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Application of Gel Chromatography to Determining Formation Constants of Inclusion Compounds of Cyclodextrins

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The gel filtration technique (Hummel and Dreyer method) was applied to determining formation constants of inclusion complexes of α- and β-cyclodextrins. As guest substances, *p*-nitrophenolate, *p*-nitrophenol, benzoate, and benzoic acid were used. Formation constants were determined at room temperature (25—30 °C) using Sephadex G-10 gel in aqueous buffered solutions of a constant ionic strength of 0.5 with NaCl or with phosphate buffer, and the results were compared with literature values. The method is applicable to such guest substances as exhibit strong adsorption onto the gel matrix.

It is generally accepted that cyclodextrins (CyDs) form inclusion complexes with a wide variety of inorganic and organic compounds in aqueous solutions. The CyD has a cavity, the interior side of which is considered to be hydrophobic, and some hydrophobic molecules can be accommodated in the cavity as guests. Thus, the CyD itself acts as a host. Several methods to determine formation constants of CyD inclusion complexes have been reported which utilize solubility,[1] kinetics,[2] pH potentiometry,[3] polarography,[4] calorimetry,[5,6] optical rotation,[7] NMR,[7,8] CD spectrum,[9,10] UV-visible spectrum,[2,4,5,7,11,12] positron annihilation,[13] and anion-exchange HPLC.[14]

A simple method of gel filtration chromatography has been successfully applied to studies on the association of macromolecules with low-molecular-weight ligands since it was first proposed by Hummel and Dreyer.[15] The method is based on the fact that associated complexes as well as free macromolecules are excluded from the gel phase, whereas small ligands are permeable into the gel, thus permitting the separation of the two. The method therefore makes use of a sieving effect of gel. In the gel chromatographic behavior, however, there have been observed some side-effects other than the sieving effect. Among them, adsorption of solute onto gel matrix is important, especially in such a case that the solute is a hydrophobic substance such as aromatic hydrocarbons.[16] The adsorption effect will be much more pronounced with the most tightly cross-linked Sephadex G-10. It was suggested, though not proved experimentally, that the interaction of ligands with gel matrix does not interfere with the determination of macromolecule-ligand association constants by the Hummel and Dreyer method.[17]

This paper is concerned with the application of gel chromatography to such a system that the ligand (guest) exhibits affinity to the gel matrix as well as to the macromolecule (host). We have chosen *p*-nitrophenolate, *p*-nitrophenol, benzoate, and benzoic acid as guests, because their formation constants with CyDs have abundantly been reported.

Experimental

Materials and Sample Preparations. Cyclodextrins (α- and β-CyDs from Wako) of reagent grade were recrystallized from water and dried over phosphorus pentaoxide. Benzoic acid and *p*-nitrophenol were of analytical reagent grade and used without further purification. Sephadex G-10 and Blue Dextran 2000 were obtained from Parmacia (Uppsala, Sweden). Phosphate buffer (Na_2HPO_4–NaOH for pH=11; NaH_2PO_4–H_3PO_4 for pH=3.5) and hydrochloric acid were used to adjust pH of eluents. Eluents, which contain guest molecules at various concentrations, $[guest]_0$, were prepared by dissolving *p*-nitrophenol or benzoic acid in buffered solutions and by applying a dilution with the same solutions. Ionic strength was adjusted to 0.5 with the buffer itself in the case of benzoate or with sodium chloride in the other cases. A suitable amount of the dried CyD was weighed out and dissolved in the eluent to prepare a CyD sample solution of known concentration.

Apparatus and Procedures. A 7.8 mm×19.5 cm glass-column packed with Sephadex G-10 gel was pre-equilibrated with the eluent. A 0.550 cm^3 aliquot of the CyD sample solution was applied to the pre-equilibrated column by using a sample injector (Kyowa Seimitsu Type KWM-4V-2) and then eluted with the eluent at a constant flow rate by means of a peristaltic pump (Atto Type SJ-1211). The absorbance of the eluant was monitored at the outlet of the column by a JASCO Type UVIDEC-100-III equipped with a flow-cell of 10 mm optical path length and recorded on a chart by a Hitachi Model 056. Both positive and negative peak areas were measured by cutting the chart and weighing the pieces.

Results

Experiments were carried out at room temperature (25—30 °C) under the conditions shown in Table 1. The formation constants of α- and β-CyDs inclusion complexes with *p*-nitrophenolate, *p*-nitrophenol, benzoate, or benzoic acid determined in this work are given in Table 2, together with literature values available. The data in Table 2 indicate that the present results are in reasonable agreement with the literature.

Discussion

Chromatograms were obtained by monitoring UV-visible absorbance of the guest in eluant, whose concentration, $[guest]_0$, is known. A typical chromatogram is shown in Fig. 1, which consists of a pair of positive and negative peaks. The negative peak reflects the guest lost by being incorporated into the CyD. It is desirable to assign the positive peak to a elution band of CyD, however this was not demonstrated in the present work because of the difficulty of detecting the eluted CyD.

Table 1. Experimental conditions to determine formation constants of cyclodextrin-guest inclusion complexes[a)]

Guest present in eluent:	*p*-Nitrophenolate	*p*-Nitrophenol	Benzoate	Benzoic acid
$[Guest]_0/10^{-5}$ mol dm^{-3}:	1.03—0.103	10.2—1.02	5.14—1.03	2.05—0.513
[α-CyD]/10^{-3} mol dm^{-3}:[b)]	2.55—10.5	5.12—18.1	5.18—16.0	5.04—15.6
[β-CyD]/10^{-3} mol dm^{-3}:[b)]	4.92—9.99	4.93—10.3	5.00—10.4	4.97—10.5
Ionic strength (with):	0.5(NaCl)	0.5(NaCl)	0.5(Na_2HPO_4)	0.5(NaCl)
pH of eluent:	11	3.5	11	0.05 mol dm^{-3}
Adjusted with:	Na_2HPO_4-NaOH	NaH_2PO_4-H_3PO_4	Na_2HPO_4-NaOH	HCl
Flow rate/cm^3 min^{-1}:	2.5	2.5	1.0	2.5
Wavelength monitored/nm:[c)]	396	327	232	237

a) Temperatures are in the range of 25—30 °C. b) The volume of CyD sample solutions introduced to the column (Sephadex G-10) is 0.550 cm^3. c) Isosbestic points.

Table 2. Formation constants of cyclodextrin-guest inclusion complexes

Cyclo-dextrin	Formation constant/mol^{-1} dm^3 [a)]	
	This work	Literature values
Guest: *p*-Nitrophenolate anion		
α-CyD	2270±230	1890(25°),[b)] 3703(14°),[c)] 2500(25°),[d)] 1587(25°),[d)] 2703(23°),[d)] 2439[e)]
β-CyD	818±146	769(20°),[e)] 1020,[e)] 476(25°)[f)]
Guest: *p*-Nitrophenol		
α-CyD	210±43	341(22°),[g)] 385(14°),[c)] 417(10°),[h)] 200(30°),[h)] 126(25°)[i)]
β-CyD	314±63	244,[j)] 1000(25°)[i)]
Guest: Benzoate anion		
α-CyD	11±2	10.5(25°),[k)] 9.69(30°),[k)] 12,[l)] 9.8(25°)[m)]
β-CyD	35±1	
Guest: Benzoic acid		
α-CyD	783±52	751(25°),[k)] 1000(25°),[i)] 800(25°),[m)] 583(30°)[k)]
β-CyD	632±59	604[j)]

a) The temperatures at which formation constants have been determined are indicated in parentheses when specified in the literature. b) Ref. 5. c) Ref. 11. d) Ref. 7. e) Ref. 4. f) Ref. 12. g) Ref. 13. h) Ref. 9. i) Ref. 6. j) Ref. 10. k) Ref. 3. l) Ref. 2. m) Ref. 8.

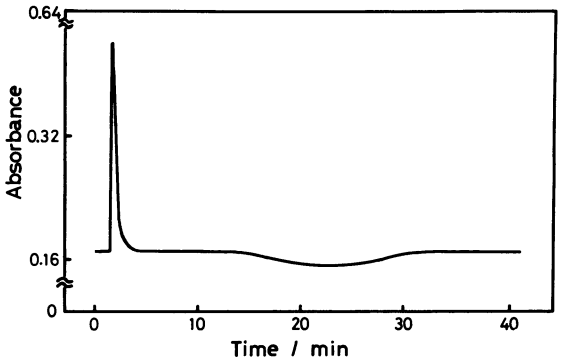

Fig. 1. Elution profile of β-cyclodextrin–*p*-nitrophenolate system.
Concentration of *p*-nitrophenolate anion in eluent: 1.03×10^{-5} mol dm^{-3}, concentration of β-CyD dissolved in eluent: 5.23×10^{-3} mol dm^{-3}, chart speed: 5 mm min^{-1}, AUFS: 0.64.

For all the CyD-guest combinations studied here, the elution volume of the positive peak was found to be nearly equal to the void volume. Based on this observation, α- and β-CyDs, with molecular weights 973 and 1135, respectively, are supposed to be completely excluded from the gel phase. Incidentally, the approximate exclusion limit of molecular weight is said to be 700, in general, for Sephadex G-10.

The formation constant, *K*, was determined for the reaction

$$\text{CyD} + \text{guest} \rightleftharpoons \text{CyD}\cdot\text{guest}. \quad (1)$$

In the calculation we assumed a stoichiometry of 1:1 for the CyD·guest based on the following two pieces of experimental evidence. Firstly, the UV-visible spectra of all the CyD-guest combinations studied here were demonstrated to have isosbestic points. Secondly, ^{13}C NMR studies have revealed that benzoate anion form a 1:1 complex with α-CyD.[3)] The formation constant can be expressed by using the amount of the CyD complex formed, $Q_{CyD\cdot guest}$, the initial amount of CyD introduced to the column, $Q_{CyD,o}$, and the concentration of guest in the eluent, $[guest]_0$, as follows:[18)]

$$K = Q_{CyD\cdot guest}/[guest]_0(Q_{CyD,o} - Q_{CyD\cdot guest}). \quad (2)$$

From the positive or negative peak area we can estimate the value of $Q_{CyD\cdot guest}$. The negative peak area always leads to $Q_{CyD\cdot guest}$, while this is not necessarily the case for the positive peak by the spectrophotometric measurement. Although CyDs have no absorbances in the UV-visible range investigated, the absorbance due to the guest changes with the concentration of the coexisting CyD. However, when the absorbance of the guest in the eluant is monitored at an isosbestic point, the positive and negative peak areas should be equal to each other. In Table 3 is shown such an example for *p*-nitrophenolate, from which formation constants have been determined.

The accuracy of the formation constant determined by the present method depends primarily on the con-

Table 3. Cyclodextrin-*p*-nitrophenolate anion complexes. formation constant and the amount of the complex formed

$10^5\times[\text{guest}]_0$[a] / mol dm^{-3}	$10^3\times[\text{CyD}]$[b] / mol dm^{-3}	$10^8\times Q_{\text{CyD·guest}}$[c] / mol		$10^{-1}\times K$[d] / mol^{-1} dm^3	
		e)	f)	e)	f)
Host: α-Cyclodextrin					
1.03	9.91	12.1	10.3	220	187
	4.96	5.74	5.47	209	199
	2.55	3.14	3.20	222	227
0.516	9.87	6.05	6.18	218	223
	5.26	3.15	3.73	213	253
	2.80	1.52	1.59	193	202
0.103	10.5	1.50	1.45	253	244
	5.04	0.727	0.769	255	267
	2.78	0.386	0.388	246	247
Host: β-Cyclodextrin					
1.03	9.64	4.13	3.81	76.2	70.3
	7.35	2.94	3.49	71.1	84.5
	5.23	2.27	2.15	77.2	73.1
0.516	9.99	2.32	2.15	82.2	76.1
	7.95	1.94	1.98	86.4	88.2
	5.04	1.10	1.57	77.2	110
0.103	9.99	0.508	0.539	89.8	95.3
	7.88	0.446	0.462	100	104
	4.92	0.144	0.162	51.7	58.2

a) Concentration of *p*-nitrophenolate anion in the eluent. b) Concentration of CyD dissolved in the eluent. c) Amount of the complex formed. d) Formation constant. e) Estimated from the positive peak. f) Estimated from the negative peak.

centration of the guest present in the eluent. That is, if $[\text{guest}]_0=K^{-1}$, a 50% of the total CyD molecules exists in the complexed form, and the most precise results are to be expected. The concentrations of CyD and guest have been fixed in this work so that the negative peak appearing on the chromatogram can be clearly recognized. The limit of Beer's law restricts the concentration of CyD as well as that of guest in the eluent. The positive peak is generally sharp, and the absorbance is apt to exceed the limit of Beer's law. The absorbance vs. concentration of guest was checked preliminarily by using the flow-cell. In the present work the product, $[\text{guest}]_0\cdot K$, falls in the order of 10^{-4} when the value of K is of the order of 10, for example, as with α-CyD-benzoate complex. This means that only 0.01% of the total CyD molecules is involved in association with the guest. Nevertheless, it is noticeable that the present results are in reasonable agreement with the literature.

Some of previous studies have suggested that several inorganic anions can form an adduct with CyDs, thereby affecting the formation of CyD inclusion complexes. In this work, we have studied effects of some electrolytes and their ionic strength on the formation of α-CyD complex with *p*-nitrophenolate. The results are shown in Table 4. As will be seen from Table 4, the formation constants measured decrease with increasing ionic strength and the effect is pronounced for NO_3^-. This is in accord with the literature.[6,11] The data of formation constants presented in this paper were obtained under the condition of ionic strength 0.5 adjusted with NaCl except for benzoate, in contrast to phosphate buffer in this case.

Table 4. Effects of electrolyte and ionic strength on the formation constant of α-cyclodextrin-*p*-nitrophenolate[a]

Electrolyte	Ionic strength	Formation constant / 10 mol^{-1} dm^3
KNO_3	0.5	119
	0.3	160
	0.1	195
$NaNO_3$	0.5	122
	0.1	176
KCl	0.5	217
	0.1	222
NaCl	0.5	227
	0.1	233
Na_2HPO_4	0.5	204
	0.3	189
	0.1	212
Phosphate buffer[b]		241

a) Concentration of *p*-nitrophenolate: 5.16×10^{-6} mol dm^{-3}; concentration of α-CyD: of the order of 5×10^{-3} mol dm^{-3}. b) Ionic strength was not adjusted. The ionic strength of buffer (Na_2HPO_4-NaOH) itself was less than 0.05.

Table 5. K_{av} values of guest substances in Sephadex G-10 gel column

Guest substance	Electrolyte used to adjust ionic strength[a]	K_{av}[b]
p-Nitrophenolate	$NaNO_3$	5.9
	NaCl	10.0
	Na_2HPO_4	20.3
p-Nitrophenol	NaCl	21.4
Benzoate	NaCl	1.2
	Na_2HPO_4	2.2
Benzoic acid	NaCl	11.7

a) The ionic strength was adjusted to 0.5. b) The K_{av} value is defined as $(V_e-V_o)/(V_t-V_o)$, where V_e, V_o, and V_t are the elution volume, the void volume, and the total bed volume, respectively.

Cyclodextrins are cyclic oligosaccharides made up of α-1,4-linked D-glucopyranose units. On the other hand, Sephadex G-type gel is prepared by closs-linking polysaccharide dextran with epichlorohydrin. In view of the chemical similarity between these two, similar associations with hydrophobic solutes are probable. Indeed, the affinity of the dextran gel matrix for aromatic and pseudoaromatic substances is particularly striking.[16] In Table 5 are given K_{av} values measured under the same experimental conditions that have been used for formation constants. Table 5 clearly indicates that the guest substances are strongly adsorbed on Sephadex G-10 gel. The order of K_{av} for a given guest may be explained in terms of hydration of the electrolyte, that is, the larger the hydration of electrolyte, the more pronounced the adsorption of the guest onto the gel. In the case of benzoate with the smallest K_{av} value, the ionic strength was adjusted with phosphate buffer,

and thereby the negative peak was made to be retarded. In this case, the ionic strength being adjusted with NaCl, the negative peak appeared so closely to the positive one that no plateau between the two peaks was observed.

The Hummel and Dreyer method is principally based on the sieving function of the gel column, that is, small ligands can be separated from both macromolecules and their associated complexes. In the present chromatography, the separation is due to the adsorption onto the gel phase rather than the sieving. The interaction of small ligands with the gel matrix does not interfere at all with the evaluation of their association with macromolecules so long as the adsorption equilibrium of ligands between the gel phase and the eluent is maintained during the course of chromatography, or it is established rapidly compared with the flow rate of the eluent.

The gel filtration method will serve as a useful technique to determine the formation constant of inclusion compounds of CyDs with various hydrophobic guest substances. However, it should be mentioned that the hydrophobic guests give rise to very broad negative peaks and often make it difficult to measure the peak area. The positive peak is preferable to the negative one if CyDs are pure enough to exhibit no absorbance at the monitoring wavelength.

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