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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).

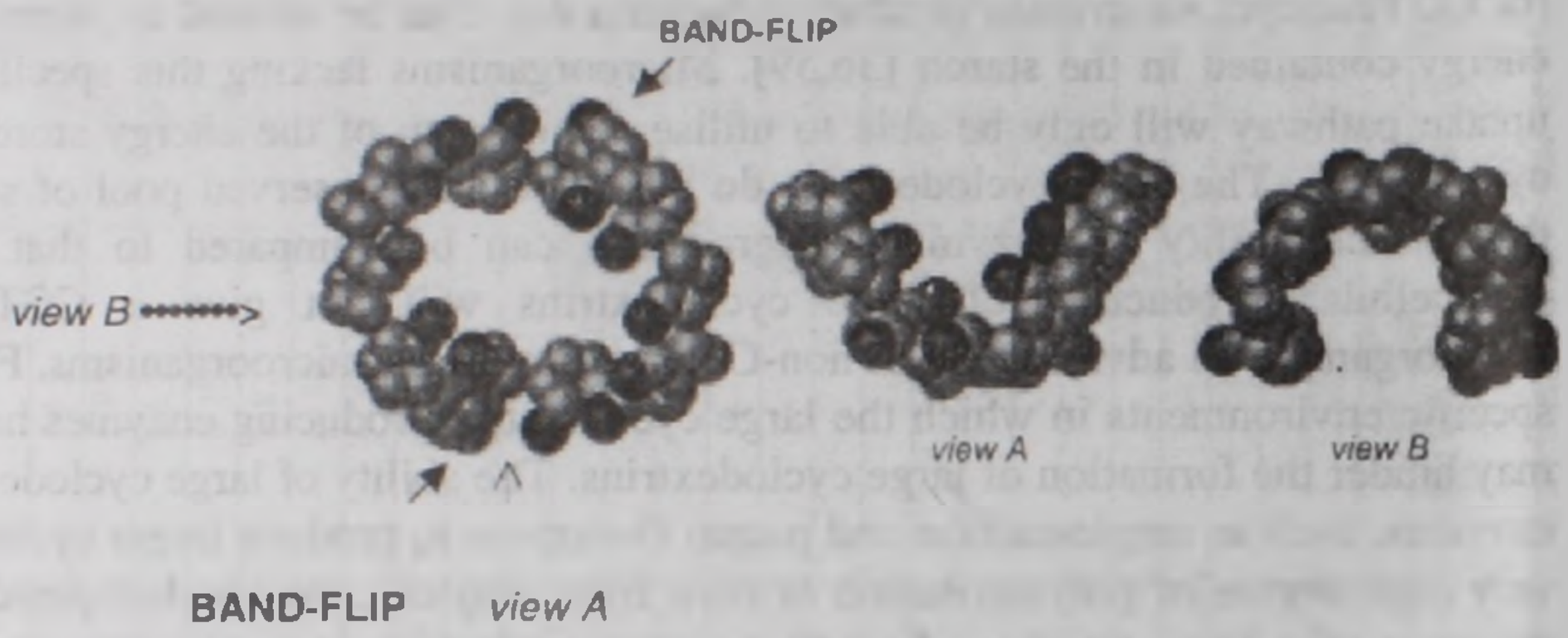

Figure 1. Solid state structure of CD_{14} showing the positions of the band-flips. Hydroxyl groups are omitted for clarity. C6 of glucose is highlighted in black.

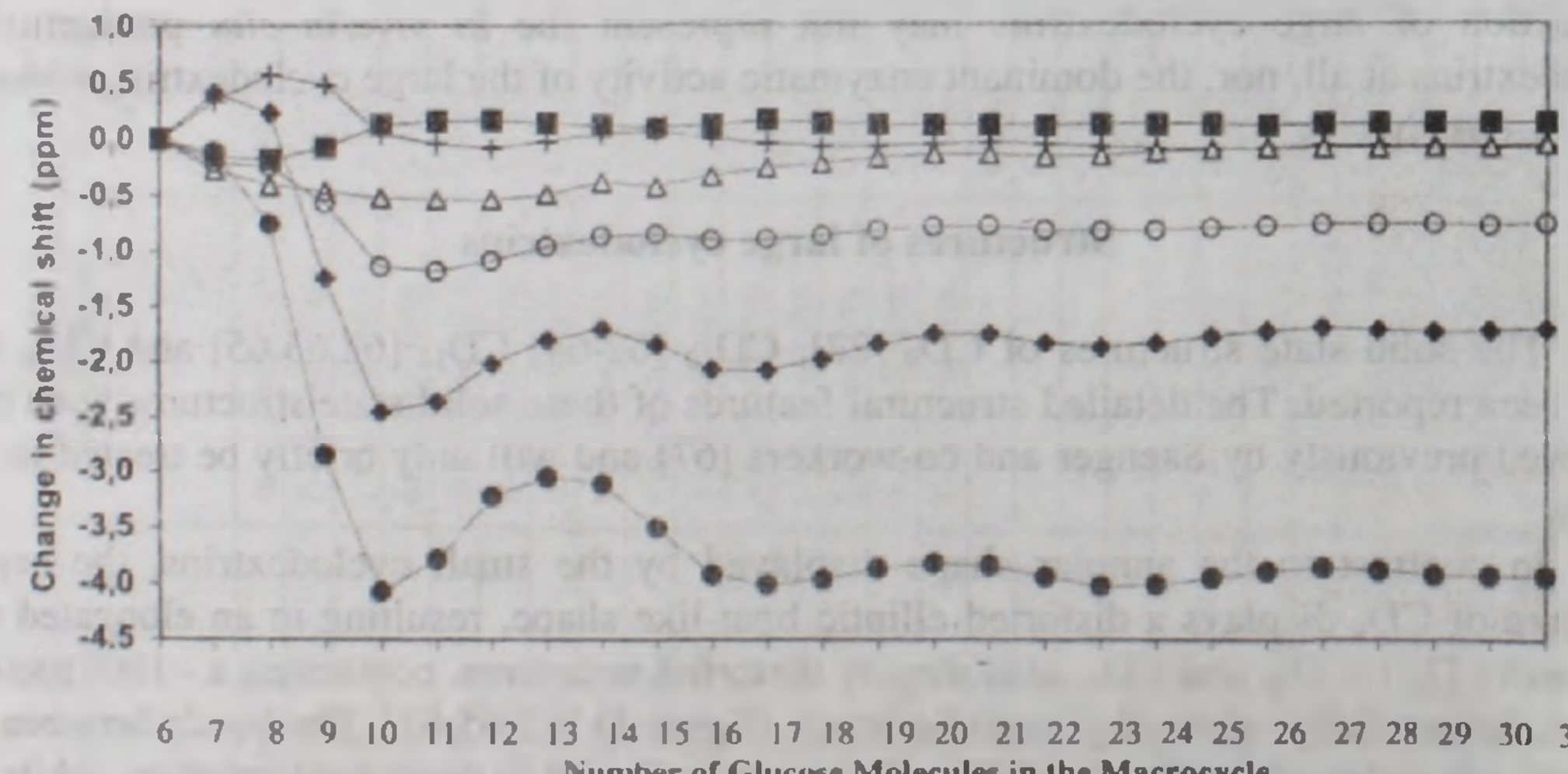

Figure 2. Changes in ^{13}C-NMR chemical shifts of cyclodextrins relative to a-cyclodextrin. •: ^{13}Cl; +: ^{13}C2; Δ: ^{13}C3; ●: ^{13}C4; ❍: ^{13}C5, ■: ^{13}C6. Data obtained from [28].

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.

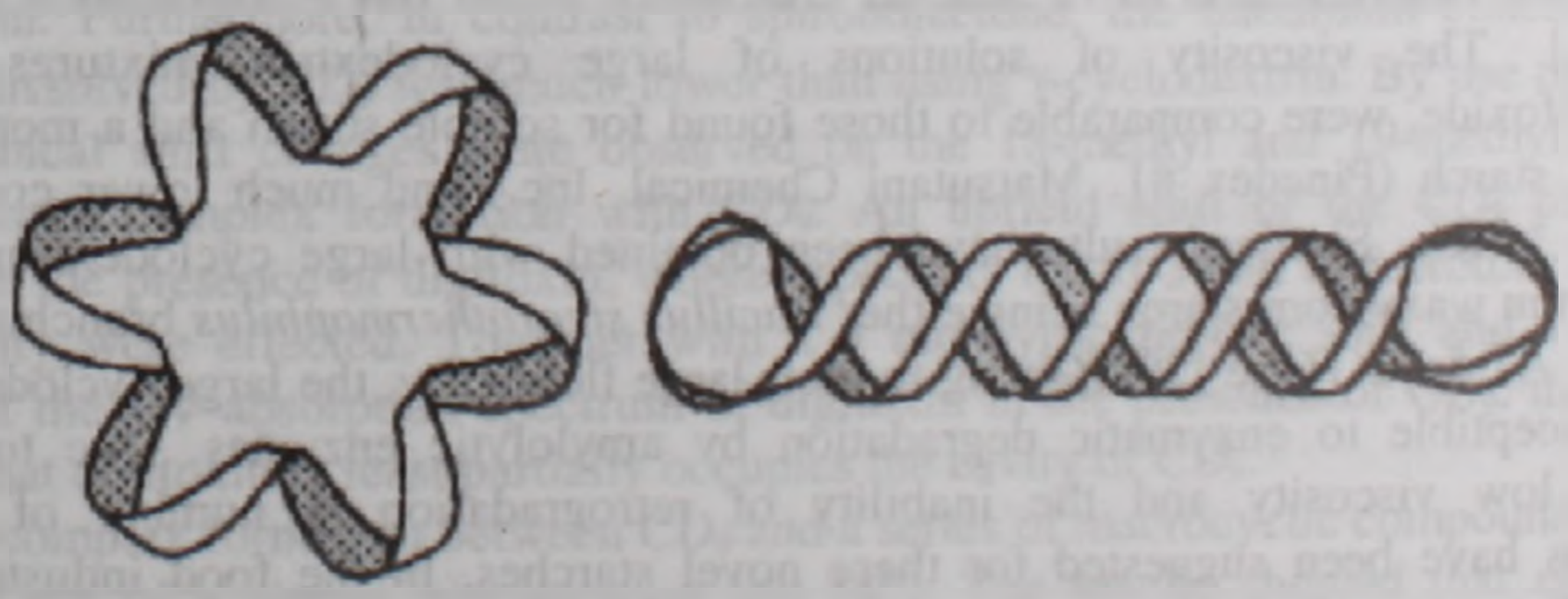

Figure 3. Schematic representations of two possible conformations of CD_{48}. Left: circularised single helical form right: anti-parallel double helical form with foldbacks at each end. Modified from [69].

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).

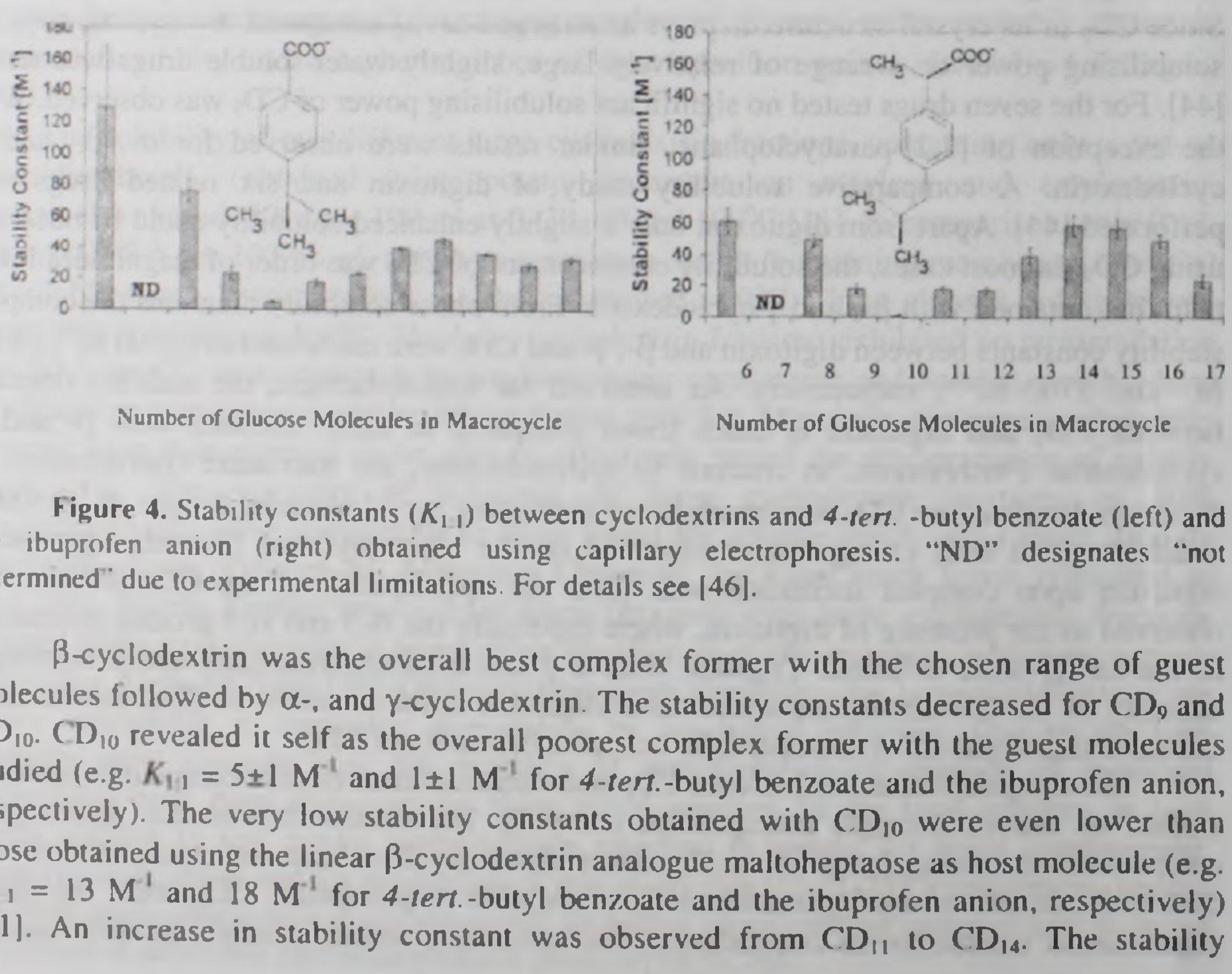

Figure 4. Stability constants ($K_{1:1}$) between cyclodextrins and *4-tert.* -butyl benzoate (left) and the ibuprofen anion (right) obtained using capillary electrophoresis. "ND" designates "not determined" due to experimental limitations. For details see [46].

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.

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