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DETERMINATION OF EQUILIBRIUM CONSTANTS BY GEL CHROMATOGRAPHY: BINDING OF SMALL MOLECULES TO CYCLODEXTRINS

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SUMMARY

Complexation of *p*-nitrophenolate or *p*-nitrophenol with α-, β- or γ-cyclodextrins (cycloamyloses) has been studied by gel chromatography by using the Hummel–Dreyer method in order to elucidate the equilibrium constants. When positive (cyclodextrin-containing) and negative (*p*-nitrophenolate-deficient) peaks were adequately separated all parameters tested indicated the suitability of this method. In addition, thermodynamic values for the cyclodextrin complexes were obtained which were in fair agreement with those reported in the literature.

INTRODUCTION

Gel filtration chromatography has been employed to measure equilibrium constants ever since the ingenious report of Hummel and Dreyer[1] in 1962. Their method is briefly as follows. An appropriate gel column is equilibrated with an eluent carrying the component (A) the equilibrium constant of which is to be determined. A sample of the other component (B) is prepared in the eluent solution and applied to the column. The sample is then eluted and the effluent is monitored. If sufficient complexation occurs and if, say, B and complex AB move faster than A, the chromatogram will show two zones where the original concentration of A is disturbed. The component eluting faster contains B and an elevated level of A, whereas the other zone has a corresponding deficiency of A. The sizes of the peaks are proportional to the extent of complexation. A prerequisite for this method is that steady-state conditions are reached in the column before the zone AB is eluted. The principles of the method are reviewed elsewhere[2,3].

The Hummel–Dreyer method has been utilized relatively little in spite of its obvious advantages. It is not laborious because only one chromatographic analysis is required for each equilibrium constant. Consumption of the reagents is also minute. This is of significance especially in biochemical work where the availability of sample components is often limiting. This and the need for the components of the complex to migrate at different velocities in the column have resulted in the method having mainly been used to determine binding of low-molecular-weight substances to biological macromolecules. However, the studies of Yoza[2] show that gel chromatography can be successfully employed to measure equilibria between metal ions and small ligands.

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Despite the fact that some of the doubts concerning the correctness of the use of gel chromatography for determining equilibrium constants are not stated firmly enough[2], there still remains a need for elucidating it more precisely[4]. Such studies require evaluation of the contribution of different chromatographic parameters and the properties of the complex forming agents to the apparent equilibrium constants. A straightforward approach would be to compare the values obtained by gel chromatography and other more generally accepted methods. This is hampered by the lack of relevant data since often only apparent values are reported.

The kinetics and equilibria of inclusion complexes formed between cyclodextrins (CDs) and certain small molecules have been studied[5]. Cyclodextrins are non-ionic cyclic carbohydrates (MW about 1000) which can readily be separated from small molecules (MW of a few hundreds) by gel chromatography. Their complexation mode is also usually relatively simple. Hence this system represents a favourable model of the more complex molecules studied by the Hummel–Dreyer method. We have adapted it to analyze the equilibria between cyclodextrins and *p*-nitrophenol (PNP-OH) or *p*-nitrophenolate ($PNP\text{-}O^-$) ion at various buffer concentrations and temperatures.

EXPERIMENTAL

Materials

The α-, β- and γ-cyclodextrins were obtained from Sigma (St. Louis, MO, U.S.A.). *p*-Nitrophenol was from E. Merck (Darmstadt, F.R.G.). Stock solutions of *p*-nitrophenol (5 m*M*) were prepared in 0.1 *M* sodium acetate, pH 4.3, or sodium carbonate, pH 10.0, buffers. The CD samples were made by dissolving solid α-, β- or γ-CD in the eluent to give 2–10 m*M* solutions.

Procedures

A 26-cm jacketted column was filled with gel (27 ml) and equilibrated with the eluent buffer (2.5–100 m*M*, containing 100 μ*M* *p*-nitrophenol) until the UV absorbance, recorded at 280 nm (Uvicord 2138; LKB, Bromma, Sweden) was constant. An Ismatec mpge peristaltic pump (usually with a flow-rate of about 20 ml/h) was used and at intervals the flow was controlled by collecting eluent (40–80 ml) in a graduated cylinder. Usually a 0.25-ml sample containing 2–10 m*M* CD was introduced with a sample applicator[6]. Calibration of the UV monitor was carried out between appropriate time intervals with a 0.25-ml sample containing 1 m*M* PNP (*p*-nitrophenol/*p*-nitrophenolate) in the place of CD. This type of calibration is apparently more accurate than mere calibration of the absorbance scale because relatively small changes in high absorbances are to be monitored.

The equilibrium constants, *K*, for formation of the CD complexes were calculated from the areas of negative peaks (cut and weigh method) after comparison with the areas of calibration peaks at appropriate flow-rates, according to[2]

$$K = \frac{Q_{PNP\text{-}CD}}{[PNP]_0 \, (Q_{CD_t} - Q_{PNP\text{-}CD})}$$

where $[PNP]_0$ = initial concentration (μ*M*) of PNP, Q_{CD_t} = total amount (μmoles)

of CD applied and $Q_{PNP\text{-}CD}$ = total amount (μmoles) of complex formed. Thermodynamic values were calculated from the K values obtained at different column temperatures achieved by jacketting the column with a MGW Lauda constant-temperature circulating bath. The temperature was monitored to ± 0.1°C by a digital thermometer.

RESULTS AND DISCUSSION

Comparison of the gel materials

The properties of the gel support may affect the kinetics and the equilibria of the complex-forming system during the gel filtration process. Fig. 1 compares the Hummel–Dreyer elution profiles obtained with various gel materials under comparable conditions. The shape of the peaks on Sephadex G-25 is distinctly sharper and more symmetric than on Sephadex G-15 gel. This effect can be explained as a result of total exclusion of CD molecules from the interstitial space of the gel particles on Sephadex G-15, whereas on G-25 gel the CD molecules can penetrate somewhat into the gel pores. The diffusional distances on G-25 gel are shorter and the equilibria are therefore achieved more rapidly, as manifested in the shapes of the peaks. A part of this effect can, however, be attributed to the larger particle sizes of Sephadex G-15 (40–120 μm) than of Sephadex G-25 (25–40 μm) gels available to us. A deviation from the gaussian form can be seen both in the positive (CD) and negative (PNP-O^- deficient) peaks in the chromatogram on Sephadex G-15 (Fig. 1). This indicates a slowness to attain equilibria between CD and guest molecules or diffusional limitations. Calculated K values were the same within experimental errors on both gels.

Under the same conditions as in Fig. 1, Bio-Gel P-2 (particle size 80–150 μm) did not adequately resolve the peaks. Therefore, this gel was not studied further.

Closer inspection of the baseline, *e.g.*, in Fig. 1, revealed an elevated absor-

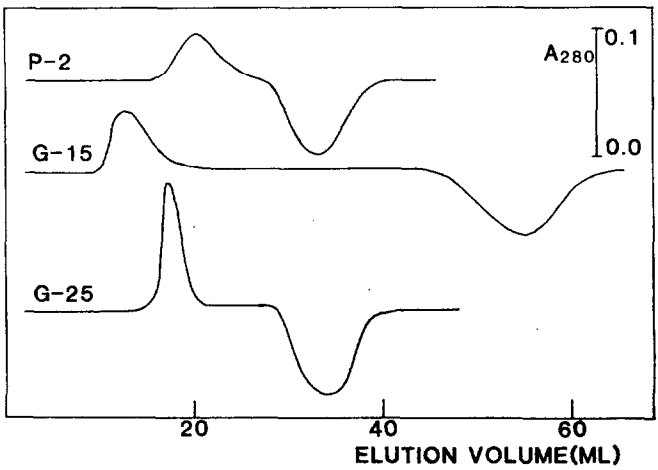


Fig. 1. Hummel–Dreyer elution profiles on Bio-Gel P-2, Sephadex G-15 and G-25 under the same chromatographic conditions. The column was equilibrated with 10 mM sodium carbonate, pH 10, containing 0.1 mM p-nitrophenolate. A 0.25-ml sample of 10 mM α-CD in the above buffer was chromatographed on a 26-cm column (27 ml of gel) at 23°C.

bance level between the positive and negative peaks. However, this occurred only occasionally and any logical deductions in respect of the experimental conditions used could not be made. Since it is difficult to imagine an equilibrium producing this kind of straight but elevated line and since the absorbance changes under consideration were only of the order of 0.002–0.004 units, the most probable explanation is simply a sluggish response of the electronics which did not occur in the optimal measuring range.

Factors affecting separation of the peaks

A prerequisite for the present method is that the positive and negative peaks can be adequately separated. In addition to the gel material itself, other experimental conditions affect the separation. Fig. 2 illustrates the effects of the concentration of the elution buffer. At low buffer concentrations the elution volume of the PNP-O^- deficient zone approximates that of the void volume of the column. This could be explained as due to insufficient mixing of the PNP-O^- deficient zone within the internal volume of the gel particles due to a Donnan equilibrium. Upon increasing the buffer concentration the Donnan equilibrium loses its significance; concomitantly, adsorption on the gel matrix increases.

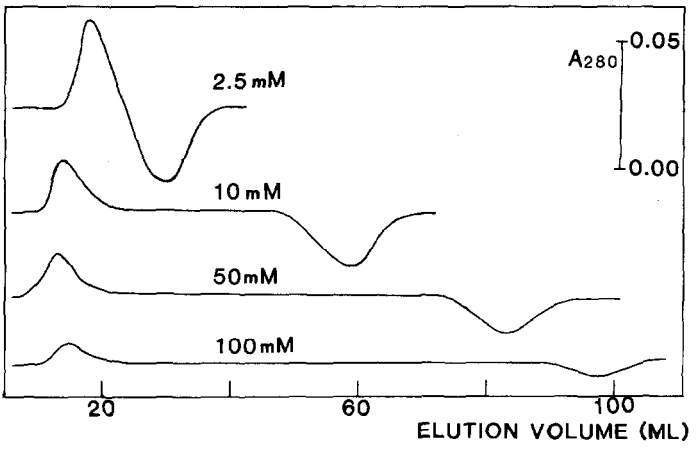


Fig. 2. Effect of the buffer concentration on Hummel–Dreyer elution profiles on Sephadex G-15. Other conditions as in Fig. 1.

The tendency of aromatic substances to be adsorbed onto Sephadex gels is well known[7] and the adsorption increases with cross-linking of the gel (see, *e.g.*, Fig. 1). The correlation between the elution volume of the negative peak and the buffer concentration, c, is very strong between 2.5 and 100 mM (Fig. 3). The elution volume seems to be a linear function of $\sqrt{\log c}$ and hence it approaches a maximum value. The solute–gel interaction is not completely hydrophobic in nature since an increase in the temperature results in a decrease in the elution volume of PNP-O^- (Fig. 4). With PNP-OH the decrease with increasing temperature is not as substantial (Fig. 4), which could indicate a more hydrophobic character of the interaction with the neutral compound.

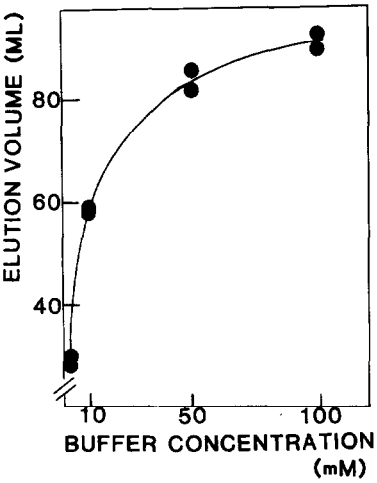


Fig. 3. The effect of the buffer concentration on elution volumes of *p*-nitrophenolate-deficient peaks. Chromatographic conditions are as in Figs. 1 and 2.

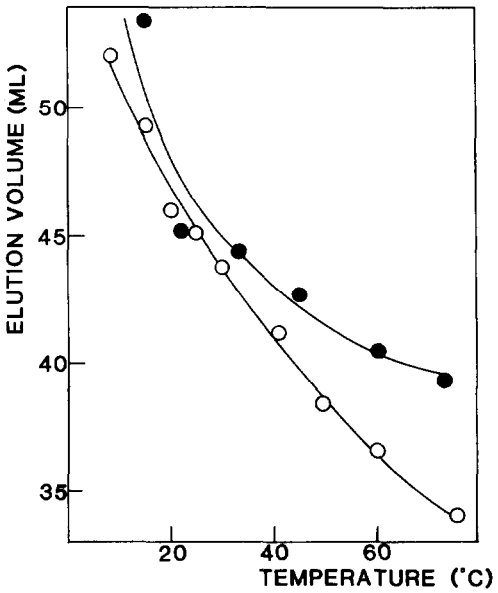


Fig. 4. The effect of temperature on the elution volumes of *p*-nitrophenol (●) and *p*-nitrophenolate (○)-deficient zones on Sephadex G-25. The eluent buffer was 10 m*M* sodium acetate, pH 4.3, or sodium carbonate, pH 10.0, respectively.

The elution volumes of α-, β- and γ-CDs are not exactly the same on Sephadex gels and this has been utilized to separate CDs[8]. The order of elution is not as expected on the basis of the molecular weights, and is apparently partly based on adsorption (see also Fig. 2). If CDs can be separated also as their PNP-O^- complexes, the present chromatographic system could be utilized as an analytical tool for the specific detection of individual CDs in the presence of non-complexing compounds.

Practical aspects of measuring equilibrium constants

In principle, either positive or negative peaks can be used to calculate equilibrium constants. The CDs did not absorb at the monitored wavelength, *e.g.*, in test runs in which the guest molecules were known not to bind to CDs a smooth line was recorded. These runs also confirm the appropriate preparation of the sample solutions. The proportion of the complex can be adjusted by changing the concentration of the guest solution[2]. If a photometer is used the accuracy may be hampered by high baseline absorbance (for low K values) of the solution. In the absence of impurities, the baseline can be lowered by measuring at a spectral region of lower absorptivity. For extreme cases the eluent can also be fractionated and diluted suitably before the measurement.

The positive peak is eluted rapidly as a relatively sharp peak whereas the negative peak is broadened. In the case of PNP-O^-, the generation of the CD complex shifts the wavelength of the absorption maximum of the intact compound (free PNP-O^-) by about 9 nm[9] and hence the areas of the positive and negative peaks are not identical (see Figs. 1, 2). The negative peak area indicates, however, in a simple manner the amount of the formed complex and this was used here.

By assuming that the dynamics of the system are in equilibrium and that the molar absorptivity of the free guest molecules is known at a certain wavelength, the molar absorptivity of the complexed guest can be simply calculated from the ratio of the areas of the positive and negative peaks. If the binding mode of the guest to CD does not alter in a series of experiments, the molar absorptivity calculated is also unchanged. However, if it changes there may be a fault in the measuring system or a change in the binding mode of the guest. In conclusion, the Hummel–Dreyer analysis yields in a single run results which are internally self-consistent. An example of the use of the ratio of the positive and negative peaks as an indication of a change in the binding mode is described below.

Flow-rate and sample concentration

The variation of the flow-rate of the eluent (0.1 mM PNP-O^- in 10 mM sodium carbonate buffer, pH 10.0 at 26°C) between 12.1 and 39.5 ml/h with α-CD and Sephadex G-25 yielded K values from 2320 to 2780 M^{-1} (mean 2580 M^{-1}, S.D. = 156 and n = 7). Within experimental errors, there was no correlation between the flow-rate and K values. The flow-rate also did not affect the elution volume of the negative peak (mean 44.3 ml, S.D. = 0.4 ml, n = 7). When the concentration of α- or β-CD was varied between 2 and 10 mg/ml there was no effect on the K values (flow-rate 20 ml/h, other conditions as above).

Effects of buffer concentration on K values

The buffer can affect the equilibrium constants either through solvent effects or directly by a competition between the buffer ions and the guest molecules. For example, an experiment in which complexation between α-CD and PNP-O^- were studied (Sephadex G-15 gel, 22°C, sodium carbonate buffer, pH 10) yielded K values (M^{-1}) of 4800 (10 mM), 2740 (50 mM) and 1000 (100 mM). Extrapolation to zero buffer of a plot of K against $\sqrt{c}$ yielded a K value of about 6700 M^{-1}. As indicated by Fig. 2, under the present conditions, *e.g.*, column length, the separation was usually not sufficient when the buffer concentration was below 10 mM. The present

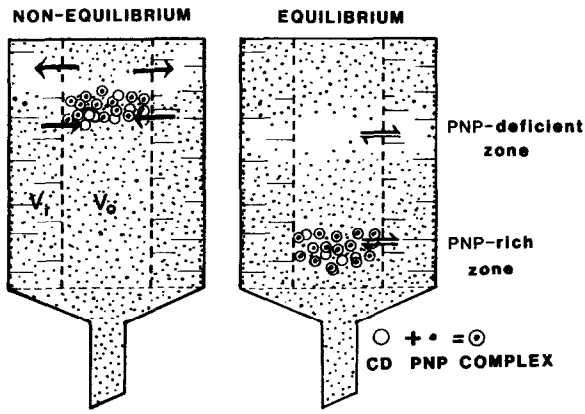
results show the importance of expressing equilibrium values of CDs at zero ionic strength. The justification of the $\sqrt{c}$ *vs.* K plots (see above) should also be studied in more detail, especially in milder buffers; therefore our results are to be regarded as pertaining mainly to 10 mM buffers.

Thermodynamics of the complexation

A change in the temperature within this system may affect the diffusional rates and equilibria of the chromatographic process itself or the rates and equilibria of the reaction between PNP and CDs. The rate constant for complex formation of small molecules like PNP with CDs is about 10^8 M^{-1} sec^{-1} which is near the diffusion-controlled rate[9] and hence cannot be limiting. Variation of the flow-rate had little effect on the elution volumes, peak shapes or the calculated equilibrium constants and therefore it is probable that the molecules in the positive and negative zones were approximately in dynamic equilibrium. Thus, we conclude that a change in the temperature only alters the capacity of CD molecules to encapsulate PNP molecules.

Scheme 1 illustrates the above situation prevailing in the column. The hypothetical column consists of two tubes, one inside the other. The wall of the inner one is assumed to contain holes permeable only to PNP molecules, *e.g.*, as in a dialysis tube. The space, V_i, between the two tubes which is not accessible to CD molecules describes the internal volume of the gel particles. In addition, a pool of adsorbed PNP on, *e.g.*, the outer tube might be assumed. So, any slow attainment of equilibrium should result in non-uniform flow-rates of PNP molecules in the inner (V_0) and outer (V_i) volumes and this should be manifested as large asymmetric peaks. In principle, a similar situation should pertain if many tubes of decreasing pore diameters are assumed and if CD molecules can enter the first of these.

Fig. 5 shows Van 't Hoff plots for the binding of PNP-O^- to the various CDs. The experimental points for α-CD lie on one line over the whole temperature range. The corresponding line for β-CD shows a point of discontinuity at 42°C. Interestingly, at the same point a change in the areas of the positive (A^+) and negative (A^-) peaks occurred. The ratio A^+/A^- was 1.1 (S.D. = 0.06, n = 3) for the line β_1 and



Scheme 1.

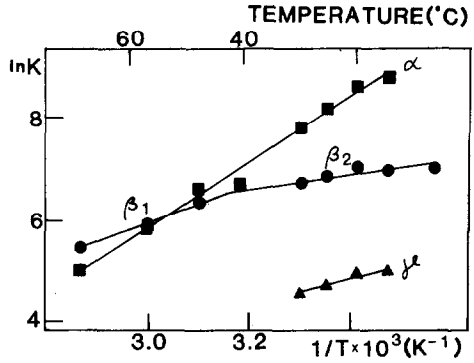


Fig. 5. Van 't Hoff plots for binding of *p*-nitrophenolate to various CDs (indicated) as measured by gel chromatography on Sephadex G-25 with 10 m*M* sodium carbonate buffer, pH 10.0.

0.9 (S.D. = 0.11, n = 5) for β_2 (Fig. 5). For α-CD the ratio A^+/A^- was 0.8 (S.D. = 0.16, n = 11) and could be considered as constant over the temperature range. The Van 't Hoff plots together with the A^+/A^- ratios strongly indicate that the binding mode of PNP-O^- changes at about 40°C, and this is also reflected in the UV-absorption properties of the complexed PNP-O^-. The complexation of γ-CD with PNP-O^- was weaker (Fig. 5). Similar curves to those presented in Fig. 5 were obtained with PNP-OH, but there was no discontinuity of the line for β-CD.

Table I shows thermodynamic values for the complexation of PNP and CDs calculated from the lines exemplified in Fig. 5. It is seen that, in general, the strength of binding decreases with increasing size of the CD cavity, *i.e.*, in the order α-, β- and γ-CD. Furthermore, PNP-OH exhibits considerably weaker binding than PNP-O^-. This is rather surprising because the driving force for the complexation by CDs has usually been assumed to be hydrophobic in nature[5]. The stronger binding of PNP-O^- could be due to an effective delocalization of free electrons over the molecule and possibly this causes the molecular structure to be more flexible and thus it can be better accommodated in the cavity of α-CD.

Table I indicates that despite the fact that the standard free energy values for

TABLE I

THERMODYNAMIC VALUES FOR BINDING OF *p*-NITROPHENOL OR *p*-NITROPHENOLATE WITH CYCLODEXTRINS IN 10 m*M* SODIUM ACETATE, pH 4.3, OR 10 m*M* SODIUM CARBONATE, pH 10, BUFFERS

For abbreviations β_1 and β_2, see Fig. 5; r is the correlation coefficient of the Van 't Hoff plot.

CD	*Ionic form*	K_{298K} (M^{-1})	$\Delta G°$ (kJ mol^{-1})	$\Delta H°$ (kJ mol^{-1})	$\Delta S°$ (J mol^{-1} °K^{-1})	r
α	PNP-O^-	3550	−20.3	−56.1	−120	0.995
α	PNP-OH	177	−12.8	−24.7	− 39.8	0.993
β_1	PNP-O^-	1560	−18.2	−32.2	− 47.0	0.995
β_1	PNP-OH	301	−14.1	−14.6	− 1.39	0.981
β_2	PNP-O^-	944	−17.0	− 9.98	+ 23.5	0.969
γ	PNP-O^-	119	−11.8	−21.8	− 33.5	0.960
γ	PNP-OH	61.9	−10.2	−43.6	−112	0.994

TABLE II

COMPARISON OF REPORTED THERMODYNAMIC VALUES FOR THE COMPLEXATION OF *p*-NITROPHENOL TO CYCLODEXTRINS

Some of the figures are calculated from literature values.

CD	*Ionic form*	K_{298} (M^{-1})	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} $°K^{-1}$)	*Remarks*	*Ref.*
α	PNP-OH	292	−14.1	−17.6	−11.8	14°C, phosphate	14
α	$PNP-O^-$	2300	−19.2	−30.1	−36.6	(pH 3.5 or 11, $I = 0.5M$), spectrophotometer	
α	$PNP-O^-$	2300	−19.2	−37.8	−62.6	25°C, phosphate	12
β	$PNP-O^-$	563	−15.7	−14.2	+ 5.06	(pH 11, $I = 0.3$ M), calorimeter	
α	PNP-OH	126	−12.1 ± 1.7	−30.5 ± 6.3	−62.8	25°C, water,	15
β	PNP-OH	1000	−17.2 ± 9.6	−43.9 ± 10.5	−87.9	calorimeter	
α	PNP-OH	18.9	− 7.28			25°C, phosphate	11
α	$PNP-O^-$	2500	−19.4 ± 0.5			(pH 6.5 or 11, $I = 0.3$ M) spectrophotometer	
α	$PNP-O^-$	1590	−18.3 ± 0.2			ORD	
α	$PNP-O^-$	2700	−19.6 ± 0.1			23°C, NMR	
α	PNP-OH	219	−13.3	−30.5 ± 1.3	−58.6 ± 4.2	24.7°C, pH 6.8,	10
α	$PNP-O^-$	2180	−19.0	−46.9 ± 1.7	−92.0 ± 4.2	spectrophotometer	

complexation of PNP-O^- and CD calculated separately for the lines β_1 and β_2 (Fig. 5) do not differ significantly from each other, the enthalpy term is advantageous for the complexation at high temperatures while the entropy term is not. At low temperatures the enthalpy decreases drastically but this is largely compensated by a positive change in the entropy. Thus, it can easily be calculated that at lower temperatures about 40% of the stabilization of the complex is due to the entropy.

Comparison of thermodynamic values with literature data

Reported thermodynamic values for the complexation of PNP with CDs are listed in Table II. It is obvious the values are not always consistent. In many cases this can be explained by the different experimental conditions employed, *e.g.*, different buffers and ionic strengths. The hydration state of the weighed CD sample can also affect the results[10].

Bergeron *et al.*[11] determined K values for the complexation of PNP-O^- and α-CD by three different methods (UV, NMR and ORD, Table II). These values are in approximate agreement with ours (3550 M^{-1}, Table I). In spite of the different ionic strength used by Takeo and Kuge[12], their values are also in fairly good accord with ours. The present results agree most closely with those of Gelb *et al.*[10], but unfortunately that report lacks details as to the buffer employed. In conclusion, the present gel chromatographic results usually correlate well with the average of those reported in the literature.

Analysis of elution volumes of guest molecules on immobilized CDs

The equilibria of CDs with various guest molecules were also studied both with the Hummel–Dreyer method and by elution analysis of the same compounds on Bio-Gel P-2 gels on which α- or β-CDs were covalently linked. Both studies were made by using 0.1 M phosphate buffer, pH 6.8, supplemented with NaCl to suppress any retardation of the compound by the ion-exchange mechanism. Plots of the elution volumes against the K values obtained by gel chromatography yielded straight lines. This result provides additional support for the validity of the Hummel–Dreyer method. The linear correlation was extended to K values over 1000 M^{-1} even though such values cannot be practically measured on immobilized CDs due to the very large elution volumes. In optimal cases, gel chromatography was applicable down to K values of about 20–50 M^{-1}. Assuming that the linear correlation can be extended to below the values of the lowest standard points, equilibrium constants as small as 1–5 M^{-1} can be obtained with immobilized CDs. Details of the above experiments are described elsewhere[13].

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