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Preferential Inclusion of Nitrophenoxide Ion over Its Conjugate Acid in Polycationic Cyclodextrin: pK_a Decrease of Nitrophenols by Host-Guest Interaction with Diethylaminoethyl-Cyclodextrins

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Abstract. Diethylaminoethyl (DEAE) groups were introduced into α-cyclodextrin (α-CD) and β-CD. The products, DEAE-CDs with various numbers of substituents, trapped *p*-nitrophenoxide and 3,4-dinitrophenoxide preferentially over their respective conjugate acids. This caused a decreased pK_a of the guest nitrophenol. Some signals of the proton-NMR of both guest nitrophenol and host DEAE-CD changed upon host-guest interaction. The relation between the number of DEAE groups and the extent of the pK_a decrease, as well as association constants, is discussed.

Key words: Cyclodextrin, diethylaminoethyl, nitrophenol, pK_a decrease.

1. Introduction

Cyclodextrins (CDs) are cyclic oligomers of α-1,4-D-glucoside; α-CD, β-CD and γ-CD being hexamer, heptamer and octamer, respectively. The CD molecule is doughnut shaped, its outside being hydrophilic and its inside hydrophobic, forming a cavity in which a number of hydrophobic as well as hydrophilic molecules of appropriate sizes are known to be trapped as guest molecules [1]. This phenomenon has been used to solubilize insoluble aromatic compounds in an aqueous medium or to confer catalytic activity on CDs [2–5]. In this study, cationic diethylaminoethyl (DEAE) groups were introduced into α-CD and β-CD to produce DEAE-CDs which are capable of lowering the pK_a of guest nitrophenol. The practical application of this phenomenon to the rate assay of enzymes of diagnostic importance will be discussed.

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2. Experimental

2.1. Introduction of DEAE Groups to CD

The procedure used to prepare DEAE-cellulose [6] was applied to derivatize CD. To a mixture of 9 g of α-CD in 20 g of 25% aqueous NaOH, 9.7 g of 64% aqueous (2-chloroethyl)diethylammonium chloride was added dropwise with vigorous stirring. The stirring was continued at about 45° C for 3 h, and the mixture directly applied to a column (22 × 400 mm) of Sephadex G-15, H_2O being the elution solvent. Elution of DEAE-CD, (2-chloroethyl)diethylamine (which may have been partially converted to diethylaminoethanol, but not confirmed), and NaOH was monitored by the pH of the effluent. Elution of DEAE-CD was also monitored by the refractive index. The DEAE-CD was eluted between 48 and 90 mL, whereas (2-chloroethyl)diethylamine and NaOH were eluted later from the column. The eluted DEAE-CD was neutralized with HCl and concentrated by ultrafiltration through a Diaflo YC-05 membrane. The concentrate was repeatedly diluted with water and concentrated to remove low molecular mass impurities. The concentrate was made alkaline with NaOH solution, rechromatographed on the same column, and vacuum dried to obtain white fluffy matter. Additional DEAE groups were introduced into the product by repeated diethylaminoethylation to obtain $DEAE_n$-α-CD of various n. $DEAE_n$-β-CDs were produced similarly. DEAE-CDs were readily soluble in water. Highly substituted DEAE-CDs were hygroscopic. $DEAE_{17}$-α-CD, but not its HCl salt, turned brown on storage. The products dried *in vacuo* contained persistent humidity which was determined by gravimetric analysis after drying at 105° C.

The number (n) of DEAE groups introduced into the CD molecule was estimated by elemental analysis and acid-base titration with 5 mM H_2SO_4.

2.2. Proton-NMR

Spectra were recorded with a JEOL GSX-400 instrument using dimethyl-d_6 sulfoxide or 20 mM phosphate buffer in ^2H_2O, and the chemical shift (δ) was expressed in ppm. The standard signals in these solvents were $\delta = 2.49$ in dimethyl-d_6 sulfoxide and $\delta = 4.80$ in ^2H_2O.

2.3. Estimation of pK_a of Nitrophenols

Nitrophenols are colorless in the nonionized (conjugate acid) form, and absorb light at 400 nm in the ionized (conjugate base) form. The millimolar absorbance differences (phenoxide-minus-phenol) at 400 nm (18.5 for nitrophenol and 13.6 for 3,4-dinitrophenol) were not greatly influenced by the addition of $DEAE_n$-CD of $n < 10$ (see Figure 2). The absorbances at 400 nm of nitrophenol solutions (0.05 mM) at various pHs in 0.1 M acetate, succinate, or citrate buffer, were determined at 20° C in the presence of DEAE-CD, the ratios of the conjugate

TABLE I. Results of elemental analysis of DEAE$_n$-CDs.

Sample n	H_2O[a] (%)	found C	H	N	calculated C	H	N
α							
1.7	2.2	46.8	7.8	2.2	47.5	7.3	2.0
2.5	2.3	48.7	7.6	2.9	49.0	7.7	2.8
3.7	5.6	48.6	8.1	3.9	49.2	8.3	3.7
4.8	5.1	50.7	8.3	4.4	51.0	8.6	4.4
6.2	9.5	51.0	8.7	4.8	50.1	9.1	4.9
11	11.0	53.0	9.6	6.3	52.9	10.0	6.6
17	12.0	55.2	10.8	8.0	54.9	10.7	7.9
β							
5.0	3.0	51.3	8.8	4.4	51.4	8.4	4.4
14.4	2.8	n.d.	n.d.	7.6			7.6

[a] H_2O content was measured gravimetrically.

base/acid concentrations calculated, and the pK_a of nitrophenol calculated with the Henderson–Hasselbach equation:

$$pK_a = pH - \log_{10}([\text{conjugate base}]/[\text{conjugate acid}]) .$$

The pK_a of p-nitrophenol in the presence of DEAE$_n$-α-CD of $n > 10$ as a host molecule was estimated similarly as above, but the absorbances at 403 nm were measured instead, at which wavelength the millimolar absorbance difference of p-nitrophenol (18.4) is not changed in the presence of the host molecule (see Figure 2).

3. Results

3.1. Estimation of the Number (n) of DEAE Groups Introduced into CD

Figure 1 shows the pH-titration curves of some products. DEAE$_n$-CDs of lower n had sharp end points, whereas those of higher n had rather diffuse end points due to the presence of spatially clouded DEAE groups of different pK_as. The number (n) of DEAE groups estimated by the titration was confirmed by elemental analysis (Table I). For DEAE$_n$-CDs of $n > 12$, the elemental analysis could not distinguish the difference of n by 1 (i.e., nitrogen contents calculated for $n = 13$, 14, and 15 of DEAE$_n$-α-CDs are 8.05, 8.30, and 8.54, respectively), and hence the results of the titration were preferred.

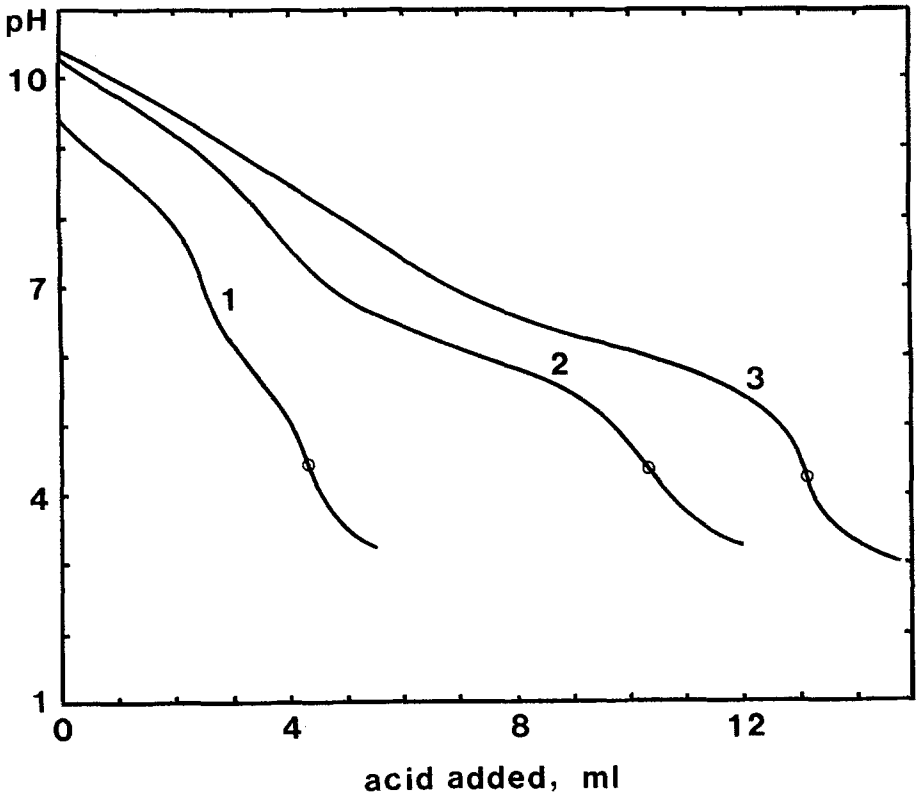

Fig. 1. pH titration curves of DEAE-α-CDs with 5 mM H_2SO_4. Curve 1: 21.4 mg of product 1 (water content: 2.3%), curve 2: 21.7 mg of product 2 (water content: 11%), and curve 3: 23.3 mg of product 3 (water content: 12%). The end points of titration are marked by circles. From these results, the number (n) of the DEAE groups of products 1, 2, and 3 were calculated to be 2.5, 11, and 17, respectively.

3.2. NMR Spectra of DEAE-CD in Dimethyl-d_6 Sulfoxide

α-CD had signals similar to those reported for β-CD [7]: δ (1-H) = 4.78 (1 proton, d, J = 3.3), δ (2-H) = 3.27 (1 proton, m), δ (3-H) = 3.76 (1 proton, t, J = 9.2; d, J = 2.9), δ (4-H) = 3.37 (1 proton, t, J = 9.0), δ (5-H) = 3.57 (1 proton, d, J = 9.9), δ (6-H) = 3.63 (2 protons, m), δ (2-OH) = 5.51 (1 proton, d, J = 7.3), δ (3-OH) = 5.42 (1 proton, d, J = 2.9), and δ (6-OH) = 4.47 (1 proton, t, J = 5.9). A signal due to humidity appeared at δ = 3.32 [8]. Of these signals, δ (1-H) is well separated from the others, and its intensity is independent of the substituents. When DEAE groups were introduced, triplet signals of methyl-H (the major signal at 0.95 and the minor one at 1.18) appeared, the integrated intensity thereof being proportional to the number of substituent groups. At the same time, δ (2-OH), δ (3-OH), and δ (6-OH) diminished and δ (1-H) split into two signals (the major signal at 4.79 and the minor one at 4.96), indicating heterogeneous and

non-regiospecific substitution of the DEAE groups to 2-OH, 3-OH, and 6-OH. The chemical shift of 6-OH of highly substituted DEAE$_n$-CDs ($n > 10$) became almost invisible, indicating complete diethylaminoethylation at 6-OH.

For DEAE$_{1.7}$-α-CD, the ratio of the integrated signal intensity of methyl-H ($\delta = 0.95$ and 1.18) to that of 1-H ($\delta = 4.79$ and 4.96) was 1.75. As the number of the methyl-H of the DEAE group and that of 1-H of α-CD are equal, n is equal to the ratio, 1.75, the value being in agreement with that obtained by elemental analysis and titration. For DEAE$_{4.8}$-α-CD, n was estimated to be 5.0 by NMR. The chemical shift of 1-H became broader for DEAE$_n$-α-CD of higher n, which prevented accurate quantitation of the integrated intensity.

β-CD had signals in agreement with an earlier report [7]: δ (1-H) = 4.81 (1 proton, d, $J = 2.5$), δ (2-H and 4-H) = 3.32 (overlapped with a signal due to humidity [8]), δ (3-H and 6-H) = 3.62 (3 protons, m), δ (5-H) = 3.55 (1 proton, d, $J = 11.2$), δ (2-OH) = 5.70 (1 proton, d, $J = 6.8$), δ (3-OH) = 5.65 (1 proton, s), and δ (6-OH) = 4.43 (1 proton, t, $J = 5.6$). The changes of NMR signals, i.e., the appearance of δ (methyl-H), the split of δ (1-H), and the decrease and broadening of δ (2-OH), δ (3-OH), and δ (6-OH) on introduction of DEAE groups to β-CD, were similar to those observed for α-CD.

3.3. NMR Spectroscopy of Host-Guest Interaction in ^2H_2O

p-Nitrophenoxide at p^{2}H 9.0 had two signals: δ (o-H) = 6.55 (2 protons, d, $J = 7.3$) and δ (m-H) = 8.09 (2 protons, d, $J = 7.3$). The NMR spectrum of α-CD was the same as that reported by other workers [8, 9]. When equimolar amounts of α-CD or DEAE-α-CD was added to p-nitrophenoxide (15 mM), δ (m-H) moved downfield significantly, but δ (o-H) moved little (Table II) as has been reported earlier [10–12]. The chemical shifts of α-CD were less influenced except for $\delta = 3.99$ (3-H) which moved upfield by 0.15, in agreement with earlier papers [9–13]. Diethylaminoethanol (170 mM, p^{2}H 9.0), i.e., unbound DEAE group, had little effect in altering the chemical shifts of aromatic protons of free p-nitrophenoxide or that trapped in α-CD.

3,4-Dinitrophenoxide at p^{2}H 8.2 had three signals: δ (2*-H) = 6.67 (1 proton, s), δ (5*- H) = 8.04 (1 proton, d, $J = 9.3$), and δ (6*-H) = 6.63 (1 proton, d, $J = 9.3$); here, the position numbers on the aromatic ring have been asterisked. β-CD had five well-separated signals similar to those of α-CD: δ (1-H) = 5.08 (1 proton, d, $J = 3.6$), δ (2-H) = 3.66 (1 proton, dd, $J = 9.8$, $J = 3.8$), δ (3-H) = 3.98 (1 proton, t, $J = 9.4$), δ (4-H) = 3.59 (1 proton, t, $J = 9.2$), δ (5-H and 6-H) = 3.89 (3 protons, m). On mixing 15 mM 3,4-dinitrophenoxide with 15 mM β-CD or DEAE-β-CD, only δ (2*-H) moved upfield in the aromatic region (Table II). Chemical shifts of 3-H and 5-H in β-CD moved upfield by 0.09 and 0.13, respectively, leaving those of the other protons little affected. Diethylaminoethanol (66 mM, p^{2}H 8.2) had little effect in altering the chemical shifts of aromatic protons of 3,4-dinitrophenoxide.

TABLE II. Change in the chemical shifts (δ in ppm) of aromatic protons upon host-guest interaction.

Free *p*-nitrophenoxide	On inclusion in		Free 3,4-dinitrophenoxide	On inclusion in	
	α-CD	$DEAE_{11}$-α-CD		β-CD	$DEAE_{5.0}$-β-CD
	$\Delta\delta$	$\Delta\delta$		$\Delta\delta$	$\Delta\delta$
o-H ($\delta = 6.55$)	+0.10	+0.04	2*-H ($\delta = 6.67$)	−0.08	−0.06
m-H ($\delta = 8.09$)	+0.30	+0.22	6*-H ($\delta = 6.63$)	+0.03	+0.01
			5*-H ($\delta = 8.04$)	+0.03	+0.01

The concentrations of the hosts and the guests were 15 mM. p^2H was 9.0 for *p*-nitrophenoxide and α-CD or DEAE-α-CD, and 8.2 for 3,4-dinitrophenoxide and β-CD or DEAE-β-CD. The temperature was 20° C.

3.4. Effect of $DEAE_n$-CD on the Absorption Spectra of Nitrophenoxide

Figure 2 illustrates the effect of CD or cationic CDs on the absorption spectra of *p*-nitrophenoxide and 3,4-dinitrophenoxide. The absorption maxima of nitrophenoxides at 400 nm were red-shifted and deepened when trapped in CD or cationic CD. The millimolar absorbances of *p*-nitrophenoxide was 18.6 (at 400 nm), 20.1 (at 408 nm with 1% α-CD), 20.1 (at 410 nm with 1% $DEAE_{4.8}$-α-CD), 20.2 (at 413 nm with 1% $DEAE_{11}$-α-CD), and 22.0 (at 418 nm with 1% $DEAE_{17}$-α-CD), and those of 3,4-dinitrophenoxide was 13.9 (at 399 nm), 14.2 (at 404 nm with 1% β-CD), and 14.6 (at 406 nm with 1% $DEAE_{14.4}$-β-CD). The millimolar absorbances of *p*-nitrophenol and 3,4-dinitrophenol at 400 nm was 0.07 and 0.26, respectively, and little influenced by the addition of cationic CDs.

3.5. Effect of n of $DEAE_n$-CD on pK_a of Guest Nitrophenol

$DEAE_n$-α-CDs (1%) of various n lowers the pK_a (7.14) of *p*-nitrophenol in 0.1 M acetate, succinate, or citrate buffers, as shown in Figure 3. $DEAE_n$-α-CDs worked best in acetate buffer. The maximum effect of pK_a lowering was observed at the highest n. Similarly $DEAE_n$-β-CDs lowers the pK_a (5.38) of 3,4-dinitrophenol to 5.04 and 4.08 in the presence of 1% β-CD and $DEAE_{14.4}$-β-CD, respectively, in 0.1 M acetate. The effects of pK_a lowering of cationic β-CDs were less prominent in succinate or in citrate buffer. Diethylaminoethanol (60 mM) had no effect on lowering the pK_as of free nitrophenols or those trapped in α-CD or β-CD.

3.6. Effect of Concentration of DEAE-CD

The pK_as of *p*-nitrophenol and 3,4-dinitrophenol in the presence of DEAE-α-CD and DEAE-β-CD, respectively, in different concentrations, were measured and are plotted in Figure 4, from which the equilibrium constants were calculated (see

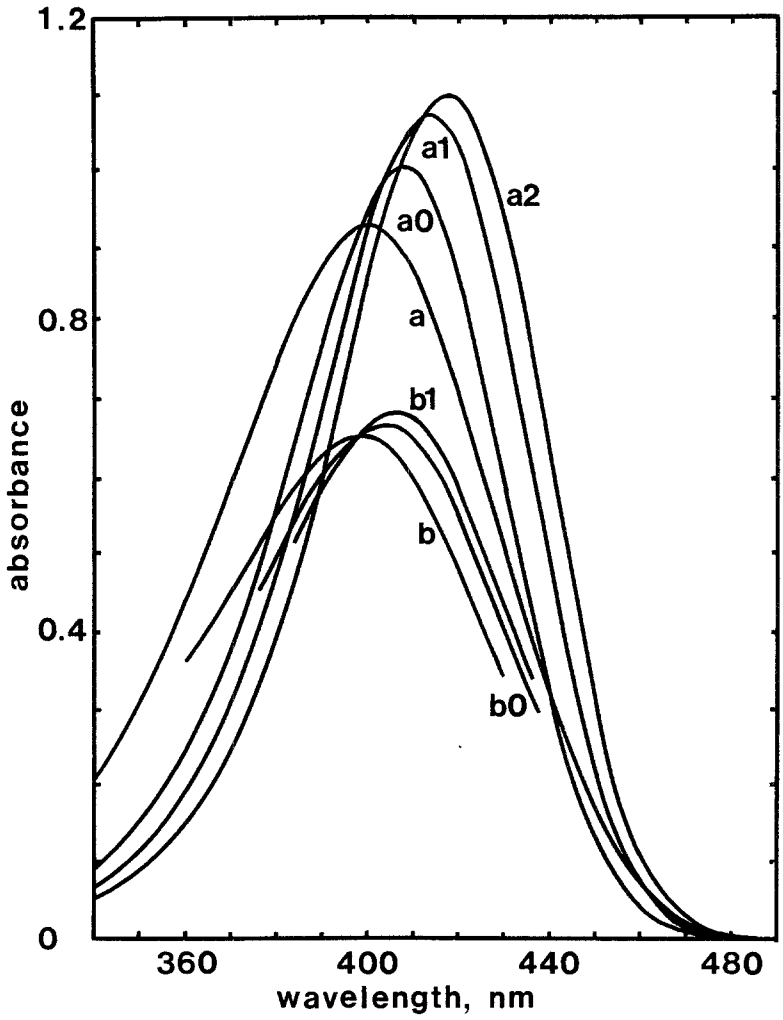

Fig. 2. Absorption spectra of nitrophenoxides in the presence of 1% DEAE$_n$-CDs of different n. Curve a: p-nitrophenoxide at pH 9.5, curve a0: with α-CD at pH 8.9, curve a1: with DEAE$_{11}$-α-CD at pH 8.3, and curve a2: with DEAE$_{17}$-α-CD at pH 8.2. Curve b: 3,4-dinitrophenoxide at pH 8.7, curve b0: with β-CD at pH 8.1, and curve b1: with DEAE$_{14.4}$-β-CD at pH 7.0. Only the curves near the peak are illustrated for curves b, b0, and b1 for clarity. All measurements were made in 0.1 M acetate buffer.

Appendix), and are shown in Table III. Figure 4 shows the pK_a curves calculated by Equation 8 (Appendix), which are in excellent agreement with the plots. The standard deviation of the plots of pK_as from the curve in Figure 4 was 0.02 pK_a unit for α-CD and less in the others.

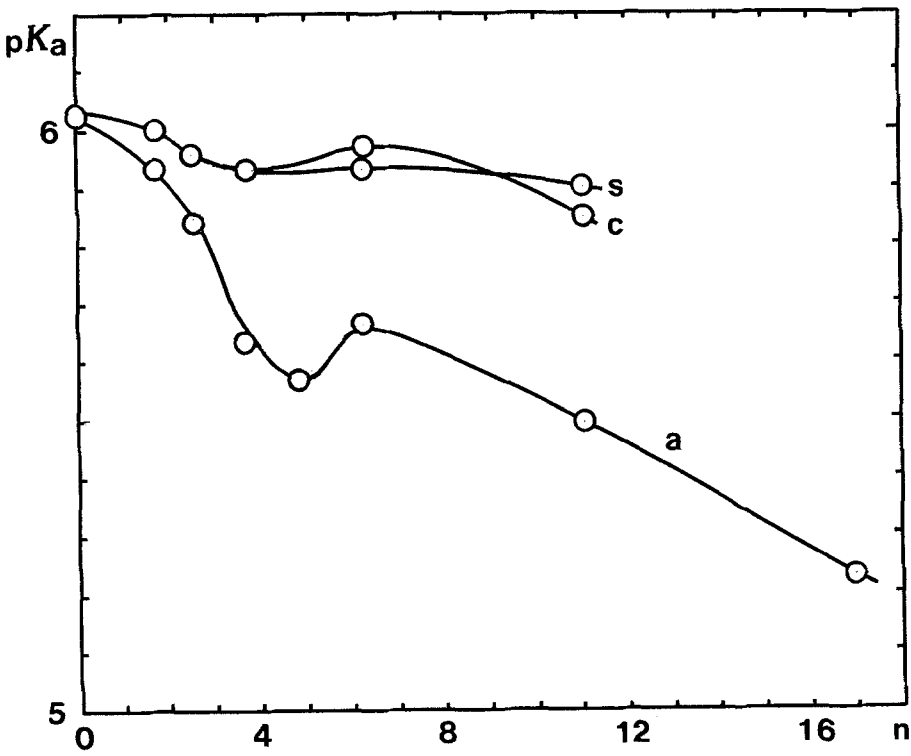

Fig. 3. pK_a of *p*-nitrophenol in the presence of 1% $DEAE_n$-α-CDs of different n, in 0.1 M acetate (a), succinate (s), and citrate (c) buffers at 20° C. The pK_a of free *p*-nitrophenol in these buffers was 7.14.

TABLE III. Association constants of nitrophenols with DEAE-CDs.

p-Nitrophenol ($pK_1 = 7.14$) with $DEAE_n$-α-CD				3,4-Dinitrophenol ($pK_1 = 5.38$) with $DEAE_n$-β-CD			
n	pK_2	K_3 (M^{-1})	K_4 (M^{-1})	n	pK_2	K_3 (M^{-1})	K_4 (M^{-1})
0	5.81	1.5×10^2	3.2×10^3	0	4.98	3.2×10^2	8.0×10^2
4.8	5.47	6.0×10^2	2.8×10^4	5.0	4.20	4.7×10^2	7.2×10^3
6.2	5.58	7.3×10^2	2.7×10^4	14.4	3.67	1.7×10^2	8.8×10^3
11	5.33	6.7×10^2	4.3×10^4				
17	5.10	1.4×10^3	1.5×10^5				

All measurements at 20° C. pK_1 is pK_a of free nitrophenol, pK_2 is pK_a of the guest nitrophenol trapped in the host, K_3 is the association constant for the guest nitrophenol and the host, and K_4 is the association constant for the guest nitrophenoxide and the host. See Appendix for details.

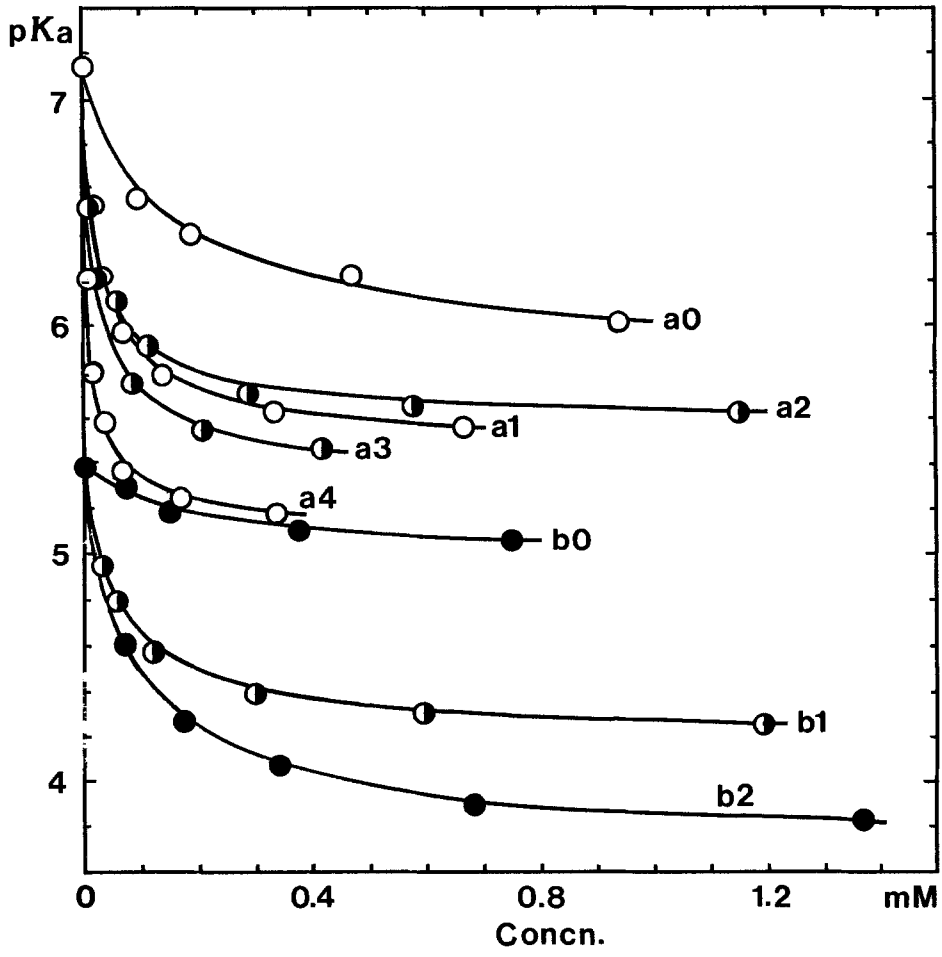

Fig. 4. pK_a of *p*-nitrophenol (○ and ◑) in the presence of α-CD (a0), $DEAE_{4.8}$-α-CD (a1), $DEAE_{6.2}$-α-CD (a2), $DEAE_{11}$-α-CD (a3), and $DEAE_{17}$-α-CD (a4), and that of 3,4-dinitrophenol (● and ◑) in the presence of β-CD (b0), $DEAE_{5.0}$-β-CD (b1), and $DEAE_{14.4}$-β-CD (b2) at various concentrations in 0.1 M acetate buffer at 20° C. Observed values are plotted, and the curves calculated by Equation 8 (Appendix) are shown for comparison.

4. Discussion

Cationic DEAE-CDs were prepared by introducing DEAE groups into α-CD and β-CD. Due to the heterogeneous nature of the number and location of the substituents, the proton-NMR signals of DEAE-CD could not be assigned, except for methyl-H in the DEAE group and the anomeric 1-H of CD.

On inclusion of *p*-nitrophenoxide in α-CD, in which the nitrophenyl moiety is located in the cavity with its phenoxide end protruding from the secondary hydroxyl side of the host molecule [11, 12, 14], δ (*m*-H) moved downfield more significantly

than δ (o-H) [11, 12, and Table II]. The inclusion geometry of p-nitrophenoxide in $DEAE_{11}$-α-CD may be similar, since δ (m-H) moved downfield more significantly than δ (o-H) as in the case with unsubstituted α-CD.

The inclusion geometry of 3,4-dinitrophenoxide in β-CD has never been reported. Proton-NMR shows that only δ (2*-H) moved upfield when the anion was trapped in β-CD or DEAE-β-CD, whereas δ (5*-H) and δ (6*-H) remained little affected (Table II). This probably means that only 2*-H is under the shielding effect of the host, and 5*-H and 6*-H are either outside the cavity or in the aquatic environment, not much different from that in the free anion. The mode of the host-guest interaction, however, may be different from that observed for p-nitrophenoxide and α-CD, where the chemical shifts of aromatic protons move downfield.

DEAE-CDs work best in acetate buffer to lower the pK_as of nitrophenols, but succinate and citrate were inhibitory (Figure 3) due to competition with the guest for the CD cavity. Acetate ion is known not to be trapped in α-CD [2].

The modifications of CDs so far reported have had modest effects in improving the affinity (K_{assoc}) of CDs to their host anions. A glucosyl or maltosyl group introduced into α-CD only slightly modified the affinity of α-CD for p-nitrophenoxide [12]. Permethylation of α-CD caused 7.6-fold and 10-fold increases in affinities to p-nitrophenoxide and p-nitrophenol, respectively [11]; hence this is not selective to the conjugate base.

Cationic groups such as 2-(2-aminoethylamino)ethylamino [15], trimethylamino [16], and imidazolyl [17] groups introduced into β-CD caused a 1.2–2.6 fold increase in affinities to aromatic anions such as p-nitrophenoxide, p-methylcinnamate, and methyl orange. Reported K_{assoc}s between p-nitrophenol or p-nitrophenoxide and unmodified α-CD shown in Table IV are comparable with those estimated in the present study for nitrophenols and nitrophenoxides with unmodified CDs ($n = 0$ in Table III). On the other hand, DEAE-CDs have a much higher affinity for nitrophenoxides than unmodified CDs (K_4 in Table III). In particular, the affinity of $DEAE_{17}$-α-CD for p-nitrophenoxide ($K_{assoc} = 1.5 \times 10^5$ M^{-1} or $K_{dissoc} = 6.5 \times 10^{-6}$ M) is comparable to, or even stronger than those of many enzymes for their specific substrates. This is the reason why the pK_a of the guest nitrophenol decreases when trapped in polycationic CD with a suitable cavity size. Since free diethylaminoethanol added to unsubstituted CD has no effect on lowering the pK_a of nitrophenol, covalent modification of CD with cationic groups is essential for this effect. The pK_a of a free functional group such as that of an amino acid side chain is known to be significantly altered when placed in an environment such as in a protein [18]. The phenomenon presented in this study can be considered as a model for discussing the effect of environment on the properties of functional groups.

In general, $DEAE_n$-α-CD of higher n exerted a stronger effect in lowering the pK_a of p-nitrophenol, but $DEAE_{6.2}$-α-CD is exceptionally less effective than $DEAE_{4.8}$-α-CD (Figure 3). This is due to the fact that additional DEAE groups introduced to $DEAE_{4.8}$-α-CD eventually strengthened the affinity for p-nitrophenol

TABLE IV. Comparison of the reported association constants for host-guest interaction of *p*-nitrophenol and *p*-nitrophenoxide with unsubstituted α-CD.

p-Nitrophenol	*p*-Nitrophenoxide	Method; Conditions	Reference
(M^{-1})	(M^{-1})		
0.2×10^2	2.5×10^3	UV-vis.; phosphate ($I = 0.5$), 25° C	10, 13
	2.7×10^3	NMR; ^2H_2O, 23° C	10
	1.6×10^3	optical rotation; 25° C	10
3.8×10^2	3.7×10^3	UV-vis.; phosphate ($I = 0.1$), 14° C	2
1.7×10^2	1.6×10^3	NMR; ^2H_2O, p^{2}H adjust without buffer	11, 12
2.0×10^2	3.4×10^3	UV-vis.; not specified	11
1.5×10^2	3.2×10^3	pK_a change; acetate (0.1 M), 20° C	This work

(K_3), without improving that for *p*-nitrophenoxide (K_4), as shown in Table III. Since the effect of the host CD in lowering the pK_a of the guest depends on the K_4/K_3 ratio, a more efficient cationic CD to lower the pK_a of guest nitrophenol may be synthesized by designing a cationic substituent which has less affinity for nonionized molecules. Lowering the pK_a of nitrophenol is of practical importance in the assay of diagnostic enzymes. It is a common practice to monitor the enzymatic liberation of nitrophenol from a colorless artificial substrate in the assay of these enzymes. In the case when the optimum pH of the enzyme is lower than the pK_a of nitrophenol, its spectroscopic estimation by the addition of alkali interrupts the enzymatic reation. Lowering the pK_a of nitrophenol by the addition of cationic CD will enable us to monitor its enzymatic liberation without interrupting the reaction. Use of DEAE-CD in the rate assay of diagnostic enzymes such as *N*-acetyl-β-D-hexosaminidase [19] or acid phosphatase will be presented elsewhere.

5. Appendix

The following equilibria are attained in the mixture containing the guest (nitrophenol) and the host (CD or DEAE-CD) molecules:

$$\mathrm{GuOH} = \mathrm{GuO^-} + \mathrm{H^+} \qquad K_1 = [\mathrm{GuO^-}][\mathrm{H^+}]/[\mathrm{GuOH}] \tag{1}$$

$$\mathrm{HoGuOH} = \mathrm{HoGuO^-} + \mathrm{H^+} \quad K_2 = [\mathrm{HoGuO^-}][\mathrm{H^+}]/[\mathrm{HoGuOH}] \tag{2}$$

$$\mathrm{Ho} + \mathrm{GuOH} = \mathrm{HoGuOH} \qquad K_3 = [\mathrm{HoGuOH}]/([\mathrm{Ho}][\mathrm{GuOH}]) \tag{3}$$

$$\mathrm{Ho} + \mathrm{GuO^-} = \mathrm{HoGuO^-} \qquad K_4 = [\mathrm{HoGuO^-}]/([\mathrm{Ho}][\mathrm{GuO^-}]) \tag{4}$$

where GuOH and GuO^- stand for the guest nitrophenol and nitrophenoxide, Ho for the host CD or DEAE-CD, and HoGuOH and $HoGuO^-$ for GuOH and GuO^- trapped in the host. There are four equilibrium constants defined, but they are related to each other by Equation 5.

$$K_4 = K_2K_3/K_1 \,. \tag{5}$$

K_1 for nitrophenol was estimated in the absence of the host, which was in agreement with K_a in the literature: 7.2×10^{-8} M ($pK_a = 7.14$) for *p*-nitrophenol [20], and 4.2×10^{-6} M ($pK_a = 5.38$) for 3,4-dinitrophenol [21]. Under the experimental conditions, [Ho] is much higher than [GuOH] or [GuO^-], and is assumed to be unchanged upon host-guest interaction. Individual values of [GuOH], [GuO^-], [HoGuOH], and [$HoGuO^-$] cannot be measured, but the total concentration of the conjugate acids, [A] (= [GuOH] + [HoGuOH]), and that of the conjugate bases, [B] (= [GuO^-) + [$HoGuO^-$]), are measured spectrophotometrically at the wavelength at which the millimolar absorbance difference (phenoxide-minus-phenol) is not affected by the presence of the host, i.e., at 400 nm for *p*-nitrophenol in the presence of $DEAE_n$-α-CDs of $n < 10$ and for 3,4-dinitrophenol in the presence of DEAE-β-CDs, or at 403 nm for *p*-nitrophenol in the presence of $DEAE_n$-α-CDs of $n > 10$ (Figure 2).

From Equations 1–5, the following equations are readily derived:

$$[A] = (1 + K_3[Ho])[GuOH] \tag{6}$$

$$[B] = (K_1 + K_2K_3[Ho])[GuOH]/[H^+] \,. \tag{7}$$

The observed K_a at a given [Ho] is defined as [H^+], at which [A] is equal to [B]. K_a is thus derived from Equations 6 and 7.

$$K_a = (K_1 + K_2K_3[Ho])/(1 + K_3[Ho]) \,. \tag{8}$$

From this, K_3 is derived.

$$K_3 = (K_a - K_1)/\Big([Ho](K_2 - K_a)\Big) \,. \tag{9}$$

Approximation of K_2 to the K_a observed at the highest [Ho] was unsatisfactory, since K_3s thus calculated at different [Ho]s were not in very good agreement with each other. Therefore the best K_2 value was sought to give the least square deviations of the individual K_3s from the mean K_3 calculated at different [Ho]s.

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