from the reaction site, can control future propagation; (5) the degree to which addition from the coordination sphere to the growing chain is by single units or by runs of units (in other words, the integrity of the coordination number during propagation); (6) the initiation and termination reactions.

Mechanisms relating the structure of the various catalysts to the observed monomer distributions can be written for the copolymerization which are described by **P** of eq 6 (or a very similar matrix) where only the last unit in the completed chain can affect growth, if suitable assumptions are made about the remaining unknown factors above (for example, assumptions about the control of propagation by monomers in the coordination sphere). Under different assumptions mechanisms can be written, described by **P**, where either the last two or the last three units in the completed chain can affect growth. At this time what can be said unambiguously is that any mechanism that is adopted is under the restriction that the monomer distributions it predicts be indistinguishable from the experimentally observed Markoffian distributions.

Acknowledgment. The authors wish to thank Dr. Allan W. Dickinson, Central Research Department, Monsanto Co., for performing the nonlinear regression analysis.

Inclusion Compounds. XIX.[1a] The Formation of Inclusion Compounds of α-Cyclodextrin in Aqueous Solutions. Thermodynamics and Kinetics

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Received July 5, 1966

Abstract: From spectrophotometric measurements it is concluded that α-cyclodextrin forms a 1:1 adduct with nitrophenol at acidic and alkaline pH. The rate of recombination of the bimolecular reaction is about $10^8\ M^{-1}\ sec^{-1}$ and, therefore, almost diffusion controlled. The binding of a series of azo dyes with α-cyclodextrin is highly stereospecific. In contrast to methyl orange, dyes of the type 4′-dimethylaminophenylazo-1-naphthalene-4-sulfonate form 1:1 complexes with cyclodextrin. The substitution of the dyes in the 4′ and 3′ positions has little influence on the equilibrium constant, while the rate of the reaction is changed by seven orders of magnitude. This behavior suggests a mechanism in which the dyes are enclosed in the cyclodextrin ring. The rate-determining step of the reaction is probably a partial melting of the water structure around the dye when it enters the cyclodextrin ring. This bond formation is compared with the enzyme substrate binding.

When starch is degraded by an amylase of *Bacillus macerans*, cyclodextrins[2,3] are formed by transglucosidation. Depending on the number of glucose residues in the molecule, the rings of these dextrins have different internal diameters (Table I). Figures

Table I

	Name	Internal diameter, A
6 glucose	α-Cyclodextrin	6
7 glucose	β-Cyclodextrin	7.5
8 glucose	γ-Cyclodextrin	9–10

1a and 1b show space-filling models of α-cyclodextrin. The cyclodextrins form a number of crystalline adducts with aromatic compounds, paraffins, and carboxylic acids, as well as nobel gases.[4,5] The crystalline iodine–cyclodextrin complex was studied by X-ray diffraction and found to be a molecular inclusion compound.[6] Also potassium acetate is accommodated in the void space of the crystalline adduct.[7]

Cyclodextrins form complexes in aqueous solution with azo dyes, nitrophenol, and other substances.[4] Although there is no direct proof for a fixation of the guest molecules within the void space of the cyclodextrin, the complexes are usually regarded as inclusion compounds in which hydrogen bonding,[8] van der Waals forces,[9] and hydrophobic interactions[10,11] are the main binding forces.

This explains the interest cyclodextrins have found as models for studying the primary step of enzyme[12] or antigen–antibody reactions.[4]

It is the aim of this work (a) to show that cyclodextrins can indeed form inclusion compounds with various

(1) (a) Part XVIII: N. Hennrich and F. Cramer, *J. Am. Chem. Soc.*, **87**, 1121 (1965); (b) W. Saenger, Thesis, Darmstadt, 1965.
(2) F. Schardinger, *Z. Untersuch. Nahrungsm. Genussm.*, **6**, 874 (1903).
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(5) F. Cramer and F. M. Henglein, *Chem. Ber.*, **90**, 2561, 2572 (1957).
(6) W. J. James and D. French, *Proc. Iowa Acad. Sci.*, **59**, 197 (1952); H. von Dietrich and F. Cramer, *Chem. Ber.*, **87**, 806 (1954).
(7) A. Hybl, R. E. Rundle, and D. E. Williams, *J. Am. Chem. Soc.*, **87**, 2779 (1965).
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(10) G. Némethy and H. A. Scheraga, *J. Chem. Phys.*, **36**, 3401 (1962).
(11) H. Zahn, *Kolloid-Z.*, **197**, 14 (1964).
(12) F. Cramer and G. Mackensen, *Angew. Chem. Intern. Ed. Engl.*, **5**, 601 (1966).

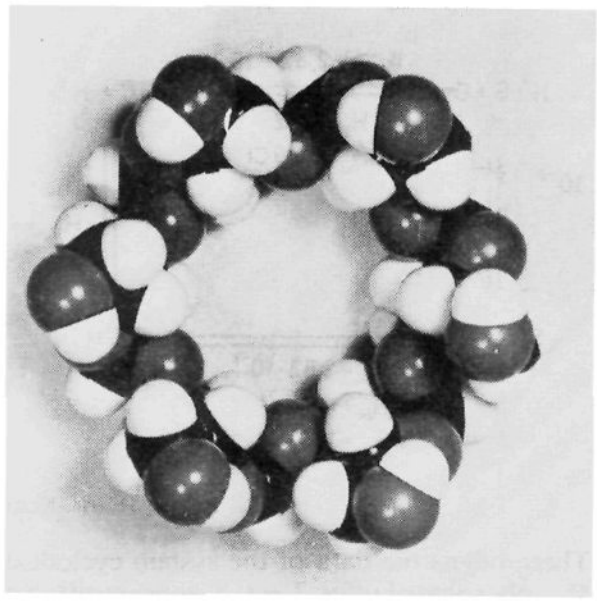


Figure 1a. Molecular model of α-cyclodextrin from the top.

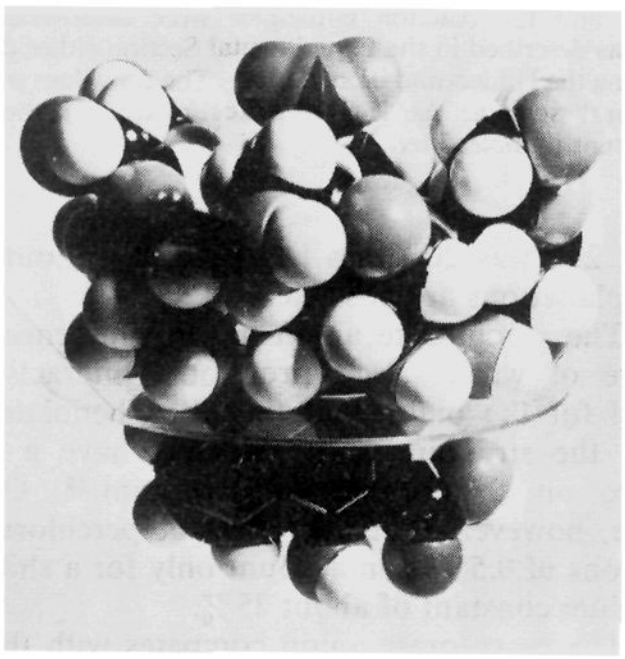


Figure 1b. Molecular model of the inclusion compound of α-cyclodextrin with 4′-nitrophenylazo-1-naphthalene-4-sulfonate from the side. The cyclodextrin ring is supported by a plastic ring. The naphthalene residue can be seen below the plastic ring, the nitro group sticking out at the top.

substrates in aqueous solutions; (b) to characterize the mechanism of the complex formation by kinetic measurements.

Experimental Section

Materials. α-, β-, and γ-cyclodextrins were prepared according to Cramer, *et al.*,[13] and purified according to French, *et al.*[14] *p*-Nitrophenol was recrystallized from water at acidic pH; the colorless crystals were dried over P_2O_5 under vacuum (mp 113°). Azo dyes were prepared by usual procedures.[15] The starting materials were analytical grade reagents. The dyes were analyzed and purity was checked by thin layer chromatography. 1-Anilino-8-naphthalenesulfonate (ANS) was a gift of Dr. L. Stryer (Stanford University).

Methods. A. Spectral Titrations. For determination of the equilibrium constants of the complexes, absorption spectra were recorded by a Cary Model 14 spectrophotometer. The cells (1 cm) were kept at constant temperature (±0.1°) with a Lauda-Kryostat.

B. Fluorescence spectra were recorded with a spectrofluorimeter developed by Stryer.[16] The excitation wave length was 365 mμ. The measurements were carried out at 23 ± 1°.

C. Kinetic measurements were carried out predominantly with the temperature-jump method developed by Eigen and de Maeyer.[17]

(13) F. Cramer and F. M. Henglein, *Chem. Ber.*, **91**, 308 (1958).
(14) D. French, *J. Am. Chem. Soc.*, **71**, 353 (1949).
(15) H. Zollinger, "Azofarbstoffe," Birkhaeuser-Verlag, Basel, 1958.
(16) L. Stryer, *J. Mol. Biol.*, **13**, 482 (1965).

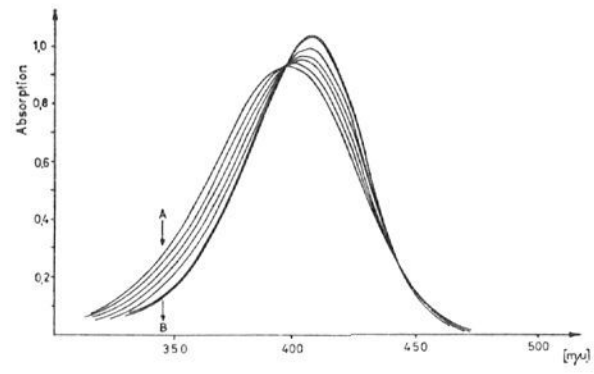


Figure 2a. Spectrum of the *p*-nitrophenol anion at varying α-cyclodextrin concentrations: solvent, phosphate buffer; pH 11.0 (I = 0.5); 20°. The cyclodextrin concentrations (M) are 0, 10^{-4}, 2.5×10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}, and 10^{-2}, read from A to B. The concentration of *p*-nitrophenol is 5×10^{-5} M.

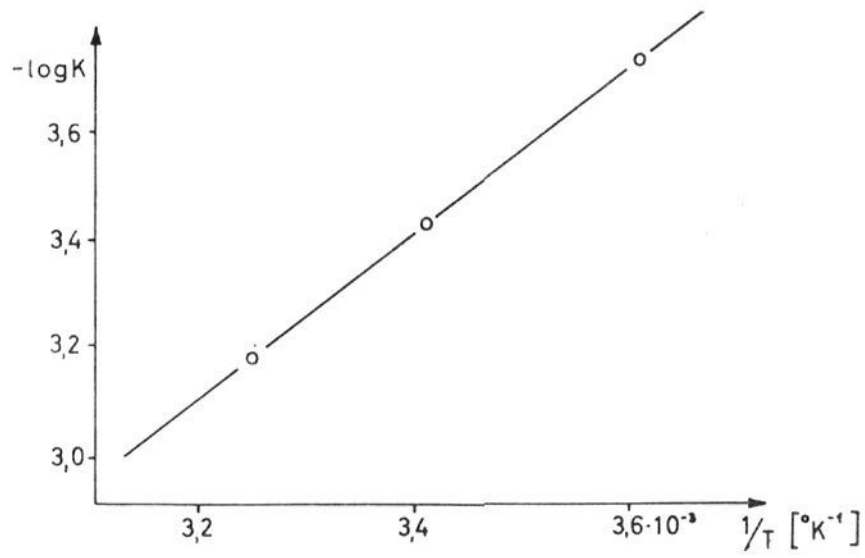


Figure 2b. Temperature dependence of the equilibrium constant of the nitrophenolate–α-cyclodextrin complex. The equilibrium constant at 20° is 3.55×10^{-4} M; H = 7.2 kcal/mole.

The complex formation of α-cyclodextrin with one of the azo dyes studied here was slow enough to be followed directly in a Cary Model 14 recording spectrophotometer. Unless otherwise stated, all rates refer to a temperature of 14 ± 1°.

Results

A. Nitrophenol. 4-Nitrophenol as well as its anion exhibit a spectral shift of about 15 mμ in aqueous solution on adding α-cyclodextrin. Figure 2a shows the spectrum of the anion of nitrophenol at varying cyclodextrin concentrations. The isosbestic points at 398 and 446 mμ indicate a 1:1 equilibrium. The equilibrium constant of dissociation at 20° for the reaction

$$\alpha\text{-cyclodextrin} + \text{4-nitrophenolate} \rightleftharpoons \text{inclusion complex}$$

was determined to be $K = 3.55 \times 10^{-4}$ M. The temperature dependence of the equilibrium constant, as shown in Figure 2b, yields an enthalpy ΔH of 7.2 kcal/mole.

The equilibrium constant of the α-cyclodextrin–nitrophenol complex was also determined by spectral titration. The concentration of the nitrophenol was 5×10^{-5} M in phosphate buffer (ionic strength I = 0.5 M). Even at the highest cyclodextrin concentra-

(17) M. Eigen and L. C. de Maeyer in "Technique of Organic Chemistry," Vol. VIII, Part 2, S. L. Friess, E. S. Lewis, and A. Weissberger, Ed., Interscience Publishers, Inc., New York, N. Y., 1963.

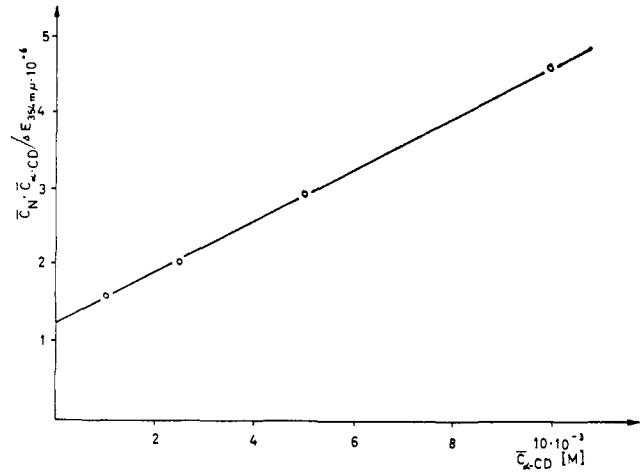


Figure 3. Determination of the equilibrium constant of the nitrophenol–α-cyclodextrin complex according to Hildebrand and Benesi.

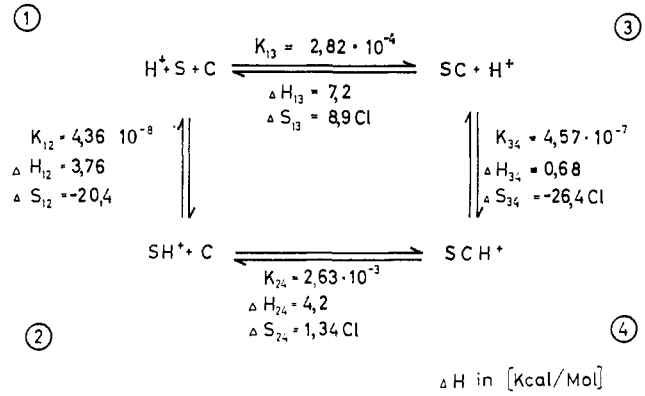


Figure 4. Thermodynamic data of the system cyclodextrin–nitrophenol at 14°: phosphate buffer, $I = 0.5$, concentration of chromophore 5×10^{-5} M. C = α-cyclodextrin, S = p-nitrophenolate, SH = p-nitrophenol, SC = nitrophenolate–cyclodextrin complex, and SHC = nitrophenol–α-cyclodextrin complex. Equilibrium constants and the reaction enthalpies were determined experimentally as described in the Experimental Section either directly or by applying the Hildebrand–Benesi plot. The ΔS values were calculated from these data; ΔH is given in kcal/mole; K is the dissociation constant in moles/liter.

tion (10^{-2} M) the nitrophenol was not completely bound. The values were therefore plotted according to the Hildebrand and Benesi relation[18]

$$\frac{\bar{C}_N \bar{C}_{\alpha\text{-CD}}}{\Delta E} = \frac{K}{\Delta\epsilon}\frac{\bar{C}_{\alpha\text{-CD}}}{\Delta\epsilon}$$

in which $\bar{C}_N$ is the total concentration of nitrophenol, $\bar{C}_{\alpha\text{-CD}}$ is the total concentration of α-cyclodextrin, K is the dissociation constant of the complex, $\Delta\epsilon$ is the difference of the molar extinction coefficients for free and complexed nitrophenol, and ΔE is the change in the extinction of the nitrophenol solution on adding α-cyclodextrin. The values are shown in Figure 3; they exhibit a linear plot; the slope yields $\Delta\epsilon = 2.9 \times 10^3$ (M^{-1} cm^{-1}); from the intercept one obtains the equilibrium constant $K = 3.5 \times 10^{-3}$ M.

Since the equilibrium constants for the complex formation are different for the phenol and the phenolate, the protolytic equilibrium constants of the free and the complexed nitrophenol must also be different. Therefore, free nitrophenol and nitrophenol in 10^{-2} M α-cyclodextrin solution were titrated spectrophotometrically. The solvent was phosphate buffer (I = 0.5 M) which by addition of phosphoric acid or sodium hydroxide was set to various pH values. Figure 4 gives the reaction scheme and summarizes the data.

If the diagram corresponds to a process in which a 1:1 complex is formed, the relations

$$K_{13}K_{34} = K_{12}K_{24}$$

$$\Delta H_{13} + \Delta H_{34} = \Delta H_{12} + \Delta H_{24}$$

must hold. These relations are exactly fulfilled ($\Delta\Delta H$ = 0.08 kcal/mole; $\Delta\Delta S$ = 1.6 eu; $K_{13}K_{34}/K_{12}K_{24} = 1.12$). The appearance of an isosbestic point (Figure 2a) together with the consistency of the thermodynamic data indicate that nitrophenol and α-cyclodextrin form a 1:1 complex.

When determining the equilibrium constant of the system nitrophenolate–α-cyclodextrin, it was found that the perchlorate ion has an influence on the equilibrium. At a concentration of 0.5 M ClO_4^- the apparent constant at 20° was 20 times larger than without ClO_4^-. Two explanations are possible.

(1) The perchlorate anion has an influence on the structure of water. If hydrophobic interactions are essential for the binding of the nitrophenolate to the dextrin, the structure of water must have a decisive influence on the equilibrium constant.[19] One can estimate, however, that this effect at perchlorate concentrations of 0.5 M can account only for a shift of the equilibrium constant of about 25%.

(2) The perchlorate anion competes with the nitrophenolate anion for the binding site in a manner similar to that described for the iodide ion.[20]

$$[\alpha\text{-CD}\cdot ClO_4^-] \underset{K_2}{\overset{-ClO_4^-}{\rightleftharpoons}} \alpha\text{-CD} \underset{K_1}{\overset{+\text{nitrophenol}}{\rightleftharpoons}} [\alpha\text{-CD}\cdot\text{nitrophenol}]$$

Neglecting the first effect and regarding the dependence of the apparent equilibrium constant on the perchlorate concentration, one can calculate the equilibrium constant for the ClO_4^-–α-cyclodextrin complex by applying the mass law in the following equation

$$[CD_{eq}] = \frac{K_1}{K_2}[ClO_4] + K_1 + \frac{\bar{C}_N}{2}$$

in which $[CD_{eq}]$ is the over-all concentration of cyclodextrin at equilibrium, *i.e.*, when half of the nitrophenol is included, $\bar{C}_N$ is the over-all concentration of nitrophenol, K_1 is the equilibrium constant for system CD–nitrophenol, and K_2 is the equilibrium constant for the system CD–ClO_4^-. For K_2 a value of 3.4×10^{-2} M is obtained.

The formation of an adduct with α-cyclodextrin was also observed with NO_3^- and I^-, while sulfate and phosphate did not have an influence on the equilibrium constants of the nitrophenol–α-cyclodextrin complex in the pH range between 2 and 11.

The determination of the kinetic data of the nitrophenol–cyclodextrin complex in acidic and alkaline

(18) H. A. Benesi and J. H. Hildebrand, *J. Am. Chem. Soc.*, 71, 2703 (1949).

(19) D. E. Gordon, B. Curnutte, Jr., and K. G. Lark, *J. Mol. Biol.*, 13, 571 (1965).

(20) H. Schlenk and D. M. Sand, *J. Am. Chem. Soc.*, 83, 2312 (1961).

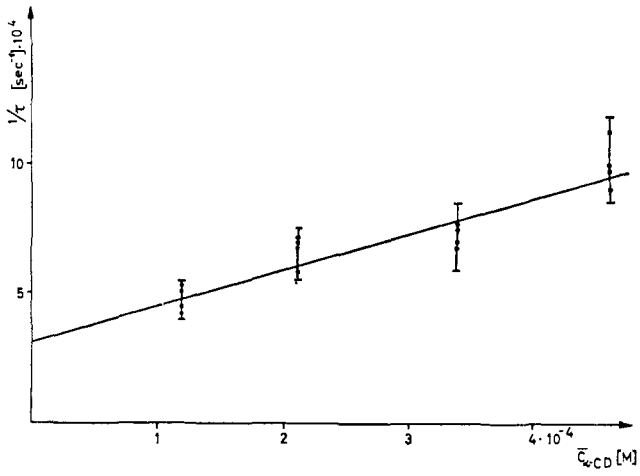


Figure 5. Reciprocal relaxation time for the nitrophenolate–α-cyclodextrin system plotted against the concentration of α-cyclodextrin.

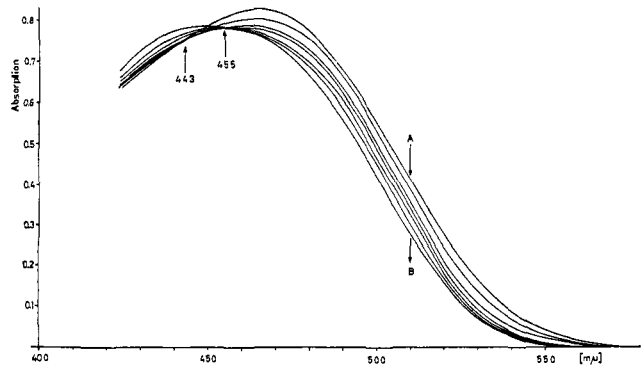


Figure 6a. Spectrum of methyl orange at α-cyclodextrin concentrations 0, 6.3×10^{-5}, 2.5×10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2}, and 2×10^{-2} *M*, read from A to B (concentration of methyl orange 5×10^{-5} *M*; phosphate buffer, $I = 0.1$, pH 9, 13°). At concentrations of cyclodextrin between 0 and 5×10^{-3} *M*, there is an isosbestic point at 443 mμ. When the cyclodextrin concentration is raised, a second isosbestic point at 445 mμ is formed.

medium was carried out with the temperature-jump method. For a 1:1 reaction the relaxation time τ is given by

$$1/\tau = k_R(C_N + C_{\alpha\text{-CD}}) + k_D$$

in which k_R is the recombination rate constant, k_D = the dissociation rate constant, C_N = the concentration of the free *p*-nitrophenol, and $C_{\alpha\text{-CD}}$ = the concentration of the free α-cyclodextrin. If one of the components is in excess (α-cyclodextrin in this case), one can determine the rate constants by plotting $1/\tau$ against $\bar{C}_{\alpha\text{-CD}}$.

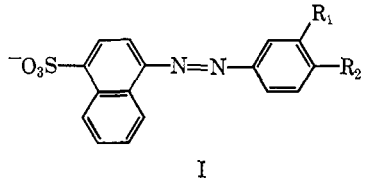
Figure 5 shows the results of the measurements at pH 11. The slope gives the recombination rate constant $k_R = 1.3 \times 10^8\ M^{-1}\ \text{sec}^{-1}$; the intercept the dissociation rate constant $k_D = 3.1 \times 10^4\ \text{sec}^{-1}$.

The quotient of the rate constants provides the equilibrium constant $K = k_D/k_R = 2.2 \times 10^{-4}\ M$ which is in good agreement with the thermodynamically measured constant. This confirms that α-cyclodextrin forms a 1:1 complex with the nitrophenolate anion.

In the case of the nitrophenol–α-cyclodextrin system at low pH, the observed relaxation times were of the order of only a few microseconds, which under our conditions is close to the limit of resolution of the temperature-jump method. The rate constants can therefore be given only as $k_R \geq 4 \times 10^7\ M^{-1}\ \text{sec}^{-1}$, $k_D \geq 10^5\ \text{sec}^{-1}$.

B. Azo Dyes. Figure 6a shows the spectrum of methyl orange at varying α-cyclodextrin concentrations. The appearance of two nonrelated isosbestic points indicates that methyl orange and α-cyclodextrin can form complexes with higher stoichiometric ratios besides a 1:1 complex. In contrast, with the similar 4′-dimethylaminophenylazo-1-naphthalene-4-sulfonate a well-defined isosbestic point is shown (Figure 6b).

The azo dyes of the general structure I offer the possibility to study the mechanism of the complex formation in greater detail. Especially interesting was the influence of various substituents, R_1 and R_2, on the thermodynamic and kinetic constants of the complex formation.



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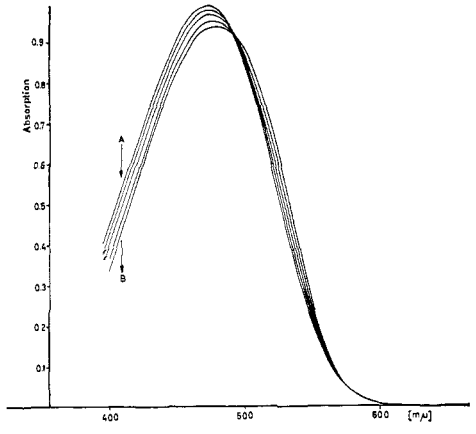
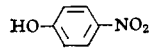
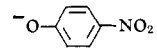
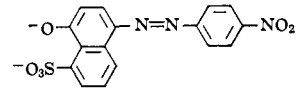
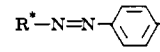
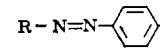
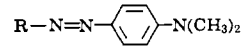
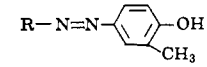
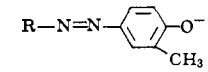
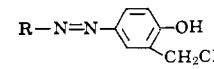
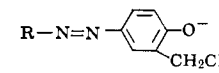
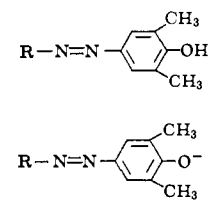


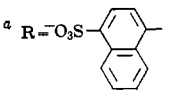
Figure 6b. Spectrum of 4′-dimethylaminophenylazo-1-naphthalene-4-sulfonate at α-cyclodextrin concentration from 0 to 10^{-2} *M*, read from A to B (phosphate buffer, $I = 0.42$, pH 6.6, 20°, concentration of azo dye 3.3×10^{-5} *M*). In contrast to Figure 6a, there is only one isosbestic point.

The equilibrium constants of several azo dyes were determined as above from a Hildebrand–Benesi plot. Table II summarizes the data. In the case of the 3′,5′-dimethyl-4′-hydroxyphenylazo-1-naphthalene-4-sulfonate, no change in the spectrum could be detected on addition of cyclodextrin. Apparently the formation of such a complex is impossible for steric reasons.

The rate of formation of the 3′-ethyl-4′-hydroxyphenylazo-1-naphthalene-4-sulfonate–α-cyclodextrin complex in alkaline medium could be measured directly. Two milliliters of a $2 \times 10^{-3}\ M$ α-cyclodextrin solution was rapidly mixed with 0.3 ml of a $3 \times 10^{-4}\ M$ solution of the azo dye in phosphate buffer, pH 11.5. The change of the extinction at 530 mμ was recorded on a Cary Model 14 spectrophotometer. Since α-cyclodextrin is present in great excess, the reaction is pseudo-

Table II

Substrate[a]	K, M[b]	ΔH, kcal/mole	k_R, M^{-1} sec^{-1}	k_D, sec^{-1}[b]	Solvent (phosphate buffer)
	2.6×10^{-3}	4.2	$\geq 4 \times 10^{7}$	$\geq 10^{5}$	$I = 0.5$, pH 3.5
	0.27×10^{-3}	7.2	1.4×10^{8}	3.1×10^{4}	$I = 0.5$, pH 11
	3.2×10^{-3}	6.3	5.2×10^{7}	1.3×10^{5}	$I = 0.1$, pH 11
	3.7×10^{-3}	7.0	1.3×10^{7}	5.5×10^{4}	$I = 0.1$, pH 3.5
	1.55×10^{-3}	6.3	1.7×10^{5}	2.6×10^{2}	$I = 0.1$, pH 11
	0.99×10^{-3}	7.1	1.1×10^{8}	1×10^{3}	$I = 0.1$, pH 11
	2.4×10^{-3}	6.4	1.2×10^{5}	3.5×10^{2}	$I = 0.1$, pH 3.5
	2.1×10^{-3}	5.8	1.5×10^{2}	0.28	$I = 0.1$, pH 11
	2.2×10^{-3}	6.5	6×10^{3}	19	$I = 0.1$, pH 3.5
	3.5×10^{-3}	7.7	2.8	1×10^{-2}	$I = 0.1$, pH 11.5
	No inclusion				

[a] R =  [b] All values refer to 14°.

monomolecular and characterized by the time constant

$$1/\tau = k_R \bar{C}_{\alpha\text{-CD}} + k_D$$

The knowledge of the equilibrium constant enables one to determine both rate constants in this equation. Figure 7 shows the temperature dependence of the rate of recombination.

Between 30 and 10° the reaction has an activation energy of 11.7 kcal/mole. Below 10° the complex formation is much more temperature dependent.

The kinetics of all the other complexes in Table I were determined with the temperature-jump method as described. Figure 8 shows the plot of the reciprocal relaxation times against the α-cyclodextrin concentration in the system 3′-ethyl-4′-hydroxyphenylazo-1-naphthalene-4-sulfonate–α-cyclodextrin in acid medium.

From the slope one obtains the rate of recombination, $k_R = 6 \times 10^3\ M^{-1}$ sec^{-1}. This is three orders of magnitude higher than that of the same substance in alkaline medium. The intercept gives a k_D value of 19 sec^{-1}. By division of the two rates, one obtains the equilibrium constant in good agreement with the thermodynamically determined value.

Table II gives a summary of the kinetic data of the complex formation of the azo dyes with α-cyclodextrin. In all temperature-jump measurements the k_D and k_R values were determined independently. In all cases there was a good agreement with the thermodynamically determined equilibrium constants. Therefore, a mechanism in which the dissociation of oligomeric azo dyes might be rate determining for the complex formation can be excluded.

C. 1-Anilino-8-naphthalenesulfonate. 1-Anilino-8-naphthalenesulfonate (ANS) shows a strong fluorescence in organic solvents such as ethyl alcohol with a quantum yield as high as 0.6, while in water it exhibits only a very low fluorescence. If ANS is bound to a protein-like apomyoglobin, the quantum yield can go up as high as 0.98.[16]

If ANS and cyclodextrin form an inclusion complex, and by this the ANS molecule is transported into a partially hydrophobic surrounding, the fluorescence of the ANS molecule should increase. Since α-cyclodextrin with an internal diameter of 6 A is capable only

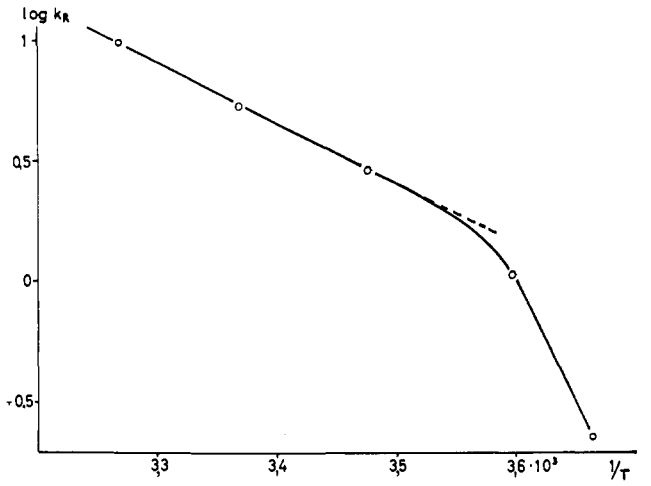


Figure 7. Temperature dependence of the rate of recombination k_R of the 3′-ethyl-4′-hydroxyphenylazo-1-naphthalene-4-sulfonate–α-cyclodextrin system.

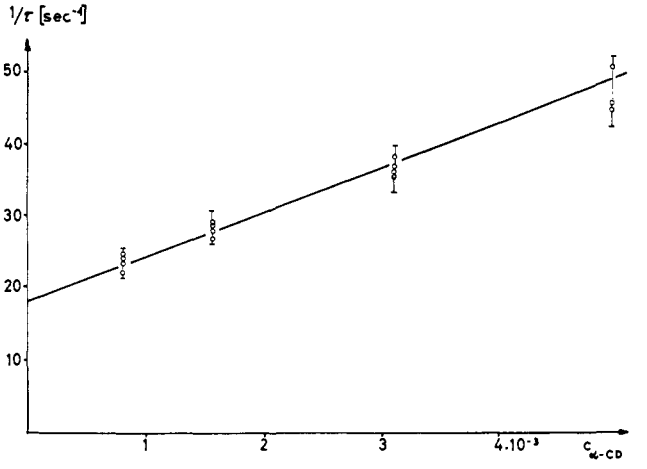


Figure 8. Reciprocal relaxation time for the 3′-ethyl-4′-hydroxyphenylazo-1-naphthalene-4-sulfonate–α-cyclodextrin system against the concentration of α-cyclodextrin at pH 3.

of including the aniline residue of ANS, it should give a much smaller increase in fluorescence than β- and γ-cyclodextrins, which are big enough to enclose the naphthalenesulfonate residue as well.

Figure 9 shows the fluorescence spectrum of ANS in 0.1 *M* phosphate buffer (pH 6.8) on addition of α- and β-cyclodextrins. α-Cyclodextrin causes only a twofold increase of the fluorescence of ANS; β- and γ-cyclodextrins, however, cause a tenfold increase.

Discussion

In principle, two structures of the cyclodextrin complexes in aqueous solution are possible: (1) an association of the substrate at the outside of the cyclodextrin, and (2) an inclusion inside the ring. Thermodynamic as well as kinetic measurements in the nitrophenol–α-cyclodextrin system show that the partners form a 1:1 complex. The same follows from the consistency of the kinetic data with the thermodynamically determined equilibrium constants of the complex formation of the azo dyes. This fact makes mechanism 2 much more likely. Equally, the stereospecificity of the complex formation indicates the existence of an inclusion compound. Methyl orange, because it does not have the bulky naphthalene residue, can probably bind two α-cyclodextrin molecules at higher cyclodextrin concentrations, whereas 4′-dimethylaminophenylazo-1-naphthalene-4-sulfonate allows only the threading of one cyclodextrin ring. The 3′,5′-dimethyl-4′-hydroxyphenylazo-1-naphthalene-4-sulfonate, in contrast, does not allow the formation of a complex at all because of its two methyl groups in *ortho* position on the phenol ring. These enlarge the diameter in this region to 6.5 A as compared with the internal diameter of the cyclodextrin of 6 A.

An especially striking feature of the complex formation with this series of azo dyes is the similarity of the equilibrium constants and reaction enthalpies, while the rate constants vary by seven orders of magnitude. An association at the outside of the ring could hardly give an explanation for this kinetic specificity. The data can only be explained by the formation of an inclusion complex.

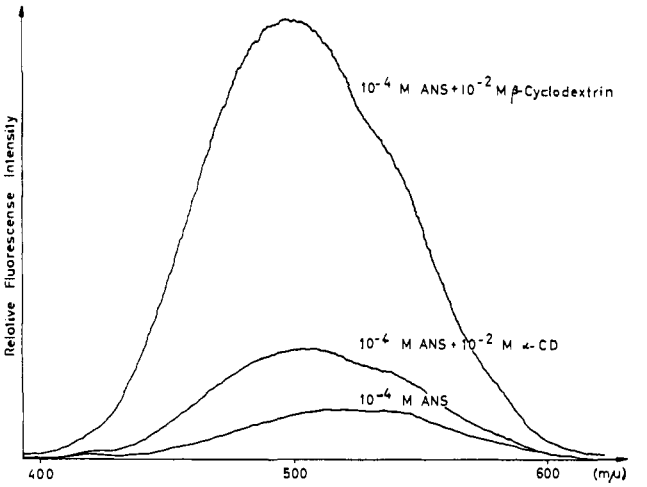


Figure 9. Fluorescence spectrum of 10^{-4} *M* ANS in 0.1 *M* phosphate buffer, pH 6.8. The spectrum of a 10^{-4} *M* ANS solution in 0.1 *M* phosphate buffer, pH 6.8 and 8×10^{-3} *M* γ-cyclodextrin, is identical with the spectrum of ANS at a β-dextrin concentration of 10^{-2} *M* shown here. The emission spectra given here are not corrected for the wavelength-dependent sensitivity of the photomultiplier (RCA 1P21).

The mechanism of the formation of this inclusion compound can be divided into several steps: (1) the approaching of the substrate to the cyclodextrin molecule; (2) breakdown of the water structure inside the cyclodextrin ring and removal of some water molecules out of the ring; (3) breakdown of the water structure around that part of the substrate molecule, which is going to be included in the cyclodextrin, and transport of some water molecules into the solution; (4) interaction of the substituents of the substrate molecules with groups on the rim or on the inside of the cyclodextrin; (5) possible formation of hydrogen bonds between substrate and cyclodextrin (formation of hydrogen bonds has been shown to be an extremely fast process and therefore cannot be rate determining for the inclusion reaction);[21,22] (6) reconstitution of the

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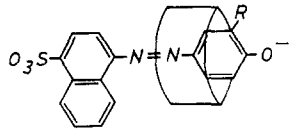

Figure 10. Proposed structure of the azo dye–α-cyclodextrin complex. The substituents R and O^- are somewhat removed from the immediate influence of the hydrophobic interior of the cyclodextrin ring.

water structure around the exposed parts of the substrate after the inclusion process.

In steps 1, 4, and 5 steric factors are involved (stability or rate of formation should be dependent on geometry); in other words "steric inhibition" might be observed.

Steps 2, 3, and 6 have to do with the water structure around the partners of the reaction. Steps 1, 2, and 6 are general steps and, therefore, should not give rise to substrate specificity of rates within the same class of compounds. When a kinetic specificity with respect to substituents is found, step 3 or 4 or less likely 5 must be rate determining.

The rate of recombination of the complex formation of nitrophenol and its anion, as well as of the 4′-nitrobenzoazo-1-naphthalene-4-sulfonate are almost of the order of diffusion-controlled reactions, *i.e.*, reactions in which the approach of the partner is rate determining. The fact that the diffusion-controlled limit value of the recombination rate of about 10^9 M^{-1} sec^{-1} [23] is not quite reached may be due to the special steric conditions of the inclusion reaction in step 1. It cannot, however, be excluded that the second step in the reaction, the removal of some water molecules from the inside of the cyclodextrin,[24] is rate determining for the complex formation of the nitrophenol with α-cyclodextrin.

Two types of water structure must be considered in principle: (1) tightly bound water-solvating charged sites or groups which are capable of hydrogen bonding [OH, $N(CH_3)_2$] and (2) "iceberg" water around hydrophobic groups (aromatic ring, CH_3, C_2H_5, NO_2).

The rate constants of the monosubstituted azo dyes (Table II) decrease in the sequence NO_2, OH, $N(CH_3)_2$, O^-. Such a sequence can be expected if the melting of the water structure of the type 1 around the part of the molecule which is going to enter the ring (step 3) is rate determining for the complex formation. The stronger the water molecules are bound, the slower they can be removed from the sphere of tight binding around the substrate.[25]

The observation that the equilibrium constants in the series of azo dyes are almost equal in spite of the enormous changes in rate constants can be explained by assuming that the hydration around the substituent is almost completely reconstituted after the inclusion in a step 6; thus the dissociation reaction is inhibited to a comparable extent. Figure 10 shows a schematic picture of the proposed structure of the complex.

The influence of the substituents in the 3′ position is unexpectedly high. A simple steric hindrance in step 1 or 4 of the inclusion reaction could hardly explain a decrease of the rate by the factor 10^3 or 10^5 when going from H over methyl to ethyl. The steric factor may, however, be amplified by the water structure around the substituent. It is possible that a substitution in the 3′ position necessitates the removal of more, or more tightly bound,[24,26] water molecules from the "iceberg" around the substrate in order for it to enter the cyclodextrin ring. On the other hand, specific interaction of the substituents with groups inside the carbohydrate ring might have an important effect on slowing down the rate of complex formation in step 4. In the case of the 3′,5′-dimethyl-4′-hydroxyphenylazo-1-naphthalene-4-sulfonate, the steric limit for being included seems to be reached. *Thus the introduction of one additional methyl group into the aromatic ring can cause a kinetic "yes" or "no" decision in complex formation.*

Since cyclodextrins can effect enzyme like catalysis,[12,27] it would appear that these results bear on the issue of enzyme substrate binding and the mechanism of template-dependent enzymes.

Acknowledgments. We are grateful to Dr. Manfred Eigen and Dr. Hans Hettler Goettingen, and Dr. L. Styrer, Stanford University, for stimulating discussions. This work was supported by Deutsche Forschungsgemeinschaft Bad Godesberg.

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