls of micobacteria for CD [6].

We believe important to elucidate if the CD-bound form of 9α-OH-AD is susceptible (subject) for microbial destruction and to estimate the efficiency of the application of CD for prevention of microbial degradation of 9α-OH-AD. For this purpose we studied the destruction of 9α-OH-AD in a set of solutions of methylated cyclodextrins (RAMEB) by cells of Mycobacterium sp. VKM. Ac-1817 D.

Materials and methods

Chemical and microorganism.

Randomly methylated β-cyclodextrins (RAMEB) (DS 1.8) was purchased from Wacker-Chemie (Germany), 9α-OH-AD was isolated under cell conversions of sitosterol and identified as described [7], Methyl Orange (MetOr) was obtained from Sigma (USA). yeast extract was obtained from Difko. Other chemicals used were of reagent grade and supplied by Reachim (Russia).

Strain Mycobacterium sp. VKM. Ac-1817 D was obtained from the All-Russian collection of Microorganisms (Institute of Biochemistry and Physiology of Microorganisms. Russian Academy of Sciences).

Microbial cultivation.

The microorganisms were maintained and cultivated as described earlier [7]. For seed culture. microorganisms on agar were suspended in sterile tap water (3-5 ml), transferred into 750 ml Erlenmeyer flasks with 50 ml nutrient medium and cultivated aerobically on a rotary shaker (220 rpm) at 30^0C for 36 h. Growing culture (10 ml) was inoculated into Erlenmeyer flask containing 100 ml nutrient medium with 0.5 g/l of sitosterol and incubated at the same conditions for 24 h. The medium for cultivation was of the same composition without agar.

Cultivation broth was centrifuged at 5000g for 15-20 min, washed with 0.01M K_2HPO_4 - KH_2PO_4 buffer, pH -7.0 and cells were used as biocatalysts for incubation with 9α-OH-AD .

Incubation of 9α-OH-AD with Mycobacterium sp. VKM. Ac-1817 D.

The microbe-mediated destruction of steroid was carried out in 0.01M K_2HPO_4 - KH_2PO_4 buffer, pH -7.0. The Erlenmeyer flasks (750 ml) were filled with 100 ml of the buffer solution, washed cells - 4.8 g/l (dry weight), 4.8 мМ 9α-OH-AD, and RAMEB. RAMEB concentration ranged from 0 to 19.2 мМ. Incubation was carried out at 30^0C and 220rpm for 100 h.

Samples of cultivation broth (1ml) were taken every 24h. Steroid was extracted with ethyl acetate (10ml). The extracts were applied to Silufol UV_{254} plates, developed in benzene/acetone (3:1, v/v). Spots of the plates corresponded to 9α-OH-AD were cut and put into flasks for elution by 5 ml of ethyl alcohol at stirring during 1 h. Solutions of 9α-OH-AD were analyzed spectrophotometrically at 240 nm following appropriate dilutions in 50% aqueous ethanol.

Determination of complex stability constant of 9α-OH-AD with RAMEB (K_{St}) by spectrometric method.

Host RAMEB solutions ($1x10^{-5}$ - $9x10^{-3}$ M) and those of guest MetOr ($2x10^{-5}$M) were prepared using 0.1 M phosphate buffer at pH 2.67. Guest steroid solutions ($1x10^{-6}$ - $5x10^{-3}$ M) were prepared in $5.0x10^{-4}$ M solutions of RAMEB in 0.1 M phosphate buffer and included MetOr. Solutions were incubated at 30°C. Absorption measurements were performed on a double-beam spectrometer Specord-M-40-UV VIS in 1 cm^2 quartz cuvette with a thermostatic cell holder. The absorption maximum chosen to determine the binding constants was 505 nm (pH 2.67, PBS 0.1 M, 30 °C).

Results and Discussion

Experiments on the dynamics of steroid decomposition in CD solutions followed independent characterization of the CD complex.

Stability constant of CD complex RAMEB - 9α-OH-AD was independently determined by the competitive spectrometric method [8]. Calculations of K_S were carried out by simultaneous fitting to two types of experimental curves. The first one was the absorbance curve of MetOr in the presence of RAMEB (fig.1,A) and the second one was the curves of displacement of the dye from the complex by 9α-OH-AD (fig.1, B). The values of stability constant of RAMEB-9α-OH-AD complex averaged 552 ± 6 M^{-1}.

The destruction of 9α-OH-AD by Mycobacterium sp. VKM. Ac-1817 D was studied in the set of RAMEB solutions with concentration range from 0 to 19.2mM. The curves of 9α-OH-AD decrease in the presence of different concentrations of RAMEB are demonstrated in Fig.2. Time interval of the destruction of a half of steroid amount in absence of RAMEB was 60 hours. Microbial destruction testifies to residual steroid-1-dehydrogenase activity of Mycobacterium sp. VKM. Ac-1817 D. With the increase of RAMEB concentration the destruction process is considerably lowered. By 60 hours of destruction the steroid concentrations varied from 50% for free form of 9α-OH-AD in absence of RAMEB to 15% in 19.2 mM RAMEB solution. This points to blocking the destruction process or at least to reducing of its rate due to conversion of the steroid into CD-bound form.. However it is unclear, if the decrease of the destruction rate is conditioned by unavailability of CD-bound form or by lower kinetic parameters of the destruction of bound for compared to the free one.

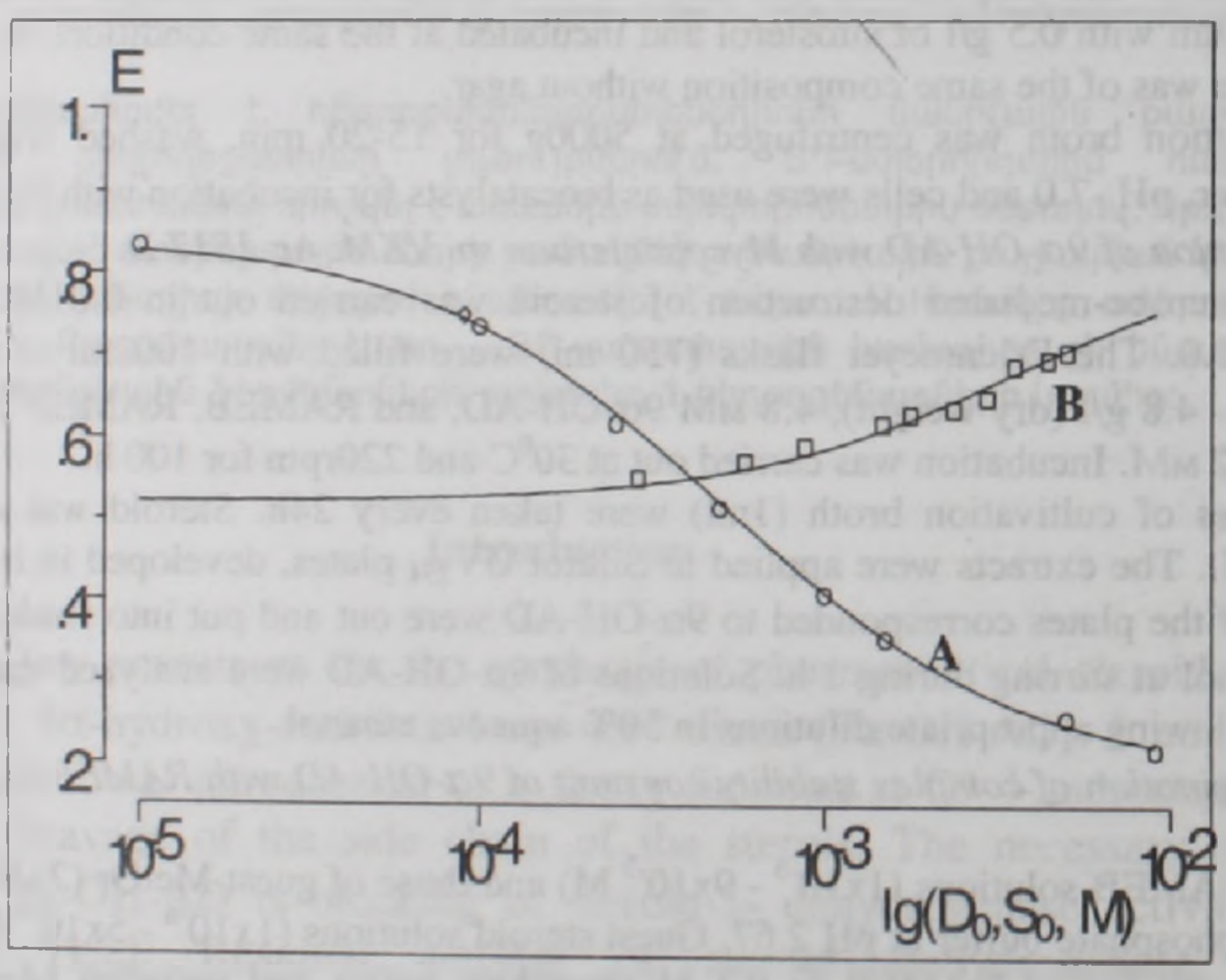

Figure 1. Dependence of absorbance (E_{abs}, 505 nm) of MetOr solution on: analytical concentration of RAMEB (D_0) with no 9α-OH-AD (A); and different 9α-OH-AD concentrations (S_0) with fixed RAMEB concentration (D_0, M): 5×10^{-4} (B). Experimental conditions: pH 2.67, PBS 0.01 M, 30°C.

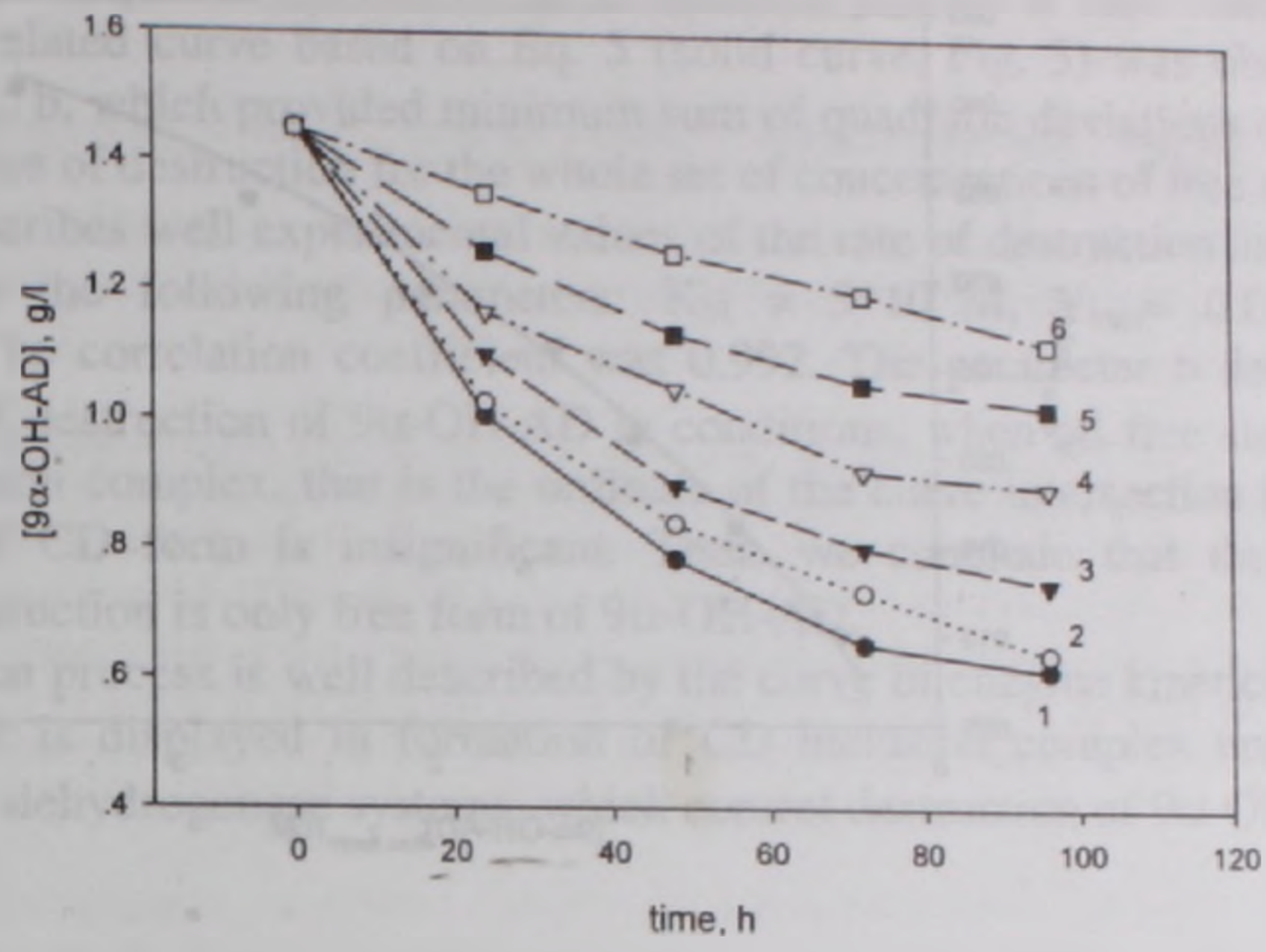

Figure 2. Effect of RAMEB on dynamic of 9-AD-OH destruction by Mycobacterium sp. VKM. Ac-1817 D cells at 30°C (RAMEB concentration : 1 - 0 mM, 2 - 1.2mM, 3 - 4.8mM, 4 - 9.6mM, 5 - 14.4mM, 6 - 19.6 mM).

To estimate the role of CD-bound form in 9α-OH-AD destruction the quantitative analysis of the curves of the change of the substrate concentration was carried out. The rate of the process was determined by the substrate decrease by 24 hours after the cell inoculation. For this time point the concentrations of free form of steroid were calculated for each RAMEB concentration, and the destruction rates were determined. The calculation was based on K_{St} values determined independently and equation for the concentration of free form of steroid in the solution. This provided independent computation of concentration of free and bound form of the steroid in CD solution and reduce the number of parameters optimized in the kinetic equation of 9α-OH-AD destruction. Indeed, in RAMEB solution the equilibrium concentration of free and bound steroid forms are related by equation for stability constant of the complex (1) and equations (2,3) of mass balance for the steroid and cyclodextrin:

$$K_{St} = [9\alpha\text{-OH-AD - RAMEB}]/([9\alpha\text{-OH-AD}]*[\text{RAMEB}]) \quad (1)$$

$$C_R = [\text{RAMEB}] + [9\alpha\text{-OH-AD - RAMEB}] \quad (2)$$

$$C_{9\alpha\text{-OH-AD}} = [9\alpha\text{-OH-AD}] + [9\alpha\text{-OH-AD - RAMEB}] \quad (3)$$

When express concentration of free form of steroid via parameters K_{St}, C_R, $C_{9\alpha\text{-OH-AD}}$, using the above equation system, we obtain the following equation:

$$K_{St}[9\alpha\text{-OH-AD}]^2 + (K_{St} C_R + K_{St} C_{9\alpha\text{-OH-AD}} + 1)[9\alpha\text{-OH-AD}] - C_{9\alpha\text{-OH-AD}} = 0 \quad (4).$$

Thus, from Eq. (4) for each steroid concentration at given values of K_{St}, C_R, $C_{9\alpha\text{-OH-AD}}$ we calculated the concentration of free form of the steroid.

The relationship of the destruction rate of 9α-OH-AD on the concentration of free form is demonstrated in Fig. 3.

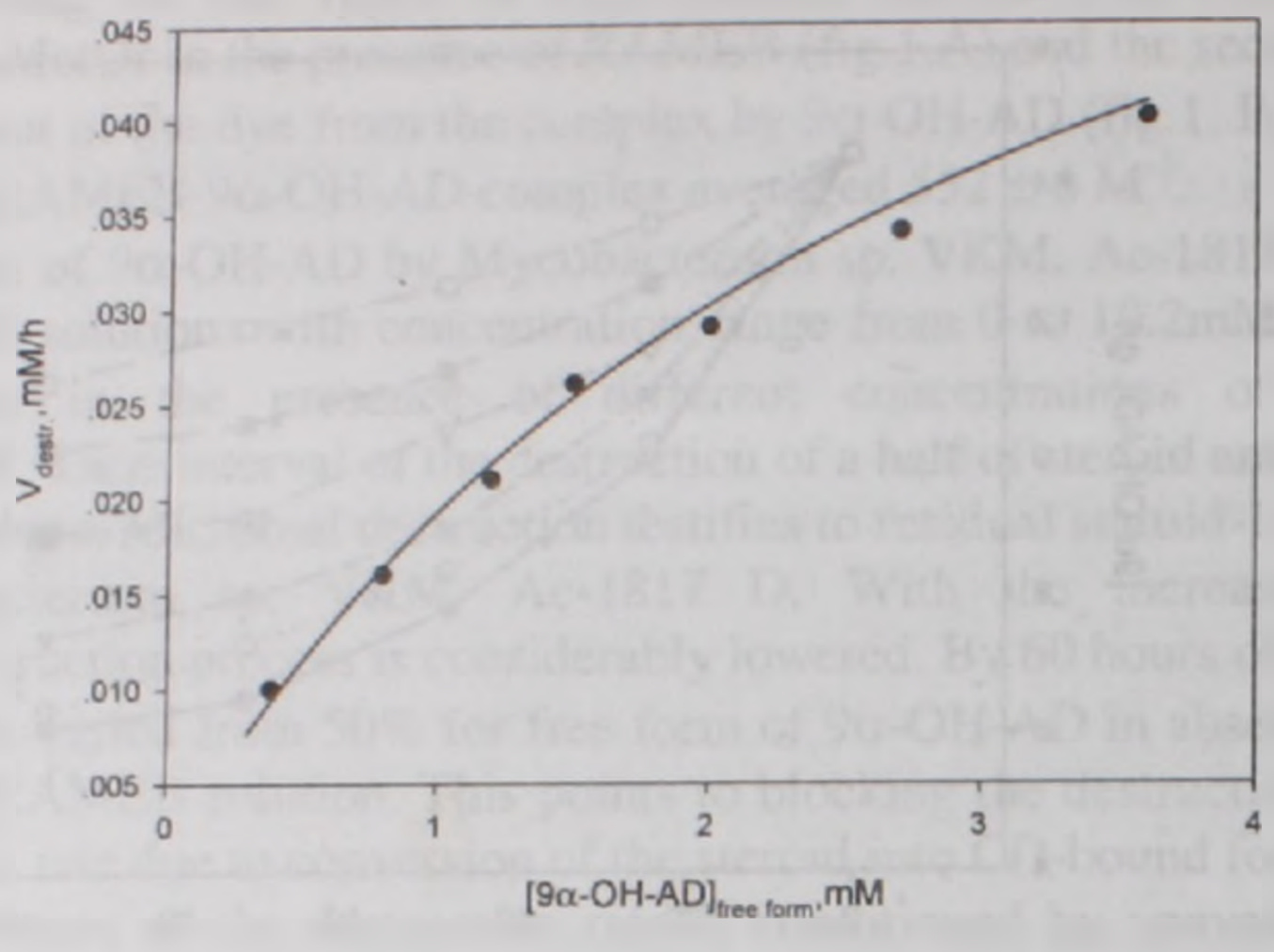

Figure 3. Dynamics of 9α-AD-OH (c=1.45g/L) destruction by Mycobacterium sp. VKM. Ac-1817 D cells at the different concentrations of free steroid form (• - experimental data, ——— calculated values of destruction rates).

Taking into consideration that in CD solutions steroid forms the inclusion complex the formal scheme of destruction may be the following:

$$9\alpha\text{-OH-AD} + \text{RAMEB} \overset{K_{St}}{\Leftrightarrow} 9\alpha\text{-OH-AD - RAMEB}$$

1-steroid- dehydrogenase activity

?

$$[\text{Intermediates}] \rightarrow CO_2 + H_2O$$

Scheme

The equation for the destruction rate is the following: $V = V_{free} + V_{comp}$,

where V_{free} is the rate of destruction of the free steroid form;

V_{comp} is the rate of destruction of the bound steroid form.

Taking into account the Michaelis-Menthen kinetics of the process, the equation for the rate of 9α-OH-AD destruction is the following:

$$V = V_{max} * C_{9\alpha\text{-AD-OH}}/(K_M + C_{9\alpha\text{-AD-OH}}) + b \quad (5),$$

where $C_{9\alpha\text{-AD-OH}}$ is the concentration of free steroid form;

V_{max} is the maximum rate of destruction of the free form;

K_M is the effective Michaelis constant of the enzymatic process, which limits the destruction rate;

b is the absolute term, which reflects the contribution of the CD form into the total destruction rate.

The value of b is equal to the rate of the destruction process at zero concentration of free form. The calculated curve based on Eq. 5 (solid curve, Fig. 3) was obtained using parameters K_M, V_{max}, b, which provided minimum sum of quadratic deviations of calculated and experimental rates of destruction for the whole set of concentrations of free steroid form.

The curve describes well experimental values of the rate of destruction and shows the stable minimum at the following parameters: $K_M = 3*10^{-3}$M, V_{max}= 0.073mM/hour, b=0.001мM/hour. The correlation coefficient was 0.992. The parameter b determines the values of the rate of destruction of 9α-OH-AD in conditions, when all free steroid form is bound in CD inclusion complex, that is the ordinate of the curve intersection (Fig. 3). The form destruction of CD form is insignificant. Thus, we conclude that the subject for microbiological destruction is only free form of 9α-OH-AD.

The destruction process is well described by the curve of enzyme kinetics. Stabilizing role of cyclodextrin is displayed in formation of CD inclusion complex unavailable for endogenic steroid-1-dehydrogenase systems, which control destruction of 9α-OH-AD.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

A KINETIC APPROACH FOR THE DETERMINATION OF HOST-GUEST BINDING CONSTANTS

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The kinetics of the alkaline hydrolysis of methyl acetate (E) was studied in the presence of β-cyclodextrin (CD), employing a conductance method. In the presence of CD, the rate of the $E + OH^-$ reaction decreases because a host-guest complex is formed between E and CD and the ester bound to the CD cavity is not accessible to the OH^- attack. The experiment consists of two steps: (1) the rate constant that describes the alkaline hydrolysis of methyl acetate is determined, and (2) the kinetics of the reaction is measured once again in the presence of CD. An algorithm is presented in the Mathematica platform to estimate the equilibrium constant for the methyl acetate/CD host-guest complex using the kinetic data.

Методом кондуктометрии изучена кинетика щелочного гидролиза метилацетата (E) в присутствии β-циклодекстрина (ЦД). В присутствии ЦД скорость $E+OH^-$ реакции снижается из-за образования "хозяин-гость" комплекса между E и ЦД, а эфирная связь в полости ЦД становится недоступной к атаке OH^- групп. Эксперимент состоит из двух этапов: (1) определение константа скорости, что представляет щелочной гидролиз метилацетата, и (2) измерение еще раз кинетики реакции в присутствии ЦД. С использованием кинетичаских данных представлен математический алгоритм для оценки константа равновесия комплекса метил ацетат/ЦД "хозяин-гость".

Կոնդուկտոմետրիայի մեթոդով ուսումնասիրվել է մեթիլացետատի (E) հիմնային հիդրոլիզը β-ցիկլոդեքստրինի (ՑԴ) ներկայությամբ: ՑԴ-ի ներկայությամբ $E+OH^-$ ռեակցիայի արագությունը դանդաղում է E-ի և ՑԴ-ի միջև «տեր-հյուր» համալիրագոյացման պատճառով և եթերային կապը ՑԴ-ի խոռոչում դառնում է անմատչելի OH^- խմբերի հարձակման համար: Գիտափորձը տարվել է երկու էտապով. (1) արագության հաստատունի որոշում, որը ներկայացնում է մեթիլացետատի հիմնային հիդրոլիզը, և (2) ռեակցիայի կինետիկայի չափում մեկ անգամ էլ ՑԴ ներկայությամբ: Օգտագործելով կինետիկական տվյալները, ներկայացվել է մաթեմատիկական ալգորիթմ, գնահատելու հավասարության հաստատունը մեթիլացետատ/ՑԴ «տեր-հյուր» համալիրի համար:

Introduction

Cyclodextrins (CDs) are water soluble molecules that posses a hydrophobic cavity which enables them to form host-guest complexes with molecules ranging widely in their physical properties and size. In particular, non-polar substrates that fit tightly inside the cavity tend to form host-guest complexes with large binding constants [1-4]. The formation of a host-guest complex may alter properties of the guest such as the fluorescence yield, the absorption extinction coefficient, and the position of NMR signals [5]. Because CDs are chiral, a substrate may exhibit the induced circular dichroism phenomenon upon interacting with the CD cavity. Monitoring changes in these properties it has been possible to study a large number of host-guest equilibria and an understanding of the physical factors underlying the formation of these complexes is emerging. More related to the present work is the fact

that CDs can reduce the accessibility of a guest with respect to a reactant or a quencher. This provides a means to control the rate of chemical reactions and to measure host-guest equilibrium constants (K_{eq}) [5-8].

Physical methods that require high concentrations of the guest may not be adequate for measuring equilibrium constants because the guest is prone to aggregate and the stoichiometry may deviate from 1:1, the host-guest complexes may precipitate out of the solution, or form homodimers [7, 8]. Clearly, if the assumption for the stoichiometry is correct, then different physical methods should yield similar values of K_{eq}. Hennrich and Cramer [9] determined the dissociation constants for different substrates by kinetic, spectroscopic and competitive inhibition methods, obtaining good agreement among the different methods. While under certain experimental conditions the use of approximations may introduce errors, to some extend this problem can be reduced employing non-linear techniques and regressions [6].

This paper describes a procedure to measure the equilibrium constant for the formation of a host-guest complex between β-cyclodextrin (CD) and methyl acetate (E) employing a conductimetric method:

$$CD + E \Leftrightarrow CD{\bullet}E \qquad (1)$$

where CD•E represents the host-guest complex [10-14]. E binds to the CD cavity through van der Waals forces and hydrophobic interactions. The formation of host-guest complexes is enthalpy driven. As implied in equation 1, in an aqueous solution containing CD the ester exists as a free species (E) as well as bound to the CD cavity (CD•E). The experiment involves various measurements. First, the rate constant that describes the alkaline hydrolysis of methyl acetate, k_f, is determined following the method presented by Crockford et al. [15]

$$CH_3COO\,CH_3 + OH^- \xrightarrow{k_f} CH_3OH + CH_3COO^- \qquad (2)$$

Second, the kinetics of reaction 2 is monitored again in the presence of a specified concentration of CD. Qualitatively, it is observed that the rate of reaction 2 decreases upon adding CD. The ester is consumed in reaction 2 and its total amount at a given reaction time is given by, $[E]_T = [E] + [CD{\bullet}E]$. In contrast, the OH^- ion exists exclusively as a free species. The rate of reaction 2 decreases upon adding CD to the reaction mixture because the ester molecules associated to the cavity of CD are inaccessible to the OH^- attack. Therefore, in the presence of CD the rate of reaction 2 also depends on the concentration of free ester, which is dictated by the following expression of K_{eq}:

$$K_{eq} = \frac{[CD \bullet E]}{[CD][E]} \qquad (3)$$

Substituting $[E]_T - [E]$ for $[CD{\bullet}E]$ in equation 3, an expression for the free ester in terms of $[E]_T$, $[CD]_o$ and K_{eq} is obtained,

$$[E] = X_E[E]_T = \frac{1}{K_{eq}[CD]_o + 1}\,[E]_T \qquad (4)$$

where $[CD]_0$ is the analytical concentration of CD. In obtaining equation 4 it was assumed, $[CD]_o >> [CD{\bullet}E]$, in order to substitute $[CD]_o$ for $[CD]$. It is important to emphasize that the

reaction rate decreases upon adding CD because [E] is reduced and not because the magnitude of k_f for reaction 2 has decreased. The magnitude of k_f only depends on the temperature of the system, which is kept constant. An interesting aspect of this method is that measuring the kinetics of reaction 2 using a simple conductimetric method one obtains thermodynamic data (*i.e.*, $\Delta G^{\circ} = -RT \ln K_{eq}$) about equilibrium 1.

Experimental Method

Methyl acetate (99+%, Aldrich) and β-cyclodextrin (Aldrich) were used as received. Deionized water was used to prepare all solutions. The experiments reported were performed at ambient temperature, although the reaction tube was placed in a water bath to minimize temperature variations. Conductance measurements were made using a YSI Model 35 conductance meter coupled with a YSI 3401 conductivity cell.

Results and Discussion

Alkaline Hydrolysis of Methyl Acetate. A task of this experiment is to measure the rate constant of reaction 2. However, strictly speaking the hydrolysis of methyl ester must be written as an equilibrium:

$$CH_3COOCH_3 + OH^- \Leftrightarrow CH_3OH + CH_3COO^- \qquad (5)$$

As stated by Crockford et al. [10], because the ionic conductivity of OH^- is larger than that of CH_3COO^- at a given temperature, it is possible to monitor the kinetics of the reaction by measuring the conductance decrease of the solution as a function of time (see Figure 1). The limiting ionic conductivities in water of Na^+, CH_3COO^- and OH^- are 50.11, 40.9 and 197.6 siemens•cm^2•mol^{-1}, respectively. Although the sodium ion contributes to the conductivity of the solution, its concentration remains constant. The kinetic equation that describes reaction 5 is,

$$-\frac{d[E]}{dt} = k_f[E][OH^-] - k_b[EtOH][Ac^-] \qquad (6)$$

where [E], [OH^-], [MeOH] and [Ac^-] represent the ester, hydroxide, methanol, and acetate ion concentrations, respectively, and k_f and k_b the corresponding forward and backward rate constants. Equation (6) can be written in terms of the reaction progress variable as follows,

$$\frac{dx}{dt} = k_f(a-x)(b-x) - k_b x^2 \qquad (7)$$

where a and b denote the ester and hydroxide initial concentrations, which we chose to be equal. In writing equation 7 it is assumed that no products are present at the beginning of the reaction. Furthermore, for short reaction times, equation 7 may be written in the following approximate form,

$$\frac{dx}{dt} = k(a-x)^2 \qquad (8)$$

Integrating equation 8 from zero to infinity one obtains,

$$kt = \frac{x}{a(a - x)} \tag{9}$$

Since a and x are proportional to $(L_0 - L_r)$ and $(L_0 - L)$, respectively, the following relations between a, x, and $a - x$ can be derived in terms of L, L_0 and L_r, which denote the conductances at time = t, 0, and infinity, respectively.

$$\frac{x}{a} = \frac{L_0 - L}{L_0 - L_\infty} \tag{10.a}$$

$$\frac{a - x}{a} = \frac{L - L_\infty}{L_o - L_\infty} \tag{10.b}$$

Inserting equations 10.a and 10.b into equation 9 leads to

$$akt = \frac{L_o - L}{L_o - L_\infty} \tag{11}$$

Thus, k can be calculated plotting $(L_0 - L)/(L - L_r)$ against time (taking into account that a is one half the concentration of the stock solutions). The inset of Figure 1 shows such a plot, as well as the linear fit from which one obtains, $k = (0.2279 \pm 0.0026)\ M^{-1}\ s^{-1}$.

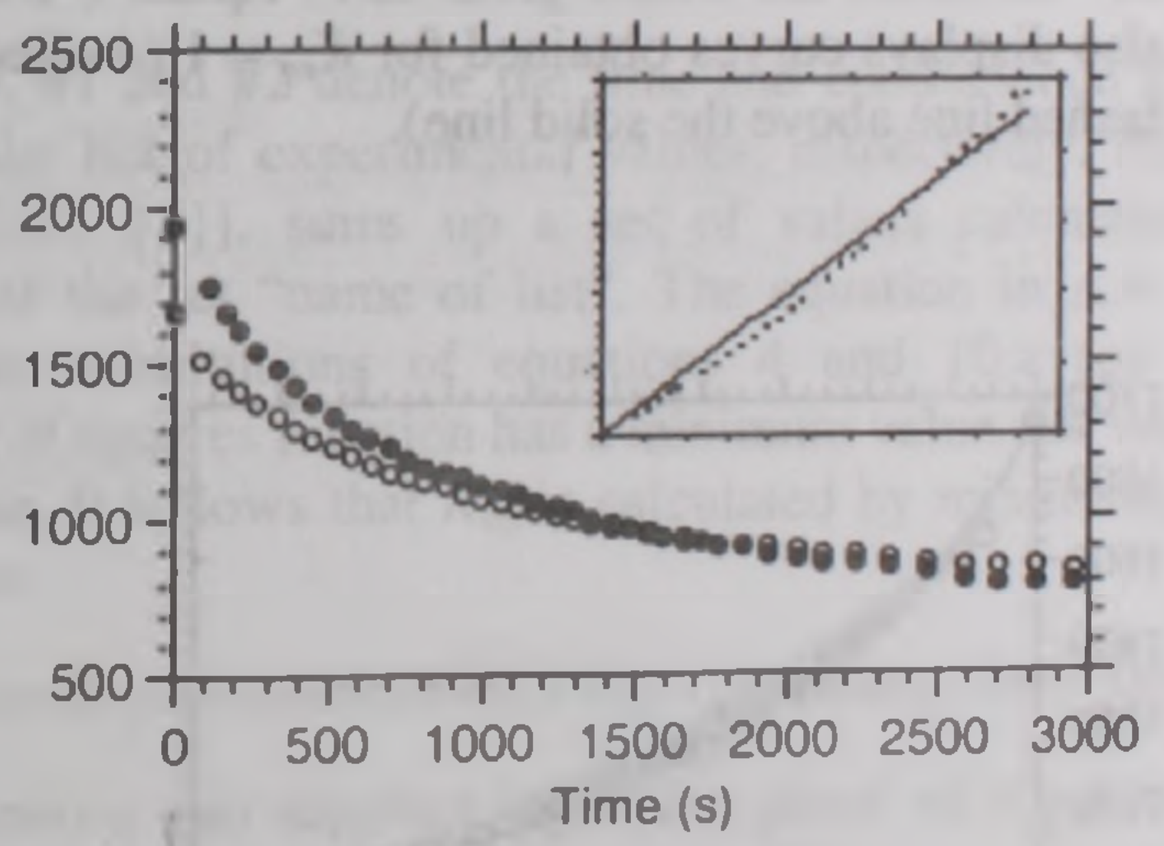

Figure 1. Conductance decays for the alkaline hydrolysis of methyl acetate with (open circles) in the presence of CD and without adding CD to the reaction medium. Not all the experimental points are shown for clarity. The following concentrations were used: 0.01M methyl acetate, 0.01M sodium hydroxide, and 0.005M CD. The inset shows conductance data for the experiment without CD expressed in linear form, along with the corresponding fit. The rate constant is estimated from the slope of the linear fit using equation 11.

Determination of the Binding Constant. As mentioned in the Introduction, the concentration of free ester available to react decreases when CD is added to the solution, as it is apparent from Figure 1. Therefore, to describe the kinetics of reaction 2 in the presence of

CD, equation 7 needs to be modified considering that a fraction of the ester molecules are forming a host-guest complex

$$\frac{dx}{dt} = k(a X_t \pm x)(b \pm x) \tag{12}$$

Letting $aX_E = a'$, and integrating the above equation using the boundary conditions employed to solve equation 8, the following standard result is obtained,

$$kt = \frac{1}{a' - b} \ln\left(\frac{b(a' - x)}{a'(b - x)}\right) \tag{13}$$

The above equation can be re-written as

$$kt = \frac{1}{E_0 X_E - E_0} \ln\left(\frac{(E_0 X_E - x)}{X_E(E_0 - x)}\right) \tag{14}$$

To calculate K_{eq}, X_E and x are substituted in equation 14, as defined in equations 4 and 10.a (or 10.b), respectively. Note that X_E introduces K_{eq} (see equation 4), which is the only parameter to be adjusted to the kinetic data. The final equation and its application into a least squares routine is explained in the Appendix. We analyzed data points up to 1000 s, which is the approximate time when the backward reaction begins to interfere significantly. Fitting the data in Figure 2 (solid curve) to our model for reaction times up to 1000 s, one obtains K_{eq} to be (11.8±1.2) M^{-1} at 295.3 K, which gives ΔG° equals (–6.05±0.60) kJ/mol. For comparison, Figure 2 also displays curves obtained for $K_{eq} = 1$ (dashed line below the solid line) or $K_{eq} = 25\ M^{-1}$ (dashed line above the solid line).

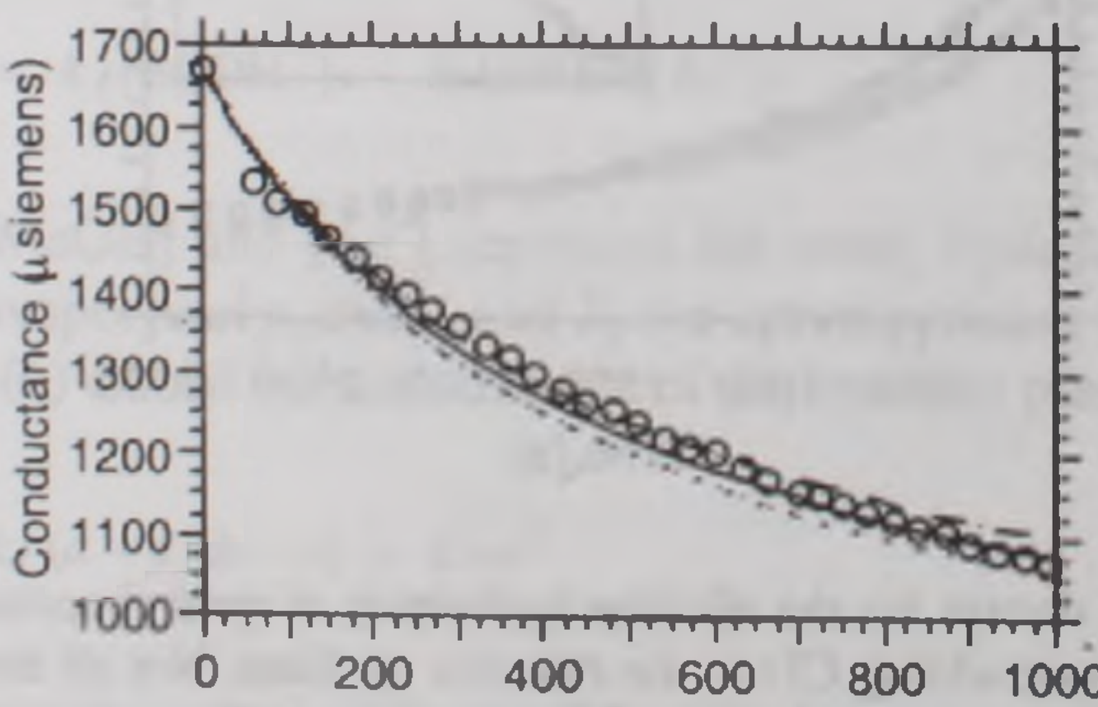

Figure 2. Conductance decay for the alkaline hydrolysis of methyl acetate in the presence of CD. The solid line represents the best fit, calculated using the estimated CD/ester binding constant. The dashed line represent the curves obtained if one puts $K_{eq} = 1$ (curve below the solid line) or $K_{eq} = 25\ M^{-1}$ (dashed line above the solid line).

Appendix

This appendix describes the algorithm developed in the Mathematica platform [16] to calculate K_{eq} using a regression. Lines in quotes mean that the respective values are chosen or determined by the user. First, an array of experimental data (the kinetic data set) is entered into the program. In the present case, times longer than 1000 seconds are not considered:

"name of list"={{t_1,L_1},{t_2,L_2},...} (A.1)

Next, a set of constants are assigned values that were previously determined in the experiment. These are the ester initial concentration (E0), the rate constant for the reaction in free water without CD (k), the CD analytical concentration (CD0), the conductance at time zero (L0), and the conductance at infinity (Li):

{E0,k,CD0,L0,Li}={"E0","k","CD0","L0","Li"} (A.2)

For instance, the input line using the values obtained in the present work would be:

{E0,k,CD0,L0,Li}={0.01,0.2279,0.005,1670,780.64} (A.3)

In order to get a regression, a function is defined for the sum of squares. It depends only K_{eq}, and is minimized to find the best estimate of K_{eq}:

```
sumofsquares[Keq_] =
Plus @@ Apply[((Log[E0]-Log[E0-(E0-(#2-Li)/(L0-Li)*E0)]-Log[E0*1/(CD0*Keq+1)]
+ Log[-(E0-(#2-Li)/(L0-Li)*E0)+E0*1/(CD0*Keq+1)])/
((E0*k)+E0*k*1/(CD0*Keq+1))-#1)^2 &,"name of list", {1}]
```
(A.4)

In equation A.4, #1 and #2 denote the time and conductance elements of each time-conductance pair in the list of experimental values, respectively. Plus @@ Apply[(t(#2)-#1)^2 &,"name of list", {1}], sums up a set of values calculated from each pair of coordinates member of the set "name of list". The equation in A.4, in this case t(#2), is equation 14 after the substitutions of equations 4 and 10.a (or 10.b) for X_E and x, respectively. The sum of squares function has a minimum value, for which the corresponding K_{eq} is the best estimate. It follows that K_{eq} is calculated by minimizing the sum of squares. This is done as follows:

FindMinimum[sumofsquares[Keq],{Keq,5},MaxIterations->100] (A.5)

where {Keq,5} represents a user supplied "educated guess" of K_{eq} for the function to initiate the iteration process. A.5 returns the best estimate for K_{eq}.

The following routine generates a set of theoretical values (fit) of conductance (L) against time (t). In this case, the routine supplies a sequence of conductance values starting at "initial L" and ending at "final L", and returns a list of time values:

```
Do[
L=i;
x=(E0-(L-Li)/(L0-Li)*E0);
t=(Log[E0]-Log[E0-x]-Log[E0*1/(CD0*Keq+1)]
+ Log[-x+E0*1/(CD0*Keq+1)])/(-(E0*k)+E0*k*1/(CD0*Keq+1));
Print[N[t]," / ",L],
{i,"initial L","final L","increase"}]
```
(A.6)

These latter values can be entered into a new list the same way as in A.1. In order to graphically compare both lists, and visually evaluate the adequacy of the fit, type the following lines:

```
g1=ListPlot["name of experimental data list"]
g2=ListPlot["name of theoretical data list"]
Show[g1, g2]                                                    (A.7)
```

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

THERMODYNAMIC INTERACTIONS OF MODEL ALLELOPATHIC COMPOUNDS (POLYPHENOLS) WITH α- AND β-CYCLODEXTRIN

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The present thermodynamic study is an initial attempt to check the possible efficiency of cyclodextrins in trapping allelopathic substances and therefore to reduce their phytotoxic effect. As a first choice, phenols have been chosen as models for complexation study. The equilibrium constants for the complexation of the polyphenols with α- and β-cyclodextrins have been determined. In many occasions the stoichiometry of the complex formed can also be obtained.

Данное термодинамическое исследование – начальная попытка проверить возможность продуктивности циклодекстринов в связывании аллелопатических веществ и таким образом привезти к снижению их фитотоксического эффекта. Первым выбором явились фенолы как модели для изучения комплексообразования. Определены константы равновесия для комплексообразования полифенолов с α- и β-циклодекстринами. Во многих случаях могут быть получены стехиометрии образованных комплексов.

Ներկայացված թերմոդինամիկական ուսումնասիրությունը առաջին փորձն է, ստուգելու ալլելոպատիկ նյութերի հետ ցիկլոդեքստրինների կապման արդյունավետության հնարավորությունը, որը այդպիսով կբերի այդ նյութերի ֆիտոտոքսիկ ազդեցության նվազմանը: Որպես համալիրագոյացման ուսումնասիրության մոդելներ առաջինն ընտրվել են ֆենոլները: Որոշվել են հավասարության հաստատունները α- և β-ցիկլոդեքստրինների հետ պոլիֆենոլների համալիրագոյացման համար: Շատ դեպքերում կարող են ստացվել առաջացած համալիրների ստեխիոմետրիաները:

Introduction

Cyclodextrins are cyclic oligosaccharides consisting of several glucopyranose units which are joined together by α(1-4) linkages. The natural cyclodextrins have six, seven, or eight glucopyranose units, respectively known as α-, β-, and γ-cyclodextrin. The oligosaccharide ring forms a torus with the primary hydroxyl groups lying on the narrow end of it, and the secondary hydroxyl groups of the glucose residues on the wider end. The cavity of the torus provides a relatively hydrophobic environment which allows to entrap (or complex) organic compounds [1-6]. The complexation is noncovalent and the driving force for the complex formation has been discussed in terms of van der Waal's interactions, hydrogen bonding, hydrophobic interactions, electrostatic effects, the release of high energy water molecules and of steric strains. To analyse these different contributions several families of guests have been the subject of systematic thermodynamic studies. Among them we can mention alcohols [7-9], phenols [10], acids [11,12], monosubstituted benzene

derivatives [13], surfactants [14], bile salts [15,16], amines [12,17], cyclohexane derivatives [18], phenethylamines, ephedrines [19], etc.

This ability of forming stable inclusion compounds has led to some technological applications of the cyclodextrins, as for instance in drug delivery systems, food technology, etc. [2] Probably because their relatively high prize till recently, less attention has been paid to the applications in agriculture, and related fields. A common problem for the propagation *in vitro* of some plants is that they become readily brown and do not germinate. Although the reasons are not clear, it has been related to the formation of allelopathic substances [20] in the cultive medium. To reduce the phytotoxic effect of these substances, active carbon has been used but it is rather ineffectiveness. Among the allelopathic substances we can mention naphtoquinones, coumarines, phenols and polyphenols, cinnamic acid and derivatives, etc.

Parallel to *in vitro* investigations, the present thermodynamic study is an initial attempt to check the possible efficiency of cyclodextrins in trapping allelopathic substances and therefore to reduce their phytotoxic effect. As a first choice, phenols have been chosen as models for the complexation study as they are very common. Furthermore, only one study has been published for the complexation of the polyphenols chosen here (see Figure 1 for structures), and only the equilibrium constants have been determined [21].

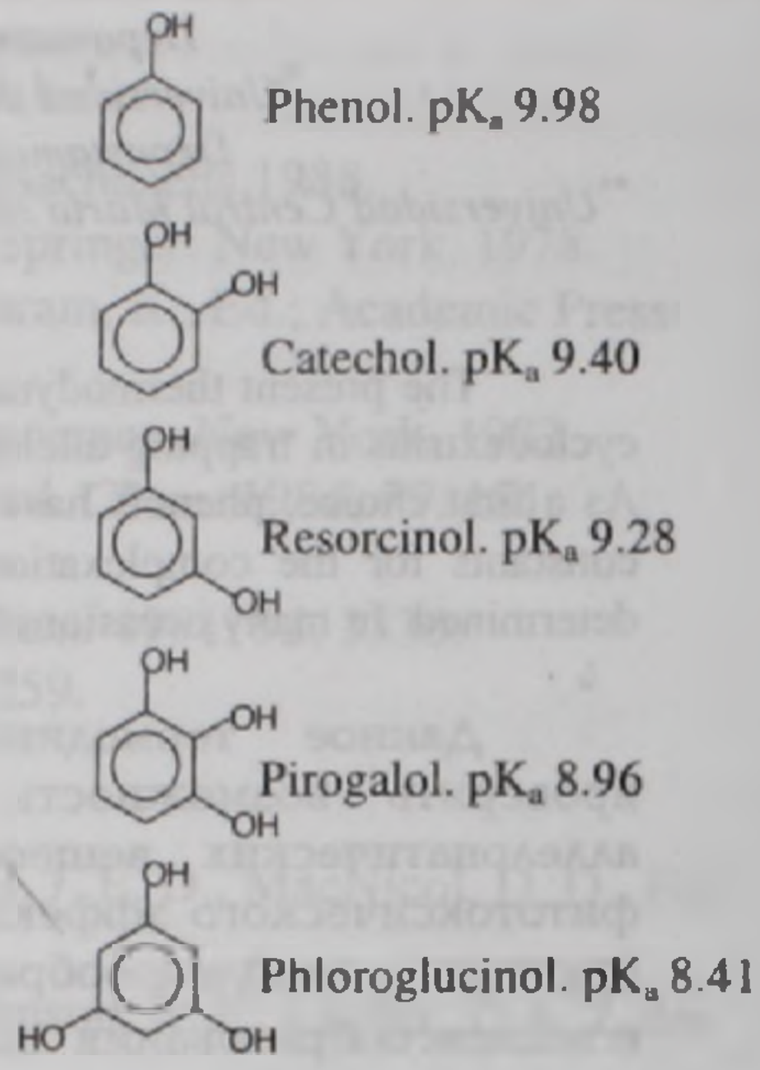

Figure 1. Structure of phenols

For the purposes of the present study, Isothermal Titration Calorimetry (ITC) has been chosen as the right experimental technique as it allows the determination of enthalpy, entropy and free energy (and the associated equilibrium constant) changes, in a single experiment. In many occasions the stoichiometry of the complex formed can also be obtained.

Calorimetry

The description of the thermodynamical background can be found elsewhere [22]. Here only the essential equations are provided.

As both host (any cyclodextrin) and guest (any phenol) have been used as titrating agents or as the sample substrates, the following nomenclature is preferred: M is the sample substrate (i.e. the reagent in the sample cell of the calorimeter), X is the titrating agent (i.e. reagent which is in the syringe) and MX is the complex formed. The association constant for the formation of a 1:1 inclusion complex (eq. 1), K can be written as in eq. 2

$$M + X \rightarrow MX \qquad [1]$$

$$K = \frac{\Theta}{(1-\Theta)[X]} \qquad [2]$$

where $\Theta = [MX]/M_t$, [X], and [MX] are the equilibrium concentrations of titrating and complex, respectively, and M_t is the initial concentration of the sample. Therefore

$$X_t = [X] + \Theta M_t$$

Combining previous equations, we arrive at

$$\Theta^2 - \Theta\left[1 + \frac{X_t}{M_t} + \frac{1}{KM_t}\right] + \frac{X_t}{M_t} = 0$$

The heat of association is

$$Q = \Theta M_t \Delta H^\circ V_0$$

where ΔH° is the enthalpy change associated to the formation of the complex and V_o is the total volume of the solution

The combination of previous equations gives

$$Q = \frac{M_t \Delta H^\circ V_0}{2}\left[1 + \frac{X_t}{M_t} + \frac{1}{KM_t} - \sqrt{\left(1 + \frac{X_t}{M_t} + \frac{1}{KM_t}\right)^2 - \frac{4X_t}{M_t}}\right]$$

The computer program Origin was used to calculate the equilibrium constant and the standard molar enthalpy of reaction from a titration experiment. The standard molar Gibbs energy of reaction, ΔG°, and standard molar entropy of reaction, ΔS°, were calculated from the measured equilibrium constant and standard molar enthalpy of reaction ΔH°, *i.e.*, by the equation

$$\Delta G^\circ = -RT \text{ Ln } K = \Delta H^\circ - T\Delta S \qquad [3]$$

The standard deviations of the thermodynamical parameters shown in Table 1correspond to the average of 4-6 independent runs, and are normally a little higher than those derived from the fit of a single experiment.

Experimental section

The phenols used in this work were purified by standard procedures. α-cyclodextrin was from Wacker and β-cyclodextrin from Roquette and were used, as well as other substances, without further purification. All solutions were freshly prepared for each experiment and degassed. Cyclodextrins and phenols were prepared in phosphate buffer 0.05 M from sodium monophosphate and sodium hydroxide. pH was checked after each calorimetric titration and no variations were observed from the initial pH. At this pH all phenols studied here are in their neutral form as the pH of 6.9 used for the experiments is well removed from the pK's of phenols (Figure 1) and therefore, the complication of having the phenols in their anionic forms is avoided. The phosphate buffer used to maintain the pH constant does not interact with cyclodextrins [19].

Enthalpies of dilution of the titrant and sample were determined in separate experiments by titrating the phosphate solution into the sample solution or by adding the

titrating reagent into phosphate solution, in exactly the same conditions of the real experiment (i.e., identical concentration of reagents, number of dilutions and volume added) and the associated calorific effects were added point by point. In a typical experiment 30 additions of 10 μL of the titrating agent (80 mM when it was a phenol or 13 mM when it was a cyclodextrin) were added over an initial volume of 1.374 mL of the sample (4 mM when it was a cyclodextrin or 1.5 mM when it was a phenol). All experiments were carried out at 303 K and at de 400 rpm.

Bruker Instruments AC300 and AMX500 were used to measure NMR spectra.

Results and Discussion

Data from a representative titration experiment (phenol 80 mM and β-CD 4 mM) are given in Figure 2. Similar curves were obtained for the rest of the systems studied here.

The shape of the titration curve [22] yields the required information concerning the thermodynamics of binding. The form is usually signalled by the dimensionless quantity C given by

$$C = K M_t$$

(K expressed in $dm^3 mol^{-1}$). For high C values (>500) almost all moles of the titrating injected into the sample cell are bond to the substrate. The curve is sigmoidal and the inflexion point correspond to the stoichiometry of the complex formed. For lower values of C, only a small fraction of the injected substrate binds to the sample and this fraction decreases with increasing in injection number. The titration plot becomes less informative and confidence in the estimates of K and enthalpy of binding decreases.

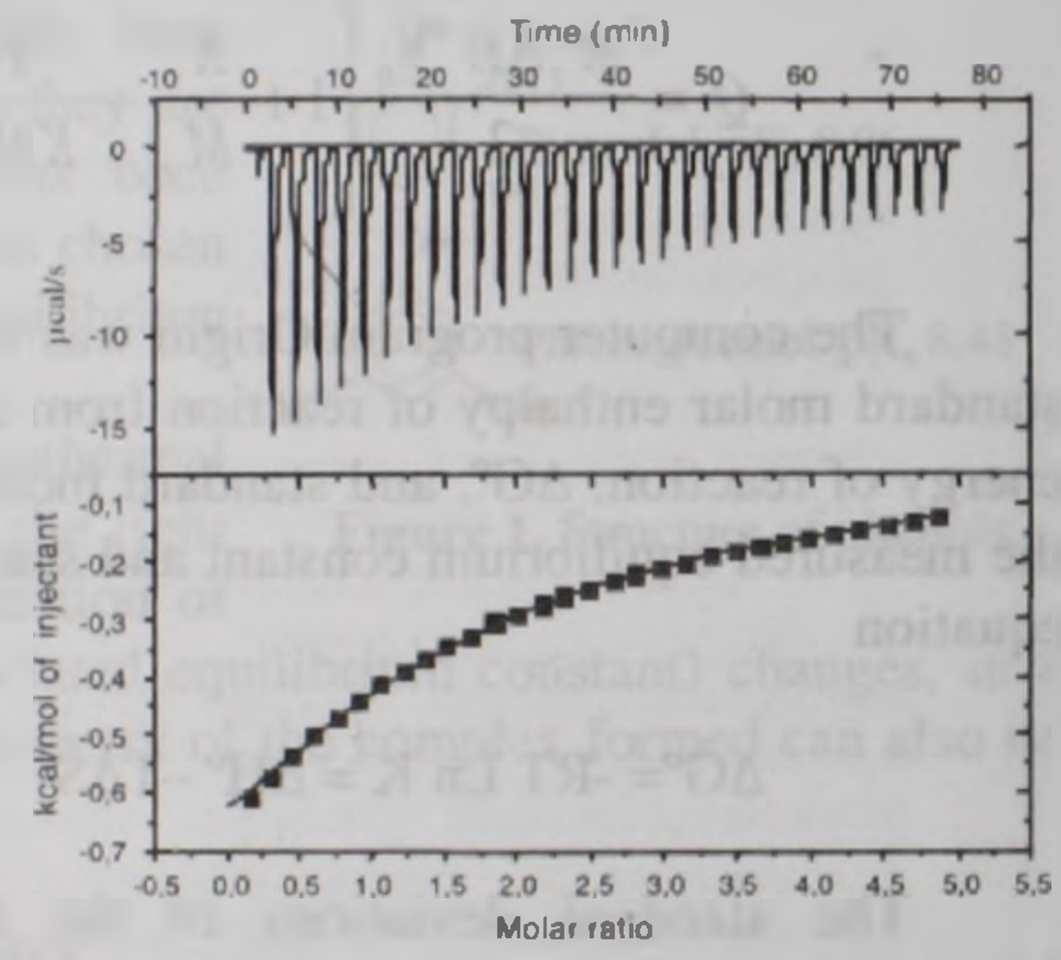

Figure 2. Calorimetric data for the titration of phenol (80 mM) into β-cyclodextrin [phosphate] = 0.05 M; pH 6.90; T=303 K. 30 injections of 10 μL.

Figure 2 clearly shows that the curve is far from being sigmoidal. By taking into account that the maximum M_t concentration used was 80 mM, it is expected that the equilibrium constant for the formation of the complex must be lower than 100 $dm^3 mol^{-1}$ (this is valid for all the phenols studied here). This is confirmed by the quantitative analysis carried out. The obtained results are presented in Table 1.

Table 1. Thermodynamic quantities for the reactions guest+cyclodextrin→complex

	β-cyclodextrin			α-cyclodextrin		
	$-\Delta H$ kJ mol^{-1}	$-\Delta S$ J $mol^{-1}K^{-1}$	K dm^3mol^{-1}	$-\Delta H$ kJ mol^{-1}	$-\Delta S$ J $mol^{-1}K^{-1}$	K dm^3mol^{-1}
Phenol	12.2 ± 0.13	5.2 ± 0.5	67.7 ± 1.1	18.4 ± 1.1	41.0 ± 3.7	10.9 ± 0.6
Catechol	17.8 ± 0.5	33.2 ± 1.7	20.4 ± 0.7	19.6 ± 5.3	54 ± 18	3.8 ± 1.1
Resorcinol	18.3 ± 0.08	24.7 ± 0.7	74.5 ± 0.6	25.0 ± 0.8	68.6 ± 2.7	5.3 ± 0.2
Pirogalol	18.6 ± 0.4	37.6 ± 1.3	17.9 ± 0.4	5.3 ± 1.0	3.2 ± 3.7	5.9 ± 1.2
Phloroglucinol	43.6 ± 1.8	112.0 ± 6.0	47.2 ± 2.3			

As can be seen from Table 1, formation of the inclusion complexes of phenols with both α- and β-cyclodextrin are exothermic driven as the enthalpies are negative and the entropies are unfavourable (negative) in all cases.

The enthalpy values in Table 1 compare favourably with those published for the complexation of p-substituted phenols with both α- and β-cyclodextrin, since values ranging from –10 (hydroquinone) to –27 kJ mol^{-1} (p-nitrophenol) have been published at pH 4.2 [10] (see Table 2). In this table, values obtained at pH > 9, where the phenols or even the cyclodextrins can be (at least partially) in their anionic forms, are omitted.

Table 2. Thermodynamic quantites for the formation of the complexes formed between p-substituted phenols and α- and β-cyclodextrin

Complex	$-\Delta H^{o}$ kJmol^{-1}	ΔS^{o} J $mol^{-1}K^{-1}$	K $dm^{3}mol^{-1}$	Reference
p-nitro–β–CD	14.9	-11.0	955	23
p-nitro-α-CD	27.1	-47	200	10
p-nitro-β-CD	13.4	-1 ± 2	260	
p-chloro-α-CD	20.1	-20	292	10
p-chloro-β-CD	11.9	10	410	
p-hydroxy-α-CD	10	-7 ± 7	24	10
p-hydroxy-β-CD	17.1	-18	113	
Phenol-α-CD	10.2	2 ± 2	37	10
Phenol–β–CD	12.2	4 ± 1	94	
p-bromo-α-CD	25.6	-31	710	10
p-bromo-β-CD	12.2	15 ± 2	860	
p-methyl-α-CD	17.7	-29	37	10
p-methyl-β-CD	12.5	4 ± 1	250	
m-nitro–β–CD	10.2	-6.3	661	23
phenol-α-CD	7.52	65	16000	24
phenol-β-CD	10.9	29	2500	

Less agreement does exist with the published values for the entropy. All the values obtained in this paper for the complexation of different polyphenols with both α- and β-cyclodextrin are negative. For the complexation of p-substituted phenols with α-cyclodextrin, the values range from 2 (phenol) to -47 (p-nitro-phenol) J mol^{-1} K^{-1}, while in the present paper values ranging from –3 (pirogalol) to –69 (resorcinol) J mol^{-1} K^{-1} have been obtained, i.e., both sets of values fall into characteristic ranges. For complexes with β-cyclodextrin Table 1 shows values ranging from –5 (phenol) to –112 (phloroglucinol) J mol^{-1} K^{-1}, while positive (from 15 J mol^{-1} K^{-1} for p-bromophenol) and negative values (-18 J mol^{-1} K^{-1} for p-hydroxyphenol) have been obtained for p-substituted phenols (Table 2) [10]. In some cases, the values are very close to zero (phenol, p-nitrophenol, p-methylphenol) with very high standard deviations. These high errors arise in part from the low values (close to zero) for the change in entropy for the complexation by cyclodextrins and by the fact that the entropy is calculated from equation 3 and therefore is greatly affected by the value used for the equilibrium constant. The value for the equilibrium constant itself is affected by a high dispersion as Table 3 illustrates for the complexation of p-nitrophenol with β-cycldoextrin for which values ranging from 130 to 1000 dm^{3} mol^{-1} have been published.

Table 3. Published Equilibrim Constants (K/ dm^3mol^{-1}) for the reaction p-nitrophenol + cyclodextrin → complex

α–CD	160		200	126		170	160	172; 204
β–CD	407	955	260	1000	130	220		185
Reference	21	23	10	24	26	27	28	29

Equilibrium constants in Table 1 are lower than those found in the literature (see Tables 2 and 4).

Table 4. Published Equilibrium constants for the formation of different complexes between the phenols studied in this paper and β-cyclodextrin

Guest	K/ dm^3mol^{-1}	Reference
Catechol, 20 °C	109	21
Resorcinol	117	21
Phenol	120; 95; 105	25
Phenol	129	26

Table 2 clearly shows that, except for pirogalol, the ΔS^o values for the complexation of polyphenols with α-cyclodextrin are more negative than those for the complexation with β-cyclodextrin. This fact is in agreement to the one observed by Rekharsky *et al* [18] for cyclohexanol, cis-1.2-cyclohexanol and trans-1.2-cyclohexanol, since negative values were observed for their complexation with α-cyclodextrin but positives ones for their complexation with β-cyclodextrin. This is also the case of cyclic alcohols (except for cyclooctanol) [12], aliphatic alcohols [7], negatively charged alkyl carboxylates and protonated amines [12], phenethylamines, ephedrines and related substances [19].

Table 1 also shows that, except for pirogalol, the ΔH^o values are more negative for the complexation of polyphenols with α-cyclodextrin than with β-cyclodextrin. As the equilibrium constants are always higher in the case of β-cyclodextrin, it is clear that the difference in ΔS^o between both cyclodextrins is the principal thermodynamic factor behind the stronger binding of these substances to β-cyclodextrin compared to α-cyclodextrin. With a few exceptions this is again in agreement to what has been observed for other systems such as cycloalcohols [12,18], phenethylamines, ephedrines, and related substances [19], and p-substituted phenols [10]. In this comparison we have only taken into account those papers in which values for the complexation of the substances with both cyclodextrins were studied. Linear alcohols are not considered as according to Masui and Mochida [7] complexation of 1-butanol, 1-pentanol and 1-hexanol with β-cyclodextrin is entropy driven (ΔH^o and ΔS^o are both positive).

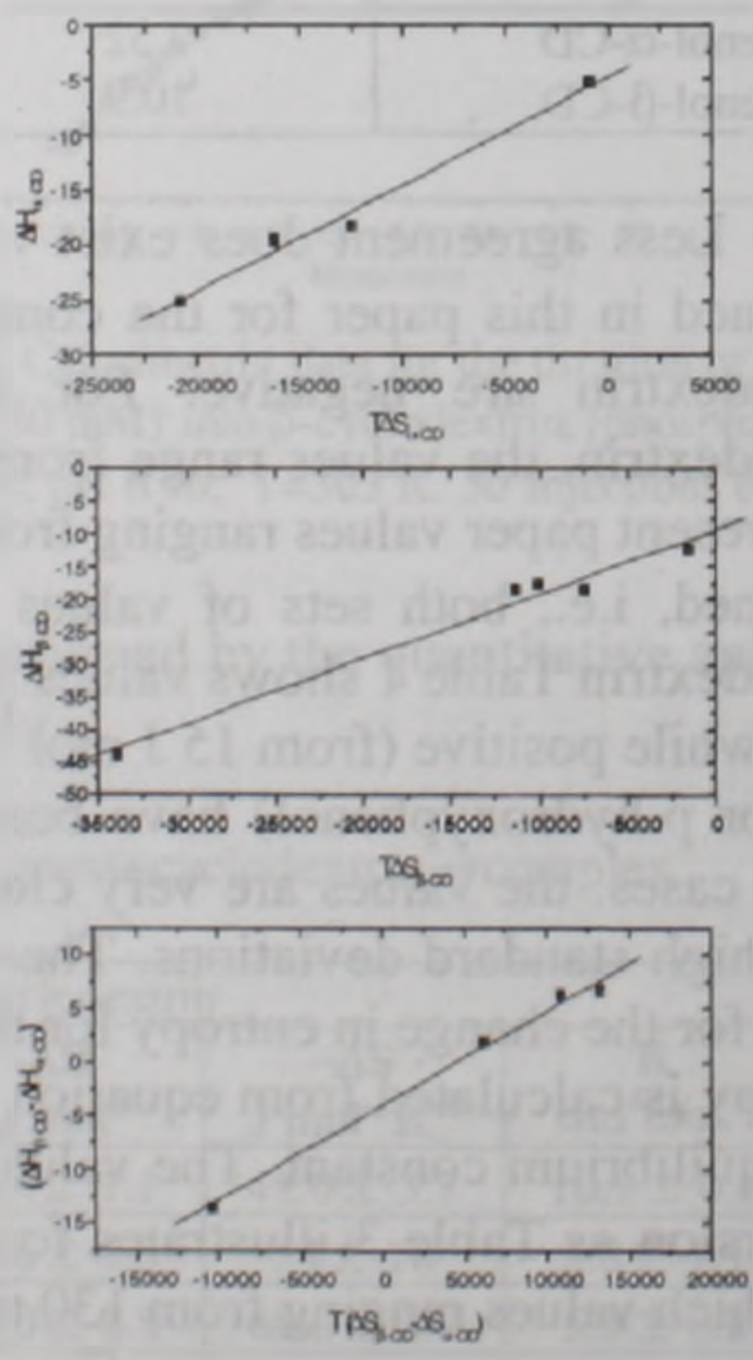

Figure 3. Enthalpy-Entropy Compensation Plot

From the entropic point of view alone, the complexation of these guests with both cyclodextrins

is unfavorable, but the entropic loss is compensated by the gain from the release of water molecules bound in and around of the cyclodextrin cavity and the guests. Highly negative values of the complexation entropy have been rationalized in terms of the decreased number of trapped water molecules that can be released upon complexation [30]. Obviously it does exist some enthalpy-entropy compensation in these complexation reactions.

Compensatory enthalpy-entropy relationships have already been observed in previous thermodynamic studies when complexing different families of compounds with cyclodextrins [10,12,14,18,19,24,30,31]. Figure 3 illustrates this relationship for the formation of complexes studied here. Slopes and intercepts are given in Table 5. Obviously, the slopes are not statistically different from 1, which means that the enthalpic gain/loss from any changes in host, guest, or solvent is perfectly canceled out by the entropic loss/gain arising from structural changes in the inclusion compound produced. This has been related to the global reorganization of the original hydrogen bond network upon complexation. Guests with hydroxyl groups, as the ones studied here, must be heavily solvated through dipole-dipole and hydrogen bonding interactions in water. The reorganization of the hydrogen-bond network in the host-guest complex has to play a significant role in the complexation [30]. This helps us to inferred the structure of the complexes, as it is commented on below.

The value equal to one observed for the slope implies that the isoequilibrium constant corresponds to the experimental temperature used in this work, *i. e.*, 303 K. This temperature is similar to other values found in the literature [10,12,18,19,30-32]. The intercept values, which correspond to hypothetical reactions without change in the entropy, are clearly negative. Close to zero values or negative ones have also been obtained by Inoue *et* al [30], Rekharsky *et al* [19], and Liu *et al* [31] for different families of compounds.

Table 5. Slope and intercept of the Enthalpy-Entropy Compensation Plots

Reaction	Intercept/ kJ mol^{-1}	Slope	r^2
guest + α-CD → α–complex	-4.8 ± 1.3	0.98 ± 0.09	0.98
guest + β–CD → β–complex	-9,4 ± 1.3	0.99 ± 0.08	0,98
α–complex + β–CD → β–complex + α–CD	-4.0 ± 0.5	0.87 ± 0.05	0.99

Following Bertrand *et al* [10] the compensation between entropic and enthalpic effect is most readily seen in terms of the exchange reaction 4, in which the effects of solvation of the free phenols in water, and the difference in solvation of the cyclodextrins is a fixed contribution for all the family of compounds.

α-complex + β-CD → β-complex + α-CD [4]

The slope and intercept of the ΔH° vs ΔS° plot are given in Table 5. The intercept is very close to the value of -3.6 kJmol^{-1} obtained by Bertrand *et al* for a set of p-substituted phenols and not far from those obtained by Rekharsky *et al* [19] (=-1.9 kJmol^{-1}) for phehethylamine, ephedrines and related substances, and cyclohexane derivatives (=-7.5 kJmol^{-1}). For primary and secondary alcohols, Rekharsky *et al* [32] have found a positive value for the intercept (4.2 kJ mol^{-1}). Again the high value for the slope indicates that the enthalpy-entropy compensation is essentially complete. The corresponding isoequilibrium temperature, 265 K is lower than those found by Bertrand *et al* [10](= 360K) or Rekharsky *et al* [12,18] but close to those obtained by Rekharsky *et al* [19,32] (= 251 K and 274, respectively).

The most frequent mode of complexation of guests to cyclodextrins consists of insertion of the hydrophobic portion of the guest into the cyclodextrin cavity while the polar groups of the ligand remain solvent exposed and near (depending on the size and structure of the guest) of the hydroxyl rims. In the present case, the hydrophobic part of the guest, *i. e.*, the phenyl ring is small enough to fit inside of both cyclodextrin cavities. On the other hand, Rekharzsky *et al* [19], have found that the OH group, provides a significant enthalpy stabilization for the ligand-cyclodextrin complex, although this stabilization can be almost cancelled by unfavorable entropy changes (see above). These authors have also found that simple additivity of the thermodynamic quantities due to the addition of an OH group to the aromatic ring only works in a few examples. Finally, in the present case the hydroxyl polar groups of the guests could form hydrogen bonds with the primary or secondary hydroxyl groups of the cyclodextrin, if the structure of the complex adopts a favorable conformation. The nature of the hydration cosphere of the guest molecule plays an important and often fundamental role in the formation of a stable inclusion complex [8] and therefore the formation and breaking of hydrogen bonds will also play an important role in the gain in entropy for water relaxed from the hydration cospheres to the bulk from both the guest and host.

On the other hand, the general tendency of the ΔH^{o} values in Table 1, clearly indicates that enthalpy change becomes more negative with the increase of the number of hydroxy groups attached to the phenyl ring. The simplest conclusion is that the formation-breaking of hydrogen bonds have to play an important role in the thermodynamics of the complexation of the polyhydroxyphenols. This is also supported by the results from Bertrand *et al* [10] who concluded that the "hydroquinone complexes seem to differ somewhat form the other p-substituted phenol complexes and that the hydroxyl group appears reluctant to enter the hydrophobic cavity of α-cyclodextrin while the strong interaction of the hydroquinone molecule with β-cyclodextrin could be due to some preferred orientation of the hydroquinone molecule penetrating the cavity and forming strong hydrogen bonds with hydroxyl groups at both the top and bottom of the cavity". Finally it is expected that, if they exist, the steric effects play a similar role all over the set of compounds.

With all these comments in mind, in order to propose a structure for the complexes, the comparison between the resorcinol and pirogalol with β-ciclodextrin plays an important role. If the hydroxyl groups of the guest remain near of the hydroxyl groups of the cyclodextrin the most obvious structure should be the one showed in Figure 4 (the structure proposed with the hydroxy phenol groups near the primary rim of cyclodextrin is supported by ^{13}C NMR and ROESY experiments commented on below). The interactions between the cyclodextrin and the hydroxy groups in relative positions 1 and 3 should be the same for both guests, while the third one in pirogalol does not interact with the β-cyclodextrin. In this way, solvent rearrangements around this third hydroxyl group should no suffer almost any change in its environment when it is transferred from the bulk solvent into the cyclodextrin cavity. At the same time, the modifications experienced by the solvent in the hydration cospheres of the other two hydroxyl groups and the apolar phenyl ring should be very similar for both phenol derivatives, and the process of squeezing out the water molecules, when the guest penetrates the cavity will be the same in both cases. The global result is that the enthalpy and entropy changes in complexation have to be very similar for both compounds as it has been observed experimentally.

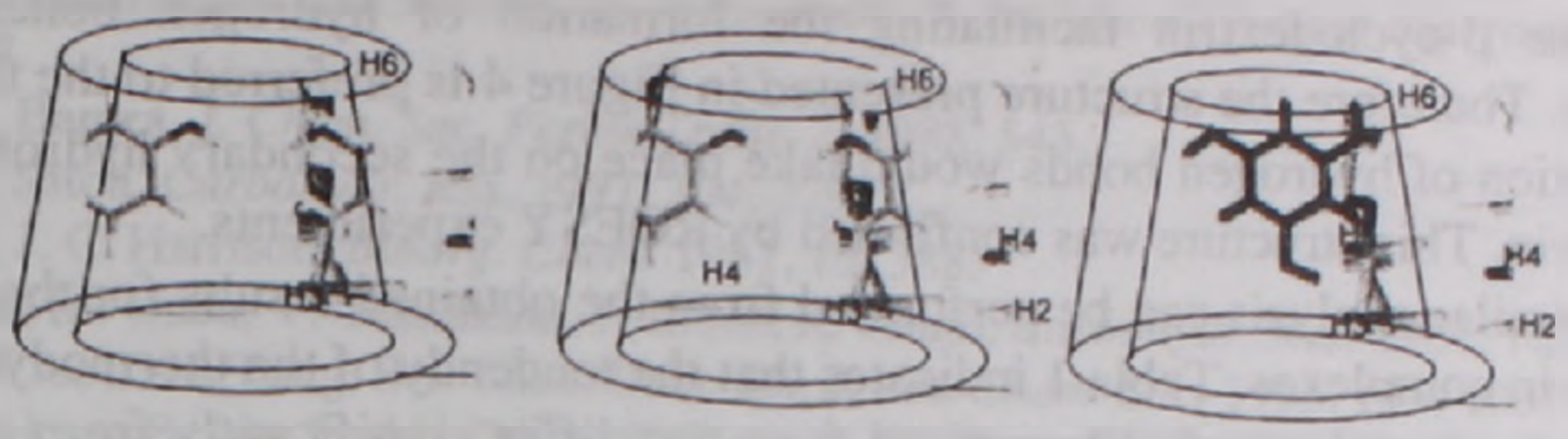

Figure 4. Schematic structures for β-cyclodextrin complexes with resorcinol, pirogalol, and phloroglucinol.

While accepting the previous structure we can propose a structure for the phloroglucinol-β-cyclodextrin complex. In this case, two of the three hydroxyl groups will present the same thermodynamic interactions as those commented on above, and the third one could form a new hydrogen bond with the other rim of the cyclodextrin in a similar way to that proposed by Bertrand *et al* for hydroquinone. Therefore, solvent rearrangements surrounding the phloroglucinol and, in less degree the cyclodextrin, must be much more important than in previous cases resulting in a much more negative entropy change. Furthermore, the phenyl ring is completely embedded into the hydrophobic cavity of the β-cyclodextrin facilitating van der Waals interactions resulting in an increase in the exothermicity of the reaction.

The similarity of the experimental thermodynamic quantities for the complexation of catechol with those of resorcinol and pirogalol (in fact, $\Delta H^{\circ}_{resorcinol} - \Delta H^{\circ}_{catechol} \approx \Delta H^{\circ}_{pirogalol} - \Delta H^{\circ}_{resorcinol}$) suggests that similar interactions and rearrangements have to be implied in the complexation process and therefore similar structures for all these complexes are expected. In this case, the structure of the guest allows the phenyl ring to enter deeper inside the cyclodextrin cavity.

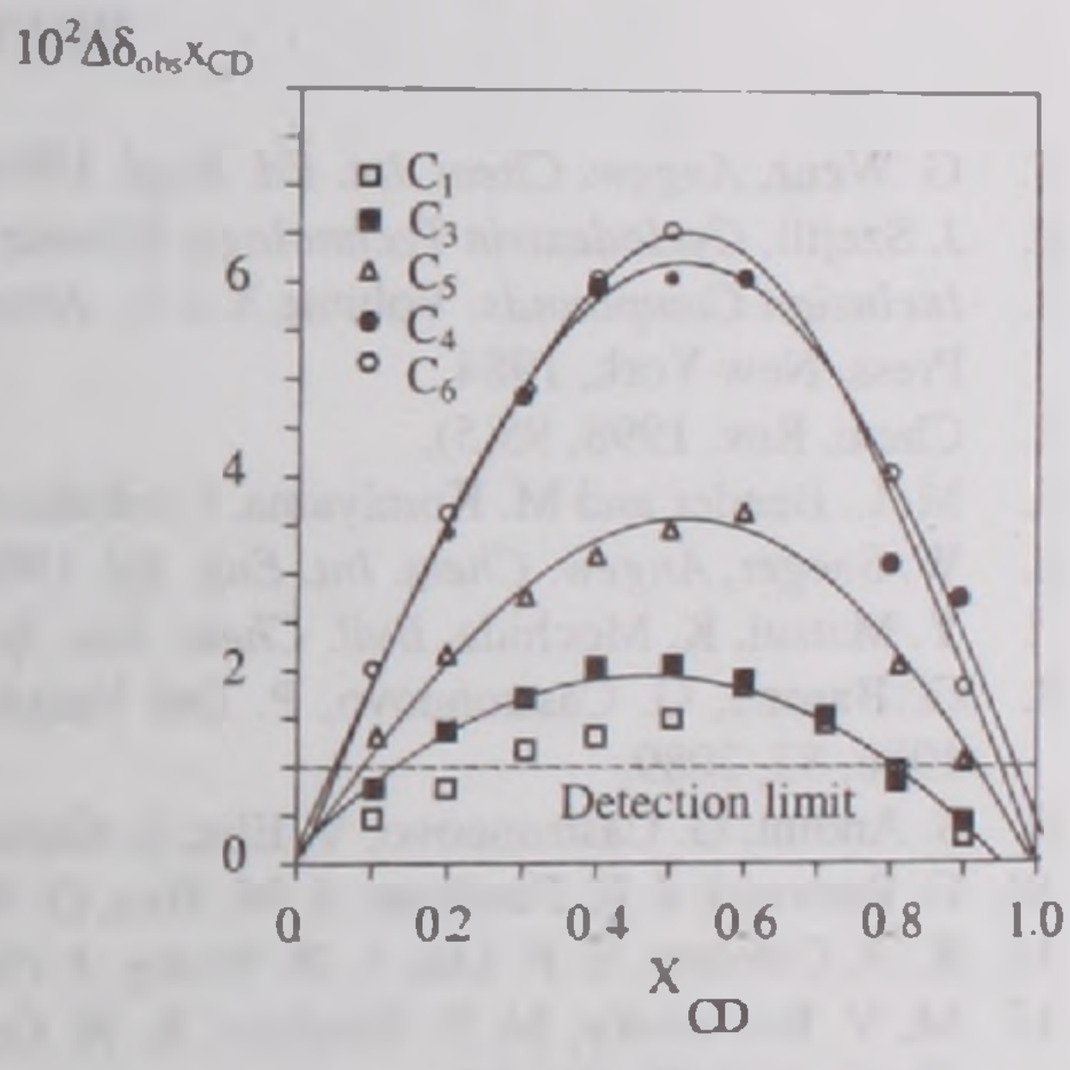

Figure 5. Job's plot corresponding to the chemical shift displacement of different carbons of β-cyclodextrin

Preliminary NMR experiments were carried out to confirm previous ideas. Figure 5 shows, in the form of a Job plot, the increments of the chemical shifts of the different carbons of the β-cyclodextrin as a consequence of the complexation of resorcinol. The maxima of the plot at $x_{\beta\text{-}CD} = 0.5$ confirm that the complex has a 1:1 stoichiometry which was used as a fixed parameter in the analysis of the experimental ITC data. These experimental data allow the determination of the equilibrium constant. However the obtained value is not shown since the standard deviation is very high as a consequence that the $\Delta\delta$ *vs* $x_{\beta\text{-}CD}$ plots are far from reaching a plateau at high β-cyclodextrin concentration, a typical finding for systems with low

equilibrium constants for the complex formation. The great chemical shift displacement for carbon 6 suggests that a strong guest-host interaction does exist near the primary hydroxyl rim of the β-cyclodextrin facilitating the formation of hydrogen bonds between both substrates. Therefore the structure presented in Figure 4 is preferred to the that one in which the formation of hydrogen bonds would take place on the secondary hydroxyl rim of the β-cyclodextrin. This structure was confirmed by ROESY experiments.

A similar analysis can be performed from the obtained results for the formation of α-cyclodextrin complexes. Table 1 indicates that the tendendy of the thermodynamic quantities in the series phenol-catechol-resorcinol does not differ significantly from the one obtained for β-cyclodextrin, the only exception being the complexation of pirogalol, since much less negative entalpy and entropy changes are observed. So in this case, it seems that the existence of a third hydroxyl group squeezes out the guest from the α-cyclodextrin cavity, resulting in a less favorable guest- α-cyclodextrin interaction and a less requirement for the rearrangement of the solvent surrounding the guest and α-cyclodextrin molecules. This hypothesis has to be confirmed by NMR experiments.

Acknowledgements. Authors thank Xunta de Galicia (Project PGIDT99PXI26201B) and CYTED (Project VIII.3) for financial support.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

BIODEGRADATION OF HYDROCARBONS IN POLLUTED SOILS USING β-CYCLODEXTRIN AS A COADIUVANT

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The biodegradation of pollutants depends on their bioavailability for soil microorganisms. Hydrocarbons are hydrophobic compounds which are poorly soluble in water and thus poorly bioavailable.

Cyclodextrins interact with hydrocarbons to produce inclusion complexes that are more water soluble, so they can be more easily degradated. Cyclodextrins, moreover, are cheap, natural and biocompatible compounds widely used for medical and food purposes.

In this paper we studied the use of cyclodextrin to enhance the degradation rate of dodecane by natural microbial strains isolated from a petroleum-polluted soil. We used three types of soil texture: sand, loamy sand and clay, in order to understand their influence on the biodegradation rate.

Probably due to the oxygen diffusion required for microbial metabolism, the biodegradation kinetic in clay is slower (half-reaction time = 199.3 h) than in loamy sand (half-reaction time = 101.7 h) and in sand (half-reaction time = 76.9 h). In all tests cyclodextrins increased the biodegradation time gain (24.2-40.9%).

It was checked the level of sorption of hydrocarbon on soil and the eluviation through a soil profile analized. Cyclodextrin did not show any effect on eluviation of hydrocarbon, that was anyway absent.

Биодеградация загрязнителей зависит от их доступности для почвенных микроорганизмов. Углеводороды – гидрофобные соединения, которые плохо растворимы в воде и таким образом слабо пригодны для микробов.

Циклодекстрины реагируют с углеводородами, образуя инклюзионные комплексы, которые более растворимы в воде, и таким образом легко разрушаемы. Циклодекстрины – недорогие, природные и биосовместимые соединения, широко используемые для медицинских и пищевых целей.

Изучено использование циклодекстринов для усиления интенсивности деградации додекана природными микробными штаммами, выделенными из почвы, загрязненной нефтью. Для выяснения степени разрушения додекана использовались 3 типа почв разной текстуры: песок, глинистый песок и глина. Вероятно, благодаря требуемой диффузии кислорода для микробного метаболизма, кинетика биодеградации в глине протекает медленно (время полуреакции = 199,3час), чем в глинистом песке (время полуреакции = 101,7час) и в песке (время полуреакции = 76,9). Во всех опытах циклодекстрины повышали период биодеградации на 24,2 – 40,9%. Проверялись уровень сорбции углеводорода в почве и элувиация в почвенном профиле. Циклодекстрин не оказал какого – либо эффекта на элувиацию углеводорода во всех испытанных случаях.

Աղտոտիչների կենսաքայքայումը կախված է նրանց մատչելիությունից հողային մանրէների համար: Ածխաջրածինները հիդրոֆոբ միացություններ են, որոնք վատ են լուծվում ջրում և այդպիսով քիչ պիտանի են մանրէների համար:

Ցիկլոդեքստրինները ռեակցիայի մեջ են մտնում ածխաջրածինների հետ, առաջացնելով ինկլյուզիոն համալիրներ, որոնք առավել լուծելի են ջրում, և այդպիսով հեշտ քայքայվող: Բացի դրանից, ցիկլոդեքստրինները էժան, բնական և կենսահամատեղելի միացություններ են, լայնորեն օգտագործվում են բժշկական և սննդային նպատակներով:

Ուսումնասիրվել է դոդեկանի քայքայման արագության մեծացման համար ցիկլոդեքստրինների օգտագործումը նավթով աղտոտված հողից անջատված բնական մանրէային շտամներով: Դոդեկանի քայքայման մակարդակի որոշման համար օգտագործվել են 3 հողային տիպեր ավազ, կավային ավազ և կավ: Հավանաբար, մանրէային մետաբոլիզմի համար պահանջվող թթվածնի դիֆուզիայի շնորհիվ կենսաքայքայման կինետիկան ընթանում է ավելի դանդաղ կավ (կես ռեակցիայի ժամանակը =199.3 ժամ), քան կավային ավազի (կես ռեակցիայի ժամանակը =101.7 ժամ) և ավազի (կես ռեակցիայի ժամանակը =76.9 ժամ) մեջ: Բոլոր դեպքերում ցիկլոդեքստրինները բարձրացնում են կենսաքայքայման ժամանակը 24.2-40.9% ով: Ստուգվել է ածխաջրածինների սորբցիայի մակարդակը հողում և հետազոտվել է էլուվիացիան հողային կտրվածքով: Բոլոր փորձարկված դեպքերում ցիկլոդեքստրինը ածխաջրածինների էլուվիացիայի վրա ազդեցություն չի թողել:

Introduction

Xenobiotic chemicals enter a specific biogeochemical cycle when discharged in an environmental compartment. Their transport and distribution not only depend upon diffusion and transport patterns within each compartment, but also on partition processes between the various compartments: air, water or land [1].

The remediation of polluted soil can be achieved by chemical, physical or biological treatment. In recent few years, bioremediation processes have been preferred because of their low cost and capability of fully degrading pollutants. In particular, *in situ* bioremediation aims at cleaning up contaminated soils, reducing health hazards and/or the ecotoxicological risks of spreading contamination, without moving the soil itself [2,3,4,5,6].

One main factor that influences the extent of pollutant biodegradation is their bioavailability; this is a priority research objective in the bioremediation field. [6,7]. Hydrocarbon bioavailability is very poor; in fact, they are hydrophobic, so they pass very slowly from a non-aqueous to the aqueous phase liquid in which they are metabolised by microorganisms [7, 8]. Moreover, in the soil they are adsorbed to clay or humus fractions [7, 9]. The addiction of surfactants can increase their bioavailability due to increased water solubility [10,11,12,13]; on the other hand, eluviation through the soil profile can also be enhanced and, as a consequence, the risk of the pollution of groundwater increased. Moreover, surfactants are frequently toxic and poorly biocompatible; sometimes they are considered as pollutant compounds.

α, β and γ cyclodextrins are cyclic oligosaccharides formed by 6, 7 or 8 α-1,4-linked glucose units respectively [14]. Since they have toroidal hydrophobic cavities with a hydrophilic shell, they are water-soluble and form inclusion complexes with hydrophobic molecules of a size compatible with their hydrophobic core. In this way, the aqueous solubility of several compounds is increased by cyclodextrins through their dynamic equilibrium exchange with guest molecules, which then dissociate from the cyclodextrin complex and become available for catabolism. Cyclodextrins are natural, non toxic compounds and harmless to microorganisms and free enzymes [15]. Due to their properties, cyclodextrins can be proposed as a surfactant to be used in soil and water bioremediation from hydrophobic xenobiotics. Schwarz and Bar (1995) found that cyclodextrins enhanced degradation of toluene and p-toluic acid [16]. Wang et al. (1998) demonstrated a better biodegradation of phenanthrene in the presence of cyclodextrins [17]. In our laboratory, hydrocarbon degradation has been investigated in the aqueous phase using microbial colonies isolated from a petroleum-polluted soil. Aliphatic (dodecane, tetracosane) and poliaromatic (naphthalene, anthracene) hydrocarbons were degraded and their degradation

kinetics were accelerated by β-cyclodextrin. Hence, β-cyclodextrin enhanced the water solubility of the hydrocarbons and consequently increased their bioavailability for microorganisms [18].

In this work, our aim was to investigate the application of β-cyclodextrin as a surfactant for *in situ* hydrocarbon polluted soil bioremediation. First, we checked the risk of increased groundwater pollution due to the increased water solubility of the hydrocarbons. We checked, in the presence and in absence of β-cyclodextrin, the sorption and the eluviation of dodecane, an aliphatic, low-molecular weight hydrocarbon, in three soils differing in texture and, consequently, in permeability and drainage properties: a sand, a loamy sand and a clay. Then we checked the degradation kinetics of dodecane in the same three soils, inoculated with a hydrocarbon degrading selected microbial population, simulating a farming system.

Materials and methods

Sorption and eluviation assays. Three mineral soils with different textures were examined: a sand (sand 97.6%, silt 1.6%, clay 0.8%), a loamy sand (sand 80.4%, silt 17.8%, clay 1.8%) and a clay (sand 3.9%, silt 60.3%, clay 35.8%). Organic matter was absent. Soils were dried in an oven at 120°C for 7 h. The amount of dodecane retained by matrix potential, due to sorption or capillary forces, was determined. 80 ml of water with 67 µl of dodecane were added to 100 g of soil and mixed for 4 hours, then the gravitational water was drained and the amount of dodecane present in it analysed. The dodecane retained by the soil was calculated as the difference between the total amount added and the amount detected in gravitational water. The same assay was carried out in each of the three soils, without cyclodextrin or with 1% (w/w) β-cyclodextrin added to the mixture.

Eluviation tests were performed in two glass columns, 3 cm diameter and 30 cm high, filled with 280 g sand. 188 µl of dodecane were added on the surface of the two columns, and 1% (w/w) of water-melted β–cyclodextrin was added to one column. Then 850 ml of water were applied at the surface of each column and left to elute by gravity. The drained water was collected in 20 ml fractions and each fraction was analysed for its dodecane content.

Biodegradation kinetics. 700 g of each soil, previously dried in an oven at 120°C for 7 h, were posed to a depth of 10 cm depth in an impermeable, open box. 470 µl dodecane and 1% (w/w) water-melted β-cyclodextrin were added, then 100 ml of microbial culture were spread as inoculum and 200 ml of mineral growth medium (LMM) were added. Daily, water was balanced and soil homogeneity and aeration were maintained by vigorous and accurate mixing. The temperature was kept at 20°C. The tests were performed by monitoring the dodecane decrease, in the presence and absence of β-cyclodextrin and with the three different soil textures (sand, loamy sand and clay).

Microbial inoculum. Microbial colonies were isolated from a petroleum-polluted soil by dispersing a sterile water suspension of soil in MMA (Mineral Medium Agar: 0.8 g/l K_2HPO_4, 0.2 g/l KH_2PO_4, 0.05 g/l $CaSO_4 2H_2O$, 0.5 g/l $MgSO_4 7H_2O$, 0.09 g/l $FeSO_4 7H_2O$, 1 g/l $(NH_4)_2SO_4$, 15 g/l agar) with 4% v/v dodecane as the sole carbon source in Petri dishes, and incubating them at 28°C for 5 days. A random pool of 54 colonies was transferred to 200 ml of LMM (Liquid Mineral Medium: as MMA, without agar) with 4% v/v dodecane in a conical flask and incubated on an oscillatory shaker at 140 rpm for 5 days at 28°C. This culture was used as inoculum for the degradation kinetic assays.

Dodecane analysis. For the biodegradation kinetics assays, 15 g of soil sample were mixed with 6 ml of water and extracted by shaking with three successive aliquots of a total volume of 30 ml of toluene. The mixture was centrifuged at 4000 rpm for 5 min and the organic phase was transferred to a fresh tube. 100 µl of a standard solution of 1% w/v naphthalene in toluene were added to 1 ml of extract. The samples were analysed by a gas chromatograph GC HP 5890 series II equipped with a flame ionization detector on a HP1 Cross-Linked Methyl Silicone capillary column, 15 m long and 0.32 mm i.d., film thickness 1.0 mµ. The operating conditions were: temperature from 100 to 250°C at

20°C/min; pressure 13 psi; injection volume 4 µl. For the sorption and eluviation assays, dodecane was extracted and analysed as previously described [18].

Results and Discussion

Sorption and eluviation assays. The amount of dodecane retained by the matrix potential of soil was 97.81% in sand, 99.42% in loamy sand and 99.80 % in clay (figure 1). Therefore, the amount of dodecane that remains in drained gravitational water is very low after just 4 hours of contact with the soil. The dodecane retained by matric potential rises from sandy to clay texture, but the difference between sand, loamy sand and clay is very low.

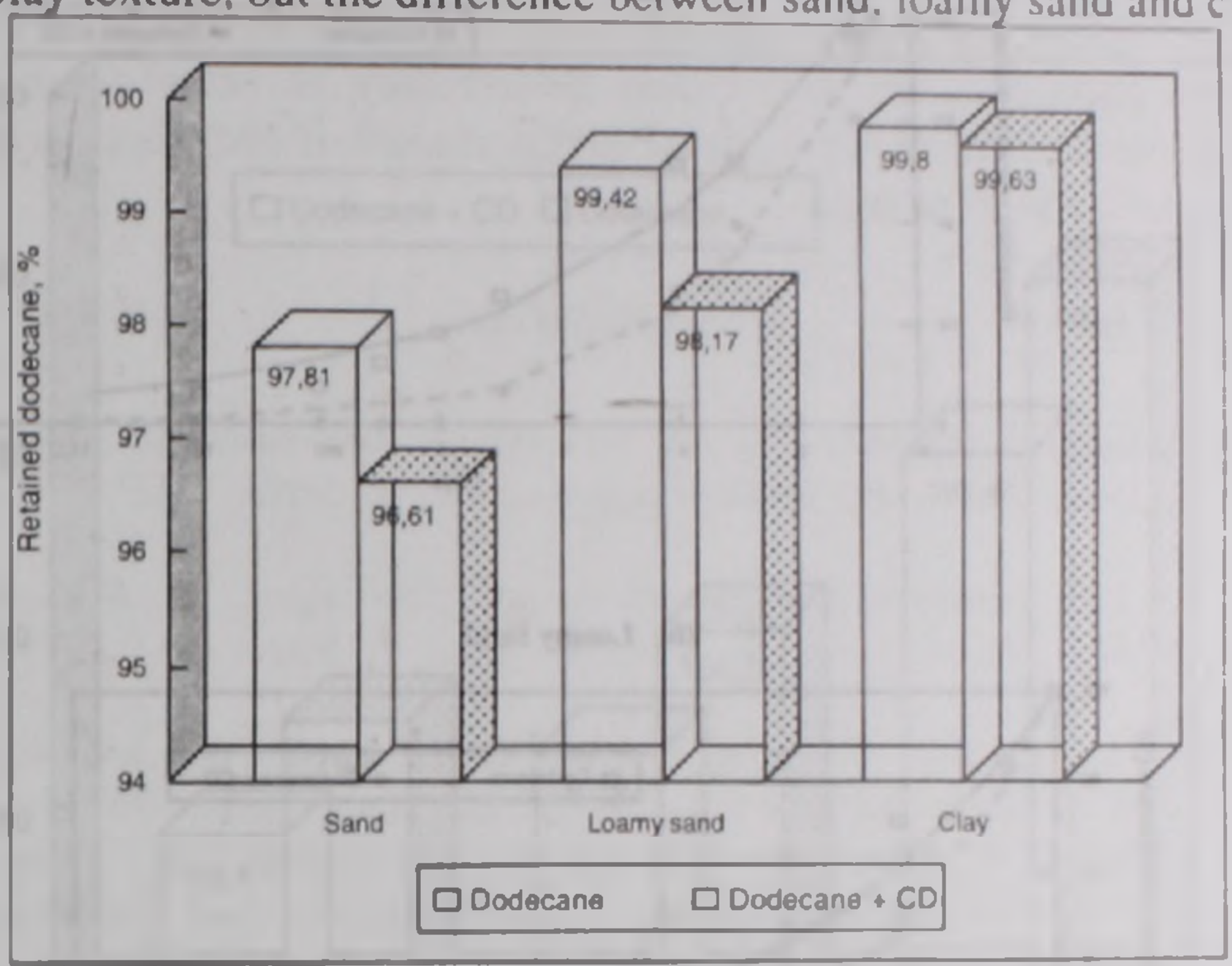

Figure 1. Retained amount of dodecane by sand, loamy sand and clay, due to sorption or capillary forces.

When β-cyclodextrin is added, the fraction of dodecane that remains in gravitational water is slightly higher: the amount retained by the soil becomes 96.61% in sand, 98.71% in loamy sand and 99.63% in clay (figure 1). It can be observed that the decrease is very small in clay (0.17%) and slightly higher in sand (1.2%). In any case, β-cyclodextrin induces a very slight increase in the solubility of the hydrocarbon in gravitational water; this solubility proved to be very low in all the chosen soils, although they were devoid of organic matter.

The eluviation assays, performed by eluting the hydrocarbon with water through the different soils in glass columns, confirmed that β-cyclodextrin does not increase the eluviation of dodecane. In these assays a water volume corresponding to the average annual rainfall in Italy was used. No traces of dodecane were found in drained water.

Hence, the risk of groundwater pollution due to β-cyclodextrin because of the increased solubility of the hydrocarbon can be excluded.

*Biodegradation kinetics.*Dodecane was fully degraded in sand in 329 hours, while this time was reduced to just 192 hours when β-cyclodextrin was added (figure 2a). The half-reaction time (T1/2), that is the time at which the xenobiotic concentration becomes half its initial value, was 76.9 h without β-cyclodextrin and 45.4 h with β-cyclodextrin (figure 3). Similar results were obtained in loamy sand: dodecane full degradation was reached in 401 h without β-cyclodextrin, while 278 h were sufficient when β-cyclodextrin was added

(figure 2b); the half-reaction time varied from 101.7 h to 67.2 h, respectively (figure 3). It is evident that in sand and loamy sand the addition of β-cyclodextrin causes a significant acceleration of biodegradation kinetics, due to the increased bioavailability of hydrocarbon that passes into the aqueous phase in the form of inclusion complexes. The degradation time gain due to β-cyclodextrin was 40.9% in sand and 33.9% in loamy sand (table 1).

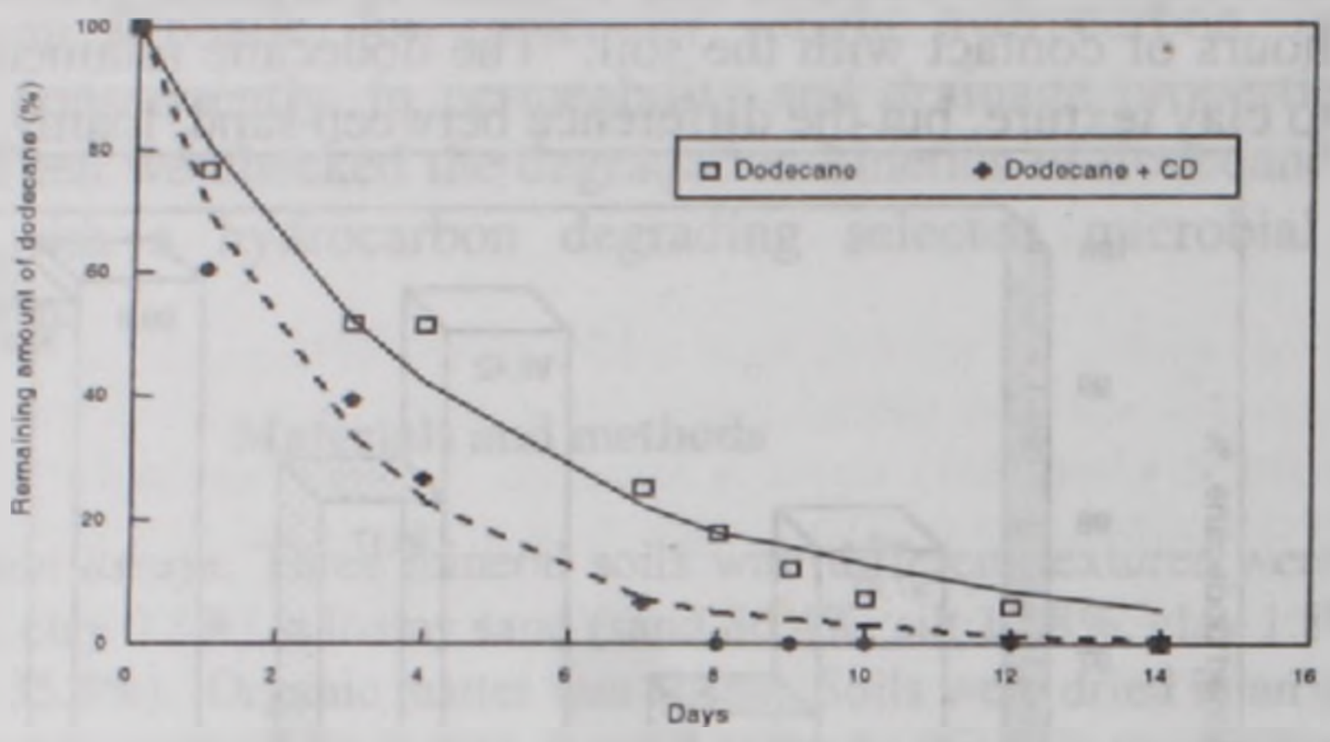

Figure 2. Percentage of remaining dodecane during the microbial degradation in sand (2a), loamy sand (2b) and clay (2c). Data are reported as media of two replicate determinations.

Table 1. Degradation time on sand, loamy sand and clay (hours)

	Sand	Loamy sand	Clay
Full degradation time without β-cyclodextrin	76.9	101.7	199.3
Degradation time with β-cyclodextrin	45.4	67.2	151.4
Degradation time gain (due to cyclodextrin)	40.9%	33.9%	24.2%
Required time	1	1.48	3.33

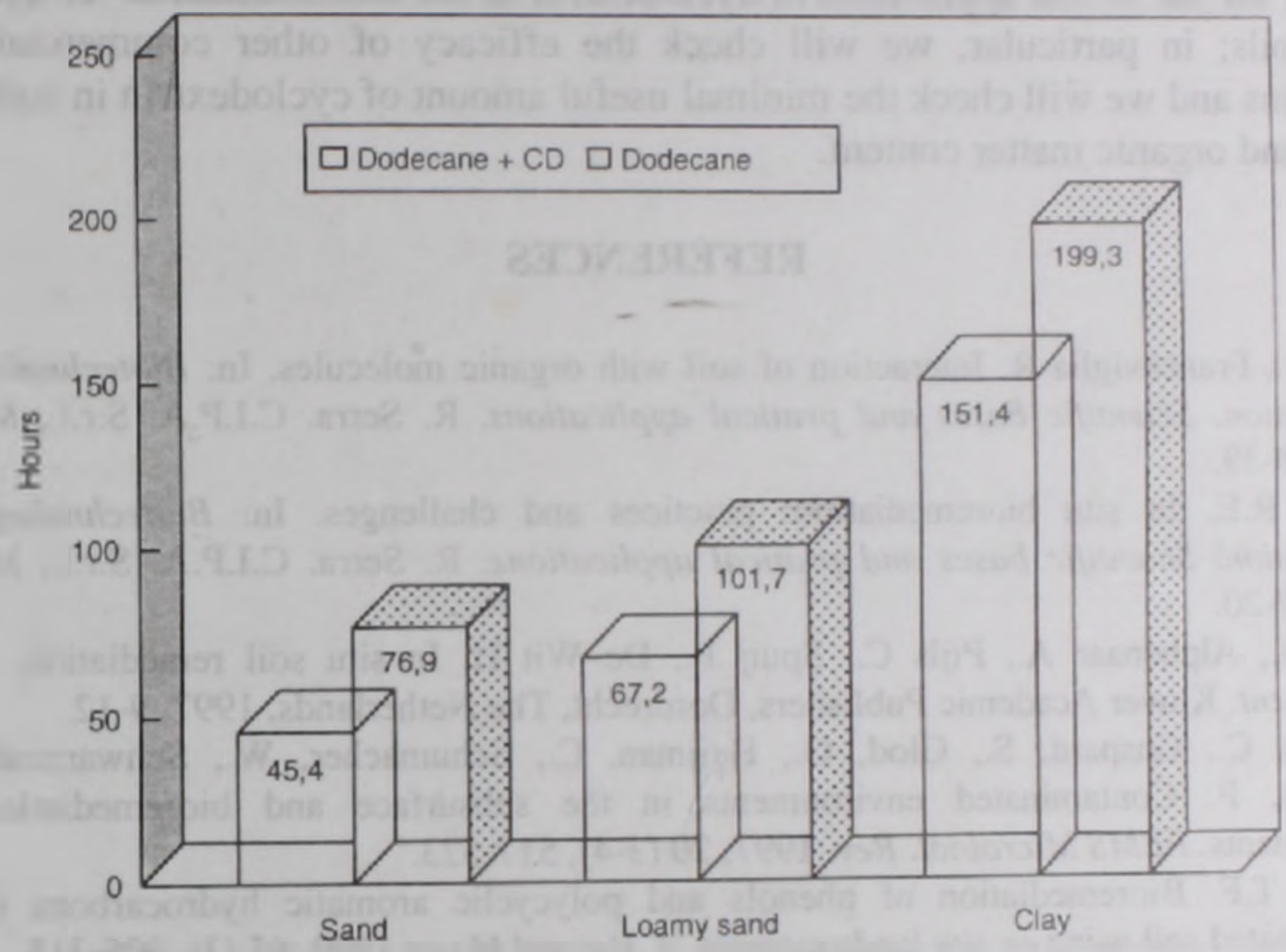

Figure 3. Dodecane half-reaction time in sand, loamy sand and clay calculated from data reported in Fig. 2

The positive effect of β-cyclodextrin was slightly less evident in clay. In fact, full dodecane biodegradation required 521 h without β-cyclodextrin and 497 h with β-cyclodextrin (figure 2c) with a 24.2% degradation time gain (table 1). Half-reaction time was reduced from 199.3 h to 151.4 h (figure 3). As expected, biodegradation was slower in clay, where the fine texture makes the diffusion of the oxygen required for microbial metabolism more difficult. Moreover, fine-textured soils have more total pore space than sandy soils, but these spaces are mostly micropores, that restrict the water flow. What may happen is that an intrinsically biodegradable compound is slowly diffused into soil micro- and nano-pores, which are so small that the compound within them is beyond microbial attack. That could be an explanation of the minor effect of β–cyclodextrin in clay, although its solubilization effect does not change. In any case, the addition of β–cyclodextrin resulted in an improvement in the biodegradation of dodecane in soil.

Conclusions

β–cyclodextrin used as a surfactant in bioremediation of hydrocarbon polluted soils enhances the bioavailability of hydrocarbons to the microorganisms; consequently, degradation kinetics are accelerated. The addition of β–cyclodextrin does not increase the risk of groundwater pollution even in mineral sandy soils. Therefore, the effectiveness of β–cyclodextrin in the improvement of biodegradation of hydrocarbons in soil has been demonstrated; its positive effect is influenced by the soil texture, but in any case the degradation time gain is always significant. Our next work will be the optimisation of several parameters for the *in situ* application of cyclodextrin in the bioremediation of hydrocarbon-polluted soils; in particular, we will check the efficacy of other commercial forms of cyclodextrins and we will check the minimal useful amount of cyclodextrin in soils differing in texture and organic matter content.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

INNOVATIVE APPROACH FOR REMOVAL AND BIODEGRADATION OF CONTAMINATED COMPOUNDS IN SOIL BY CYCLODEXTRINS

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Hydroxypropyl-β-cyclodextrin (HP-β-CD) was used for the remediation of contaminated soil. Very highly efficient removal was obtained by using the kneading method. A simple calculation model for the equilibrium amount of dissolution of biphenyl was proposed on the basis of the equilibrium adsorption assumption. The decomposition of the biphenyl by the activated sludge depended on both the type of CD and the degree of inclusion of biphenyl with CD. The direct decomposition of biphenyl in the kneaded soil slurry was applied to the bioremediation of the contaminated soil treatment by activated sludge.

Использован гидроксипропил – β – циклодекстрин для обработки и оздоровления загрязненных почв. Достигнут очень высокий результат с применением метода смешивания. Предложена простая расчетная модель для равновесия количества растворения бифенила на основе уравнения адсорбции. Разложение бифенила активированным илом зависит как от типа ЦД, так и от степени включения бифенила в ЦД. Использовано прямое разложение бифенила в размешанной почвенной жиже с помощью обработки активированным илом для биообработки и оздоровления почв.

Օգտագործվել է հիդրօքսիպրոպիլ- β – ցիկլոդեքստրինը (ՀՊ- β – ՑԴ) աղտոտված հողերի մշակման և առողջացման համար: Ստացվել է շատ բարձր արդյունք խառնման մեթոդի կիրառմամբ: Ադսորբցիոն հավասարության հիման վրա առաջարկվել է պարզ հաշվարկային մոդել բիֆենիլի լուծելիության քանակական հավասարության համար: Ակտիվացված տիղմի բիֆենիլի քայքայումը միջոցով կախված է ինչպես ՑԴ տիպից, այնպես էլ ՑԴ-ի հետ բիֆենիլի ներառման աստիճանից: Հողերի կենսաբուժման և մշակման համար կիրառվել է հողային խառնված զանգվածում բիֆենիլի ուղղակի քայքայում ակտիվացված տիղմով մշակմամբ:

Introduction

Contamination of soil by PCB and dioxin has been one of the serious pollution problems. The compounds are poorly soluble and toxic substances and have a high affinity with soil, so that the effective removal of the compounds from the soil is an important technical problem [1]. Various methods have been applied for the remediation of contaminated soil [2, 3]. The remediation process is very complex, but commonly divided into two steps; the removal of strongly adhered compounds from soil, and the decomposition of the removed compounds.

Cyclodextrins (CDs) are cyclic oligosaccharides from the enzymatic treatment of starch. The compounds have a relatively apolar cavity in which hydrophobic organic compounds can be stably included. Cyclodextrins can help to solubilize poorly soluble

substances by formation of inclusion complexes. Molnár *et al.* [4] reported that randomly methylated β-cyclodextrin is very effective for treating polluted soils. Fenyvesi [5] has reviewed extensively the application of CDs for the decontamination of soil and the ground water, and suggested that a great advantage of applying CDs is their biodegradability by the soil microflora. Wang and Brusseau [6], and Fenyvesi *et al.* [7] used hydroxypropyl-β-cyclodextrin (HP-β-CD) for the solubilization of low-polar organic compounds, and transportation of these substances adhered on the soil surface. Fava *et al.* [8] developed soil reactors to study the intrinsic *ex-situ* bioremediation of chronically PCB-contaminated soil, indicating that both HP-β-CD and γ-CD significantly enhanced the biological degradation of the soil contaminated compounds. Furuta *et al.* [9] kneaded biphenyl polluted soil with HP-β-CD and found that the removal and biodegradation of biphenyl is markedly enhanced by kneading soil with HP-β-CD.

The aim of this study was the development of a process for the removal of the contaminated compounds from soil by including the compounds selectively in the cavity of cyclodextrin. In this work, to obtain the fundamental knowledge for the remediation of soil by means of cyclodextrin, biphenyl was used as a model contaminating compound. We carried out the fundamental studies such as the enhancement of the solubility of biphenyl by cyclodextrin, the desorption of biphenyl from the soil by kneading the mixture of the contaminated soil and cyclodextrins using a twin-screw kneader, and the biodegradation of the included biphenyl in an aerobic system by activated sludge. The effect of kneading and the concentration of HP-β-CD on the rate of desorption of biphenyl from the soil was investigated by measuring the dissolution rate of biphenyl into the solution. On the basis of the solubility and the adsorption isotherm of the biphenyl on the soil, the amount of the removal of biphenyl was estimated and compared with the experimental values.

Experimental Methods

Materials. Biphenyl was obtained from Wako Chemicals (Tokyo, Japan). Hydroxypropyl-β-cyclodextrin (HP-β-CD), methyl-β-CD, Maltosyl-β-CD, and β-CD were purchased from Ensuiko Sugar Refining Co. (Tokyo, Japan). β-LCD was an oligomer of about five β-CDs purchased from Katayama Chemical Co. (Osaka). Other chemicals were of reagent grade. The model soil ("Andisol") was gathered in the campus of Tottori University. The content of N, C, and H in the Andisol was 0.226, 5.61, and 1.16 %, respectively.

Measurement of solubility of biphenyl in various CD solutions. Various concentrations (0.2 – 2 mmol/L) of CD solutions of 100 mL were prepared in 300 mL Erlenmeyer flasks with a screw cap. Ten mg of biphenyl was added to the CD solution, and incubated at 30 ℃ for 72 hours in a rotary shaker. Then, 5 mL of the aqueous solution was sampled in a test tube and centrifuged at 3,000 rpm for 10 min. Three mL of the supernatant was put a mixture of chloroform and water (1:4 v/v), and the included biphenyl was extracted into the chloroform phase by heating and mixing at 80 ℃ for 15 min. The concentration of biphenyl in chloroform was determined by gas chromatography. The analytical conditions were: glass column (2 m × 2 mm I.D.) packed with PEG-20M on 80/100 mesh Chromosorb W, column temperature 190 ℃, injection temperature 200 ℃, and nitrogen as the carrier gas.

Adsorption isotherm of biphenyl with Andisol. Four grams of Andisol was mixed with aqueous biphenyl solution of various concentrations in a 100 mL Erlenmeyer flask, followed by incubation at 30 ℃ for 72 hours. Six mL of the solution was centrifuged at 3,000 rpm. Four mL of the supernatant was sampled and biphenyl was determined by the same procedure as described above. The adsorbed quantity of biphenyl on the Andisol was calculated from the mass balance of biphenyl.

Preparation of the model contaminated soil. Forty grams of the vacuum-dried Andisol was mixed with 4 mg of biphenyl and 16 mL of water. The mixture was put into a twin screw kneader [10]

and kneaded at 30 °C for 30 min to obtain a model contaminated soil, in which 100 μg of biphenyl was adsorbed on a unit gram of dry soil (called as the contaminated soil of 100 ppm).

Inclusion and removal of the adsorbed biphenyl from the contaminated soil by kneading. Forty grams of the model contaminated soil was mixed with HP-β-CD at concentrations of 5 to 50 molar-folds to the initial biphenyl content in the soil. Then, the mixture was kneaded in the twin screw kneader for 30 min at a water content of 40 %, including the adsorbed biphenyl into HP-β-CD. Five grams of the kneaded sample was mixed with 10 mL water in a 100 mL Erlenmeyer flask with a screw cap, and shaking at 30 °C and 72 rpm in a rotary shaker. At prescribed time intervals, 7 mL of water was sampled and the concentration of biphenyl was determined by the same procedure as described above.

Aerobic treatment of the included biphenyl solution. In order to investigate the effect of the extent of inclusion on the biodegradation of biphenyl, two types of solution were prepared. One was a fully inclusion complex solution of 100 ppm biphenyl with 50 molar-folds of HP-β-CD by mixing at 30 °C for 72 hours. In the other case solutions of the two compounds were simply mixed at the same mixing ratio. Twenty-five mL of each solution was mixed with the same volume of concentrated aerobic sludge, which was a gift from Kurita Kogyo Co. Ltd. (Tokyo), and incubated at 30 °C. The concentration of biphenyl in the mixture was measured at prescribed intervals by gas chromatography as described above. For practical application of biodegradation by the activated sludge method, contaminated soil kneaded with HP-β-CD was directly incubated with the activated sludge. A model contaminated Andisol of 300 ppm (300 μg of biphenyl adsorbed on a unit gram of dry soil) was kneaded with HP-β-CD of 50 molar-fold, followed by mixing and incubating with the aerobic sludge of 50 mL at 30 °C and 85 rpm. At prescribed intervals, 1 mL of the slurry was sampled, and the total concentration of biphenyl in the slurry was measured by a similar procedure described above.

Results and Discussion

Enhancement of solubility of biphenyl in various cyclodextrin solutions. The solubility of biphenyl (mmol/L) in various CD solutions against the concentration of CD in the solution (mmol/L) is illustrated in Figure 1. β-CD, HP-β-CD, and methyl-β-CD markedly increased the solubility of biphenyl, while for Maltosyl-β-CD the solubility was slightly lower than with the other three CDs. The solubility changed linearly with an increase in the concentration of CD. However, above 1000 mg/L of the CD, the solubility in β-CD began to decrease because of the crystallization and the sedimentation of the inclusion complex. Assuming that a one-to-one complex was formed, the association constant K_c of biphenyl for each CD can be calculated from the slope s of the solubility line as [10]:

$$K_c = \frac{s}{b(1-s)} \tag{1}$$

where b is the interception of the line with y-axis in mg/L. Table 1 shows the association constant K_c of biphenyl for the various types of CD. Though β-CD has the highest value of K_c, it could not be used because of the sudden decrease of solubility above the CD concentration of 1000 mg/L. HP-β-CD and methyl-β-CD have also a higher value of K_c, indicating that these CDs have the potential ability of removing biphenyl from the contaminated soil. Therefore, we concluded that HP-β-CD was the most suitable among all the CDs.

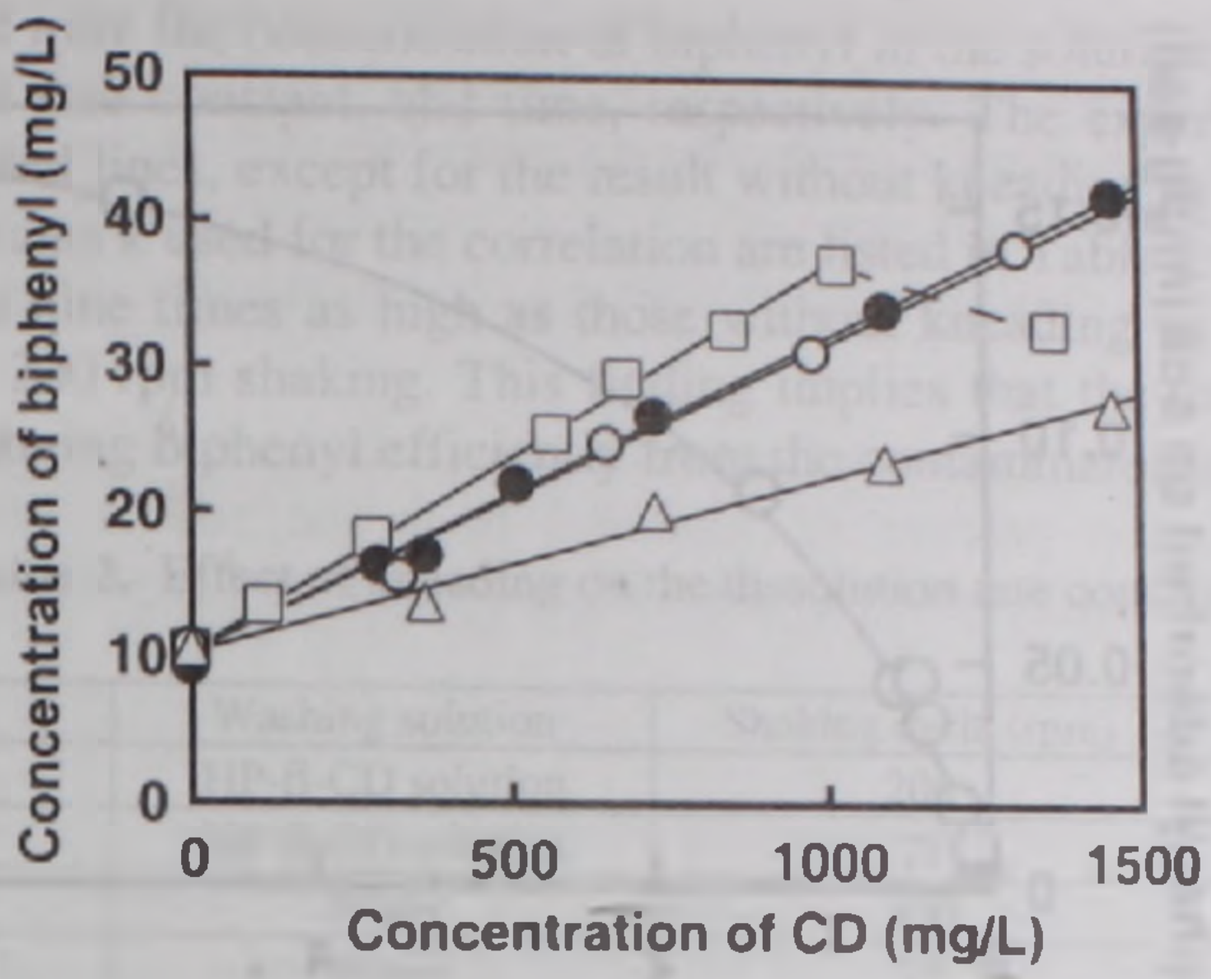

Figure 1. Solubility of biphenyl in various kinds of CD solutions. ●: HP-β-CD, ○: Methyl-β-CD, □: β-CD, ▽: Maltosyl-β-CD. Temperature was 30 °C. Solid lines are the linear correlation lines. Association constants of biphenyl to CD can be calculated by the slope and the y-interception of the line.

Table 1. The association constant of biphenyl with various CDs

	HP-β-CD	Methyl-β-CD	β-CD	Maltosyl-β-CD
K_c (L/mg)	0.00257	0.00247	0.00305	0.00142

Adsorption isotherm of biphenyl with Andisol. Figure 2 shows the adsorption isotherm of biphenyl with Andisol at 30°C. The amount of adsorbed biphenyl was markedly dependent on the content of organic compounds in the soil. The experimental data could be correlated well with the following Langmuir equation:

$$q = \frac{0.21C}{1 + 2.1C} \tag{2}$$

where q represents the amount of adsorbed of biphenyl (mg/g-dry soil) and C the concentration of biphenyl in the solution (mg/L) at equilibrium conditions.

Effective removal of biphenyl from the contaminated soil by kneading. The effectiveness of the kneading on the removal of biphenyl from the contaminated soil was investigated by measuring the dissolving rate of the included biphenyl from the soil into water. Figure 3 shows the dissolving time-course of biphenyl from the soil kneaded with HP-β-CD and the soil without kneading. A HP-β-CD solution of equal concentration (50 molar-fold to the initial biphenyl concentration in soil) was used as a dissolving medium in case of the soil without kneading. For the soil kneaded with HP-β-CD, biphenyl released and dissolved markedly faster, particularly at the lower shaking rate of 72 rpm. This implies that during kneading biphenyl in the soil was effectively included in HP-β-CD and removed from the soil. However, the equilibrium concentration of dissolution showed the same value regardless of the kneading, though the equilibrium concentration for the soil without

kneading could not be reached at 72 rpm shaking. It was also found that kneading for 60 min was enough to reach the equilibrium concentration at both 72 and 200 rpm shaking.

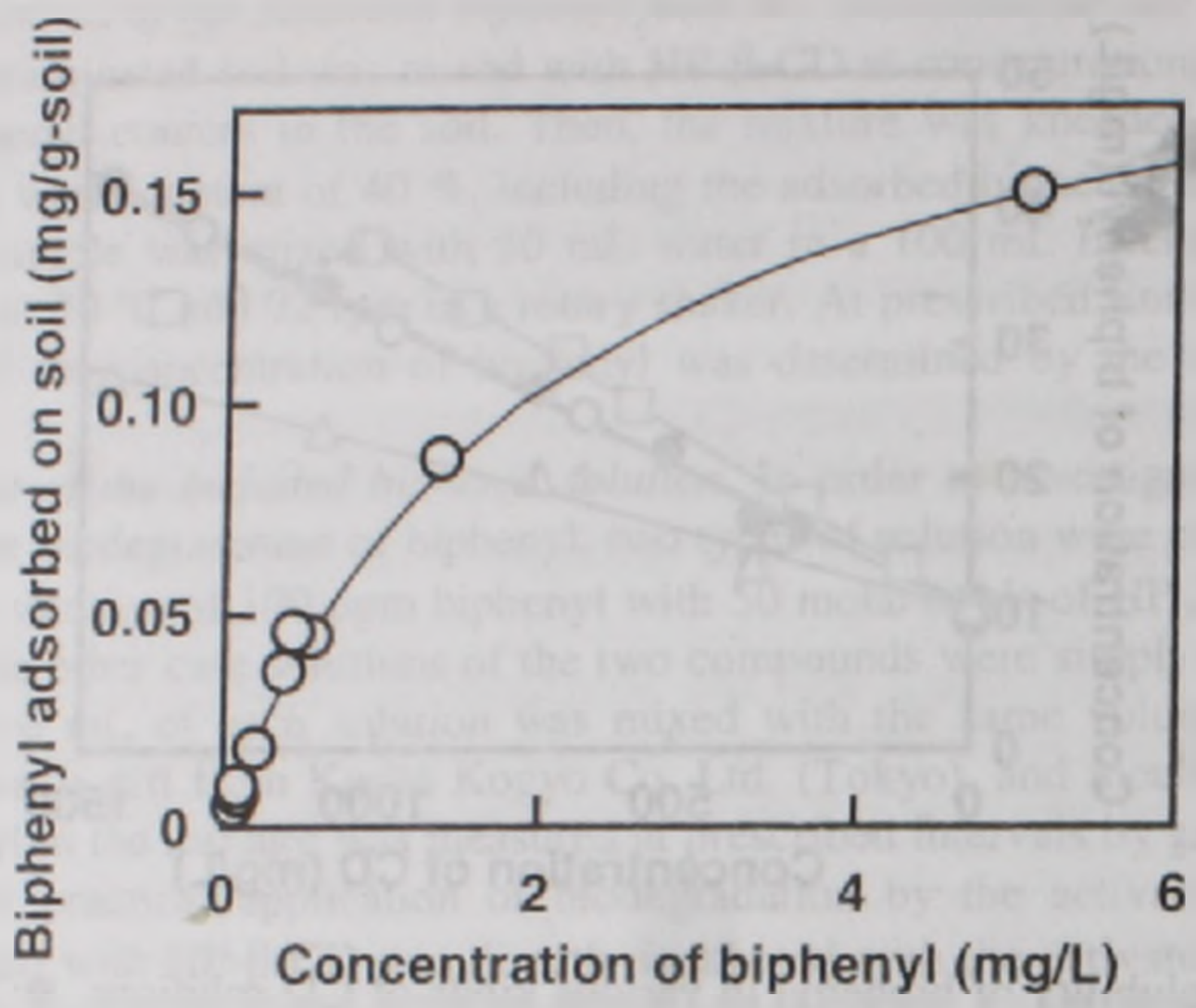

Figure 2. Adsorption isotherms of biphenyl with Andisol. ○: Equilibrium amount of biphenyl adsorbed on Andisol. Solid line is the correlation curve by equation (2).

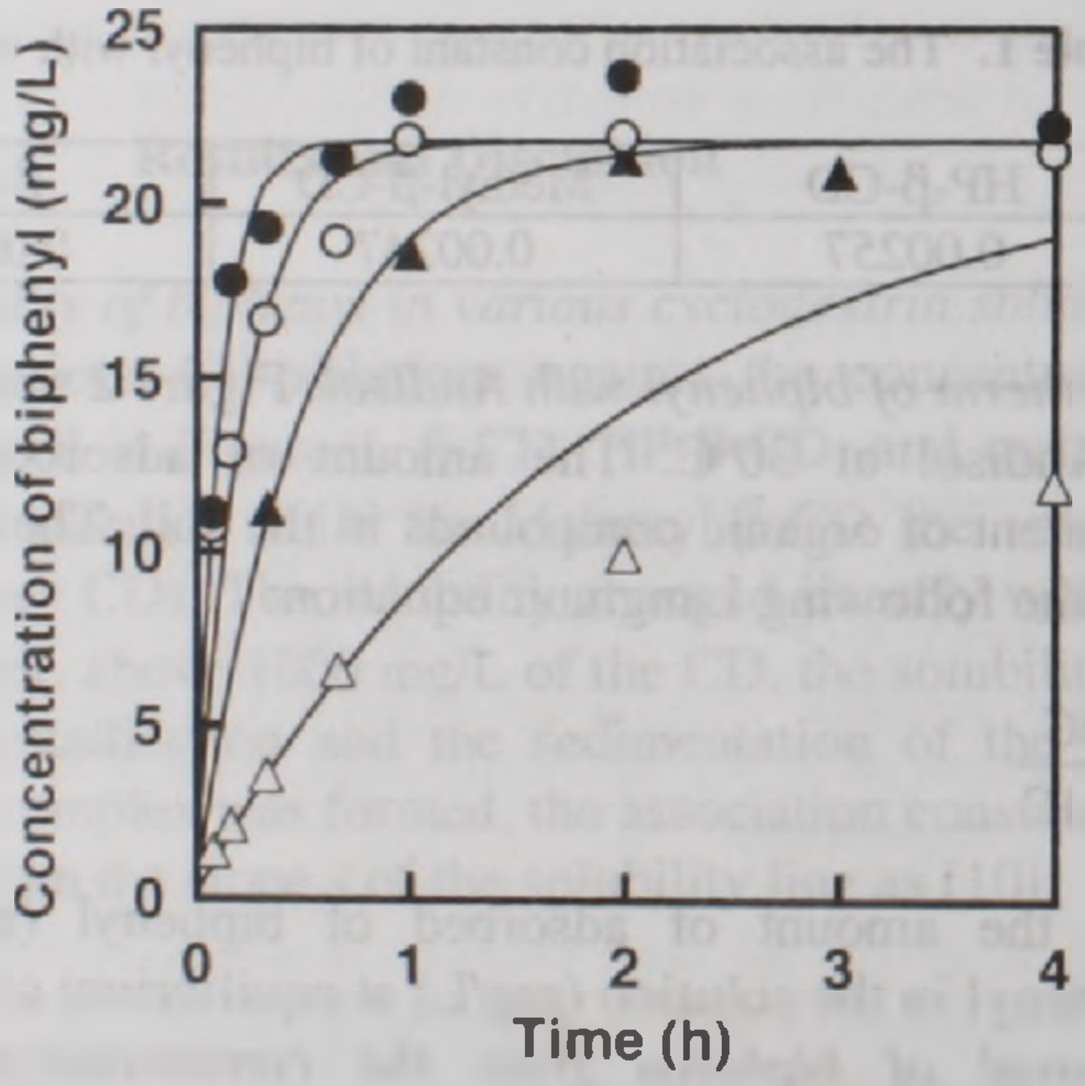

Figure 3. Effect of kneading on the rate of removal of biphenyl. ○, ●: Kneading with HP-β CD. △,▲: Without kneading. Closed symbols represent shaking at 200 rpm, and open symbols at 72 rpm. The solid lines are the correlation lines by equation (3). Initial biphenyl concentration in soil was 100 μg/g-dry soil. The molar ratio of the added HP-β-CD to the initial amount of biphenyl was 50. The water content during kneading was 40 % on dry basis.

The solid lines in Figure 3 represent the correlation curves by the following equation of the step response:

$$C = C_{max}(1 - e^{kt}) \tag{3}$$

where C, C_{max}, k, and t are the concentration of biphenyl in the solution, the maximum value of C, the dissolution rate constant, and time, respectively. The experimental values fitted well with the calculated lines, except for the result without kneading at 72 rpm shaking. The dissolution rate constants k used for the correlation are listed in Table 2. The values of k with kneading were about nine times as high as those without kneading at 72 rpm shaking, and 4.5 times as high at 200 rpm shaking. This finding implies that the use of a kneader has a high potential in removing biphenyl efficiently from the contaminated soil.

Table 2. Effect of kneading on the dissolution rate constant k

	Washing solution	Shaking cycle (rpm)	k (1/s)
Kneaded soil	HP-β-CD solution	200	2.43×10^{-3}
Kneaded soil	HP-β-CD solution	72	1.25×10^{-3}
Natural soil	Water	200	0.57×10^{-3}
Natural soil	Water	72	0.14×10^{-3}

The rate of removal of biphenyl from the soil under various concentrations of HP-β-CD. The rate of removal of biphenyl from soil was also investigated by changing the amount of HP-β-CD in kneading. As shown in Figure 4, both the dissolution rate and the maximum concentration of the included biphenyl increased considerably as the concentration of HP-β-CD increased. The solid lines in Figure 4 represent the correlation curves according to the equation (3) for $k = 1.25 \times 10^{-3}$ (1/s). The experimental values fitted well with the calculated lines using a unique value of k, indicating that the amount of HP-β-CD added during kneading did not affect the dissolution rate k but the maximum concentration of dissolution C_{max}.

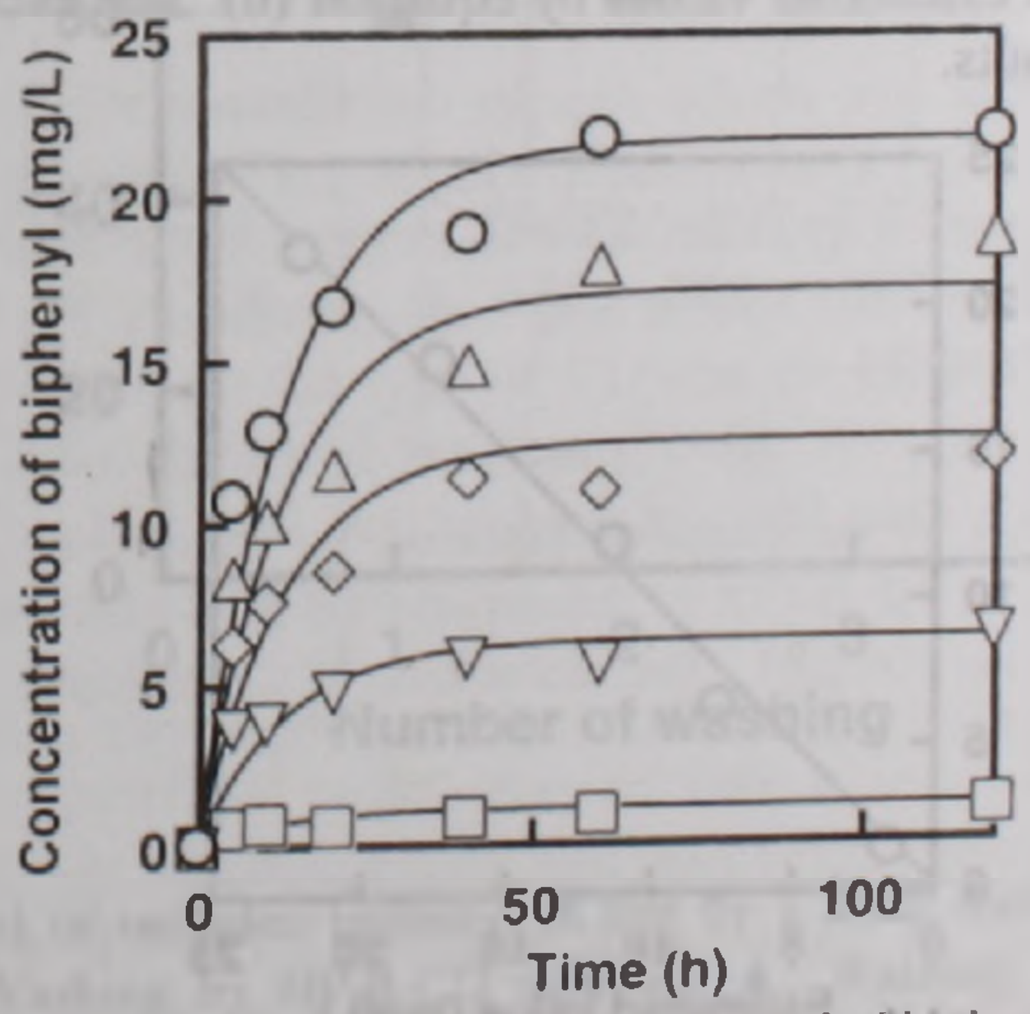

Figure 4. Effect of HP-β-CD concentration on the removal of biphenyl. The initial biphenyl concentration in soil, the added amount of HP-β-CD, and the water content during kneading are the same as in Figure 3. The solid lines are the correlation curves by equation (3). The dissolved rate constant k was the same value of 1.25×10^{-3} (1/s) for all the values of HP-β-CD added. The kneading conditions were the same as in Figure 3.

Estimation of the maximum concentration of biphenyl in solution. The maximum concentration of biphenyl in solution was estimated with the following assumptions:

i) In aqueous solution, cyclodextrin C_{CD}, biphenyl C_B and the inclusion complex C_{BCD} are equilibrated with each other, as given by equation (4);

$$C_B + C_{CD} \xleftrightarrow{K_c} C_{BCD} \tag{4}$$

where K_c is the association constant of equation (1).

ii) The dissolution process of biphenyl from the soil is assumed to be in an equilibrium. Therefore, equation (2) should hold between the adsorbed biphenyl and the free biphenyl in the solution.

Under these assumptions, the mass balance equation of biphenyl could be obtained as given by equation (5):

$$mq_0 = (K_c C_B C_{CD} + C_B)V + m\frac{k_1 C_B}{1 + k_2 C_B} \tag{5}$$

where m, q_0, V, k_1, and k_2 are the mass of the dry soil, the biphenyl adsorbed initially on the soil, the liquid volume, and the Langmuir constants in equation (2), respectively. Substituting equations (1) and (2) into equation (5), one could obtain a quadratic equation of C_B, which could be solved to give (6):

$$C_B = \frac{-\{(K_c C_{CD} + 1)Vk_2 + mk_1 - mq_0\} + \sqrt{\{(K_c C_{CD} + 1)Vk_2 + mk_1 - mq_0\}^2 - 4(K_c C_{CD} + 1)Vk_2 mq_0}}{2(K_c C_{CD} + 1)V} \tag{6}$$

Figure 5 shows the comparison between the equilibrium concentration of biphenyl C_B at 60 min shaking and the calculated values by equation (6). The estimated values fitted well with the experimental results.

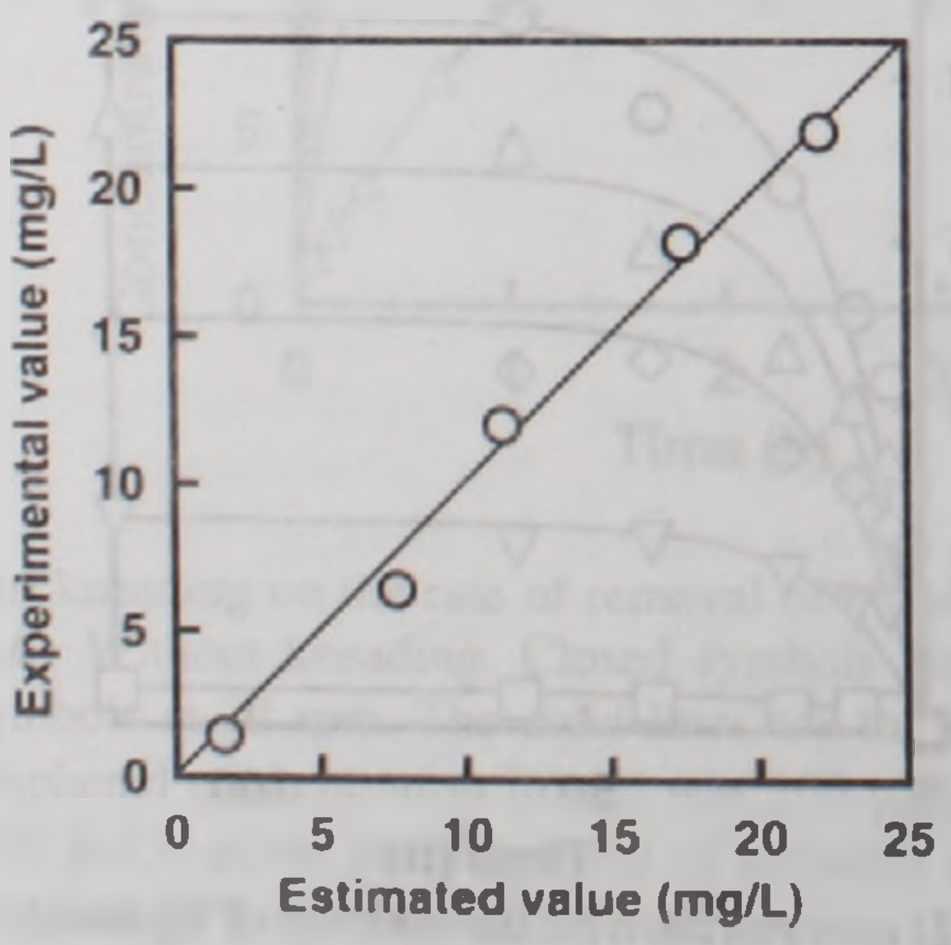

Figure 5. Estimation of the maximum concentration of biphenyl in solution. ○: 60 min shaking. The kneading conditions were the same as in Figure 3.

Washing out of the included biphenyl in the soil by a batch multi-stage washing operation. Since the included biphenyl by kneading exists in the soil, one needs the washing operation for several times to decrease the biphenyl concentration below an adequate level. The kneaded soil with HP-β-CD was washed three times by using distilled water or HP-β-CD solution with shaking at 200 rpm for 60 min. The concentration of biphenyl in the solution was measured after each washing step. At the same time, the biphenyl concentration at the i-th washing step C_{Bi} was estimated by a modified equation (6) as:

$$C_B = \frac{-\{(K_c C_{CD}+1)Vk_2 + nk_1 - mq_{i-1} - \alpha_{i-1}\} + \sqrt{\{(K_c C_{CD}+1)Vk_2 + nk_1 - mq_{i-1} - \alpha_{i-1}\}^2 - 4(K_c C_{CD}+1)Vk_2(mq_{i-1} - \alpha_{i-1})}}{2(K_c C_{CD}+1)V} \quad (7)$$

where q_i is the biphenyl adsorbed on the soil after the i-th washing and α_i ($\alpha_0 = 0$) is the amount of biphenyl remaining in the solution after the i-th washing in the void of the soil layer. Figure 6 shows the total removal of biphenyl against the number of washings. After washing 3 times, biphenyl was totally removed from the soil when HP-β-CD solution was used as the washing solution. On the other-hand, the total removal efficiency remained unchanged when water was used. In Figure 6, the removal by shaking for 5 min is also shown. The total removal efficiency increased markedly between the first and the second washing, though the removal was not perfect after washing 3 times. The solid and dotted lines in Figure 6 are the estimates of the removal by equation (7), indicating satisfactory results obtained by a simple calculation.

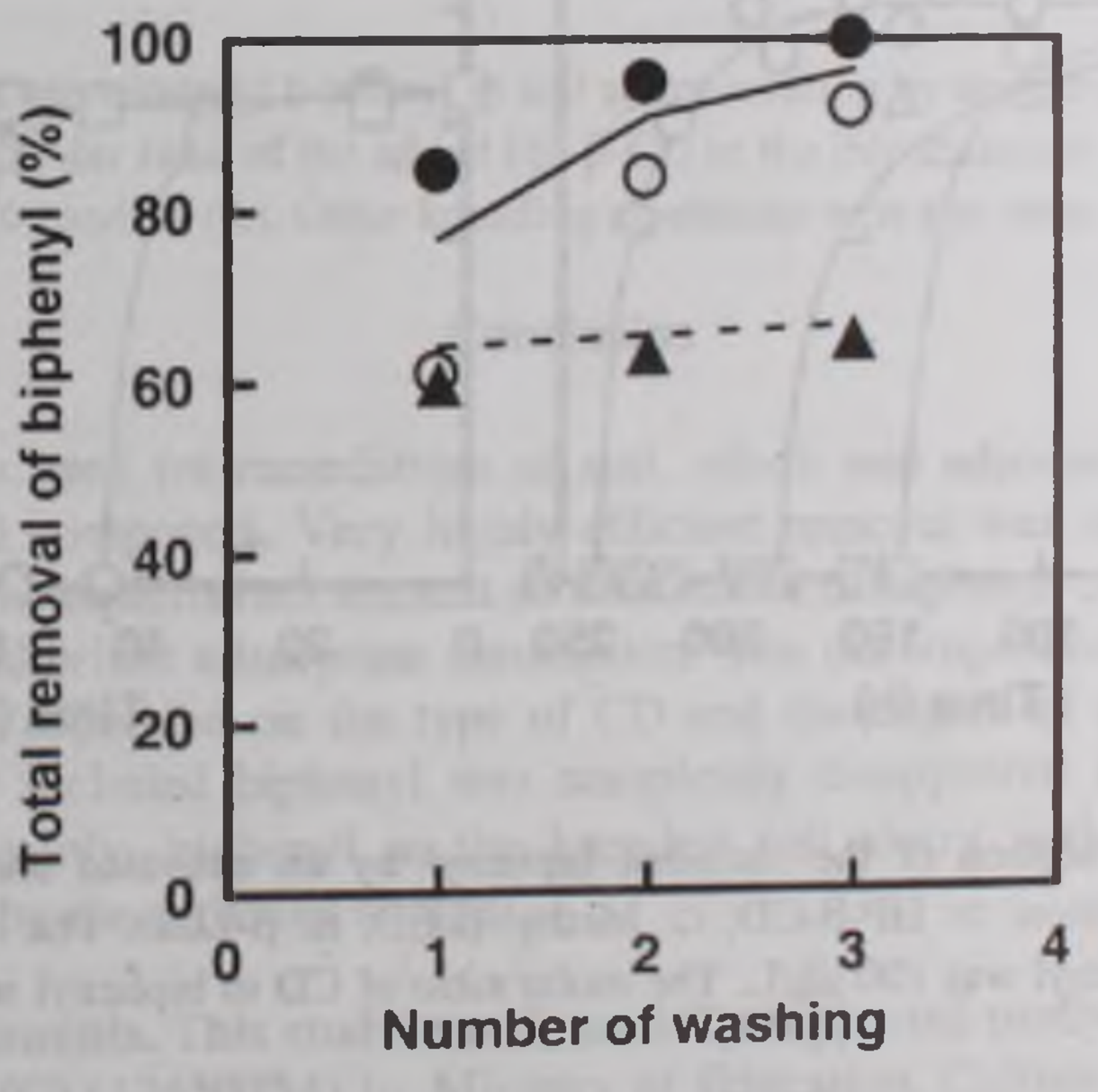

Figure 6. Removal of included biphenyl in soil by a batch multi-stage washing operation. ○, ●: Washing by HP-β-CD solution. ▲: Washing by water. Closed symbols represent shaking for 60 min, and open symbols for 5 min. The solid and dotted line are the estimated removal curves by equation (7). The kneading conditions were the same as in Figure 3.

Biodegradation of biphenyl solution by an activated sludge under aerobic conditions. Two types of biphenyl solution were incubated with an activated sludge under aerobic conditions. One was a perfectly included biphenyl solution with CD (called as "included solution"). In the other case, biphenyl and CD were simply mixed in the activated sludge solution just before the incubation (called as "simply mixing solution"). Three kinds of CDs such as HP-β-CD, methyl-β-CD, and β-LCD were used for the inclusion of biphenyl. The time-courses of biphenyl in both solutions are illustrated in Figure 7, (a) for the simply mixing solution and (b) for included solution. For the simply mixing solution, sudden decomposition of biphenyl was observed after 100 to 200 hours following inoculation as shown in Figure 7. Biphenyl mixed with methyl-β-CD could be decomposed fastest, and totally degraded in 150 hours. The rate of decomposition using HP-β-CD was the slowest and 240 hours were needed for nearly complete degradation. On the other hand, biphenyl in the included solution could be degraded surprisingly fast for all kinds of CDs. Contrary to the simply mixing case, HP-β-CD was the most suitable one, and biphenyl in the included solution completely disappeared in 45 hours, about five times faster than in the simply mixing case. For the other CDs, the deradation time decreased less. These findings imply that the degree of inclusion of biphenyl with CD was very important for the effective biodegradation by activated sludge.

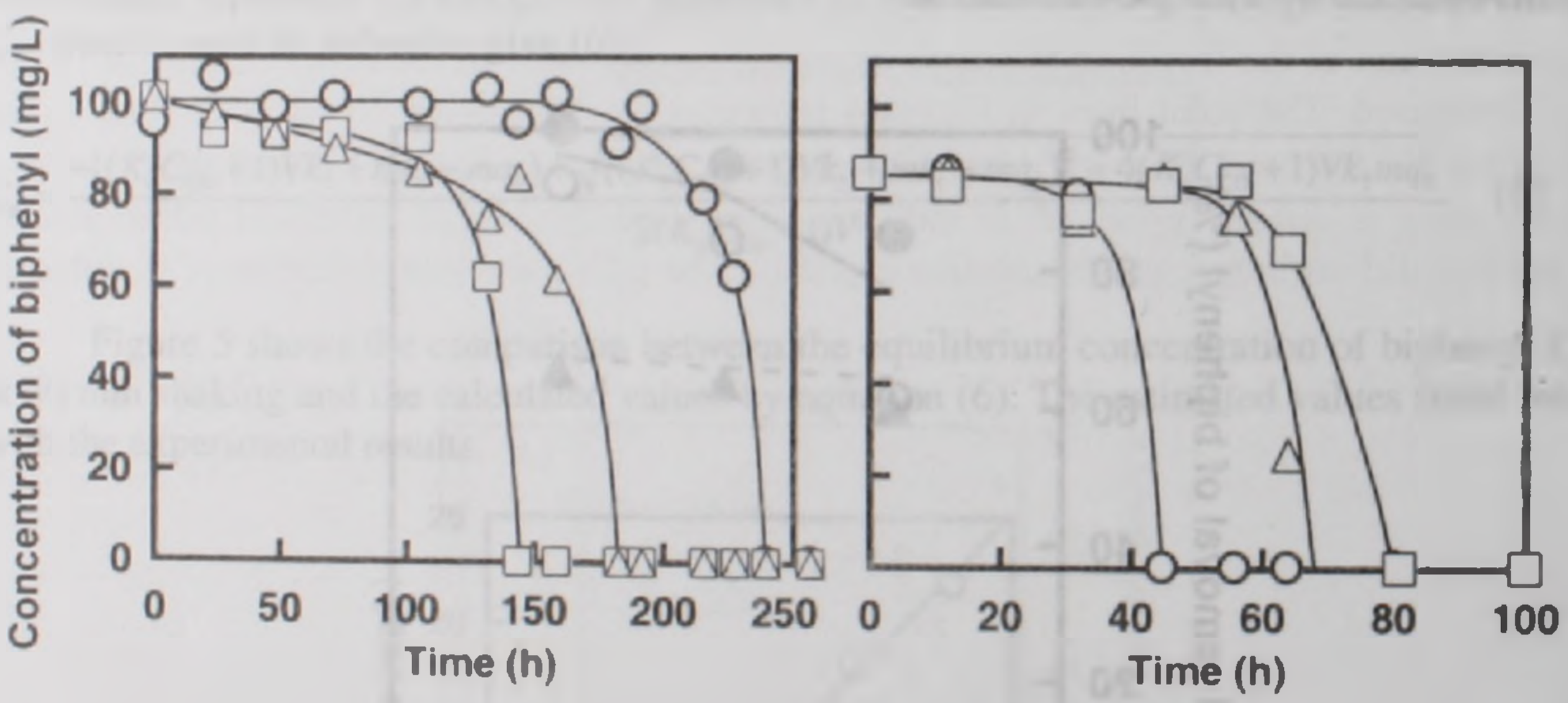

Figure 7. Degradation of the included biphenyl by an activated sludge under an aerobic condition. ○: HP-β-CD, □: Methyl-β-CD, Δ: β-LCD. The initial concentration of biphenyl was 100 μg/L. The molar ratio of CD to biphenyl was 50. Incubated at 30 °C.

Biodecomposition of biphenyl in soil slurry solution by an activated sludge. As mentioned above, biphenyl in the kneaded soil with HP-β-CD should be washed several times for complete removal from the soil, resulting in a large amount of the waste water. To simplify the treatment process and reduce the waste water, biphenyl in the kneaded soil with HP-β-CD was directly treated by the activated sludge. Figure 8 illustrates the degradation time-course of biphenyl in the kneaded soil with the addition of HP-β-CD of 10 and 50 molar-folds to the initial biphenyl content. The degradation rate of biphenyl was proportional to the added amount of HP-β-CD. Biphenyl concentration decreased gradually from the

beginning of the incubation, and no sudden decrease of the concentration could be observed as can be seen in Figure 7(b). However, the time needed for the complete disappearance was nearly the same in both cases. This implies that biphenyl in the kneaded soil would be perfectly included during kneading with HP-β-CD, and that the direct treatment of the kneaded soil by the activate sludge would be practical for the bioremediation of the contaminated soil.

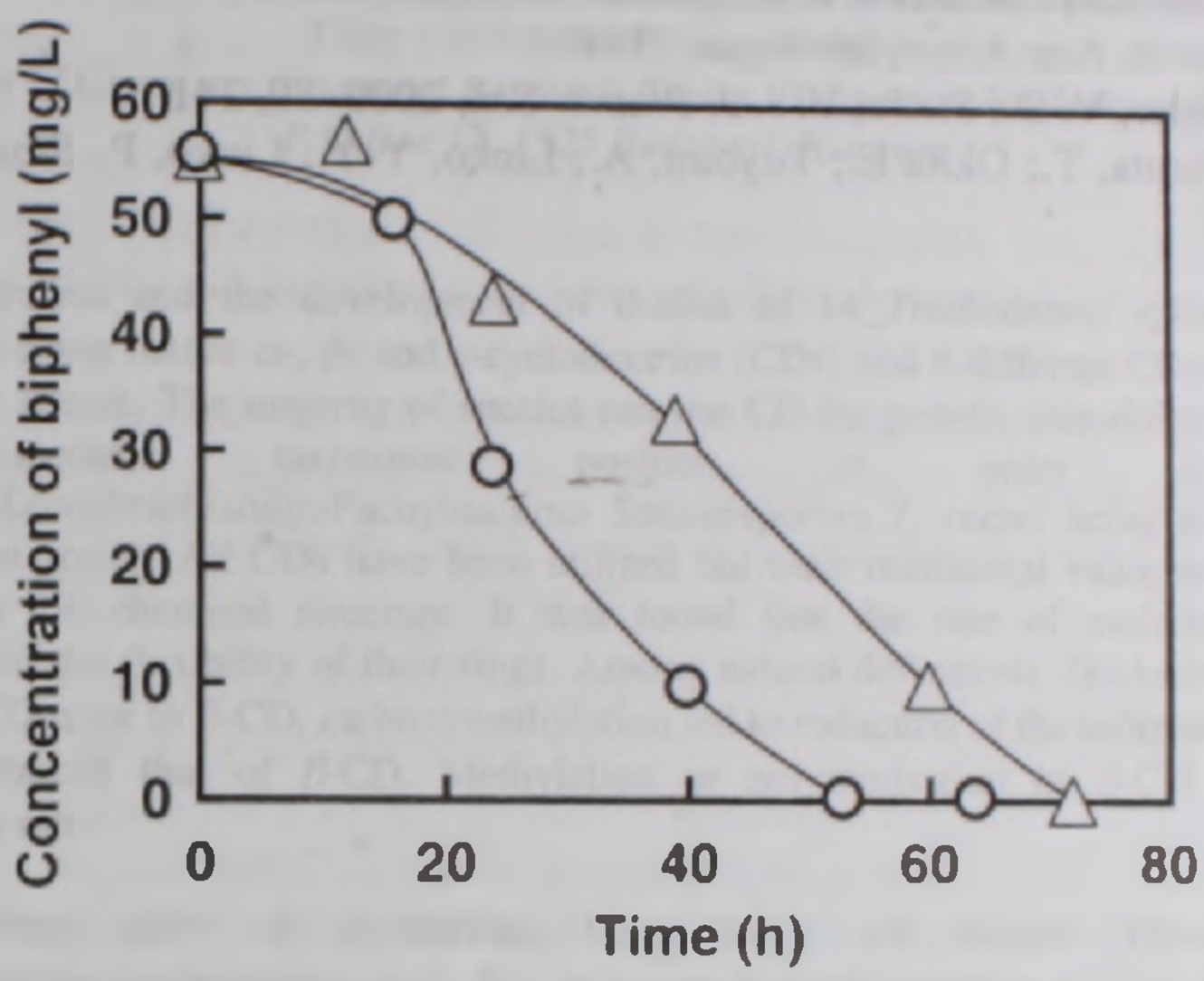

Figure 8. Decomposition of biphenyl in soil slurry solution by an activated sludge. The molar ratio of the added HP-β-CD to the initial amount of biphenyl in soil was 50 (○) and 10 (Δ). Other kneading conditions were the same in Figure 3.

Conclusins

HP-β-CD was used for remediation of soil, which was adsorbed with biphenyl as a model contaminated compound. Very highly efficient removal was obtained by using the kneading method. The equilibrium amount of dissolution of biphenyl could be calculated on the basis of the equilibrium adsorption assumption. The decomposition of the biphenyl by the activated sludge depended on the type of CD and the degree of inclusion of biphenyl with CD. The fully included biphenyl was completely disappeared in 40 to 80 hours of incubation. Consequently, biphenyl in the kneaded soil slurry with HP-β-CD could be successfully treated by the activated sludge.

Acknowledgements. This study was financially supported partly by a grant-in aid for Scientific Research (C) (12650754) by Ministry of Education, Culture, Sports, Science and Technology, and partly by Research Institute of Innovative Technology for the Earth (RITE).

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DECOMPOSITION OF NATIVE CYCLODEXTRINS AND CYCLODEXTRIN DERIVATIVES BY VARIOUS *TRICHODERMA* SPECIES

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The growth and the development of thallus of 14 *Trichoderma* species have been determined by using native α-, β- and γ-cyclodextrins (CDs) and 8 different CD derivatives as a unique carbon source. The majority of species can use CD for growth, this ability was related to their intrageneric taxonomic position in order of sections Trichoderma>Longibrachiatum>Pachybasium> Saturnisporum *T. reesei* being the most and *T. virens* the least active. All CDs have been utilized but their nutritional value was significantly influenced by the chemical structure. It was found that the size of molecules had major importance than the flexibility of their rings. Among natural derivatives *Trichoderma*s preferred the α- and γ-CD prior to β-CD, carboxymethylation led to reduction of the utilization of α- and γ-CDs and enhanced that of β-CD. Methylation or polymerisation of β-CD also modified decomposition rate.

Изучены рост и развитие таллома у 14 видов *Trichoderma* с использованием природных α−, β− и γ−циклодекстринов (ЦДы) и 8 разных производных ЦД в качестве единственного источника углерода. Большинство видов грибов может использовать ЦД для роста. Эта способность относится к их межродовому таксономическому положению, поскольку в порядке секций *Trichoderma>Longibrachiatum>Pachybasium> Saturnisporium*, вид *T.reesei* − самый активный, а *T.virens* − самый слабый. Все ЦДы усваивались, но их питательная ценность значительно зависит от химической структуры. Выявлено, что размеры молекул имеют более важное значение, чем пластичность их колец. Из природных производных культуры *Trichoderma* более предпочитают α− и γ−ЦД, чем β−ЦД. Карбоксиметилирование ведет к снижению утилизации α− и γ−ЦД, но повышает использование β−ЦД. Метилирование и полимеризация β−ЦД также изменяет процесс их разложения.

Ուսումնասիրվել է *Trichoderma*-ի 14 տեսակների աճը և թալոմի զարգացումը, օգտագործելով բնական α−, β− և γ−ցիկլոդեքստրինները (ՑԴներ) և 8 տարբեր ցիկլոդեքստրինների ածանցյալները որպես ածխածնի միակ աղբյուր: Տեսակների մեծամասնությունը կարող է օգտագործել ՑԴ-ն աճի համար: Այս հատկությունը վերաբերվում է նրանց միջցեղային տաքսոնոմիական դիրքին, քանի որ ըստ բաժինների *Trichoderma>Longibrachiatum>Pachybasium> Saturnisporium* հերթականության, *T.reesei*−ին ամենաակտիվ, իսկ *T.virens* ամենաթույլ տեսակն է: Բոլոր ՑԴները յուրացվել են, բայց նրանց սննդառական արժեքը բավականին կախված է քիմիական կառուցվածքից: Բացահայտվել է, որ մոլեկուլների չափերը ունեն առավել կարևոր նշանակություն, քան նրանց օղակների ճկունությունը: Բնական ածանցյալներից *Trichoderma*-ի կուլտուրաները առավել նախընտրում են α− և γ−ՑԴ, քան β− ՑԴ: Կարբօքսիմեթիլացումը տանում է α− և γ−ՑԴ -ի յուրացման նվազմանը, բայց բարձրացնում է β− ՑԴ-ի օգտագործումը: β− ՑԴ-ի մեթիլացումը և պոլիմերիզացիան նույնպես փոխում են քայքայման արագությունը:

Introduction

Trichoderma, an anamorphic Hypocreaceae (class Ascomycetes), is a cosmopolitic group of fungi, common in the environment, especially in soils. Species of this genus are important members of detritus (Papavizas, 1985), and they have been used in many human activities including commercial applications for the production of cellulolytic and hemicellulolytic enzymes and for the biological control of plant diseases (Sundheim, 1977; Naár and Kecskés, 1995, Naár and Kecskés, 1998), for the biodegradation of chlorophenols (Fava et al., 1998) and polynucleated aromatic compounds (Wang et el., 1998), and for soil bioremediation (Ye et al., 1996; Esposito and da Silva, 1998; Bardi et al., 2000). *Trichoderma* species may cause severe losses in commercially produced mushrooms (Samuels, 1996), and have been identified as causal agents of disease in immunosuppressed humans (Furukawa et al. 1998).

Starch and cyclodextrins (CDs) can be used for the formulation of biologically active chemicals in solid state dispersons and liquid state (Schierbaum and Vorwerg, 1997). CDs are cyclic oligosaccharides commonly composed of six, seven or eight α-D-glucose units (α-, β- and γ-CD, respectively) which have an overall shape reminiscent of a truncated cone. On account of their relatively hydrophobic interiors, CDs have the ability to form inclusion complexes with a wide range of substrates in aqueous solution or heterogenic systems (Zsadon and Fenyvesi, 1981; McCray and Brusseau, 1998). This property of CDs has led to their application in areas as varied as enzyme mimics (Breslow et al., 1991; Yuan et al., 1998; Lee and Ueno, 2000), catalysis (Komiyama, 1993; Jarho, Van der Velde et al., 2000; Chou et al., 2001) and the encapsulation of drugs (Loftsson and Brewster, 1996; Thompson, 1997; Merkus et al., 1999; Loftsson and Jarvinen, 1999) and pesticides (Szejtli, 1997; Cserháti et al., 1984). Although they are frequently employed in various industrial pocesses as carriers (Duchene et al, 1999; Redenti et al., 2000) few attention has been paid to the study of their fate in biological systems (Szejtli, Gerloczy and Fonagy, 1980; Gerloczy et al., 1981; Szabo et al., 1981). It has been established that they are rapidly excreted by kidney when applied intravenously, and the rate of their intracorporal degradation is very low (Kubota et al., 2000). Bacteria associated to mammals and plants, as well as members of soil microbiota can utilize CDs (Oros et al., 1990; Pedersen et al., 1996).

The objectives of this work were the determination of the decomposition rate of native CDs and various CD derivatives by *Trichoderma* species, and the elucidation of the effect of chemical structure of CDs on the capacity of *Trichoderma* species to use them as unique carbon source.

Materials and Methods

Native α-, β- and γ-CDs (compounds I-III), their carboxymethylated derivatives (compounds IV-VI), hydroxypropylated-β-CD (VII, HP-β-CD), Dimeb (VIII = heptakis(2,6-di-O-methyl)-β-CD), Trimeb (IX = heptakis(2,3,6-tri-O-methyl)-β-CD), water-soluble β-CD polymers (X,XI) (EGP = prepared by crosslinking native β-CD molecules with ethylene glycol bis[epoxypropylether]; BGP = prepared by crosslinking native β-CD molecule with buthylene glycol bis[epoxypropylether]) were the gift of Dr.József Szejtli (CYCLOLAB Research and Development Laboratory, Budapest, Hungary). D-glucose, cellulose (Merck, Darmstadt, Germany), starch (amylum solubile), analytical grade mineral salts (Reanal, Budapest, Hungary) were used as received. The agar-agar (Oxoid No.3, Basingstoke, UK) was purified prior to use as follows: 100 g of agar-agar was suspended and steeped in 1 L of NaOH (1 N) for 18 hrs, and after washing with distilled water until neutral pH it was dried at 60-70°C.

Strains of 14 species belonging to four sections of *Trichoderma* genus: Trichoderma: *T. koningii* Oudemans [T-1], *T. aureoviride* Rifai [T-2], *T. viride* Persoon ex Gray [T-3], *T. atroviride* Karsten [T-4]; Longibrachiatum: *T. reesei* Simmons [L-5], *T. pseudokoningii* Rifai [L-6], *T. longibrachiatum* Rifai [L-7], *T. parceramosum* Bissett [L-8], *T. citrinoviride* Bissett [L-9], Pachybasium: *T. harzianum* Rifai [P-10], *T. polysporum* (Link ex Persoon) Rifai [P-11], *T. virens* (Miller, Giddens, et Foster) von Arx [P-12], *T. hamatum* (Bonorden) Bainier [P-13] and Saturnisporum: *T. saturnisporum* Hammill [S-14] were maintained on potato dextrose agar as previously described (Oros et al., 1999). Species L-5, L-8, L-9, and S-14 were taken from the collection of the Plant Protection Institute of Hungarian Academy of Sciences while species T-1, T-1, T-3, T-4, L-6, L-7, P-10, P-11, P-12, and P-13 were the gift of Dr Zoltán Naár (Esterházy Károly College, Department of Botany, Eger, Hungary). Conidia used for inoculation were produced on glucose mineral agar (GMA) amended with starch and casein (10 and 2 gL^{-1}, respectively) slains. The medium for utilization tests contained purified agar-agar, NH_4Cl, KH_2PO_4, K_2HPO_4, $MgSO_4 \times 7H_2O$ and NaCl (12, 1.0, 1.0, 0.5, 0.5 and 0.5 g per litre of tap water, respectively). Cyclodextrins or their derivatives as well as glucose were incorporated as sole carbon source into the melted medium at a concentration of 5 g per litre, prior to dispensing in 5 mm deep layer into Petri dishes (125 mm of diameter for GMA and 90 mm for the other carbon sources). The use of Petri dishes of different diamter was motivated by the different velocity of growth on glucose and CD carbon sources.

The response of fungi was characterized by measuring the linear growth of hypha (colony diameter in mm) at each day up till seven days and by evaluating the status of the thallus after 120 hrs incubation by a six fold scale (0 = no growth, 1 = tiny, non-sporulating thallus, diameter less than 10 mm, 2 = tiny thallus bearing some tufts of conidia, 10-50 mm of diameter, 3 = well developed but non-sporulating thallus, 10-50 mm of diameter, 4 = well developed sporulating thallus, diameter over 50 mm, but less intesively grew than observed on glucose, 5 = the thallus grews like on glucose containing medium). All incubations were performed at 25±2°C.

Each experiment was performed in triplicates. Two-way analysis of variation was applied for distinguishing averages of species(A)×carbon source(B) combinations. The nutritive value (bioavailability) of native CDs and CD derivatives was calculated by the spectral mapping technique (Lewi, 1976) the nutritive value of glucose being considered to be 100%. In order to compare the information content of the two methods of evaluation linear correlation was calculated between the data obtained by the methods.

Results and Discussion

Three *Trichoderma* species (*parceramosum*, *reesei* and *longibrachiatum*) produced yellow pigments on GMA (Csiktusnádi Kiss et al. 2000), and only *T. parceramosum* exhibited this property when grown on CDs. It was further established that the formation of aeral mycelium by *T. virens* was strongly depressed on media containing CDs as sole carbon source.

The average of scores characterizing the development of thallus of *Trichoderma* species in different media are compiled in Tables 1 and 2. The standard deviation of species x CD pairs was lower than 1 proving the good reproducibility of the method. The calculated F value characterizing the differences among the species x CD pairs was 347.7 indicating that the differences observed are highly significant the significance level being over 99.9%. It was not possible to reveal any connection between the intrageneric taxonomic position of *Trichoderma* species and their ability to consum CDs. The variability in activity of species depended considerably on the structure of carbon source and on the type of species as demonstrated by the relatively high coefficients of variation (C.V.(%) values).

Table 1. Development of thallus of *Trichoderma* species grown on glucose and on β-cyclodextrin derivatives as sole carbon sources. Average score values. For abbreviations see Materials and Methods

Species (A)	Carbon sources (B)					
	Glucose	β-cyclodextrin derivatives				
		Dimeb	Trimeb	HP-β-CD	EGP	BGP
T-1	3	1	1	2	1	2
T-2	4	1	1	1	1	2
T-3	5	1	1	1	1	1
T-4	5	1	1	1	1	2
L-5	3	0	1	2	1	2
L-6	5	1	1	1	1	2
L-7	4	1	1	2	1	2
L-8	5	0	1	0	0	2
L-9	3	0	1	0	0	1
P-10	4	0	1	1	1	2
P-11	3	1	1	1	1	1
P-12	5	1	1	1	1	1
P-13	3	1	1	1	1	1
S-14	3	0	1	1	1	1
C.V.(%)	23	77	0	57	42	63

C.V.(%) =variability of utilization

Table 2. Development of thallus of *Trichoderma* species grown on native and carboxymethylated α-, β- and γ-cyclodextrins as sole carbon sources. Average score values.

Species (A)	Carbon sources (B)					
	Native			Carboxymethylated		
	α-CD	β-CD	γ-CD	α-CD	β-CD	γ-CD
T-1	2	0	2	1	1	1
T-2	2	1	2	1	2	1
T-3	2	0	2	1	2	1
T-4	2	2	2	1	2	1
L-5	2	2	3	1	2	1
L-6	2	2	3	1	2	1
L-7	1	1	1	1	1	1
L-8	1	2	3	1	1	1
L-9	2	2	2	0	1	1
P-10	1	1	3	1	1	1
P-11	1	2	1	1	1	1
P-12	1	1	1	1	1	1
P-13	0	2	1	1	1	1
S-14	1	1	1	1	2	1
C.V.(%)	45	55	43	29	36	0

C.V.(%) =variability of utilization

The dependence of the colony diameter of *Trichoderma viride* on the incubation time and the character of carbon source is shown in Fig.1. It can be established that the decomposition of β-CD starts after a considerable lag period similarly to other natural carbohydrate polymers. Carboxymethylation markedly modified the rate of colony growth the highest effect was observed in the case of β-CD. These effect are less marked for native α-

and γ-CDs. This discrepancy can be tentatively explained by the fact that in the β-CD molecule a complete secondary belt of hydrogen bonds is formed between the C-2 hydroxyl group of one glycopyranose unit and the C-3 hydroxyl group of the neighbouring glucopyranose unit, so that β-CD is a rather rigid structure making the molecule less liably to enzymatic attack.

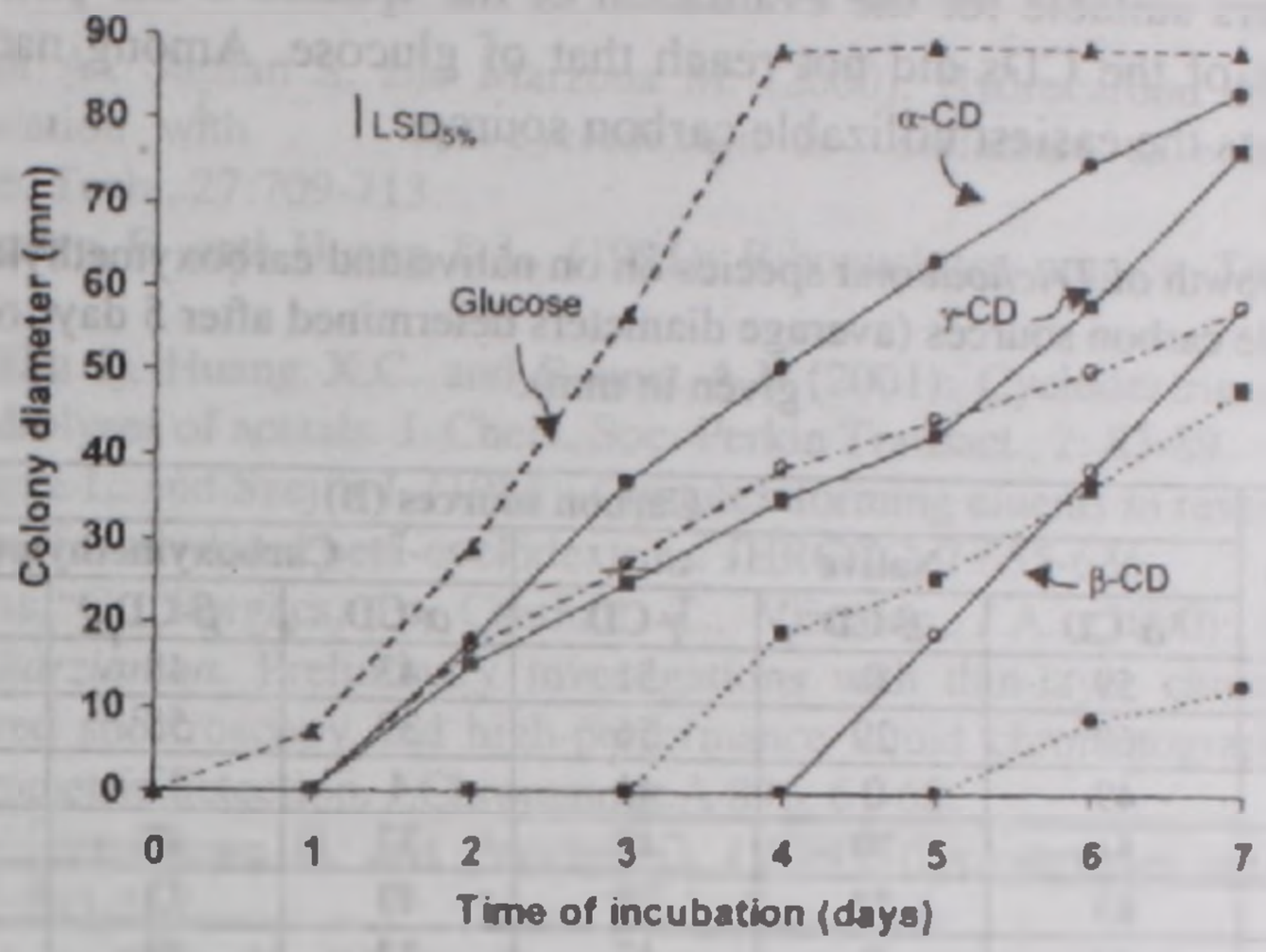

Figure 1. Dependence of the colony diameter *Trichoderma viride* on the incubation time and character of carbon source. Natural α-, β- and γ-cyclodextrins and their carboxymethylated derivatives are depicted by solid and dotted lines, respectively.

Table 3. Linear growth of *Trichoderma* species on glucose and on β-cyclodextrin derivatives as sole carbon sources (average diameters determined after 5 days of incubation are given in mm). For abbreviations see Materials and Methods.

Species (A)	Carbon sources (B)					
		β-cyclodextrin derivatives				
	Glucose	Dimeb	Trimeb	HP-β-CD	EGP	BGP
T-1	59	27	29	38	55	64
T-2	99	25	30	39	43	76
T-3	89	37	13	47	33	4
T-4	73	0	22	40	45	49
L-5	99	0	33	57	55	69
L-6	116	26	37	39	45	74
L-7	114	33	49	51	47	69
L-8	97	0	6	0	13	2
L-9	121	0	10	0	39	29
P-10	103	37	39	37	43	77
P-11	35	24	38	31	37	43
P-12	89	3	25	39	7	60
P-13	73	0	24	49	49	56
S-14	27	0	25	27	37	34
C.V.(%)	23	104	44	48	36	50
PBA (%)	100	16	25	27	22	40

C.V.(%) = variability of utilization; PBA (%) = potencial of bioavilability (related to the utilization of glucose, calculated by the spectral mapping technique).

The average colony diameters of *Trichoderma* species determined after five days of incubation in different media are compiled in Tables 3 and 4. The standard deviation of species x CD pairs was 5.1, higher than in the case of the average score values. The calculated F value characterizing the differences among the species x CD pairs was high also in this case ($F_{A\times B}$ = 160.6, significance level over 99.9%) indicating that the measurement of the colony diameters suitable for the evaluation of the species x CD pair intercations too. The nutritive value of the CDs did not reach that of glucose. Among native CDs and CD derivatives γ-CD was the easiest utilizable carbon source.

Table 4. Linear growth of *Trichoderma* species on on native and carboxymethylated α-, β- and γ-cyclodextrins as sole carbon sources (average diameters determined after 5 days of incuba- tion are given in mm).

Species (A)	Carbon sources (B)					
	Native			Carboxymethylated		
	α-CD	β-CD	γ-CD	α-CD	β-CD	γ-CD
T-1	59	0	51	43	48	19
T-2	69	29	56	39	58	18
T-3	49	0	41	4	43	21
T-4	54	39	49	37	49	9
L-5	63	22	75	49	73	37
L-6	49	0	47	33	49	45
L-7	73	49	46	47	57	8
L-8	64	0	59	20	55	43
L-9	46	45	19	3	59	4
P-10	73	49	63	56	59	49
P-11	39	56	37	14	42	14
P-12	66	50	35	20	44	14
P-13	59	45	39	33	39	30
S-14	18	72	29	57	18	64
C.V.(%)	45	55	43	29	36	0
PBA (%)	36	35	49	24	36	25

C.V.(%) = variability of utilization; PBA (%) = potencial of bioavilability (related to the utilization of glucose, calculated by the spectral mapping technique).

Significant linear relationship was found between the average score values and the colony diameters determined after 5 days of incubation the coefficient of correlation being 0.7455 indicating a significance level higher than 99%. This finding indicates that the information content of the two methods of evaluation are similar. However, the ratio of variance explained is fairly low (55.58 %) suggesting that the inclusion of both sets of data in future quantitative structure-decomposition rate studies is justified.

Conclusions

It has been established that *Trichoderma* species are potent deteriorators of native CDs and their derivatives and they may promote the elimination of this class of compounds from any organic and inorganic matrices. As the decomposition rate considerably depends on both the type of *Trichoderma* species and the chemical structure of CDs an adequate selection of species for the decomposition of a given CD is necessary.

Acknowledgement. The authors express to their gratitude Dr.József Szejtli and Dr.Zoltán Naár for supplying the cyclodextrins and *Trichoderma* isolates, respectively.

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Biolog. J. Armenia, *Special issue: Cyclodextrins*, 2001

CYCLODEXTRINS PRODUCING ACTIVITY OF DIFFERENT SPECIES OF AEROBIC SPOREFORMING BACTERIA

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A comprehensive and systematic study of cyclodextrin producing activity of 572 strains of bacilli has been carried out. The predominant distribution of cyclodextrins producing activity among thermophilic, alcalophilic and certain species of mesophilic bacilli has been shown. As a rich source for cyclodextrins can be used from thermophiles - *Bacillus stearothermophilus*, *B.circulans "subsp. thermophilus"* and *B.macerans "subsp. thermophilus"*, from alcalophiles - *B.alcalophilus* and *B.alcalophilus "subsp. halodurans"* and among mesophiles *B.macerans*, *B.circulans*, *B.licheniformis* and *B.coagulans*. Thermophilic and mesophilic bacilli synthesize the mixture of α-, β-, γ -cyclodextrins whereas alcalophiles and halophiles mainly β-cyclodextrin.

Проведено систематическое изучение продуцирования циклодекстринов на материале 572 хорошо изученных и идентифицированных штаммов спорообразующих бактерий. Установлено преобладающее распространение продуцентов этих соединений среди термофильных, алкалофильных и некоторых видов мезофильных бацилл. Богатыми источниками циклодекстринов могут служить среди термофилов *Bacillus stearothermophilus, B.circulans "subsp. thermophilus", B.macerans "subsp. thermophilus"*, из алкалофилов *B.alcalophilus* and *B.alcalophilus "subsp. halodurans"*, а из мезофилов – *B.macerans, B.circulans, B.licheniformis, B coagulans*. Термофильные и мезофильные штаммы бацилл продуцируют в основном смесь α–, β–, γ–циклодекстринов, тогда как алкалофилы и галофилы – в основном β – циклодекстрин.

Կատարվել է սպորառաջացնող բակտերիաների լավ ուսումնասիրված և իդենտիֆիկացված 572 շտամների մոտ ցիկլոդեքստրինների արտադրման սիստեմատիկ ուսումնասիրում: Հաստատվել է այդ միացությունների արտադրիչների գերիշխող տարածումը թերմոֆիլ, ալկալոֆիլ և որոշ տեսակների մեզոֆիլ բացիլների մոտ: Ցիկլոդեքստրինների հարուստ աղբյուր կարող են ծառայել թերմոֆիլների մոտ *Bacillus stearothermophilus, B. circulans "subsp. thermophilus"* և *B. macerans "subsp. thermophilus"*, ալկալոֆիլներից *B. alcalophilus* և *B. alcalophilus "subsp. halodurans"*, իսկ մեզոֆիլներից *B. macerans, B. circulans, B. licheniformis, B. coagulans*: Բացիլների թերմոֆիլ և մեզոֆիլ շտամները արտադրում են հիմնականում α, β, γ-ցիկլոդեքստրինների խառնուրդ, այն ժամանակ երբ ալկալոֆիլները և հալոֆիլները միայն β-ցիկլոդեքստրին:

The unique cyclic compounds α-, β-, γ-cyclodextrins (CDs) are formed from starch under the action of enzyme cyclodextrin glucanotransferase (CGTase). The ability of these substances to form the complex inclusions is used for practical application in foods, pharmaceutical and chemical industry, as well as in production of cosmetics, pesticides, to increase the efficiency of biocatalytic processes, etc.

Microorganisms are the only source for obtention of CGTases. The great number of bacilli and other microorganisms possessing CGTase activity have been summarized in V.Abelian's monography [1]. In spite of a great number of works in study of peculiarities of

CGTases from different taxons of bacilli the search of new perspective cultures - producers of these enzymes is actual yet. The extremophilic forms of microorganisms capable to grow in extreme conditions of environment are of a great importance for their well-known industrial potential.

This work aimed to study the distribution of CGTase activity in different species of bacilli including their extremophilic forms.

Material and methods

The well studied and identified bacterial strains, representing the Culture Collection of the Republican Centre for Deposition of Microorganisms (RCDM) as well as newly isolated bacillar cultures have been used. In total 572 bacillar strains were tested for the CGTase activity, they include mesophilic and extremophilic strains of aerobic sporeforming bacteria and thermophilic actinomycetes (Table 2).

The screening of strains for cyclodextrin glucanotransferase (CGTase) activity was carried out by microscopic iodine test of Tilden a.Hudson [2] and also by plate express-method on solid nutrient medium with starch and stains proposed by Korean investigators [3] for alcalophilic bacteria has been also applied. The method is based on the use of stain specifically decolorized due to formation of CDs inclusions. Decolorization is correlated by CGTase activity what is possible to detect by colorless zones formed around the material tested.

To reveal the CGTase activity of mesophilic and thermophilic cultures by plate method Burtseva's medium [4] was used (in per cent): K_2HPO_4 - 0,05, KH_2PO_4 - 0,05, $MgSO_4x7H_2O$ - 0,02, $(NH_4)_2SO_4$ - 0,02, soluble starch - 1,0 and yeast extract - 0,5, agar - 1,5, congo red stain - 0,02, distilled water - up to 100 ml. The CGTase activity of alcalophilic forms of bacilli was studied by the same method on Horikoshi medium [5], having the following composition, g/l: soluble starch -10,0, peptone - 5,0, yeast extract - 5,0, NaCl - 5,0, KH_2PO_4 - 1,0, $MgSO_4x7H_2O$ - 0,2, agar -20,0, distilled water - up to 1000 ml, stains - phenolphthalein - 0,03%, methyl orange - 0.01%. After the sterilization to increase pH of medium to 9.5 10 ml of 10% solution of Na_2CO_3 was added. The CGTase activity by the plate method was controlled after the growth of bacilli tested on liquid medium. Medium 1 is the same as Burtseva's medium, but without agar and stains. Medium 2 is the same as Medium 1, but with 0.7% of soluble starch. Medium 3 was proposed by Abelian [6] for thermophiles and has the composition (in per cent): soluble starch - 0,7, corn extract - 0,5, NH_4Cl - 0,53, $MnSO_4$ - traces, $CaCO_3$- 0.5, pH - 7.0. Horikoshi [5] medium (without agar) was used for alcalophilic strains.

The CGTase activity of halophilic forms of bacilli was studied by the same method on Zvyagintseva's medium [9], having the following composition, g/l: NaCl - 150,0, KCl - 2,0, $C_6H_5Na_3O_7$ - 3,0, $MgSO_4 \cdot 7H_2O$ - 20,0, $CaCl_2$ - traces, solube starch - 20,0, peptone - 10,0, yeast extract - 1,0, distilled water - up to 1000 ml. The final pH 7,2. The CGTase activity of psychrophilic and mesophilic bacilli was studied on medium with fish paste - 20,0g, yeast extract - 5,0 g, soluble starch - 20,0 g, agar - 20,0 g, distilled water - up to 100 ml, pH 7,0. To the medium has to be added: phenolpthalein - 0,03% and methyl orange - 0,01%.

Incubation: for thermophiles - 56°C (for some strains - 52°) 18-20 h under aerobic conditions, alcalophiles and halophiles were grown under similar conditions at 37° - 24 h, psychrophiles - at 20°.

CGTase activity was determined in reaction mixture (2% soluble starch 1 ml + culture liquid - 1 ml), incubation at 50°, beginning from 5 min after each 10 min before the end of reaction.

The activity of the strains tested was revealed by cyclization and production of CDs and expressed in min, what is detected by change of iodine color of starch from violet to brown.

The synthesis of CDs types was determined as described in [7].

Results and discussion

Taking into the consideration some diversity in application of agar plate screening for CDs producers, the experiments have been simultaneously carried out with the use of liquid medium. The results obtained indicate that in the case of use of 0,7 % of starch (medium 2 and 3) there is almost complete coincidence of the data on CGTase activity (Table 1).

Table 1. CGTase activity of thermophilic bacilli (incubation in liquid medium)

Strains	Species	CGTase activity, min			
		56°			50°
		Medium M-1	M-2	M-3	M-3
4002	*B.stearothermophilus*	-	20-40	40-60	
4012	- " -	-	20-40	-	
4014	*B.coagulans*	-	+	40-60	
4025	*B.licheniformis*	-	20-40		
4029	*Bacillus sp.*	-	40	40-60	
4033		-	20-40		
4017	*B.circulans*	-	20		
4018		12-20			
4023	*B.circulans*	-	-	15-20	
4024	- " -	-	60	15-20	
4030	*B.stearothermophilus*	-	20-40		
4035	- " -	-	20-40		
4041	*B.alvei*	-	30		
4071	*B.circulans*	-	40		
4088	*B.stearothermophilus*	-	-	60	
4111	- " -	15-30			
4115	- " -	-	60		
4188	- " -	15-30			
4070	*Bacillus sp.*	-	-	60	
4019	*B.stearothermophilus*	12-20			
4250	*B.coagulans*	-	-	20	
4251	*B.circulans*	-	60		
4253	*B.macerans*	-	-	60	
4254	*B.coagulans*	-	-	-	40
4258	*B.macerans*	-	-	90	
4259	- " -	-	-	40-60	
4260	- " -	-	-	20	
4261	- " -	-	-	20-40	
4091	*B.stearothermophilus*	-	-	60	

*) *The strain numbers are presented in accordance of the Culture Collection of RCDM.*

Comments. M-1 (Burtseva's medium) with 1% of soluble starch; M-2 the same medium with 0,7% of starch; M-3 (Abelian's medium) with 0,7% of starch.

For example, if with the medium 1 could be possible to detect 4 strains with CGTase activity, on medium 2 and 3 such activity possible to reveal in significantly more bacillar cultures.

As it is shown from data on the Table 2 CGTase activity was detected in all groups of extremophilic forms of bacilli except psychrophiles.

Table 2. CGTase activity of extremophilic groups of microbes

Extremophilic groups	Number of strains studied	Producers revealed	CDs predominant formation
Thermophiles	170	37	β-,γ-, less α
Thermoactinomycetes	15	3	β
Halophiles	67	3	β
Psychrophiles	17	0	
Alcalophiles	140	30	β-, less γ
Mesophiles	163	17	α
Total	572	90	

Table 3. Summary data on screening of CGTase activity among different species of extremophilic forms of bacilli

Extremophiles	Species tested	Number of strains	Number of active strains
Thermophiles			
Obligate	*B.stearothermophilus*	33	15
	B.circulans "subsp.thermophilus"	23	8
	B.macerans "subsp.thermophilus"	8	4
	Thermoactinomyces sp.	12	
	-"- vulgaris	3	3
Facultative	*B.macerans*	12	4
	B.circulans	19	5
	B.licheniformis	12	6
	B.subtilis	9	4
	B.coagulans	8	6
	B.firmus	3	1
	B.alvei	1	1
	B. polymyxa	29	0
	B. megaterium	24	1
	B.sphaericus	2	0
Halophiles	*B.halophilus*	67	3
Alcalophiles			
Obligate	*B.alcalophilus*	36	9
	B.alcalophilus "subsp.halodurans"	26	13
Facultative	*B.megaterium*	3	2
	B.subtilis	4	0
	B.circulans	2	0
	B.firmus	3	0
	B.cereus	2	0
	B.polymyxa	2	0
	B.licheniformis	2	0

Summing up our researches we may conclude that there are some specificity in the production of CDs types, thermophilic strains are characterized by biosynthesis mainly of β and γ CDs, where as alcalophiles, halophiles and to some extent thermoactinomycetes produce chiefly β type of CDs. Mesophilic forms of bacilli produce a mixture of CDs with the predominance of α type.

As the most important bacillar species for production of CDs can be recommended obligate thermophilic species *Bacillus stearothermophilus, B.circulans "subsp. thermophilus"* and *B.macerans "subsp. thermophilus"*, from alcalophiles - *B.alcalophilus* and *B.alcalophilus "subsp. halodurans"*.

As the characteristic features it is necessary to underline that thermophilic, alcalophilic and mesophilic bacilli intensively assimilate maltose [8].

The formation of yellow pigment is the characteristic pattern of alcalophilic bacillar strains.

The results presented indicate the presence of some regularities not only on the species specificity in CDs production by different species of sporeforming bacteria but also on biosynthesis of certain types of these substances by certain species and groups of bacilli.

The authors wish to express their thanks to V.Abelian and L.Manukian for the assistance and useful consultations.

The researches have been carried out within the framework of International Science and Technology Center Project A-326.

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