Interaction of 6-*p*-Toluidinylnaphthalene-2-sulfonate with α-Cyclodextrin

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The interaction of 6-*p*-toluidinylnaphthalene-2-sulfonate (TNS) with α-cyclodextrin was investigated in a 0.1 M phosphate buffer at pH 7.4 by fluorescence spectrophotometry. Using the fact that the fluorescence intensity of TNS increases in the presence of α-cyclodextrin, the thermodynamic parameters for the inclusion complex formation were determined as follows; $\Delta G° = -2.21$ kcal/mol at 25 °C, $\Delta H° = -3.0$ kcal/mol, $\Delta S° = -2.6$ e.u. The driving force for the inclusion complex formation was considered to be the van der Waals-London dispersion force and hydrogen bonding between the nitrogen atom of TNS and the secondary hydroxyl groups of α-cyclodextrin.

Also, from the measurements of proton nuclear magnetic resonance spectra and the model building with Corey Pauling Koltum models, the most probable structure was determined.

Keywords 6-*p*-toluidinylnaphthalene-2-sulfonate; α-cyclodextrin; fluorescence; inclusion complex; thermodynamic parameter; ^{1}H-NMR

6-*p*-Toluidinylnaphthalene-2-sulfonate (TNS) is known to be a fluorescent probe for exploring hydrophobic regions.[1] The fluorescence of this agent is quenched in water, but in hydrophobic environments which is augmented significantly with shifts of emission maximum toward shorter wavelength.[2] In addition, biological substances, such as proteins, having intramolecular hydrophobic regions can interact with TNS to result in the changes in the fluorescence spectrum of TNS. Taking advantage of this property, TNS has been used for clarifying the structures and functions of biological substances.[3]

It has been reported that fluorescence emitted by TNS in water increases markedly when cyclodextrin such as β- or γ-cyclodextrin,[4,5] which are cyclic molecules produced from starch by transglucosidation catalyzed by Bacillus amylase, were added to the aqueous solution. The fluorescence has been considered to originate in including TNS in the cavity of the cyclodextrin molecule. Consequently, the changes of the fluorescence spectra offer one of the means of evidence of hydrophobicity of the cavity. Thus, the hydrophobic bonding is suggested as an important driving force in the formation of the inclusion complex between the dye and cyclodextrin. However, it was reported recently that the enthalpy term contributes more than the entropy term to the formation of the inclusion complex with a molar ratio of 1 : 1 between the dye and β-cyclodextrin.[6]

In the present study, the authors investigated the interaction between α-cyclodextrin selected as a representative of cyclodextrins, and the dye, by measuring the change of fluorescence spectra in a phosphate buffer solution. α-Cyclodextrin was selected because its cavity is smallest in the family of cyclodextrins and, therefore, the structure of the inclusion complex can be investigated most easily. Then, the changes in enthalpy and entropy due to the formation of the inclusion complex were determined so that the driving forces acting between the components of the complex could be clarified. Further, the probable structure of the inclusion complex was studied by using Corey Pauling Koltun (CPK) models and by considering the changes of the proton nuclear magnetic resonance (^{1}H-NMR) spectra due to the complexation.

Experimental

Materials Reagent-grade α-cyclodextrin supplied by Nakarai Chemicals Ltd. was recrystallized twice from water and dried over P_2O_5 for 5 h at 110 °C in a vacuum before use. TNS supplied by Nakarai Chemicals Ltd. as potassium salt was recrystallized twice from water and dried in vacuum for 2 d. Water used was obtained by distilling twice water purified with ion-exchange resin.

^{1}H-NMR Spectra ^{1}H-NMR experiments were all measured in deuterium oxide at 30 °C. ^{1}H-NMR experiments for determining the change of chemical shift were conducted on a Varian XL-200 (200 MHz) NMR spectrometer with tetramethylsilane (TMS) as an external reference. Aquisition time, 3 s; pulse angle, 90°; delay time, 2 s; number of transients, 96. ^{1}H-NMR spectra of the others were recorded on a Varian VXR-500 (499.8 MHz) spectrometer. Two-dimentional nuclear Overhauser enhancement spectroscopy (NOESY) experiments were performed using the State–Haberkorn method (2D hypercomplex method). The mixing time of 0.6 s and the relaxation delay, D_1 of 2 s were used. Two-dimensional rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments were performed in the phase-sensitive mode methods using the State–Haberkorn method. Spectra were acquired with 256 t_1 increments and each increment consisted of 1 kilo data points from 16—32 transients. The spinlock mixing pulse of 250 ms was used.

Fluorescence Spectra Fluorescence spectra were measured with a Shimadzu RF-503A fluorescence spectrophotometer at 5, 15, 25, and 40 °C. To obtain a given temperature, water regulated at a constant temperature from a thermostat was circulated through the cell holder. The change of temperature during the measurements was within 0.1 °C. The lamp emission intensity changed only slightly during measurements. In order to obtain reliable data, the spectra were corrected by repeating the measurement of the standard sample after that of each sample.

Absorption Spectra Absorption spectra were taken on a Shimadzu UV 300 spectrophotometer at 25 °C. The measurements were carried out in a 0.1 M sodium phosphate buffer of pH 7.4.

Results

Figure 1 shows the effects of α-cyclodextrin on the absorption spectra of TNS. These spectra were measured, using buffer solution containing the same concentration of α-cyclodextrin as the sample, as reference. The absorption spectra of TNS showed the bathochromic shifts by addition of α-cyclodextrin although the shifts were only slight. The shift at the maximal wavelength of 258 nm assigned to ^1L_a was most remarkable.[7]

Figure 2 shows a part of the fluorescence spectra of 1.0×10^{-5} M of TNS measured in the presence of α-cyclodextrin at different concentrations in 0.1 M phosphate buffer (pH 7.4) at 25 °C. The concentrations of α-cyclodextrin were in the range of 200 to 3000 times higher than that of TNS. The fluorescence intensity increased with the shift of fluorescence maximum to shorter wavelength as the concentration of α-cyclodextrin increased. The

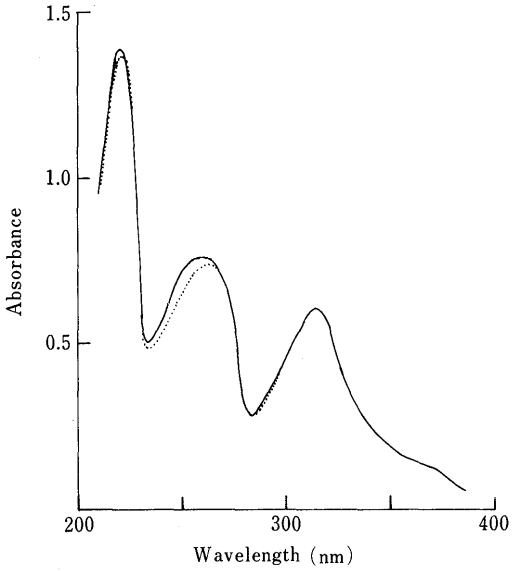

Fig. 1. Absorption Spectra of TNS in the Presence of α-Cyclodextrin in 0.1 M Sodium Phosphate Buffer of pH at 25 °C

—, TNS alone (3.0×10^{-5} M), ---, TNS (3.0×10^{-5} M) + α-cyclodextrin (2.0×10^{-2} M).

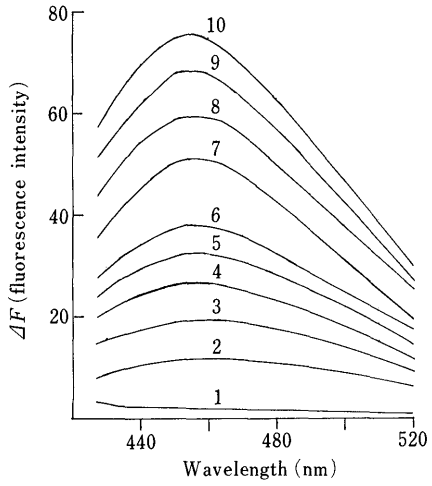

Fig. 2. Fluorescence Spectra of 1.0×10^{-5} M TNS in the Presence of α-Cyclodextrin in 0.1 M Phosphate Buffer of pH 7.4 at 25 °C

The concentrations of α-cyclodextrin are: 1, 0; 2, 2.0×10^{-3} M; 3, 4.0×10^{-3} M; 4, 6.0×10^{-3} M; 5, 8.0×10^{-3} M; 6, 1.0×10^{-2} M; 7, 1.4×10^{-2} M; 8, 2.0×10^{-2} M; 9, 2.4×10^{-2} M; 10, 3.0×10^{-2} M. The excitation wavelength is 365 nm.

changes of the fluorescence and absorption spectra were not seen when D-glucose or D-maltose was added in place of α-cyclodextrin. Therefore, it is clear that the interaction of TNS with α-cyclodextrin is closely related to the cavity of the oligosaccaride.

As described above, the fluorescence intensity of TNS increases significantly in the presence of α-cyclodextrin. By taking advantage of this effect of α-cyclodextrin, the formation constant K was estimated. Assuming a 1:1 complex, when the large excess α-cyclodextrin is added to TNS, Eq. 2 applies.

$$\mathrm{CD}+\mathrm{TNS}\leftrightarrows\mathrm{CD\text{–}TNS} \tag{1}$$

$$K=\frac{X}{C_o(T_o-X)} \tag{2}$$

$$\Delta F=\Delta F_t+\Delta F_c \tag{3}$$

Table I. Thermodynamic Parameters for Inclusion Complex Formation of TNS with α-Cyclodextrin

Temp. (°C)	$K^{a)}$ (M^{-1})	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° (e.u.)
5	57.8	−2.24		
15	45.9	−2.19	−3.0±0.2	−2.6±0.3
25	41.9	−2.21		
40	31.0	−2.14		

a) These values are averages which were obtained from 5 repetitive runs. Average probable errors are ±6% for K.

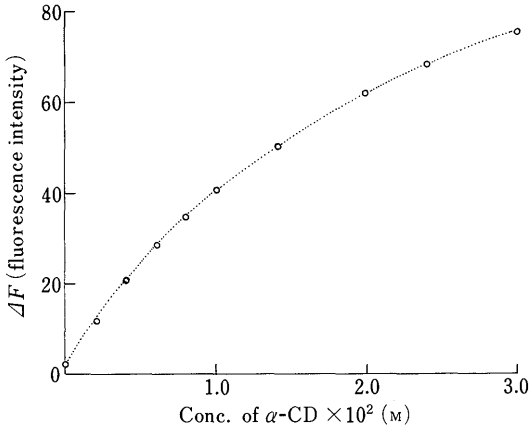

Fig. 3. Binding Curves of TNS with α-Cyclodextrin

○, observed fluorescence intensity; ---, theoretical curve, which was obtained from Eq. 4, using the calculated parameters.

$$\Delta F=\frac{1}{1+C_oK}\Delta F_T-\frac{1}{1+C_oK}\Delta F_{\infty 1:1}+\Delta F_{\infty 1:1} \tag{4}$$

Hence, C_o, T_o, and X represent total concentration of α-cyclodextrin, total concentration of TNS, and the concentration of TNS–α-cyclodextrin complex, respectively. Also, CD represents α-cyclodextrin. The fluorescence intensity (ΔF) observed is a sum of those of free TNS (ΔF_t) and complex (ΔF_c) (Eq. 3). Where ΔF_T and $\Delta F_{\infty 1:1}$ denote the fluorescence intensities which should be observed when all TNS is free and all TNS has formed TNS–α-cyclodextrin complex, respectively, Eq. 4 is valid. Formation constant K can be estimated from Eq. 4 using a nonlinear squares program MULTI.[8] The values obtained at 5, 15, 25, and 40 °C are shown in Table I. Also, Fig. 3 shows the binding curve for α-cyclodextrin with TNS together with the theoretical curves obtained using the calculated parameters. The van't Hoff plots obtained by plotting log K against the absolute temperature are shown in Fig. 4. The changes in enthalpy (ΔH°) and entropy (ΔS°) accompanying the complexation were determined in the usual way. The results obtained are shown in Table I.

^{1}H-NMR spectra and CPK model were used to estimate the structure of the inclusion complex between TNS and α-cyclodextrin. In Fig. 5a, a ^{1}H-NMR spectrum of 3.0×10^{-3} M TNS in deuterium oxide at 30 °C is shown. The assignments of the proton signals of TNS were undertaken on the basis of COSY measurements and NOESY measurements. The proton signals of TNS showed downfield shifts by adding α-cyclodextrin. However, it is considered that TNS molecules self-associate in aqueous

solution at a concentration of 3.0×10^{-3} M by vertical stacking and the interaction of TNS with α-cyclodextrin induces the decrease in the concentration of uncomplexed TNS, resulting in the dissociation of the self-association form. Thus, the proton signals of TNS would show the downfield shifts due to the decrease in the concentration of the self-association form in the presence of α-cyclodextrin. To investigate this effect, ^{1}H-NMR spectra were recorded in deuterium oxide at 30 °C, at the various concentrations. The downfield shifts of the proton signals of TNS were observed with the decrease of the concentration. The results are shown in Fig. 6. It can be seen from Fig. 6 that the 5-H signal shifts downfield most significantly, and 3-H and methyl-H signals most slightly with the decrease of TNS concentration. In the presence of 1.2×10^{-2} M α-cyclodextrin, for example, as shown in Fig. 5b all the signals due to the protons of TNS shifted downfield. At the benzene ring, the downfield shifts of the signals due to 3′-H and 5′-H were most prominent, followed by the signals due to 2′-H, 6′-H, and methyl-H. At the naphthalene ring, the downfield shift of signals due to 4-H was most prominent, followed by 5-H and 7-H.

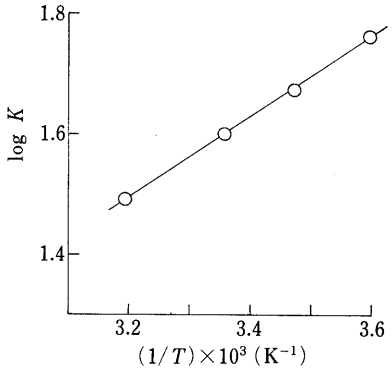

Fig. 4. Van't Hoff Plot of Data in Table I

When 1.2×10^{-2} M α-cyclodextrin is added to 3.0×10^{-3} M TNS, the concentration of TNS uncomplexed is estimated as 2.1×10^{-3} M using the formation constant K, 37 M^{-1} at 30 °C, obtained from Fig. 4. The downfield shifts of the proton signals due to TNS, which are induced by the

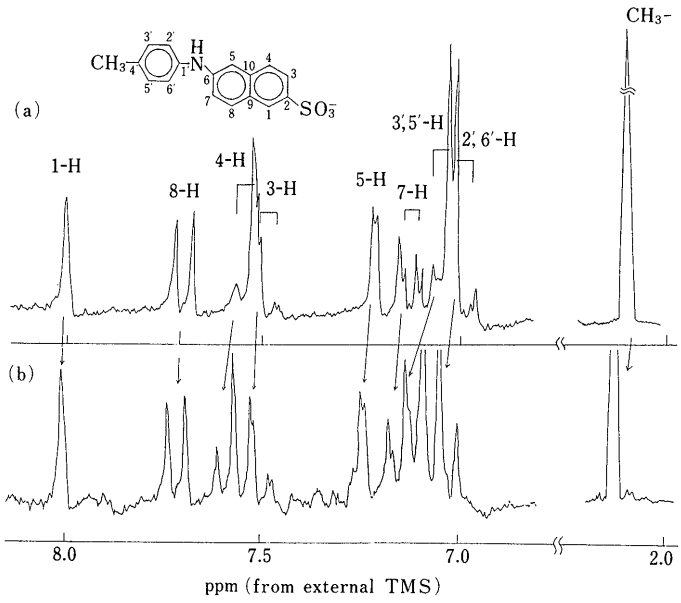

Fig. 5. ^{1}H-NMR Spectra of TNS in the Presence of α-Cyclodextrin in Deuterium Oxide at 30 °C

(a) TNS alone (3.0×10^{-3} M), (b) TNS (3.0×10^{-3} M) + α-cyclodextrin (1.2×10^{-2} M).

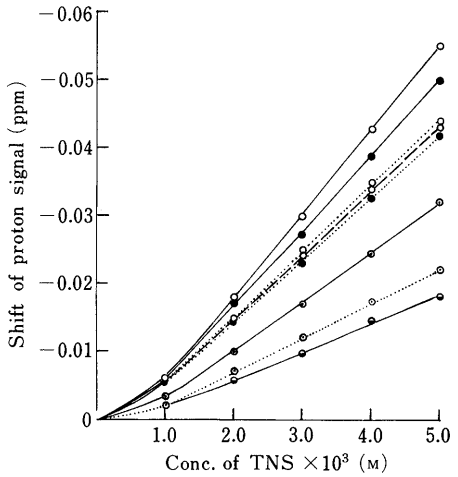

Fig. 6. Concentration Dependence of TNS Proton Chemical Shifts

—○—, 5-H; —●—, 7-H; ---○---, 8-H; -○——, 2′-H, 6′-H; ---●---, 4-H; —⊙—, 3′-H, 5′-H; ---⊙---, 1-H; —◒—, 3-H, methyl-H.

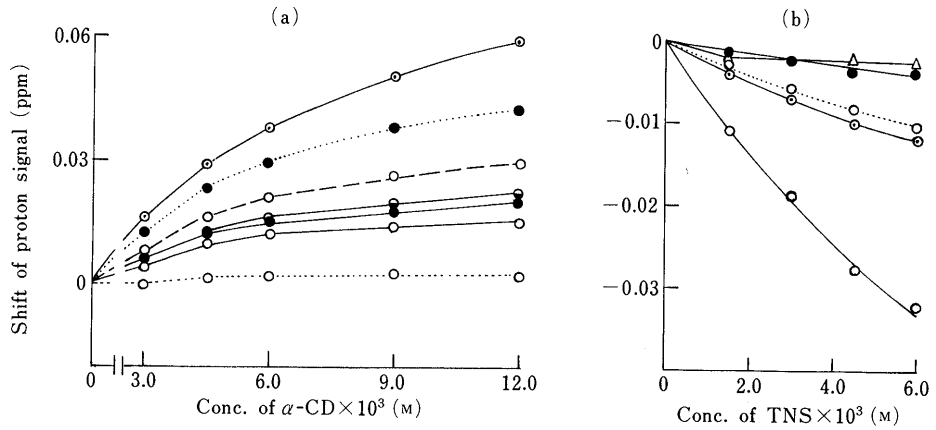

Fig. 7. (a) Induced ^{1}H-NMR Chemical Shifts of TNS (3.0×10^{-3} M) in the Presence of α-Cyclodextrin

—⊙—, 3′-H, 5′-H; ---●---, 4-H; -○——, 2′-H, 6′-H; —◒—, methyl-H; —●—, 7-H; —○—, 5-H; ---○---, 8-H. 1-H and 3-H did not shift.

(b) Induced ^{1}H-NMR Chemical Shifts of α-Cyclodextrin (3.0×10^{-3} M) in the Presence of TNS

—△—, 5-H; —●—, 6-H; ---○---, 4-H; —⊙—, 1-H, 2-H; —○—, 3-H.

decrease of the concentration of uncomplexed TNS (from 3.0×10^{-2} M to 2.1×10^{-2} M), are determined using Fig. 6. The magnitudes of the complexation shifts are obtained by substracting these shifts from the those shown in Fig. 5 and are illustrated in Fig. 7a. But, it was found that the order of the magnitudes of the upfield shifts remained unaltered by the corrections, although the differences of the magnitudes of the upfield shifts changed. The magnitudes of the shifts of the proton signals due to TNS in the presence of α-cyclodextrin at various concentrations are shown in Fig. 7a in the same manner as above.

A ^{1}H-NMR spectrum of 3.0×10^{-3} M α-cyclodextrin solution is shown in Fig. 8a. The spectrum is different from that (Fig. 8b) in the presence of 6.0×10^{-3} M TNS. The comparison of these spectra revealed that the signals due to protons of all types in α-cyclodextrin molecule shifted upfield in the presence of TNS. The shift of the signal due to 3-H on the inner surface of the cavity at the secondary hydroxyl group side was most prominent, followed by the signals due to 1-H, 2-H, and 4-H which lie on the outer surface of the cavity. On the other hand, the upfield shifts of the signals due to 5-H and 6-H lying on the inner surface of the primary hydroxyl group side were not so prominent as those due to the other protons of α-cyclodextrin. The magnitudes of the shifts of the proton signals due to α-cyclodextrin in the presence of TNS at various concentrations are shown in Fig. 7b.

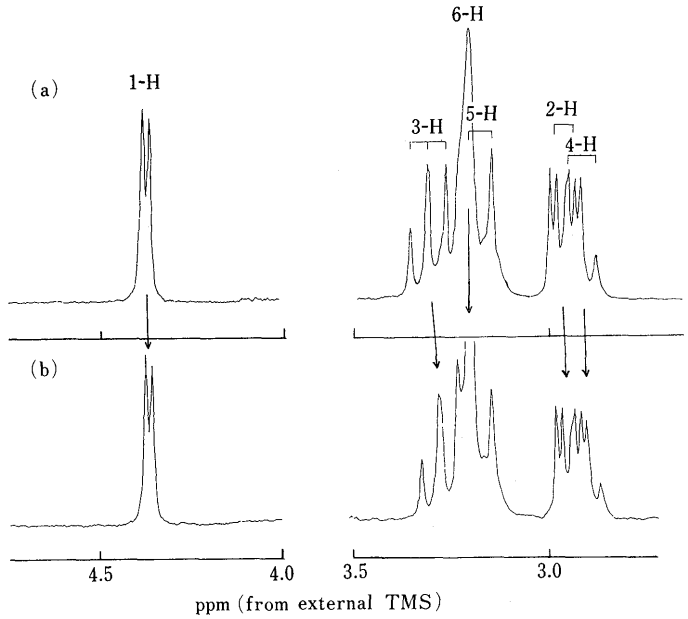

Fig. 8. ^{1}H-NMR Spectra of α-Cyclodextrin in the Presence of TNS in Deuterium Oxide at 30 °C

(a) α-cyclodextrin alone (3.0×10^{-3} M), (b) α-cyclodextrin (3.0×10^{-3} M) + TNS (6.0×10^{-3} M).

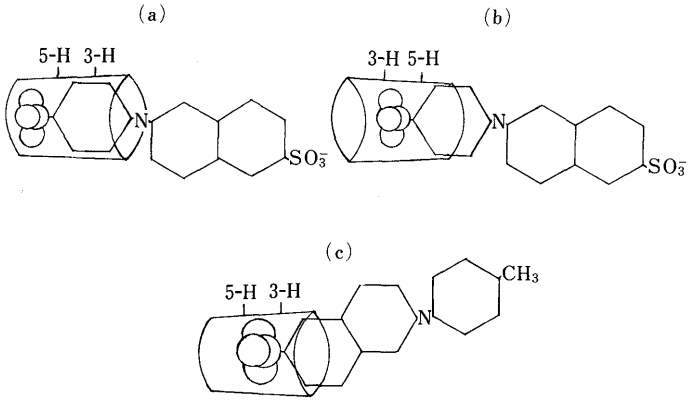

Fig. 9. Possible Structures of the Inclusion Complex of TNS with α-Cyclodextrin in Aqueous Solution Based on CPK Space-Filling Models

As the possible structures for the inclusion complex judged from the investigation by using CPK model, three kinds of structures described next are considered (Fig. 9), (a): the toluidinyl group of TNS is enclosed in the cavity of α-cyclodextrin from the side of the secondary hydroxyl group in considerable depth, the naphthalene ring being almost outside the cavity, (b): the toluidinyl group is included shallowly in the cavity from the primary hydroxyl group side, a part of the toluidinyl group and the naphthalene ring being left outside the cavity, (c): the naphthalene ring enters shallowly at the head of the sulfonyl group into the cavity from the side of the secondary hydroxyl group, a great part of the naphthalene ring and the toluidinyl group being left outside the cavity.

It is considered that upfield shifts of the proton signals lying on the inner surface of α-cyclodextrin result mainly from the magnetic anisotropy of the benzene or the naphthalene ring of the TNS molecule. Therefore, the results that the magnitude of the upfield shifts of the signals due to the protons on the inner surface of α-cyclodextrin are in the order of 3-H > 6-H ≧ 5-H, suggest that the structure shown in Fig. 9b can not occur. In the structure described in Fig. 9c, it may be expected that the 3-H signal shifts upfield and the signals of 5-H and 6-H do not shift. But it was found that 5-H and 6-H signals shifted upfield, although only slightly. Hence, it is suggested that the aromatic ring eneters the cavity more deeply than that in Fig. 9c, and the structure shown in Fig. 9c does not seem likely. If the complex has the structure described in Fig. 9a, it

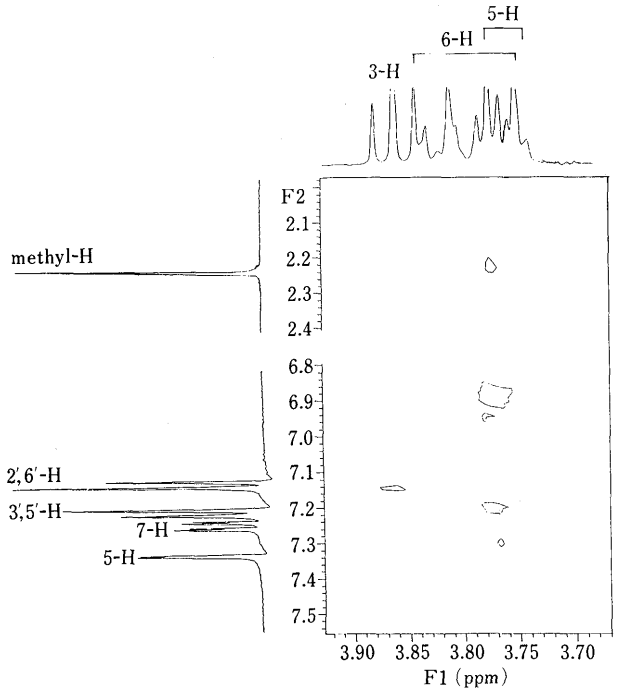

Fig. 10. ROESY Spectrum of Solution Containing TNS (3.0×10^{-3} M) and α-Cyclodextrin (3.0×10^{-3} M) in Deuterium Oxide

is not surprising that the upfield shift of the 3-H signal is prominent and those of 5-H and 6-H signals are only slight. Also, the upfield shift of the 5-H signal was almost the same as that of 6-H signals. This may be because 5-H contracts with the toluidinyl group which entered into the cavity and, therefore, the upfield shift of the 5-H signal is compensated by the steric compression effect bringing about the downfield shift.[9] Consequently, it is reasonable that TNS is estimated to be enclosed in α-cyclodextrin in the manner illustrated in Fig. 9a.

To confirm the structure of the inclusion complex, ROESY spectrum of the solution containing α-cyclodextrin (3.0×10^{-3} M) and TNS (3.0×10^{-3} M) was measured in deuterium oxide (Fig. 10). The cross peaks were observed between the 5-H of α-cyclodextrin and the 3′-H, 5′-H, methyl-H of TNS, and also between the 3-H of α-cyclodextrin and the 2′-H, 6′-H of TNS. Therefore, the structure in Fig. 9a was supported.

Discussion

Further evidence for the structure described in Fig. 9a and more detailed structure containing hydrogen bonding between TNS and α-cyclodextrin will be discussed. If the complex has the structure shown in Fig. 9a, the shifts of the signals due to the protons of TNS included can be explained as follows. The signals due to methyl-H, 3′-H, 5′-H, 2′-H, and 6′-H of the benzene ring shifted downfield as mentioned above. These shifts are considered to have occurred mainly because these protons contact strongly with the atoms lying on the inner surface of the cavity of α-cyclodextrin, namely by the steric compression effect.[9] As 3′-H and 5′-H are most strongly subjected to the steric compression effect in Fig. 9a, it is reasonable that 3′-H and 5′-H signals shift downfield most significantly.

Then, the proton signals of the naphthalene ring shifted downfield except for those of 1-H and 3-H in the presence of α-cyclodextrin. In particular, the downfield shift of 4-H was prominent. It can be presumed that these downfield shifts of the signals due to naphthalene ring protons occur as a result of the hydrogen bonding between >NH of TNS and the secondary hydroxyl group of α-cyclodextrin, because the secondary hydroxyl group of cyclodextrin has the property of proton donor ($pK_a = 12$[10]), and >NH of TNS has that of proton acceptor.[11] However, it cannot be explained by hydrogen bonding that the signal of 4-H shows a most prominent downfield shift. From consideration with CPK models, it was assumed that when the toluidinyl group is included from the side of the secondary hydroxyl group of α-cyclodextrin, 4-H of the naphthalene ring and the secondary hydroxyl group tend to come into contact. Therefore, it was assumed that the prominent downfield shift of the 4-H signal results from the contact, namely, the steric compression effect. It is not reasonable to consider that the prominent downfield shift of the 4-H signal occurs from the magnetic anisotropy effect of the benzene ring because only the 4-H signal cannot structurally[12] shift downfield significantly by the magnetic anisotropy effect of the benzene ring. From the discussion mentioned above, the detailed structure of the inclusion complex containing hydrogen bonding is proposed as shown in Fig. 11.

In the case of complexation in aqueous solution, van der Waals–London dispersion force, hydrogen bonding, hydrophobic interaction, release of high-energy water molecules from the cavity of cyclodextrin, and release of strain energy in a macromolecular ring of cyclodextrin have been reported to play a big part in the interaction.[13] However, according to Tabushi *et al.*[14] the van der Waals–London dispersion force and hydrophobic interaction are major driving forces for the inclusion complexation between cyclodextrin and a guest molecule. In the present study, it was found that complexation is accompanied by negative changes in both entropy and enthalpy, suggesting that van der Waals–London dispersion force and hydrogen bonding between >NH of TNS and the secondary hydroxyl group of α-cyclodextrin are mainly responsible for the inclusion complex formation. When the structure shown in Fig. 11 was examined again using the CPK model, it was found that the benzene ring moiety of TNS is tightly packed. Consequently, it seems reasonable that the changes in enthalpy and in entropy are both negative, neither large nor small. In addition, it is considered that the hydrophobic interaction contributes to the inclusion complex formation,[15] judging from the fact that α-cyclodextrin has a hydrophobic cavity, but the contribution of the hydrophobic interaction may be minor because the change in entropy accompanying the inclusion complex formation has a negative value of −2.6 e.u.

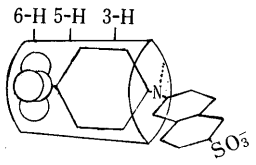

Fig. 11. Most Probable Structure of the Inclusion Complex of TNS with α-Cyclodextrin

It has been reported that there are two cases in which the fluorescence intensity of 8-anilinonaphthalene-1-sulfonate (ANS) increases.[5] First is the case in which the coplanarity of the aromatic rings increases more than in the aqueous solution, as is observed in nonaqueous solvent. Second is the case in which the motion of the ANS molecule is restricted more strongly than in the aqueous solution, as is encountered in the viscous solution. It is considered that these two cases apply to the increase in fluorescence intensity of TNS, because TNS has a similar structure to ANS and shows an extremely analogous fluorescence behavior to ANS. It is apparent from the structure shown in Fig. 11 that the coplanarity of the aromatic rings of TNS does not increase much more in the inclusion complex than in the uncomplex form. On the other hand, it is clear that the motion of the TNS molecule may be restricted more strongly in the inclusion complex than in the uncomplex form. Therefore, the increase in fluorescence intensity can be attributed more to the latter, the restriction of motion, rather than the former, the coplanarity of the aromatic rings.

Acknowledgement We are grateful to Dr. M. Sugiura for her measurements of ^{1}H-NMR spectra.

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