Properties and the Inclusion Behavior of 6-*O*-α-D-Galactosyl- and 6-*O*-α-D-Mannosyl-cyclodextrins[1)]

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The novel heterogeneous branched cyclodextrins (CDs), 6-*O*-α-D-galactosyl-α, -β, and -γCDs (Gal-α, -β, and -γCDs) and 6-*O*-α-D-mannosyl-α, -β, and -γCDs (Man-α, -β, and -γCDs) dissolved sufficiently in water and in 10—50% (v/v) methanol aqueous solutions, as did the homogeneous branched CDs, 6-*O*-α-D-glucosyl-α, -β, and -γCDs (Glc-α, -β, and -γCDs). The solubilities of heterogeneous branched CDs were higher than those of each parent non-branched CDs. The hemolytic activities of heterogeneous and homogeneous branched CDs were lower than those of each parent non-branched CDs and the hemolytic activity became weaker in the order of non-branched CD>Man-CD>Glc-CD>Gal-CD in each series of α, β, and γCD. A_L type solubility-phase diagrams were displayed in the formation of inclusion complexes of the guest compounds of small size (methyl benzoate, estriol, and dexamethasone) with Gal-, Man-, and Glc-CDs, and marked differences among the three kinds of branched CDs could not be detected. However, solubility-phase diagrams between these branched CDs and the insoluble guest compounds of large cyclic structure (cyclosporin A, tacrolimus, and amphotericin B) showed A_P type, and the improvement of water solubilities of these guest compounds with three kinds of branched CDs was enhanced in the order of Man-CDs>Glc-CDs>Gal-CDs.

Key words heterogeneous branched cyclodextrin; inclusion complex; hemolytic activity; solubilization

The three most common naturally occurring cyclodextrins (CDs) are αCD, βCD, and γCD consisting of six, seven, and eight α-1,4-linked D-glucopyranose units, respectively. CDs have been investigated in many fields including pharmaceuticals, food, cosmetic, agricultural medicine, *etc.*, mainly as solubilizing and stabilizing agents for lipophilic compounds and labile materials against light and air oxidation. The natural CDs, in particular βCD, have limited solubility in water and their complexes with lipophilic water-insoluble guest compounds often precipitate from water.[2,3)] Therefore various homogeneous branched CDs such as 6-*O*-α-D-glucopyranosyl-α, -β, and -γCDs (Glc-α, -β, and -γCDs) (Fig. 1) and 6-*O*-α-maltosyl-α, -β, and -γCDs (Mal-α, -β, and -γCDs) which have glucose and maltose side-chain α-1,6-linked to CD rings, respectively, and possess high solubility in water, were first enzymatically synthesized.[4—10)] After that, 6-*O*-α-D-galactopyranosyl-α, -β, and -γCDs (Gal-α, -β, and -γCDs) (Fig. 1) were prepared from α, β, and γCDs and melibiose by transgalactosylation with coffee beans α-galactosidase [EC 3.2.1.22], respectively.[11)] Further, recently, 6-*O*-α-D-mannopyranosyl-α, -β, and -γCDs (Man-α, -β, and -γCDs) (Fig. 1) were also obtained from a mixture of mannose and α, β, and γCDs by the reverse action of jack bean α-mannosidase [EC 3.2.1.24], respectively.[12)] These novel heterogeneous branched CDs have a side-chain of galactose and mannnose which are known to be recognized by animal lectins.[13,14)] Therefore, they are expected to be useful as drug carriers in targeting a drug delivery system.

This paper deals with the solubilities of heterogeneous branched CDs in water and in various concentrations of methanol aqueous solutions, the hemolytic activities on human erythrocytes, and the inclusion behavior for several poorly water-soluble (slightly soluble or insoluble) compounds of different molecular sizes, in comparison with those of the parent non-branched CDs and Glc-CDs.

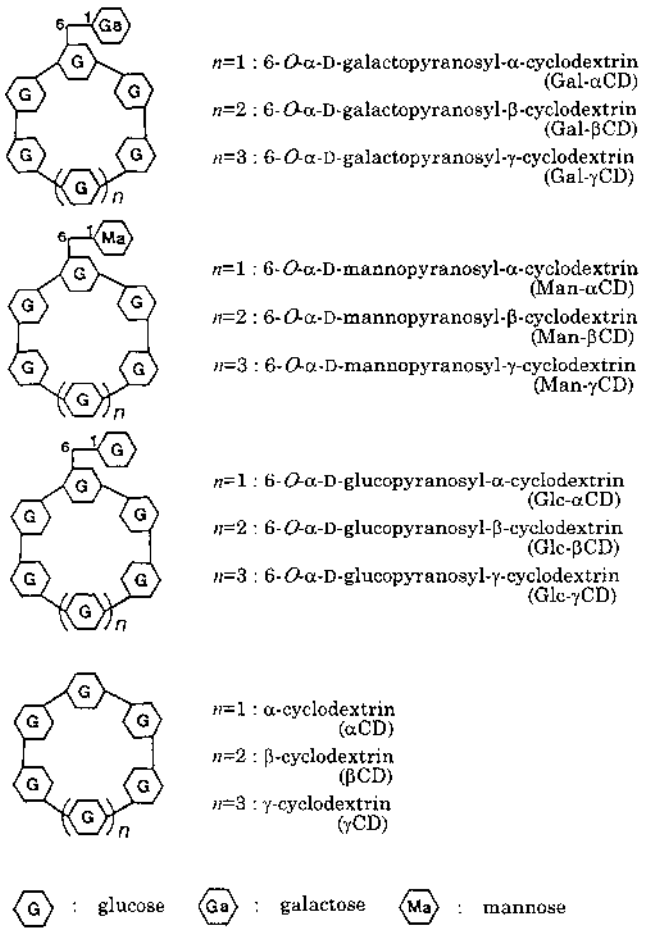

Fig. 1. Schematic Structures of Branched CDs

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Table 1. Analytical Conditions of Guest Compounds by HPLC

Guest compound	Column	Eluent	Column temperature	Wavelength (nm)	t_R[a] (min)
Mbe	M80[b]	CH_3OH–H_2O (70 : 30)	30 °C	273	10.6
CyA	M80	CH_3OH–H_2O (83 : 17)	45 °C	210	11.2
Est	M80	CH_3OH–H_2O (55 : 45)	30 °C	280	10.6
FK506	L80[c]	CH_3OH–H_2O (78 : 22)	45 °C	210	11.5
Dex	M80	CH_3OH–H_2O (68 : 32)	30 °C	240	10.8
AmB	M80	CH_3OH–H_2O (80 : 20)	30 °C	363	11.6

a) Retention time, *b*) J'sphere ODS-M80 (150×4.6 mm i.d.), *c*) J'sphere ODS-L80 (150×4.6 mm i.d.); flow rate, 0.5 ml/min.

Experimental

Materials Non-branched α, β, and γCDs, and Glc-α, -β, and -γCDs were the preparations of Bio Research Corporation of Yokohama (Yokohama, Japan). Non-branched α, β, and γCDs were used after recrystalization from water. Glc-α, -β, and -γCDs were purified on a YMC-Pack SH-343-5 ODS column (250×20 mm i.d.) (YMC, Kyoto, Japan). Gal-α, -β and -γCDs, and Man-α, -β and -γCDs were prepared according to the previous papers.[11,12] These heterogeneous branched CDs were purified by semi-preparative HPLC. Each mixture of reaction products was separated on the basis of molecular weight on a TSK gel Amide-80 column (300×21.5 mm i.d.) (Tosoh, Tokyo, Japan) with 53 : 47 acetonitrile–water as the eluent, at a flow rate of 3 ml/min, followed by further purification on a YMC-Pack SH-343-5 ODS column at a flow rate of 3 ml/min and a column temperature of 30 °C, using a methanol–water system containing the individually optimum concentration of methanol, that is, 6 : 94 methanol–water for branched αCDs and for branched γCDs, and 8 : 92 methanol–water for branched βCDs. Each fraction was analyzed by HPLC on a YMC-Pack A-312 ODS column (150×6 mm i.d.) (YMC) with 5 : 95 methanol–water for branched αCDs and for branched γCDs, and 6 : 94 methanol–water for branched βCDs as the eluent, respectively, at a flow rate of 0.7 ml/min and a column temperature of 30 °C. Tacrolimus (FK506) used as a guest compound in inclusion complexation was kindly given by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). All other compounds used were obtained from commercial sources. Deionized and doubly distilled water was used throughout this experiment. Reagent-grade organic solvents used for HPLC were freshly distilled and filtered through a 0.45 μm filter.

General Method A UVIDEC 610C double beam spectrophotometer (Jasco, Tokyo) was used for the determination of absorbances. HPLC was performed with a PU-980 pump (Jasco), a Rheodyne 7125 injector, a UV-970 variable-wavelength UV spectrophotometer (Jasco), and a Shodex RI-71 refractive index monitor (Showa Denko, Tokyo). HPLC analyses at constant temperature were carried out with an SSC 3510C column oven (Senshu Scientific, Tokyo). The columns employed for analyses of the guest compounds in inclusion complexes were a J'sphere ODS-M80 column (150×4.6 mm i.d.) (YMC) and a J'sphere ODS-L80 column (150×4.6 mm i.d.) (YMC).

Measurement of Solubility The solvent (water or various concentrations of methanol aq. soln.) was carefully added in portions of 0.01—0.1 ml to a glass vessel containing 500 mg of the dry CD, and the volume of solvent required for complete dissolution of the CD within 30 min at 25±1, 40±1, and 55±1 °C, respectively, was measured by vigorous shaking for 30 s periods at 5 min intervals.

Determination of Hemolytic Activity A 0.2% (v/v) human erythrocyte suspension (1 ml) in 100 mM isotonic phosphate buffer (pH 7.4, PBS) was added to 1 ml of PBS containing various concentrations of CDs. The mixture was incubated at 37 °C for 30 min and centrifuged at 1300×***g*** for 10 min. Percent hemolysis was expressed in terms of the ratio of the absorbance at 541 nm of hemoglobin released from erythrocytes with CDs to the absorbance after the complete hemolysis of erythrocytes in water. The inclusion complexation between cholesterol and branched βCD was examined according to the method mentioned in the next section. The concentration of cholesterol was determined by HPLC on a DAISOPAK SP-120-5-C4-P column (150×4.6 mm i.d.) (DAISO, Osaka, Japan) with 95 : 5 acetonitrile–water as the eluent, at a flow rate of 0.8 ml/min and a column temperature of 40 °C.

Inclusion Behavior of Branched CDs Complex-forming abilities of CDs for poorly water-soluble compounds were estimated according to the solubility method.[15] Excess amounts of the guest compounds were added to the solutions containing various concentrations of CDs and the suspensions were shaken at 30 °C. Non-branched βCD has low solubility in water, and therefore, βCD at over saturated concentration was used in states of suspensions. After equilibrium was attained, an aliquot was pipetted through a 0.2 μm membrane filter, and the amounts of the guest compounds in the CD solutions were measured by HPLC. HPLC conditions for analyses of the guest compounds are shown in Table 1.

Table 2. Solubility of CDs in Water at Various Temperatures

CD	Solubility (mmol/100 ml)		
	25 °C	40 °C	55 °C
αCD	18	21	47
Glc-αCD	80	103	119
Gal-αCD	105	134	138
Man-αCD	70	135	166
βCD	1.6	3.1	4.4
Glc-βCD	77	77	133
Gal-βCD	68	90	97
Man-βCD	71	80	89
γCD	20	43	64
Glc-γCD	98	101	118
Gal-γCD	82	95	111
Man-γCD	69	74	96

The data is the average of three experiments.

Results and Discussion

Solubility Table 2 summarizes the solubilities of Gal-CDs and Man-CDs in water at 25, 40, and 55 °C together with those of Glc-CDs and the parent non-branched CDs. Every member of the heterogeneous and homogeneous branched CDs has high aqueous solubility, compared with each of the parent non-branched CDs. In particular, the solubilities of Gal-, Man-, and Glc-βCDs rose markedly; for example, they dissolved about 40-fold over the parent non-branched βCD in water at 25 °C. Although the solubilities of all CDs increased with rising temperature, this tendency was observed more distinctly in the cases of non-branched CDs than branched CDs. Further, the solubilities of Gal-CDs, Man-CDs, and Glc-CDs in 10, 30, and 50% (v/v) methanol aqueous solutions were examined at 25 °C (Table 3). The solubilities of the non-branched CDs steeply decreased with increasing methanol concentrations, while those of the branched CDs remained relatively high even in 50% (v/v) methanol aqueous solution.

Hemolytic Activity Figure 2 shows the hemolytic effects of heterogeneous branched CDs on human erythrocytes in isotonic solution, compared with those of Glc-CDs and the parent non-branched CDs. The hemolytic activities of the branched CDs were lower than those of each parent non-branched CDs, and became weaker in the order of Man-CD>Glc-CD>Gal-CD for each series of α, β, and γCD.

Table 3. Solubilities of CDs in Methanol Aqueous Solutions of Various Concentrations (% (v/v)) at 25 °C

CD	Solubility (mmol/100 ml)		
	10%	30%	50%
αCD	4.1	1.3	0.7
Glc-αCD	108	108	108
Gal-αCD	100	116	76
Man-αCD	118	119	100
βCD	1.0	0.5	0.3
Glc-βCD	92	86	77
Gal-βCD	76	84	73
Man-βCD	89	91	91
γCD	15	4.8	1.7
Glc-γCD	80	76	69
Gal-γCD	84	67	56
Man-γCD	70	71	58

The data is the average of three experiments.

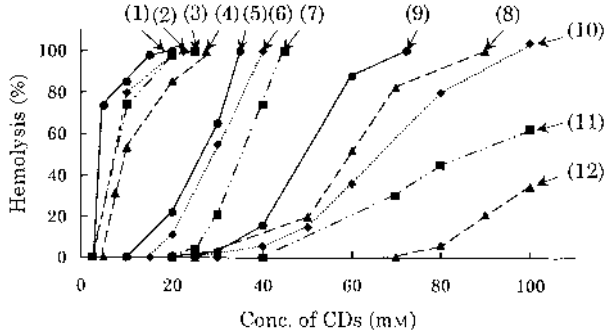


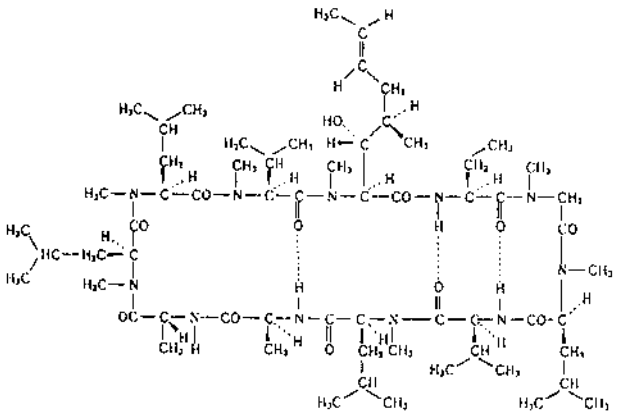
Fig. 2. Hemolytic Effects of Branched CDs and Non-branched CDs on Human Erythrocytes in 100 mM Isotonic Phosphate Buffer (pH 7.4)

(1) βCD, (2) Man-βCD, (3) Glc-βCD, (4) Gal-βCD, (5) αCD, (6) Man-αCD, (7) Glc-αCD, (8) Gal-αCD, (9) γCD, (10) Man-γCD, (11) Glc-γCD, (12) Gal-γCD.

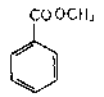
The hemolytic activities of non-branched CDs are known to increase in order of βCD>αCD>γCD[16] and this order is contrary to that of the solubilities of CDs in water. In addition, we have already reported the water solubilities and hemolytic activities of three positional isomers of 6^1,6^n-di-*O*-α-D-glucopyranosyl-β-cyclodextrins (1,*n*-$(Glc)_2$-βCDs; n=2—4).[17] That is, 1,4-$(Glc)_2$-βCD which had significantly lower water solubility than 1,2- and 1,3-$(Glc)_2$-βCDs, showed the strongest hemolytic activity in three isomers. Therefore, a reason that the hemolytic activities of the branched CDs are lower than those of the parent non-branched CDs may be due to the higher solubilities of the branched CDs in water. In addition to this view, it can be considered that the saccharide side-chain linked to the CD is closely related to the decrease in hemolytic activity. The hemolysis effect of CD on human erythrocytes was profoundly correlated to the extraction of the various components of erythrocytes, cholesterol, phospholipids, proteins from membrane with CD.[16] Previously it was suggested that the potencies of non-branched CDs for solubilizing the various components of erythrocytes were βCD≫γCD>αCD for cholesterol, αCD>βCD≫γCD for phospholipids, and βCD≫γCD>αCD for proteins, and the extraction of cholesterol may be of more importance than those of phospholipids and proteins.[18] Therefore, the inclusion complexation between cholesterol and the three kinds of branched βCDs were examined in order to elucidate the influence of the saccharide side-chain on the hemolytic activity. The solubilities of cholesterol in 20 mM Gal-βCD, Man-βCD, and Glc-βCD solution were 0.68, 1.65, and 0.81 mM, respectively, and it was proved that the solubilizing ability for cholesterol was enhanced in order of Man-βCD>Glc-βCD>Gal-βCD. This order is the same as that of the hemolytic activity in branched βCDs. This result suggests that the saccharide side-chains of branched CDs play an important role in formation of inclusion complexes between the components of erythrocytes and branched CDs.

Inclusion Behavior The formation of inclusion complexes of heterogeneous branched CDs for several poorly water-soluble (slightly soluble or insoluble) compounds in water was studied by the solubility method[15] and were compared with that of the parent non-branched CDs and Glc-CDs. Cyclosporin A (CyA), tacrolimus (FK506), and amphotericin B (AmB) were employed as the guest compounds, because these drugs hardly dissolve in water and recently their solubilization has been a subject of great interest. These insoluble guest compounds have a large cyclic moiety in their structures. Furthermore, the formation of inclusion complexes of CDs with methyl benzoate (Mbe), estriol (Est), and dexamethasone (Dex) which have smaller molecular size, was also examined for comparison. The structures of the guest compounds used in this study are shown in Fig. 3. CD forms inclusion complexes with many compounds by taking up the whole compound molecule, or more frequently, some hydrophobic part of it, into the cavity. In the case of inclusion complexation of these guest compounds with the non-branched CDs, the best host compounds for CyA and Mbe, for FK506 and Est, and for AmB and Dex, were αCD, βCD, and γCD, respectively. Figure 4 shows the solubility-phase diagrams obtained for the six kinds of guest compounds with the respective three kinds of branched CDs (Gal-α, -β, or -γCDs, Man-α, -β, or -γCDs, and Glc-α, -β, or -γCDs) and non-branched α, β, or γCDs in water at 30 °C. In the systems with the non-branched CDs, the solubility-phase diagrams were Bs type for the guest compounds other than CyA and AmB, because of the formation of the insoluble inclusion complexes at higher concentrations of the host compounds. On the contrary, A_L or A_P type solubility-phase diagram due to formation of water-soluble inclusion complexes, were displayed by all guest compounds in each branched CDs solution, which resulted in a dramatic increase in the water solubility of all guest compounds.

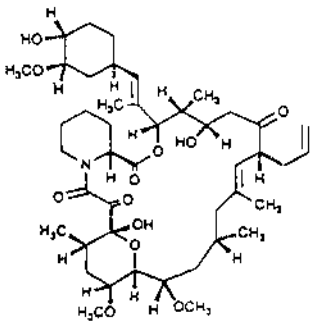
In the cases of the insoluble guest compounds of large size (CyA, FK506, and AmB), their solubility curves can be classified as A_P type except FK506–non-branched βCD system, suggesting high-order complex formations. The ascending curvatures were quantitatively analyzed according to the optimization technique to obtain the stability constants of high-order complexes.[19—21] Unfortunately, the exact stability constants could not be determined, because of the narrow range of the host compounds concentrations tested. Interestingly, Fig. 4a shows that the enhancement of water solubilities of CyA, FK506, and AmB by forming inclusion complexes with branched αCDs, βCDs, and γCDs, respectively, is different among the three kinds of saccharide side-chain of the branched CDs. The saccharide side-chain of the branched CDs contributed in the order of Man-><Glc-><Gal- to solubilization of CyA, FK506 and AmB, and in particular, Man-



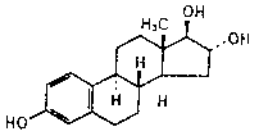
cyclosporine A(CyA)



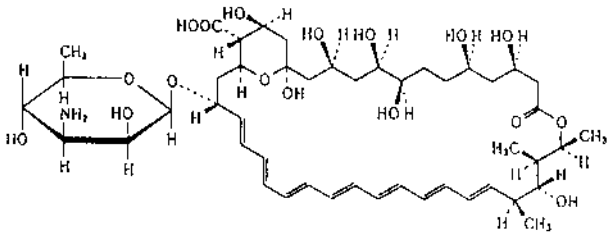
methyl benzoate (Mbe)



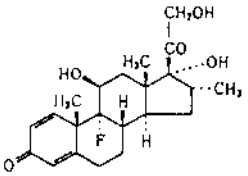
tacrolimus (FK506)



estriol (Est)



amphotericin B (AmB)



dexamethasone (Dex)

Fig. 3. Structures of Guest Compounds

CDs were thought to be feasible solubilizers for these guest compounds. CyA and FK506, and AmB are widely used as immunosuppressants and as an antifungal, respectively. However, hydrogenated castor oil as a solubilizing agent is contained in the injections of CyA and FK506, and therefore, the possibility of anaphylatic shock is present. In this study, CyA, FK506, and AmB were sufficiently dissolved in branched CDs solutions and the concentrations were adequate for clinical use. For example, the solubilities of CyA, FK506, and AmB rose to 0.74, 0.091, and 0.36 mM in 100 mM solution of Man-αCD, Man-βCD, and Man-γCD, respectively. The use of Man-CDs solutions having higher concentration than 100 mM is expected to result in further increases in the water solubilities of these drugs.

On the other hand, in the case of the guest compounds of small size, the solubility of Mbe, Est, and Dex increased linearly as a function of each branched CDs concentration (Fig. 4b). No pronounced difference in the inclusion behavior among the three kinds of branched CDs (Gal-, Man-, and Glc-CDs) for the respective guest compounds of small size (Mbe, Est, and Dex) was observed, and it was confirmed that the apparent stability constants (K) in each system were nearly same. According to Eq. 1, K values of the 1 : 1 complexes were calculated from the solubility-phase diagrams,[15] and they were found to be *ca.* 1500 M^{-1} for Mbe–Gal-, Man-, and Glc-αCDs systems, *ca.* 30000 M^{-1} for Est–Gal-, Man-, and Glc-βCDs systems, *ca.* 12000 M^{-1} for Dex–Gal-, Man-, and Glc-γCDs systems, respectively.

$$K=\frac{\text{slope}}{\text{intercept}\times(1-\text{slope})} \quad (1)$$

Conclusions

The solubilities of heterogeneous branched CDs (Gal-CDs

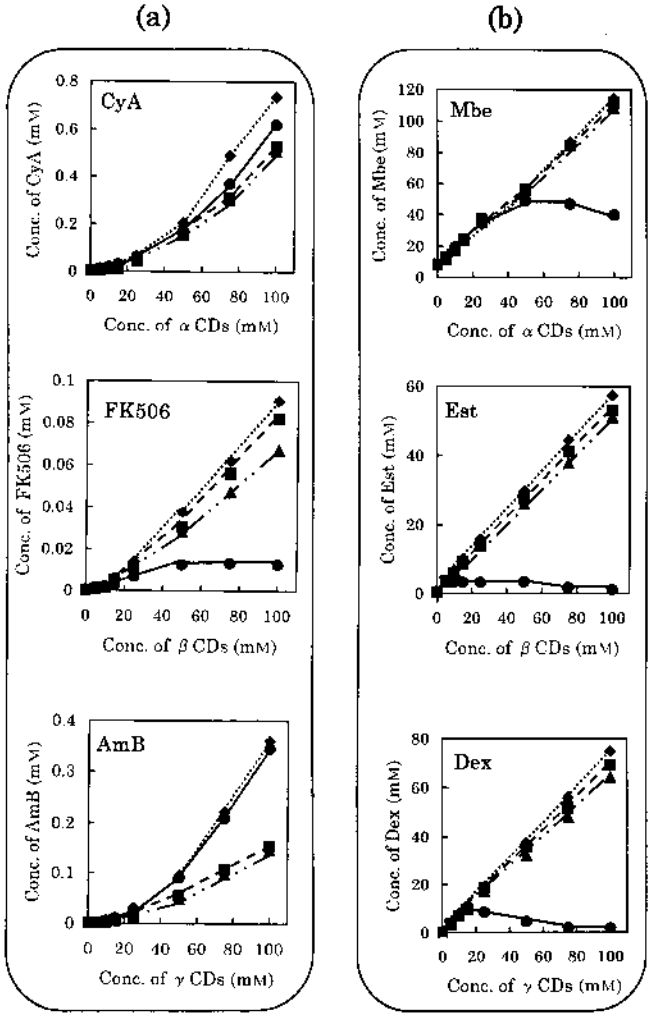


Fig. 4. Phase-Solubility Diagrams of Various Guest Compounds with Branched CDs and Non-branched CDs in Water at 30 °C

▲, Gal-CDs; ◆, Man-CDs; ■, Glc-CDs; ●, non-branched CDs.

and Man-CDs) and of homogeneous branched CDs (Glc-CDs) did not differ greatly. They were higher than those of the parent non-branched CDs and were not appreciably influenced by temperature or the polarity of solvent. The hemolytic activities of heterogeneous and homogeneous branched CDs were lower than those of each parent non-branched CDs, and the hemolytic activity became weaker in the order of non-branched CDs>Man-CDs>Glc-CDs>Gal-CDs in each series of α, β, and γCD. It was suggested that Gal-CDs were the most safe host compounds *in vitro* among three kinds of branched CDs. From model building with Corey Pauling Koltun (CPK) atomic models, it was thought that the inclusion behavior of branched CDs for the guest compounds of small size (Mbe, Est, and Dex), which can be taken up as a whole molecule into the cavity of CDs, was nearly the same regardless of the kind of saccharide side-chain. On the other hand, in the cases of the molecules of large cyclic structure where only a part of the structure is taken up into the cavity (CyA, FK506, and AmB), the solubilizing abilities of the branched CDs for those guest compounds became higher in the order of Man-CDs>Glc-CDs>Gal-CDs. It is highly expected that these three kinds of branched CDs possessing greater water solubility and lesser toxicity will be effectively utilized in widespread fields.

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