THE INVESTIGATION OF COLLAGEN TOPOLOGY BY USING THE HYDROPHOBIC FLUORESCENT PROBE, 2-p-Toluidinylnaphthalene-6-Sulfonate

by

Hsin-Chou Chiang

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1-27-76
date

Aaron Lukton
Chairman of Examining Committee

1-27-76
date

Aaron Lukton
Executive Officer

Morton D. Glantz

Fred Naider

Carl Haberfield

Robert Bittman
Supervisory Committee

The City University of New York

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GENERAL INTRODUCTION

The fluorescent probe, 2-p-toluidinylnaphthalene-6-sulfonate (TNS) and related compounds have been extensively used to monitor the conformational changes of proteins and biomembranes[1-4]. This spectroscopic technique which provides information on the higher order structures of macromolecules is primarily based upon the finding that fluorescent properties of the Frank-Condon excited state of these compounds are sensitive to the solvent polarity[2]. In general, the emission maximum of TNS becomes blue shifting with increasing quantum yield in lower polarity solvents. By measuring solvent polarity dependent changes in the emission spectrum as well as other fluorescent properties, including excited state lifetime, it is possible to probe the hydrophobic region of macromolecules. The method is sensitive and it can provide valuable information which can not be easily obtained by other methods.

The original purpose of this research work is to use TNS to investigate the hydrophobic characteristics of collagen. The vertebrate collagen molecule has a molecular weight in the vicinty of 300,000 and exists in solution as a rodlike, triplet-helical particle approximately 2800Å in length and 15Å in diameter. It consists of three polypeptide chains (α chains) which may be covalently cross-linked to an extent which depends upon the origin and age of the collagen. A repeating tripeptide sequence, -Gly-X-Pro-, -Gly-Pro-X- and

-Gly-X-Hyp-, is frequently occurring in the primary structure α chains. Part I of this thesis indicates the results of using TNS to study the hydrophobic characteristics of the triple-helical structure of collagen molecule.

One conclusion of this investigation was the binding force for the interaction of collagen molecule with TNS was mainly hydrophobic in nature. This result was not quite in agreement with Beyer et al's suggestion that charge neutralization was required for the binding of TNS with some antibiotic peptides[5]. Since TNS is an amphiphilic compound, carrying a negatively charged sulfonate group and an aromatic ring system of toluidinylnaphthalene in the molecule, the question arises as to the relative importance of the two binding forces. To test the critical question, whether TNS could bind with negatively charged molecules only by the hydrophobic interaction regardless of possible charge repulsion, sodium dedecyl sulfate (SDS) was found to be a good model. SDS forms micelles at the concentration greater than the critical micelle concentration (cmc). The formation of the SDS micelle is caused by the hydrophobic interaction which is strong enough to compensate for the charge repulsion between SDS monomers. Part II of this thesis shows the results of the interaction of TNS with SDS micelle in aqueous solution of various ionic strengths. The result strongly suggests that the toluidinylnaphthalene group of TNS is a hydrophobic bonding contributor. Since hydrophobic bond, like the ionic interaction, is a nonspecific force, the

result implies that the bonding between TNS with proteins, may only involve hydrophobic interaction.

To further investigate the nature of both the sulfonate group and aromatic rings of TNS is bonding, the interaction of TNS and alkyltrimethylammonium halide (ATAH) with various alkyl chain length was examined by using the salt effect. The results of part III of this thesis show that, although ionic interaction seen in the interaction of ATAH monomer-TNS complex, there is a hydrophobic interaction between the alkyl group of ATAH with the aromatic rings of TNS. Many interesting and exciting results have been obtained in this study.

The results of Part I provided the stimulus for the experiments in Part II and III. The results of these three parts are united as a whole to establish that the aromatic rings of TNS is a hydrophobic bonding contributor in binding with other molecules. The significance of these results is that hydrophobic fluorescent probes such as TNS may bind with the hydrophobic area of a macromolecule without charge neutralization.

I was so deeply attracted by this fundamental molecular force problem that I felt tireless in the pursuit of this research. Science is a combination of philosophy, challenge and enjoyment. I can feel some kind of harmonic beauty in this universe from the scientific research presented here.

References

1. W. C. McClure and G. M. Edelman, Biochemistry, 5, 1908 (1966)
2. L. Brand and J. R. Gohlke, Ann. Rev. Biochem., 41, 843 (1972)
3. L. Stryer, Science, 162, 526 (1968)
4. T. Takagi, Tampakushitsu Kakusan Koso, Bessatzu, 143 (1974)
5. C. F. Beyer, L. C. Craig, and W. A. Gibbons, Biochemistry, 11, 4920 (1972)

PART I

The Interaction of Collagen with the Hydrophobic Fluorescent Probe, 2-p-toluidinylnaphthalene-6-sulfonate*

* This manuscript was published in Biopolymers, 14, 1651-1666 (1975)

Abstract

Three kinds of fluorescence enhancement result from the interaction of 2-p-toluidinylnaphthalene-6-sulfonate and calf-skin collagen. They are negatively cooperative, independent, and highly cooperative fluorescence enhancement. In the independent region at pH 3.7, the binding number is about 36 moles of 2-p-toluidinylnaphthalene-6-sulfonate per mole of tropocollagen with a binding constant of 2.0×10^4 M^{-1}; with $\Delta G = -5.7$ kcal/mole, and $\Delta S = 6$ e.u. The pH dependence of fluorescence of native collagen shows that the deprotonated forms of the β and γ carboxyl groups of aspartic and glutamic acid decrease the intensity, possibly by charge repulsion of the negatively charged sulfonate group of 2-p-toluidinylnaphthalene-6-sulfonate. The positive charge of lysine is found to be unimportment in the interaction of 2-p-toluidinylnaphthalene-6-sulfonate with collagen. Fluorescence enhancement is caused mainly by the hydrophobic interaction of 2-p-toluidinylnaphthalene-6-sulfonate and collagen. Salt bridge formation between basic and acidic side chains in very low salt concentration may be detectable by 2-p-toluidinylnaphthalene-6-sulfonate fluorescence.

Introduction

The fluorescent probe 2-p-toluidinylnaphthalene-6-sulfonate (TNS) and its related substituted derivatives have been extensively used to study the conformational changes

of proteins[1-3] and biomembranes[3-5]. The compounds are practically nonfluorescent in water but become highly fluorescent, with blue shifts in the emission spectra, both in organic solvent and when bound to certain proteins. The method is sensitive, and it provides valuable information concerning the hydrophobic and hydrodynamic properties of proteins.

Although fluorescent probes, which are noncolvently bound to proteins, have been applied to studies of conformation changes of proteins, the exact binding environment is still obscure. Hydrophobic interaction was found to be a major contribution to the binding energy, when 1-anilino-8-naphthalene sulfonate (ANS) bound to apomyoglobin and to apohemoglobin[6]. Recently, Beyer et al.[7], revealed that although the hydrophobic environment of the tyrocidine peptides was essential for enhancing the fluorescence of TNS, charge neutralization between the sulfonate group of TNS and a basic group on the peptide was also required for binding. Other studies have shown that only the β-sheet[8,9] of the uncharged form of polylysine resulted in TNS fluorescence. Nevertheless, the energy-dependent ANS fluorescence changes in submitochondrial particles have been attibuted to the pK_a of charged groups, such as carboxylate anion, at a polar-nonpolar interface inside the membrane[10]; and, the electrostatic repulsion between ANS and phospholipid appears to be important for ANS binding[11].

Collagen and some related peptides, as rodlike polymers[12,13],

have not been investigated with respect to their hydrophobic characteristics. The collagen molecule provides a good model for studying the binding requirements of TNS to protein since the amino acid residue side chains are regularly exposed outside of the rigid triple helix peptide backbone. In this study, we examine the TNS-collagen binding parameters, ultimately to correlate them with the amino acid sequence of calf-skin collagen which has been recently determined[14-20].

Experimental Section

The potassium salt of TNS was purchased from Sigma Chemical Co. (lot no. 80c-5130). It was homogeneous, as shown using silica gel thin layer chromatography[7]. The salt was stored in the dark at 4°C.

The $(Pro\text{-}Ala\text{-}Gly)_n$ ($M_r \simeq 12,400$) and $(Pro\text{-}Ser\text{-}Gly)_n$ ($M_r \simeq 18,000$) were the gift of Prof. E.R. Blout, Harvard Medical School. Poly (L-glutamic acid), with M_r 50,000 - 80,000, was the product of Schwarz/Mann (lot no. Y3117).

The acid-soluble calf-skin collagen was the product of Sigma Chemical Co. (lot no. 43C-8212). The collagen was dissolved in 0.01N acetic acid (pH 3.7) or diluted HCl (pH 3.7) with stirring for 1 day at 4°C. The solution was centrifugated at 0°C for 1 hr at 40,000 × g to remove undissolved proteins. Using the disc electrophoresis method of Nagai et al.[21], the supernatant was homogeneous. That is, only two bands corresponding to the α and β forms were observed. The collagen concentration was determined using the microbiuret reaction[22].

It followed Beer's law to the maximum collagen concentration of 1.7 mg/ml.

Fluorescence measurements were done on a Perkin-Elmer Fluorescence Spectrophotometer MPF-2A with both excitation and emission band widths being set at 4 nm. The temperature of the sample was maintained at 15°C. Because the absorption band maximum changed from 350 nm to 383 nm at increasing TNS concentrations and at constant collagen concentration, the emission spectra were obtained by exiting at the largest absorption maximum. The excitation spectra were measured at the emission wavelength 440 nm.

The binding constant for collagen with TNS at the Hill coefficient C = 1 region was calculated from the following equation[23]:

$$\frac{\text{Collagen}}{xD} = \frac{1}{n} + \frac{1}{nK} \frac{1}{(1 - x)D}$$

where x is the fraction of the TNS bound, n is the number of binding sites, D is the total TNS concentration, and K is the statistical association constant. For each TNS concentration, x was calculated from the relation:

$$x = \frac{(I_{obs} - I_{TNS})}{(I_{max} - I_{TNS})}$$

where I_{obs} is the observed fluorescence intensity of the sample; I_{max} is the fluorescence intensity at infinite collagen concentration obtained by plotting $1/I_{obs}$ vs. $1/[\text{collagen}]$. I_{TNS} is the fluorescence intensity of TNS in free solution.

The enthalpy for the binding between TNS and collagen

was calculated according to the following equation:

$$\Delta H = -R \, d \ln K_T / d\left(\frac{1}{T}\right) = -R \, d \ln I_T / d\left(\frac{1}{T}\right)$$

where K_T is the association constant at temperature T; I_T is the relative fluorescence intensity at temperature T. The equation was derived with the assumption that $K_T = \alpha I_T$; α is a constant which is valid if the quantum yield of fluorescence is constant. We assume this to be the case since the emission wavelength of maximum intensities and band widths of emission spectra did not change when the temperature was increased from 6^{o} to 26^{o}C. The circular dichroism spectrum of the collagen solution also does not change in this temperature range, suggesting that the number of binding sites remain essentially constant.

The succinylation of collagen was done by the method of Klapper and Klotz[24]. Succinic anhydride (0.3 g) was added in small increments to 10 cc of collagen (concn 0.85 mg/ml). The pH of the solution was maintained between 8-10 by adding 1N NaOH. The solution was stirred at 0^{o}C for 1 hr. The reaction conditions were the same for the control sample of collagen except that 0.3 g of sodium succinate solution at pH 8 instead of succinic anhydride was added to collagen. After the reaction, the succinylated collagen and the control were subjected to exhaustive dialysis against distilled water at 4^{o}C.

The circular dichroism was measured on a Durrum-Jasco spectro-Polarimeter Model J-20.

The sample of TNS-treated collagen for study in the electron microscope was prepared as follows: the TNS-collagen solution (1.1×10^{-6}M collagen in 0.01M acetic acid with 2.7×10^{-4}M TNS) was centrifugated after glutaraldehyde fixation. The pellet was post-fixed with Palade's fluid and aqueous uranyl acetate. After dehydration, the pellet was embedded in Maraglas and was stored at 37°C for 1 week before sectioning. The section was stained in aqueous lead citrate solution for 5 min. and was observed under a Zeiss EM 95-2 electron microscope. The native collagen (1.1×10^{-6}M in 0.01 M acetic acid) was also studied with the same procedure.

Results

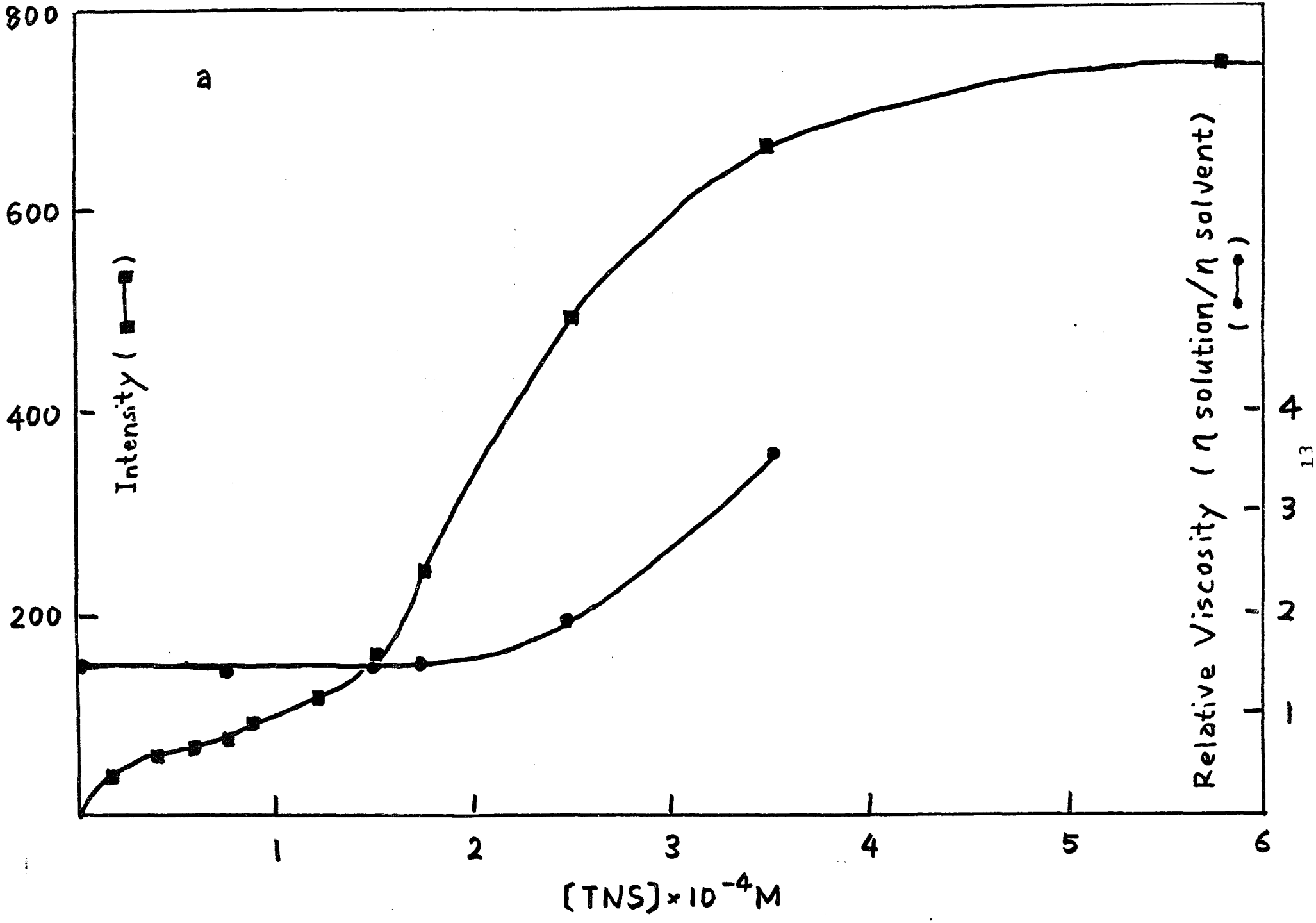
Fluorescence of the Interaction between TNS and Collagen.

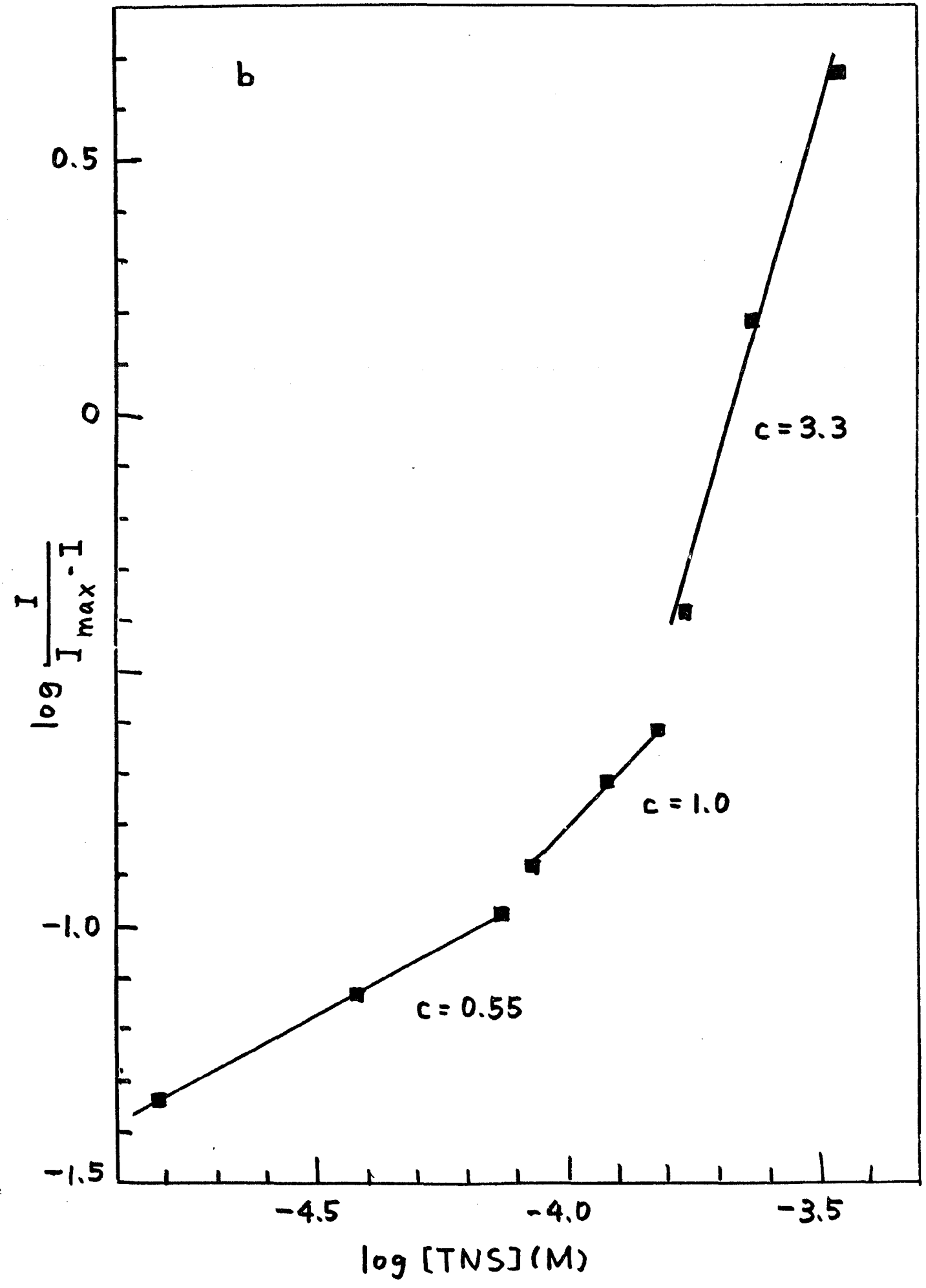
The fluorescence and the viscosity changes of TNS at constant collagen concentration is shown in Fig. 1a. The fluorescence intensity changes seem heterogeneous. Although the viscosity increased at TNS concentrations greater than 1.75×10^{-4}M, the fluorescence intensity increase was not due to the increase of viscosity since the quantum yield did not change significantly as the TNS concentration increased. There is, however, a slight viscosity-induced fluorescence enhancement. As measured, using TNS-sucrose solutions with viscosities equivalent to those of TNS-collagen solutions, the viscosity contribution to fluorescence enhancement never exceeded 10% of the total enhancement. The effect of TNS on

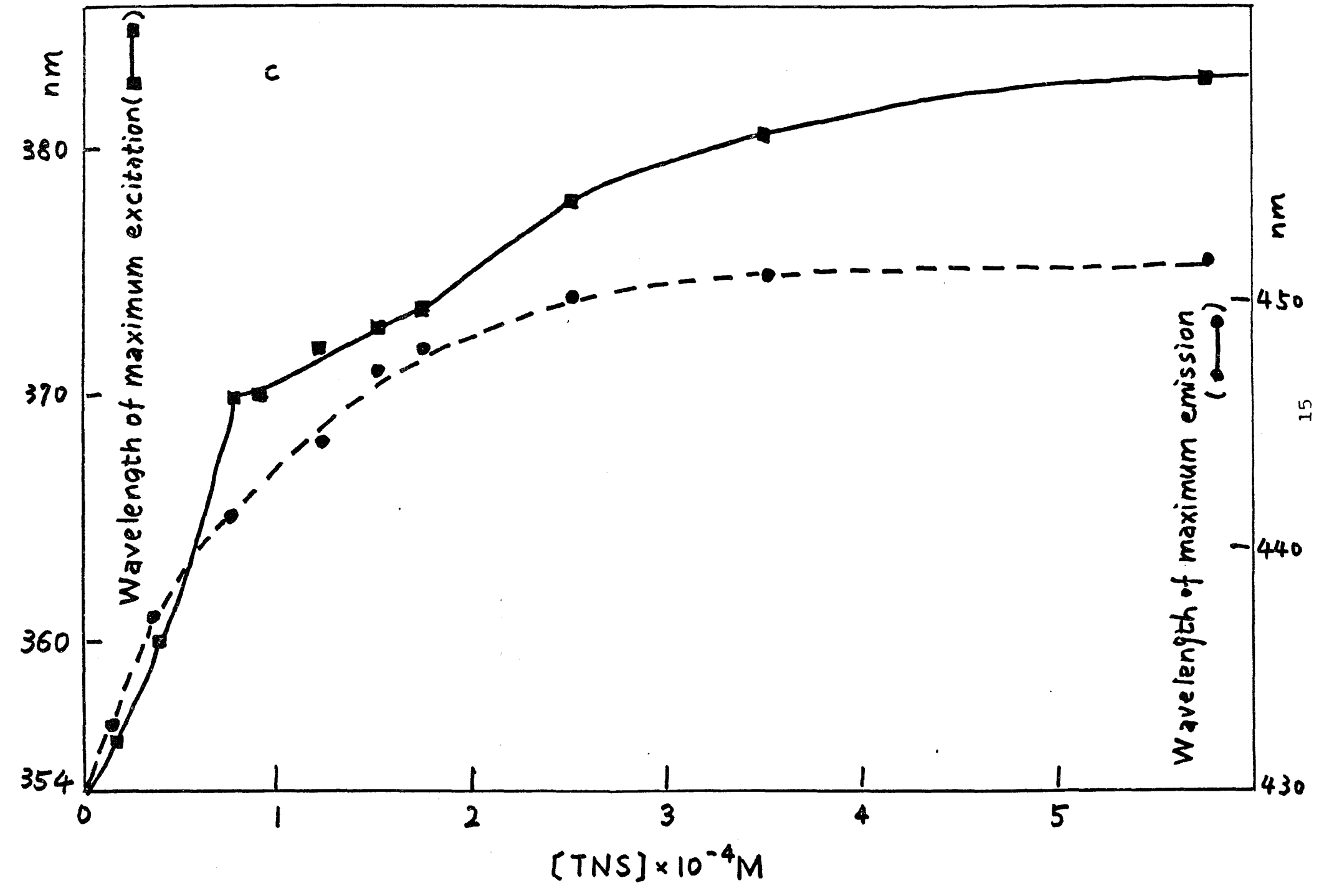
Fig. 1. (a) Fluorescence (▪—▪—▪) and viscosity (•—•—•) change as a function of [TNS] . Collagen concentration is 1.1×10^{-6}M in 0.01 acetic acid, pH 3.7. Fluorescence was measured at 15^{o}C.

(b) Hill plot of Fig. 1a I_{max} is 800.

(c) Wavelengths of maximum excitation and emission of Fig. 1a.


800
600
400
200
a
Intensity (■—■)
1
2
3
4
5
6
[TNS] × 10⁻⁴M
Relative Viscosity (η solution/η solvent) (●—●)
1
2
3
4

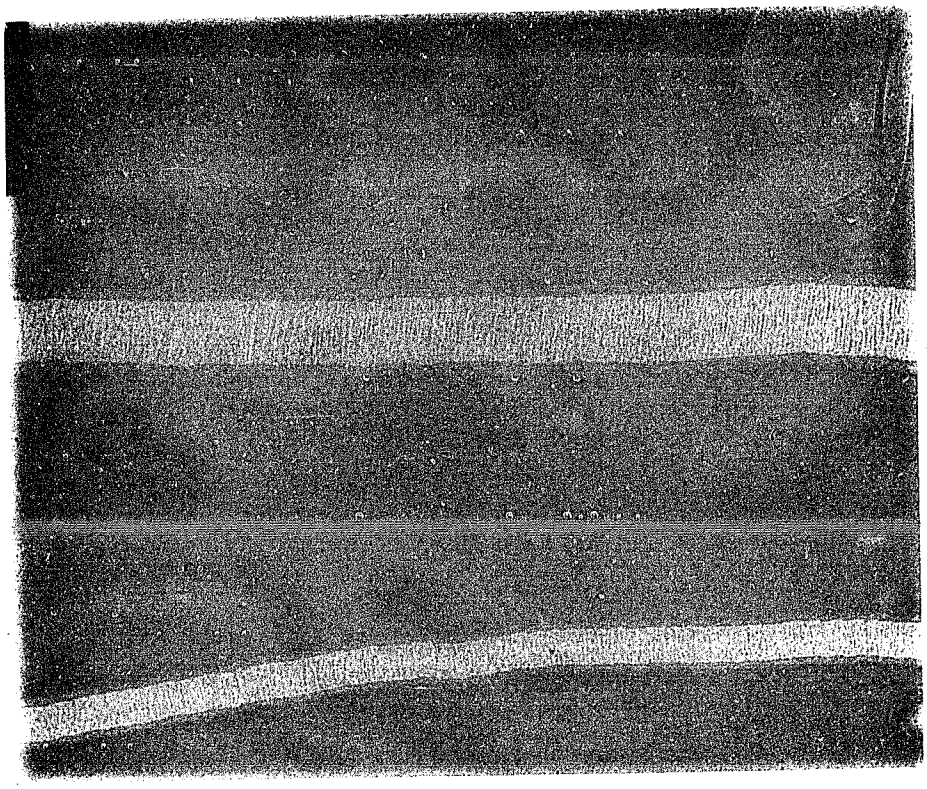

b
0.5
0
-1.0
-1.5
$\log \frac{I}{I_{max}-I}$
c = 3.3
c = 1.0
c = 0.55
-4.5
-4.0
-3.5
log [TNS] (M)


c
nm
380
370
360
354
Wavelength of maximum excitation (■—■)
Wavelength of maximum emission (●—●)
450
440
430
nm
0
1
2
3
4
5
[TNS] × 10⁻⁴M

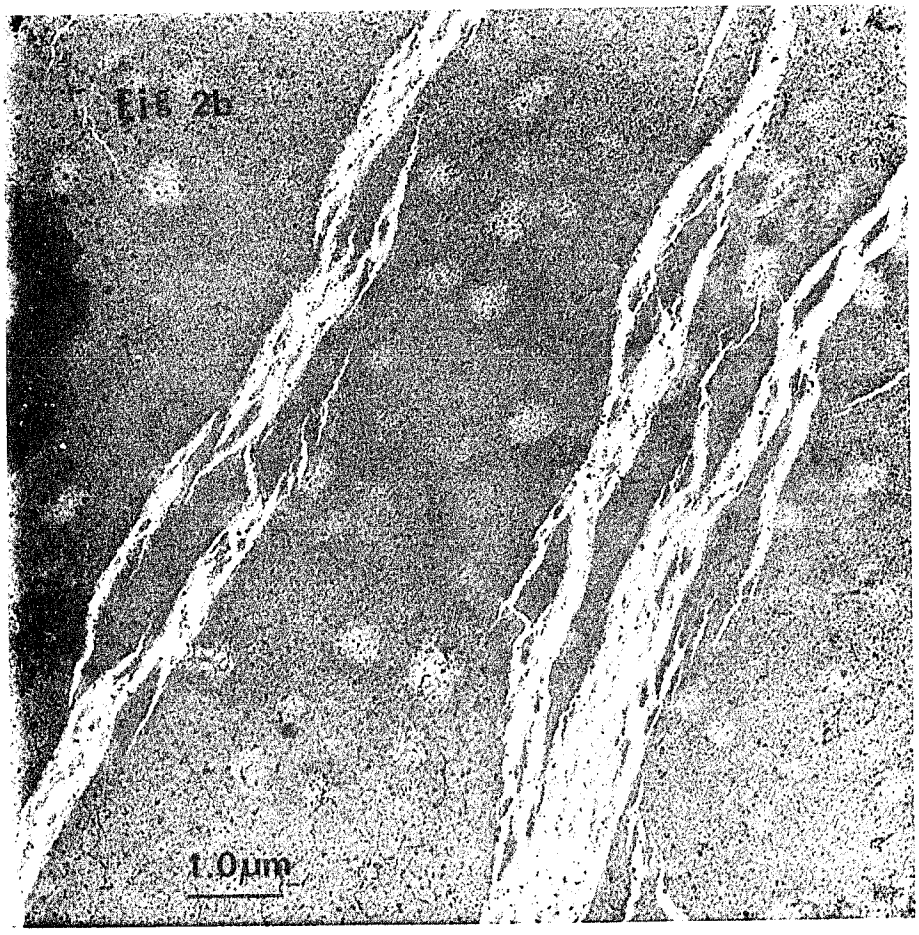
collagen fiber formation can be seen by comparing the electron micrograph of collagen without and with TNS (Fig. 2a and 2b, respectively). It is apparent that TNS caused the disaggregation of the fiber structure, suggesting that the viscosity increase at high TNS concentrations may have been caused by hydrodynamic property changes of TNS-bound tropotum yield was 0.073 ± 0.002 in the entire binding range as calculated by the Parker and Rees's method[25]. The Hill plot of the Figure 1a fluorescence enhancement data is given in Figure 1b; it shows three significantly different kinds of fluorescence enhancements. They are negatively cooperative enhancement for C = 0.55, independent enhancement for C = 1.0, and highly cooperative enhancement for C = 3.3. The three kinds of fluorescence enhancements correspond to three regions with different degrees of red shifts of the excitation maxima (Fig. 1c) as TNS concentrations changed from 0 to 8.8×10^{-5}M, from 1.0×10^{-4}M to 1.8×10^{-4}M, and from 2.6×10^{-4}M to 5.7×10^{-4}M.

At the Hill coefficient C = 1.0 region, the binding number was about 36 moles of TNS per mole of collagen (M_r = 3.0×10^5) with the association constant $2.0 \times 10^{-4}M^{-1}$ (Fig. 3). The enthalpy of the binding, as calculated from the data in Fig. 4, was -4 0 kcal/mole. From this ΔH value and the association constant ΔG = -5.7 kcal/mole, ΔS = 6 e.u. was obtained. The thermodynamic data is very close to that obtained for the interaction of polyvinylpyrrolidone with aromatic

Fig. 2. Electron micrographs of native collagen (a) and TNS-treated collagen (b) (x 51,250). The preparative procedure is given in the text.



(a)



(b)

Fig. 3. The binding of collagen and TNS in 0.01 acetic acid (pH 3.7) at 15°C. Collagen concentration, 1.66×10^{-6}M.

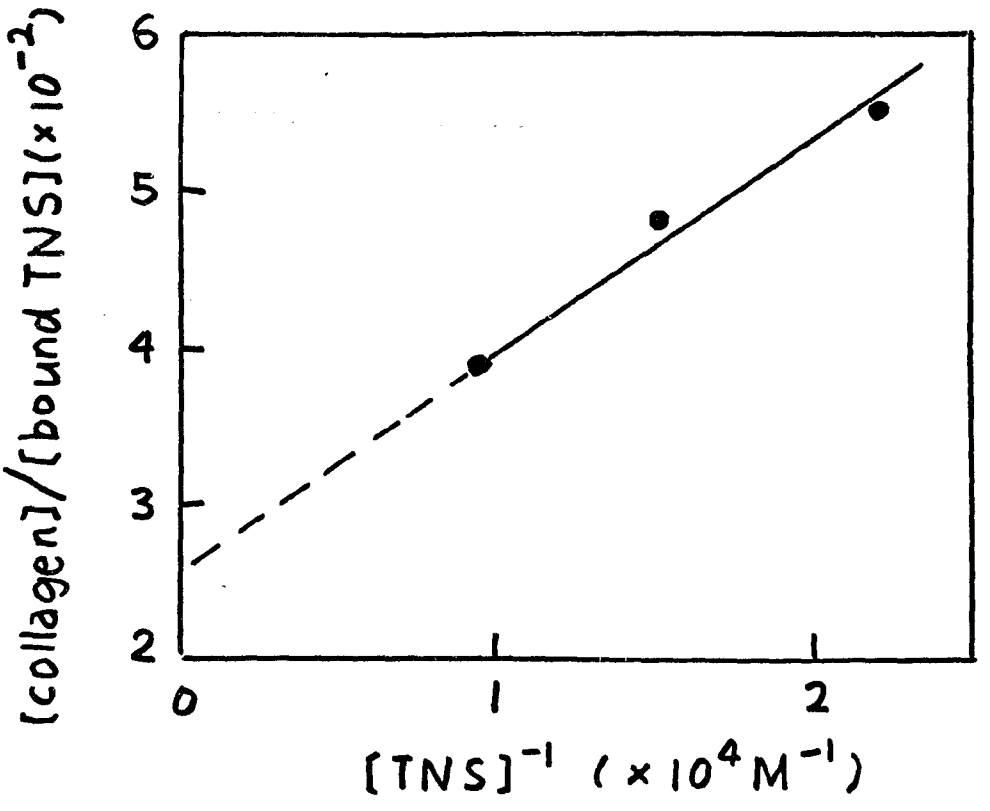
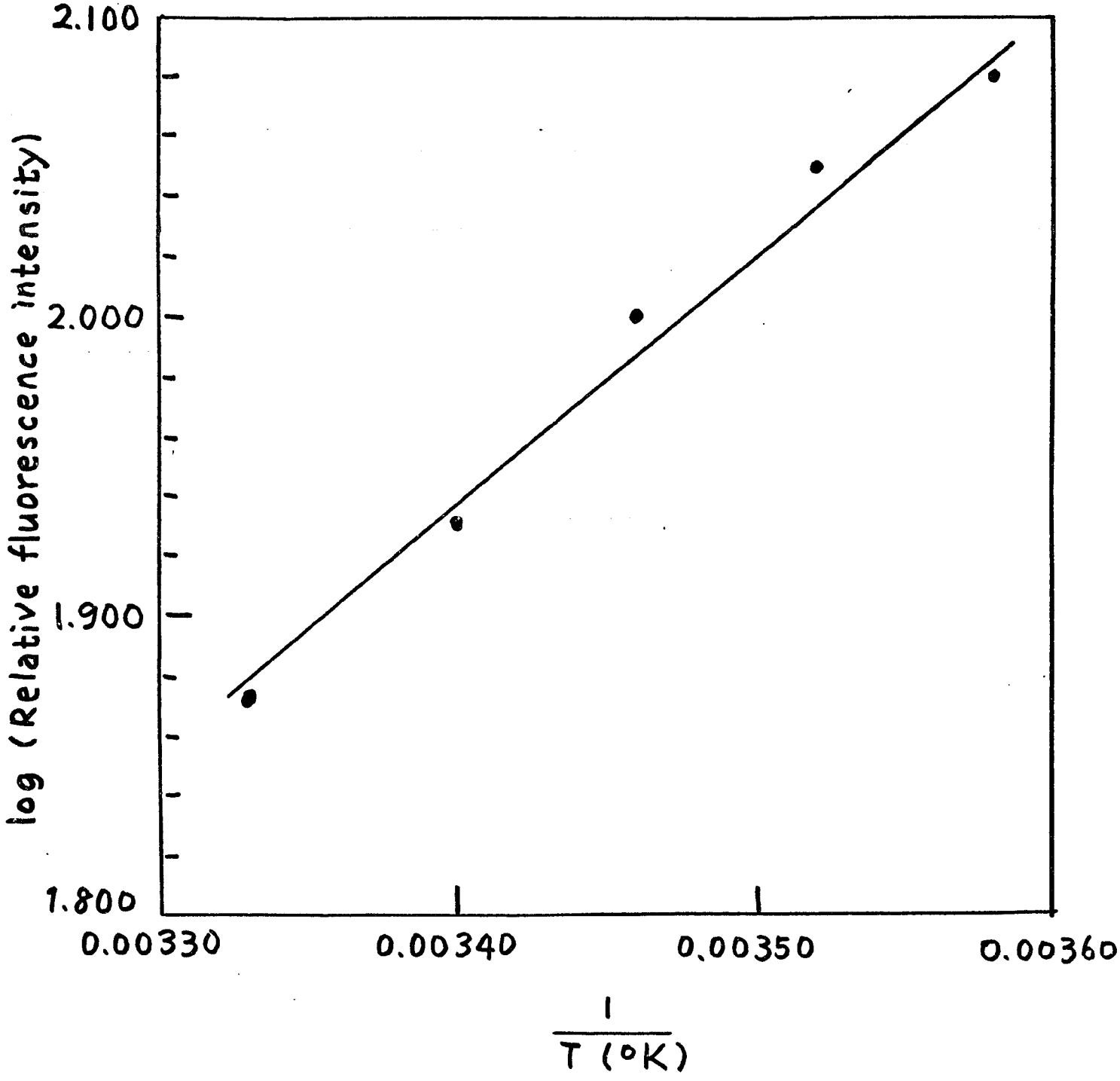

[collagen]/[bound TNS](×10⁻²)
6
5
4
3
2
0
1
2
[TNS]⁻¹ (×10⁴M⁻¹)

Fig. 4. Log [relative fluorescence intensity] vs. $1/T(^{o}K)$.
The relative fluorescence at $15^{o}C$ was taken as 100.
Collagen concentration: 1.1×10^{-6}M, TNS : 1.5×10^{-4}M.


2.100
2.000
1.900
1.800
log (Relative fluorescence intensity)
0.00330
0.00340
0.00350
0.00360
1
T (°K)

compounds in aqueous solution[26] and is similar to the data for the interaction of ANS with apomyoglobin[6]. The temperature-sensitive binding and small entropy gain suggest that the fluorescence enhancement was probably due to the hydrophobic interaction between TNS and collagen.

The fluorescence polarization as shown in Fig. 5a decreased as [TNS] increased; however, the polarization attained a negative value at [TNS] greater than 1.5×10^{-4}M. According to Weber's equation[27] of concentration-dependent polarization, which holds to [TNS] = 8.8×10^{-5}M, the limiting polarization was 0.33 (Fig. 5b). The small value of polarization as shown in Fig. 5a suggests that the binding sites for TNS in collagen are not rigid. It is not known whether the decreasing fluorescence polarization [28, 29] was caused by energy transfer of close TNS binding sites or by the change of rotation relaxation time of collagen. However, polarization was apparently not caused by a decreasing lifetime of excited bound TNS since the quantum yields were constant.

The binding of TNS with collagen caused not only a red shift of the excitation spectra and emission spectra as shown in Figure 1c, but it also resulted in a large change of the excitation spectra as indicated in Fig. 6. Thus, at [TNS] less than 2.33×10^{-4}M, the intensity of three excitation bands at about 280 nm, 335 nm, and 370 nm increased continuously as the [TNS] increased. Also at [TNS] greater than 2.33×10^{-4}M, the intensity of the 370 nm band increased as the [TNS] increased; however, the intensity of the 335 nm decrease

Fig.5. (a) Polarization (p) vs. [TNS]. Collagen concentration is 1.1×10^{-6}M in acetic acid (0.01 M, pH 3.7).
(b) (1/p - 1/3) vs. [TNS] of Fig. 4a at [TNS] less than 0.88×10^{-4}M.

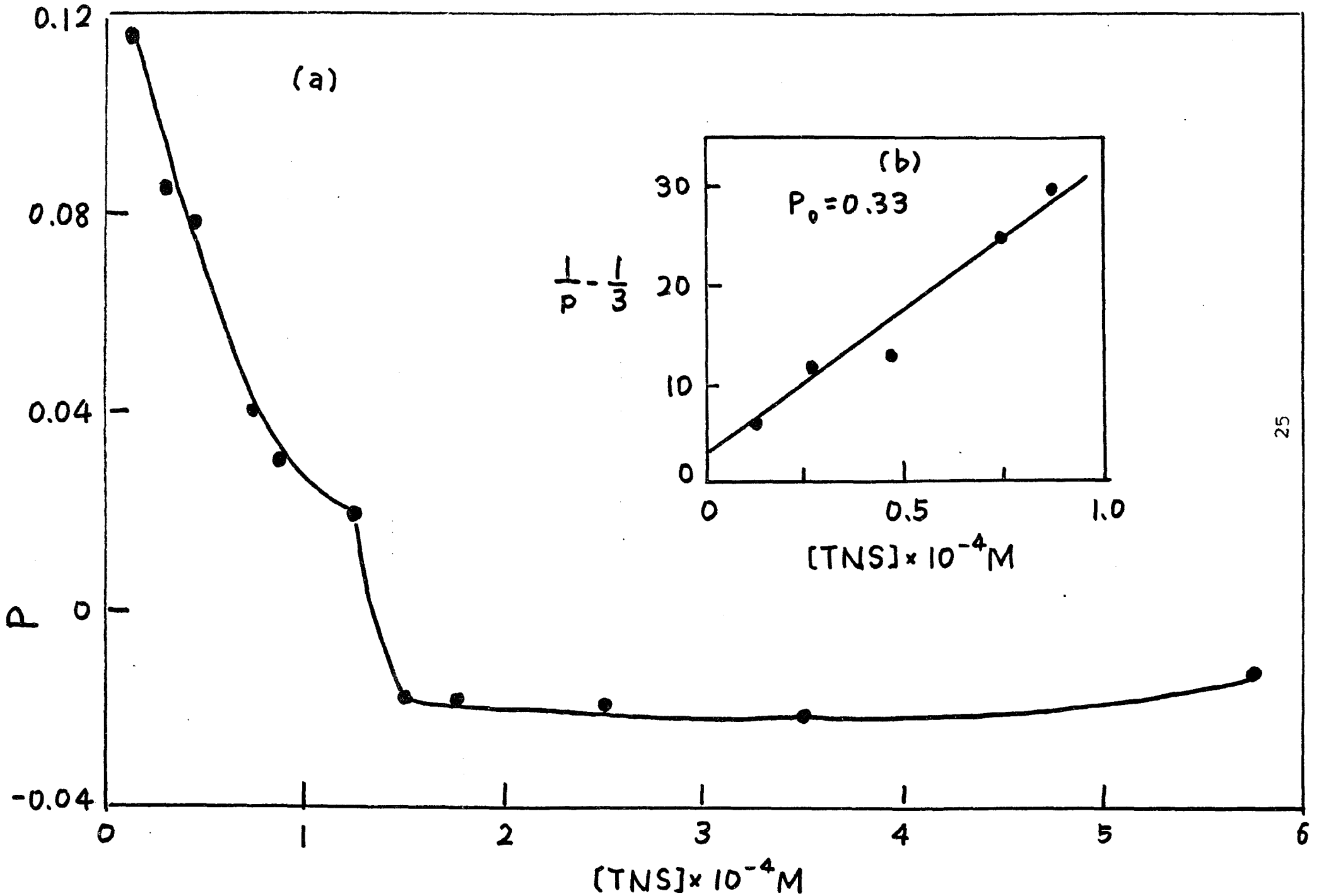
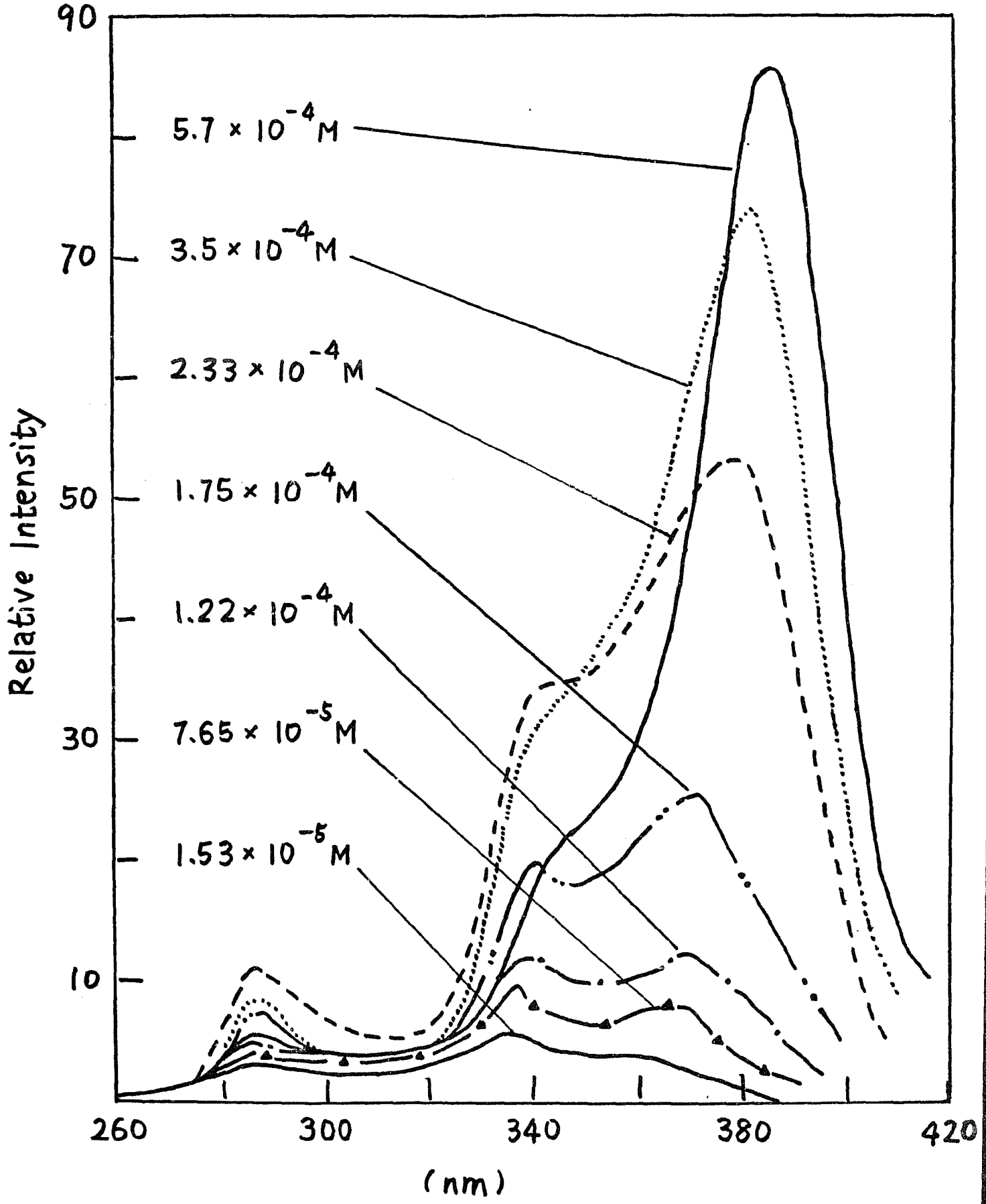

(a)
0.12
0.08
0.04
0
-0.04
P
0
1
2
3
4
5
6
[TNS]× 10⁻⁴M
(b)
P₀=0.33
1/P - 1/3
30
20
10
0
0.5
1.0
[TNS]× 10⁻⁴M

Fig. 6. The excitation spectra at different TNS concentrations. Collagen concentration is 1.1×10^{-6}M. Fluorescence was measured at 15^{o}C in pH 3.7.

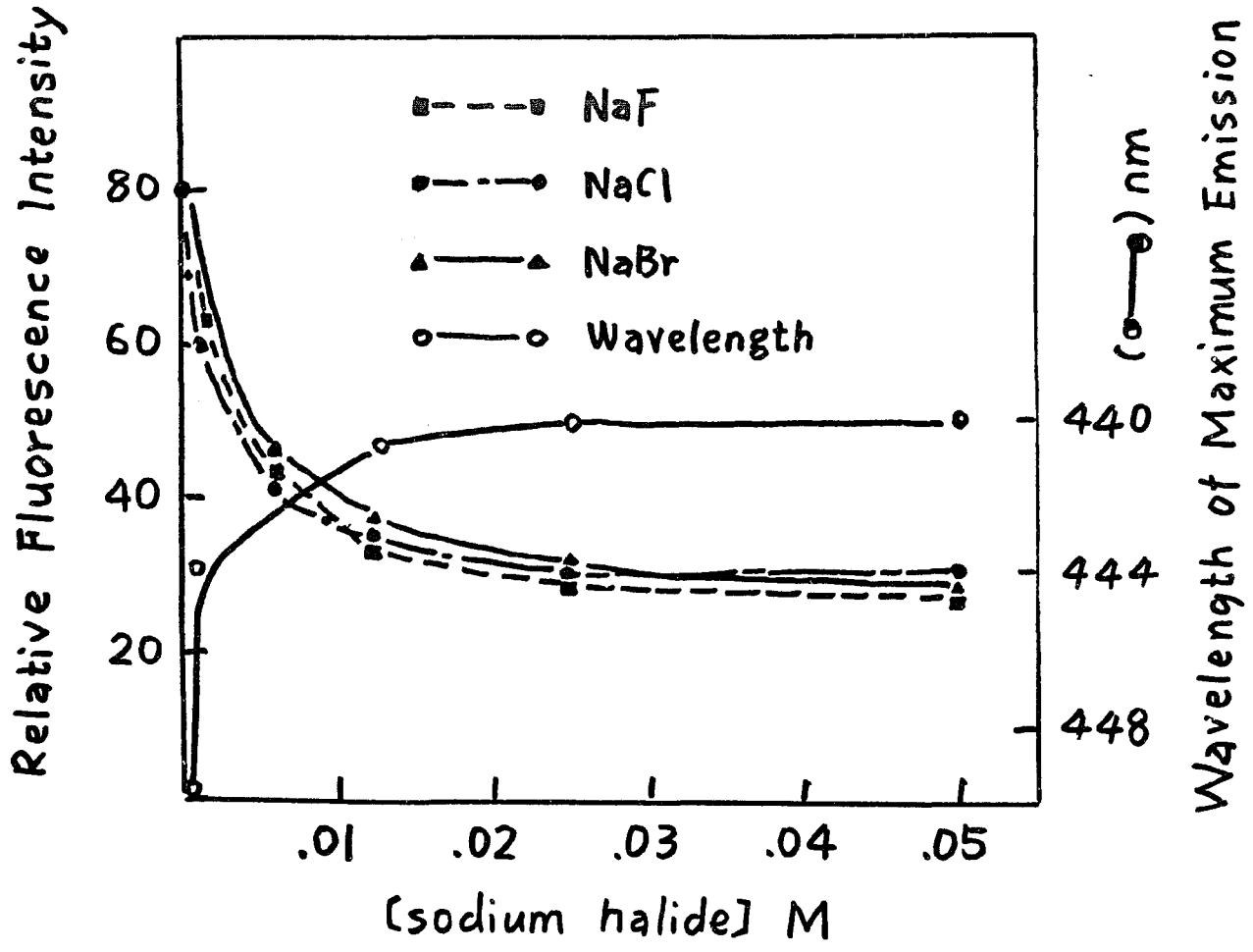

90
70
50
30
10
Relative Intensity
5.7 × 10⁻⁴M
3.5 × 10⁻⁴M
2.33 × 10⁻⁴M
1.75 × 10⁻⁴M
1.22 × 10⁻⁴M
7.65 × 10⁻⁵M
1.53 × 10⁻⁵M
260
300
340
380
420
(nm)

as the [TNS] increased. Seliskar and Brand[30] have determined that the $a_{\pi} \leftarrow \ell$ transition is the main contribution to the 370 nm and 335 nm absorption band. Our results indicate that the assignment of both excitation transitions to be $a_{\pi} \leftarrow \ell$ should be conservative.

Salt Effects on Fluorescence

Fig. 7 shows a sharp drop in fluorescence and a blue shift of the maximum emission wavelength at salt concentrations between 0 and 0.0125M, thus revealing a marked salt sensitive property of the collagen surface which disappears at salt concentrations greater than 0.025M. This supports the idea that the TNS and collagen interaction is hydroplobic and that it could not be eliminated by high ionic strength. No significant change of viscosity was found in the salt concentration range used, thus ruling out the possibility that the fluorescence change was caused by the association of collagen. The striking fluorescence decrease at salt concentrations between 0 and 0.01 M may be the result of increasing internal salt bridge formation between adjacent acidic and basic amino acid residues in collagen in the lower ionic strength. The increasing salt bridge formation would make the deprotonated and carboxyl groups of glutamic acid and aspartic acid less available to repel the binding of TNS to collagen with the result of increasing of fluorescence at lower ionic strength. The amino acid sequence analysis shows that many basic and acidic amino acid residues are close to each other; thus a salt bridge can

Fig. 7. The salt effect on fluorescence. Collagen concentration is 1.0×10^{-6}M. TNS : 1.5×10^{-4}M. The pH of the solution was 3.7. The fluorescence spectra was measured at 15^{o}C.


Relative Fluorescence Intensity
80
60
40
20
NaF
NaCl
NaBr
Wavelength
440
444
448
Wavelength of Maximum Emission (nm)
.01
.02
.03
.04
.05
[sodium halide] M

be formed between them and also between residues on different chains in the triple helix.

pH dependence of Fluorescence

Collagen structural changes appear to be minimal from pH 2.4 to 7 since the circular dichroism spectra of collagen[31-33] is independent of pH in that range. At pH 1 and 12, the triple helix of collagen is partially disrupted. These data are shown in Fig. 8.

The effect of pH on the binding of TNS with native collagen and heat-denatured collagen, in the absence of salt, is given in Fig. 9a and the effect of adding 0.0125M NaCl is shown 9b. The difference of fluorescence dependence at pH values below 3 in solutions with and without 0.0125M NaCl indicates that charge participates in the interaction of collagen and TNS below pH 3. The decreasing fluorescence intensity below pH 3 in zero ionic strength solution may have been caused by the charge repulsion of basic amino acid residues with the protonated secondary amine of TNS (pK = 1.5)[11]. Such charged repulsion could be reduced in 0.0125M NaCl solution.

The decrease of fluorescence intensity at pH above 3 in both no salt and 0.0125M NaCl solutions (Fig. 9a and 9b) may reflect the charge repulsion between the ionized carboxyl groups of glutamic and aspartic acid residues and the negatively charged sulfonate group of TNS. The apparent pK value of the carboxyl groups as determined from the curves is about

Fig. 8. The pH dependence of ellipticity of calf-skin collagen.

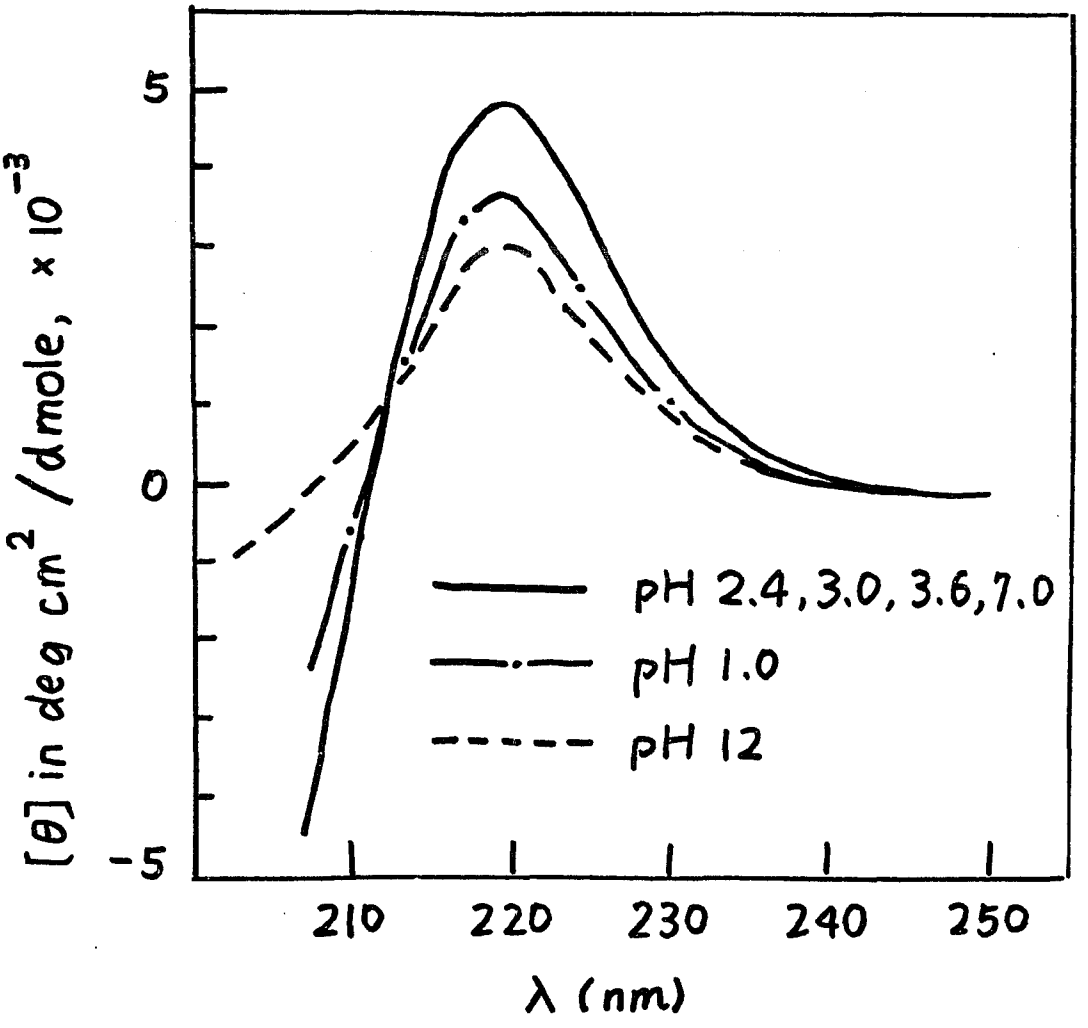
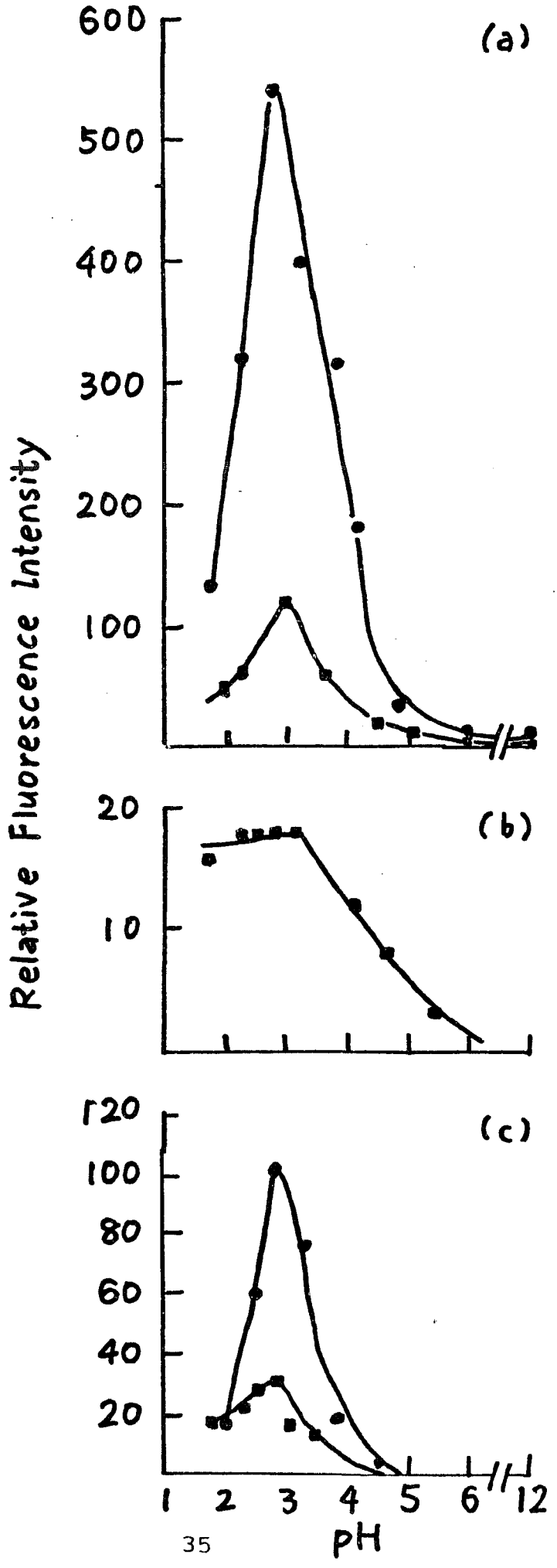

5
0
-5
[θ] in deg cm² /d mole, × 10⁻³
210
220
230
240
250
λ (nm)
pH 2.4, 3.0, 3.6, 7.0
pH 1.0
pH 12

Fig. 9. (a) pH dependence of fluorescence intensity of TNS binding with native collagen (▪—▪—▪) and heat-denatured collagen (•—•—•). Collagen 8.4×10^{-7} M was in HCl solution (pH 3.7). TNS: 1.5×10^{-4}M. The mixture of collagen and TNS was dissolved in pH 3.7 HCl solution and was adjusted to different pH values by adding dilute HCl or NaOH.

(b) pH dependence of fluorescence intensity of TNS binding with native collagen in 0.0125M NaCl. Collagen: 8.4×10^{-7}M, TNS: 1.5×10^{-4}M.

(c) pH dependence of fluorescence intensity of TNS binding with control collagen (▪—•—▪) and succinylated collagen (•—•—•). Collagen concentration : 5.0×10^{-7} TNS: 1.5×10^{-4}M.


600
500
400
300
200
100
(a)
20
10
(b)
120
100
80
60
40
20
(c)
Relative Fluorescence Intensity
1
2
3
4
5
6
12
pH

3.7 ± 0.1 in zero ionic strength and 4.2 ± 0.1 in the 0.0125M NaCl solution. This data strongly suggests that salt bridges form between carboxylate and nearby positively charged amino acid residues in very low salt concentration solution and that they can be detected with the fluorescent probe.

Evidence in support of the contribution of the carboxyl group to fluorescence intensity was provided by modified collagen studies. The number of acidic groups relative to basic groups increases by succinylation of collagen. The result of this collagen modification as shown in Fig. 9c was a greater TNS fluorescence enhancement as compared to the control collagen solution. The pH data suggest not only that the binding sites of TNS are dependent upon the carboxyl group of collagen, but also that the positive charge of the lysine residues is unimportant in fluorescence. It should be noted that there was no TNS fluorescence in any solution at pH above 6, including heat-denatured collagen, succinylated collagen, and native collagen.

Binding of TNS with synthetic polypeptides

The relative TNS fluorescence values with two collagen-like polypeptide, $(Pro\text{-}Ala\text{-}Gly)_n$ and $(Pro\text{-}Ser\text{-}Gly)_n$, and with poly(L-glutamic acid) are listed in Table I. The binding of TNS with the triple helix form of $(Pro\text{-}Ala\text{-}Gly)_n$ resulted in a small but definite fluorescence enhancement as compared with that of the random coil form. However, in both cases, charge neutralization was not important in the binding process. On the other hand, an interruption of the hydrophobic

Table I

Relative Fluorescence of Calf-Skin Collagen and Some Synthetic Peptides

Protein or Peptide	Conformation	Relative Fluorescence[a]	Condition(15°C)	
			[TNS] × 10^4 M	protein mg/cc
Calf-skin collagen	triple helix (pH 3.7 HCl solution)	160(450 nm)	3.0	0.20
$(Pro\text{-}Ala\text{-}Gly)_n$	triple helix (pH 3.7 HCl solution)	15(460 nm)	3.0	0.20
$(Pro\text{-}Ala\text{-}Gly)_n$	random coil[b] (pH 3.7 HCl solution)	5(460 nm)	3.0	0.20
$(Pro\text{-}Ser\text{-}Gly)_n$	random coil (pH 3.7 HCl solution)	0	3.0	0.19
Poly(L-glutamic Acid)	-helix (pH 4.1 HCl solution)	0	1.5	0.31
Poly(L-glutamic Acid)	random coil (pH 12)	0	1.5	0.31

a. The value in the bracket is the wavelength of maximum emission.
b. The triple helix $(Pro\text{-}Ala\text{-}Gly)_n$ (pH 3.7 15°C) was treated in a 60°C waterboth for 10 min.

region with a polar residue as in the $(Pro\text{-}Ser\text{-}Gly)_n$ case restricted fluorescence altogether; thus, the TNS fluorescence may require an extended hydrophobic region at least larger than two residues.

It has been found that only the β sheet[8,9] form of polylysine and not the α-helix or random coil forms can cause the fluorescence of TNS in aqueous solution. These findings may suggest that, in the random coil and α-helix structures, the positively charged side chains of lysine favor binding with the sulfonate groups of TNS, yet these structures cannot provide a sufficiently hydrophobic space for binding bulky aromatic TNS rings. The same interaction may prevail for these forms of poly(L-glutamic acid) since neither causes TNS fluorescence.

Discussion

Fluorescence and the binding of TNS with collagen.

The thermodynamic data and the observed salt effect support the view that the fluorescence which results when TNS binds with collagen is caused by hydrophobic interactions. Ionic attractive forces appear not to be essential. Thus, the binding of TNS with $(Pro\text{-}Ala\text{-}Gly)_n$, an uncharged molecule, except at the terminal residues, resulted in fluorescence enhancement. The pH dependence of fluorescence, however, suggests that binding sites of TNS in collagen are near carboxyl groups and that when they are negatively charged they repel the TNS sulfonate groups and thus limit the fluorescence of

TNS. These results are in agreement with those of Flanagan and Hesketh[11], indicating that charge repulsion between TNS and the phospholipids eliminate the binding and fluorescence of TNS at pH above 4.

In the cyclic and linear peptide models, Beyer et al.[7] found that a positive charge on the peptide was necessary for the binding of negatively charged TNS. Flanagan and Ainsworth[11] showed, however, that if the hydrophobic moiety of the fluorescent probe were large enough, for example, ANS, a stable nonpolar bond could be formed with the sulfonate ion sufficiently excluded from the neutral micelle to retain its hydration shell. Adsorption of other ligands, for example, DNS (N, N-dimethyl-1-aminonaphthalene-8-sulfonate), with a smaller hydrophobic region, could only take place in the micelle where charge neutrality could be achieved within the nonpolar medium[34]. Brand et al.[35], show that 1,5-ANS, but not 1,5-DNS, could bind to alcohol dehydrogenase, suggesting that the hydrophobic interaction between the aromatic moiety of the fluorescent probe and the hydrophobic site of the protein plays an important role in binding. It appears then that the binding of TNS to protein may not need charge neutralization at the binding site since hydrophobic interactions could provide enough force for binding. However, charge repulsion between TNS and a negatively charged group at or near the hydrophobic site would be expected to restrict TNS binding and thus its fluorescence.

The possibility of formation of internal weak salt bridges between the adjacent carboxyl groups of aspartic and

glutamic acid and basic amino acids of tropocollagen is favored by the finding that the pK value of carboxyl groups increased from 3.7 ± 0.1 without salt to 4.2 ± 0.1 in 0.0125M NaCl solution. Such salt bridges would be spatially allowed according to the partial collagen amino acid sequence[17,20].

Heterogeneous binding sites of TNS in collagen.

The heterogeniety of the binding sites of TNS in collagen was reflected in the different Hill coefficients in Fig. 1b and in the magnitude of the red shifts as affected by TNS (Fig. 1c). The binding began at Hill coefficient C = 0.55 region followed by the C = 1.0 and C = 3.3 binding region. The hydrophobic characteristic decreased in the order C = 0.55 C = 1.0 C = 3.3 as revealed by the longer maximum emission of the larger Hill coefficients. In the C = 0.55 region a restricted binding site space may cause the first bound TNS to limit the following TNS bindings. At the C = 3.33 region, which is less hydrophobic, a larger space would allow the first TNS to facilitate binding of the next TNS possibly by hydrophobic interaction between the aromatic ring system of adjacent TNS molecules. The ligand binding distance is assumed to be close in the C = 0.55 region since the degree of polarization in C = 0.55 was greater than that in the C = 3.3. Several cases of cooperative binding have been reported. Cooperativity has been observed for the binding of ANS and bovine serum albumin[36] and negative cooperativity has been reported for the binding of ANS and

human serum prealbumin[37]. The binding of β-lactoglobulin A with hexane, iodobutane, or cyclohexane was negatively cooperative while the binding of β-lactoglobulin A with butane and cyclohexane was, respectively, independent and cooperative[38].

Red shifts of the emission and excitation spectra.

It has been shown that the emission and excitation spectra of TNS in nonpolar solvents will undergo blue shift[39] and red shift respectively[40]. The result of excitation maxium changes shown in Fig. 1a seems opposite what is expected. One possible explanation of this anomoly is that there is a hydrogen bonding formation between the aninilo group of TNS and carboxyl group of aspartic and glutamic acids. This hydrogen bonding may stabilize the ground state of TNS in the C = 0.55 region of collagen more than that of TNS in the other Hill coefficient regions of collagen.

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References

1. G. M. Edelman & W. C. McClure, Accounts Chem. Res., 1, 65 (1968)
2. L. Stryer, Science, 162, 526 (1968)
3. L. Brand & J. R. Gohlke, Ann. Rev. Biochem., 41, 843 (1972)
4. S. K. Phillips & W. A. Cramer, Biochemistry, 12, 1170 (1973)
5. W. A. Cramer, S. K. Phillips & T. W. Keenan, Biochemistry, 12, 1177 (1973)
6. L. Stryer, J. Mol. Biol. 13, 482 (1965)
7. C. F. Beyer, L. C. Craig & W. A. Gibbons, Biochemistry, 11, 4920 (1972)
8. J. Lynn & G. D. Fasman, Biochem. Biophys. Res. Commun., 33, 327 (1968)
9. G. Witz & B. L. van Duurren, J. Phys. Chem., 77, 648 (1973)
10. G. K. Radda, Biochem. J., 122, 385 (1971)
11. M. T. Flanagan & T. P. Hesketh, Biochim. Biophys. Acta, 298, 535 (1973)
12. A. G. Walton & J. Blackwell, "Biopolymers" B. Horecker, N. O. Kaplan, J. Marmur, and H. A. Scheraga, Eds., Academic Press, New York, p. 145, 1973
13. P. H. von Hippel, "Structure and Stabilization of the Collagen Molecule in Solution" in Treatise on Collagen, Vol. 1, G. N. Ramachandran, Ed., Academic Press, New York, p 253, 1967
14. J. Rauterberg, Timpel, R., & H. Furthmayer, Eur. J. Biochem. 27, 231 (1972)
15. P. P. Fietzek, I. Kell & K. Kuehr, FEBS Lett. 26, 66 (1972)
16. P. P. Fietzek, P. Wendt, I. Kell & K. Kühn, FEBS Lett, 26 74 (1972)

17. P. Wendt, v.d. Mark, K., F. W. Rexrodt & k. Kühn, Eur. J. Biochem., 30, 169 (1972)

18. P. P. Fietzek, F. W. Rexrodt, P. Wendt, M. Stark & K. Kühn, J. Biochem. 30, 163 (1972)

19. J. Ranterberg, P. P. Fietzek, F. W. Rexrodt, J. Becker, M. Stark & K. Kühn, FEBS Lett, 21, 75 (1972)

20. P. P. Fietzek, F. W. Rexrodt, K. E. Hipper, & K. Kühn, Eur. Biochem., 38, 396 (1973)

21. Y. Nagai, J. Gross & K. A. Piez, Ann. N. Y. Acad. Sci., 121, 494 (1964)

22. S. Zamdnhof, Methods Enzylmol., 3, 696 (1957)

23. D. B. Bloxham, Biochemistry, 12, 1602 (1973)

24. M. H. Klapper & I. M. Klotz, Methods Enzymol., 25, Part B 531 (1972)

25. C. A. Parker & W. T. Rees, Analyst, 85, 587 (1960)

26. P. Molyneux & H. P. Frank, J. Am. Chem. Soc., 83, 3167 (1961)

27. G. Weber, Trans. Faraday Soc. 50, 552 (1954)

28. G. Weber & E. Danil, Biochemistry, 5, 1900 (1966)

29. S. R. Anderson & G. Weber, Biochemistry, 8, 371 (1968)

30. G. J. Seliskar & L. Brand, J. Am. Chem. Soc., 93, 5405 (1971)

31. M. G. Tiffany & S. Krinn, Biopolymers, 8, 347 (1969)

32. S. N. Timasheff, H. S. N. Susi, R. Townsend, L. Mesnati, M. J. Gorbunoff & T. F. Kumosinski, "Comformation of Biopolymers" G.N . Ramachandran, Ed., Academic Press, New York, 1967.

33. F. H. Chu & A. Lukton, Biopolymers, 13, 1427 (1974)

34. M. T. Flanagan,& S.Ainsworth, Biochim. Biophys. Acta, 168, 16 (1968)

35. L. Brand, J. R. Gohlke, & D. S. Rao, Biochemistry, 6, 3510 (1967)

36. E. Danniel & G. Weber, Biochemistry, 6,1893 (1966)

37. R. N. Ferguson, H. Edelhuch, H. A. Sarnott & J. Rubbins, Biochemistry, 14, 282 (1975)

38. K. A. Robillard & A. Wishnia, Biochemistry, 11, 3835 (1975)

39. W. C. McClure & G. M. Edelman, Biochemistry, 5, 1908 (1966)

40. F. C. Greene, Biochemistry, 14, 747 (1975)

PART II

Interaction of Sodium Dodecyl Sulfate with the Hydrophobic Fluorescent Probe, 2-p-Toluidinylnaphthalene-6-sulfonate*

* This manuscript was published in J. Phys. Chem. 79, 1935-1939 (1975) with some corrections presented here.

Abstract

The critical micelle concentration (cmc) of sodium dodecyl sulfate at various NaCl concentrations can be followed by the increasing fluorescent intensity of 2-p-toluidinylnaphthalene-6-sulfonate (TNS). The thermodynamic parameters of the interaction of TNS and the SDS micelle have been obtained. Binding is exothermic and involves a negative entropy change. The salt (NaCl) increases the association constant between TNS and micelle of SDS by increasing the positive entropy change. The results suggest that the binding force between TNS and the micelle of SDS is hydrophobic. The nature of hydrophobic fluorescent probe binding with proteins is discussed.

Introduction

It is well know that the quantum yield of fluorescence of 2-p-toluidinylnaphthalene-6-sulfonate (TNS) and related compounds, such as 1-aninilo-8-naphthalenesulfonate (ANS), is very much higher in a nonpolar than in a polar solvent. Also the emission spectrum in a nonpolar solvent is blue shifted relative to the spectrum in polar solvents[1-3]. This characteristic is, therefore, widely used to detect protein conformational change assuming that the environments of dye binding sites change as protein conformation changes. This kind of research using fluorescent probes to follow protein conformation changes and to study cell membrane phenomena is leading to a better understanding of the hydrophobic

characteristic of proteins and membranes with respect to the binding of substrate and inhibitors[2,4].

Although such fluorescent probe research has been extensive, the actual probe binding environment is still obscure, and the nature of the binding forces are not well defined. In 1972, Beyer et al. suggested that, although a hydrophobic environment is essential for enhancing the fluorescence of TNS, charge neutralization is required to provide binding between peptide and TNS[5]. The studies on the interaction of polylysine and TNS show that only the β-sheet conformation of polylysine. The form which exists without charges, results in TNS fluorescence[6,7]. The negative charge repulsion between anionic ANS and negative phosphate or carboxylate groups of phosphatide[8] or of submitochondrial particles[9] is considered to be critically important for the binding of a dye to a phospholipid or a biomembrane. The same finding, namely, that charge repulsion but not charge neutralization is important, has been revealed in studies of the binding between TNS and collagen[10].

There is extensive evidence indicating that hydrophobic interactions, not charge neutralization, provide the binding force, including the following. A positively charged derivative of ANS, N-(1-anilinonaphthalene-4-sulfonyl)-1,6-diaminohexane, has been observed to bind as well as ANS itself to heavy chains of human γG immunoglobin[11]; N-phenyl-1-naphthalene (NPN) without a charge was found to bind more tightly to Escherichia coli cell envelope than negatively charged

ANS[12]; Brand et al. showed that 1,5-ANS but not N,N-dimethyl-1-aminonaphthalene-5-sulfonate (DNS) could bind to alcohol dehydrogenase indicating that the hydrophobic interaction between the aromatic moiety of the fluorescent probe and the hydrophobic site of the plays an important role in binding; Flanagan and Ainsworth[14] showed that a negative dye can absorb to neutral micelles if the nonpolar moiety of the probe is sufficiently large, thus ANS, but not DNS or 1-naphthylamine-5-sulfonate, was absorbed by polyoxyethylene lauryl ether micelle. These data indicate that the hydrophobic characteristic of nonpolar moiety determines the binding.

Sodium dodecyl sulfate (SDS) micelles, like proteins, provide hydrophobic and charged regions for interaction with amphiphilic molecules such as TNS. The formation of the SDS micelle in aqueous solution is caused by hydrophobic interaction[15]. TNS molecules could be incorporated into the SDS micelle if the hydophobic interactions are not disrupted by negative charge repulsions between TNS and SDS. Thus, the study of SDS micelles binding with negatively charged TNS should contribute to an understanding of TNS-protein binding parameters.

In this report, the use of TNS as a probe for detecting SDS micelle formation is presented. The thermodynamic parameter of the SDS micelle-TNS interaction at various NaCl concentrations suggest that hydrophobic forces are principal contributors to the binding .

Experimental Section

Materials. The potassium salt of 2-p-toluidinylnaphthalene-6-sulfonate was a product of Sigma Chemical Co. (lot no. 80c-5130) and it was stored in the dark at 5oC. The compound was found pure according to the results of TLC[5]. Sodium dodecyl sulfate was purchased from Bio-Rad Laboratories, Richmond, Calif., and it was used without further purification.

Fluorescence measurements. Fluorescence measurement were done on a Perkin-Elmer fluorescence spectrophotometer MPF-2A equipped with a thermostat cell holder. Both emission and excitation slits were set at 4 nm. The emission spectra were obtained by exciting at 370 nm. The mixtures of SDS and TNS were at first incubated in a thermostat at the desired temperature for at least 0.5 hr. The samples were then transfered to the cell holder controlled at the same temperature.

Thermodynamic parameters. The association constant of TNS and micelles of SDS was calculated according to eq. 7, derived as follows:

$$M_n + TNS \underset{}{\overset{K_T}{\rightleftharpoons}} M_n - TNS \qquad (1)$$

where M_n is the micelle of SDS (M is the SDS monomer). K_T is the association constant at temperature T. Then

$$K_T = \frac{M_n - TNS}{M_n \quad TNS} \qquad (2)$$

If $[M_n] >> [M_n - TNS]$, eq. 3 can be obtained where $[TNS_{total}] = [TNS] + [M_n - TNS]$ and $[M_{n\ total}] = [M_n] + [M_n - TNS]$.

$$\frac{1}{[M_n - TNS]} = \frac{1 + K_T [M_{n\ total}]}{K_T [M_{n\ total}] [TNS_{total}]} \quad (3)$$

At $[M] >> cmc^{\#}$(critical micelle concentration)

$$[M_{n\ total}] = \frac{[M]_{total}}{n} \quad (4)$$

substituting (4) into (3) gives

$$\frac{1}{[M_n - TNS]} = \frac{1}{[TNS_{total}]} + \frac{n}{K_T [M_{total}][TNS_{total}]} \quad (5)$$

Assume

$$I = \alpha_T [M_n - TNS] \quad (6)$$

where I is the fluorescence intensity and α_T is a coefficient which is temperature dependent. Then

$$\frac{1}{I} = \frac{1}{\alpha_T [TNS_{total}]} + \frac{n}{\alpha_T K_T [M]_{total} [TNS_{total}]} \quad (7)$$

The enthalpy of the binding was calculated according to the following equation:

$$\Delta H = -R \frac{d \ln K_T}{d(1/T)} \quad (8)$$

Results

Critical micelle concentration (cmc)

The micelle formation at SDS concentrations larger than the cmc was accompanied by a sharp increase in the fluorescence of

the added TNS. Figure 1 shows how fluorescence changes with SDS micelle formation in water. In agreement with the cmc value obtained by other methods[16-18] without added salt, it was found that the cmc obtained by this method occurred at an SDS concentration of 8.0×10^{-3}M. This value was independent of added TNS concentrations. However, at or below a TNS concentration of 6×10^{-6}M, micelle formation could not be accurately detected by this method (Figure 1). The cmc at various concentrations of added salt (NaCl) compared with literature values are listed in Table I. It has been established that there is a linear relation between log cmc and log (cmc + molarity of added salt)[16]. Such a linear relationship is exhibited by a plot (Figure 2) of our values of log cmc vs. log (cmc + molarity of added salt). The formation of micelles from SDS monomers also causes an increase of polarization of fluorescence as shown in Figure 3. Salt increases the polarization of fluorescence of TNS (Figure 3). This suggests that salt may increase the rotational relaxation time[20] of TNS due to the salt-induced rigidity of the binding site of TNS in the micelle.

If $\frac{\text{cmc}}{M_{\text{total}}} > 5\%$, then equation 4 should be changed to $[M_{n\ \text{total}}] = \frac{1}{n}([M]_{\text{total}} - \text{cmc})$ with the result that the $[M]_{\text{total}}$ term of eq 7 should be changed to $([M]_{\text{total}} - \text{cmc})$. The $([M]_{\text{total}} - \text{cmc}) = [M]_{\text{total}}$ can be assumed when $[M]_{\text{total}}$ is very large, which was the case in the experiments of Figure 5 (a-c), or when cmc is very small so that the ratio is $\frac{\text{cmc}}{[M]_{\text{total}}} < 5\%$.

Figure 1. Critical micelle formation in water. The TNS concentration ranged from 6.15×10^{-6} to 1.23×10^{-4} M.

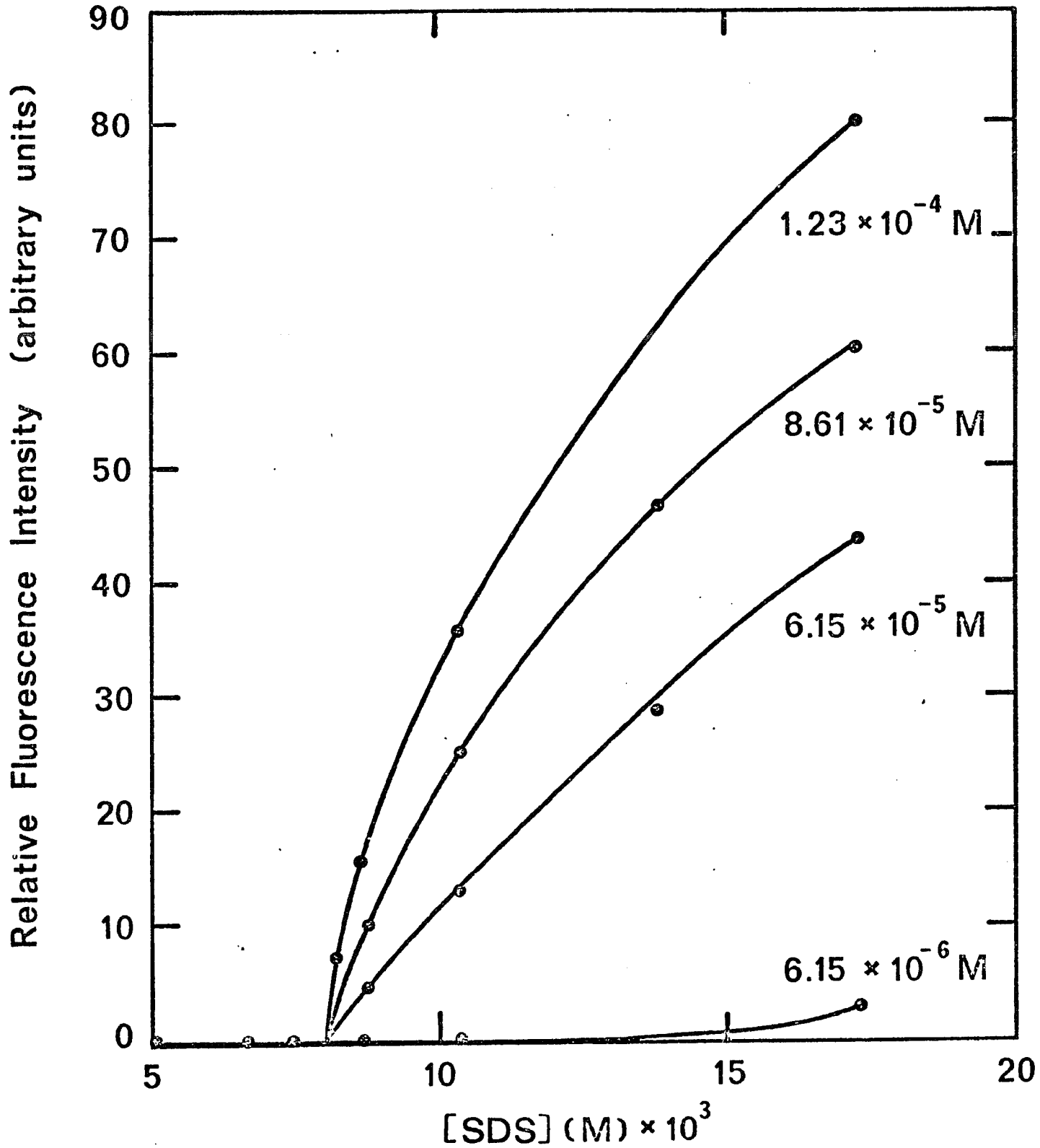

Relative Fluorescence Intensity (arbitrary units)
90
80
70
60
50
40
30
20
10
0
5
10
15
20
[SDS] (M) × 10^3
1.23 × 10^-4 M
8.61 × 10^-5 M
6.15 × 10^-5 M
6.15 × 10^-6 M

Table I. Comparision of critical micelle concentrations obtained with literature values at 25°C

Ionic Strength	cmc, M 10^3 (lit. values)	cmc, M 10^3 (obsd)
water	8.0[a], 8.13[b], 8.1[c]	8.0
0.033	2.15[d], 3.09[c]	2.25
0.050	2.3[a]	1.55
0.10	1.39[c]	0.85
0.20	0.94[a], 0.83[c]	0.61
0.40	0.52[c]	0.50
0.50	0.51[a]	

a Reference 16. b Reference 17. c Reference 18.
d Reference 19.

Table II. Thermodynamic parameters of binding between SDS micelle and TNS at different ionic strengths and temperatures.

Temp. °C		Ionic strength of added salt 0	0.05	0.10
15	ΔG^a	-4.565	-4.687	-5.240
	ΔH^a	-7.473	-7.473	-7.473
	ΔS^b	-10.10	-9.049	-7.753
25	ΔG^a	-4.642	-4.802	-5.237
	ΔH^a	-7.473	-7.473	-7.473
	ΔS^b	-10.10	-8.963	-7.503
35	ΔG^a	-4.407	-4.734	-5.106
	ΔH^a	-7.473	-7.473	-7.473
	ΔS^b	-9.946	-8.893	-7.685

a The unit of ΔG and ΔH is kcal/mol.
b The unit is eu.

Figure 2. The plot of log [cmc(M)] vs. log [cmc(M) + added salt(M)] at 25°C.

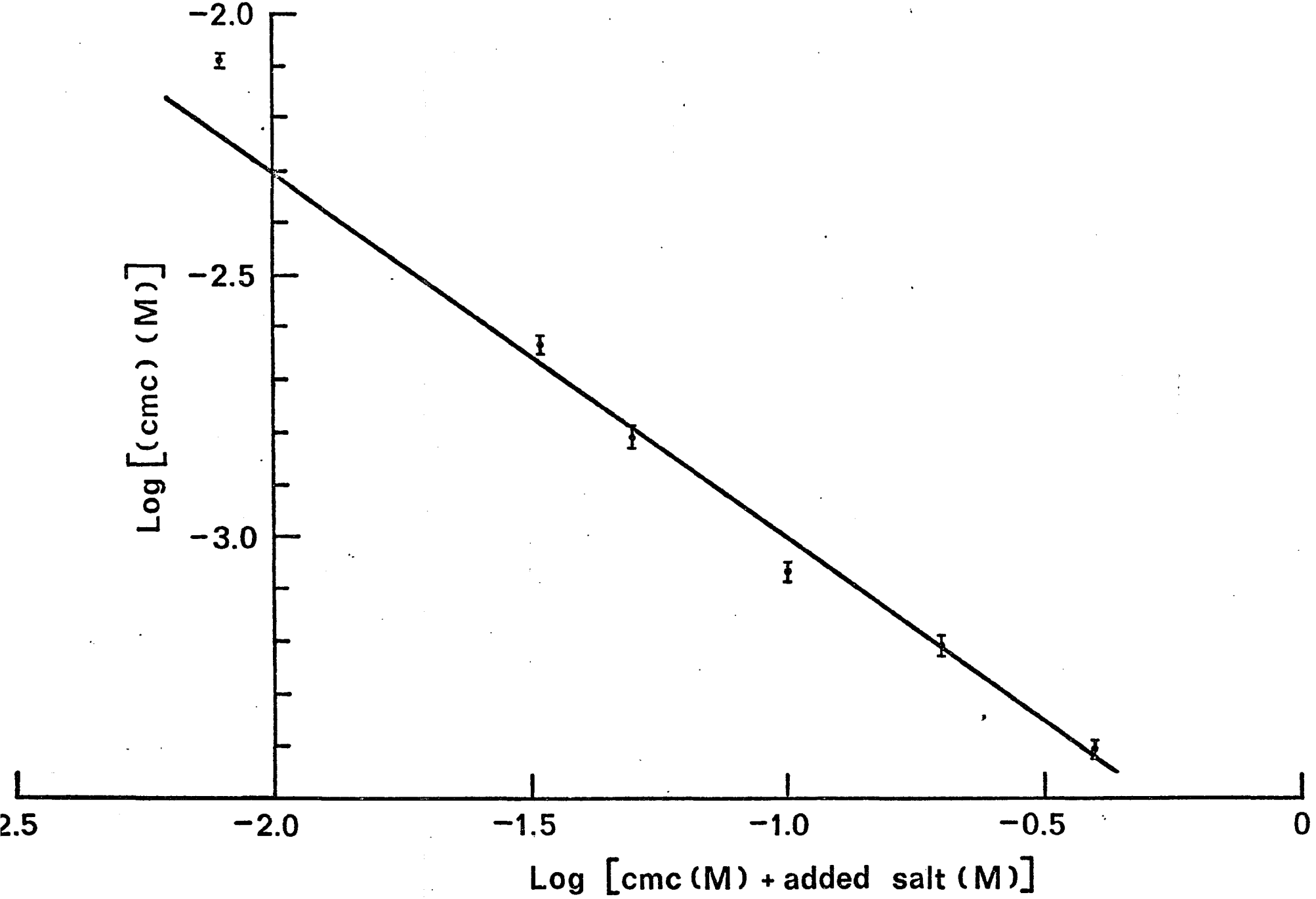
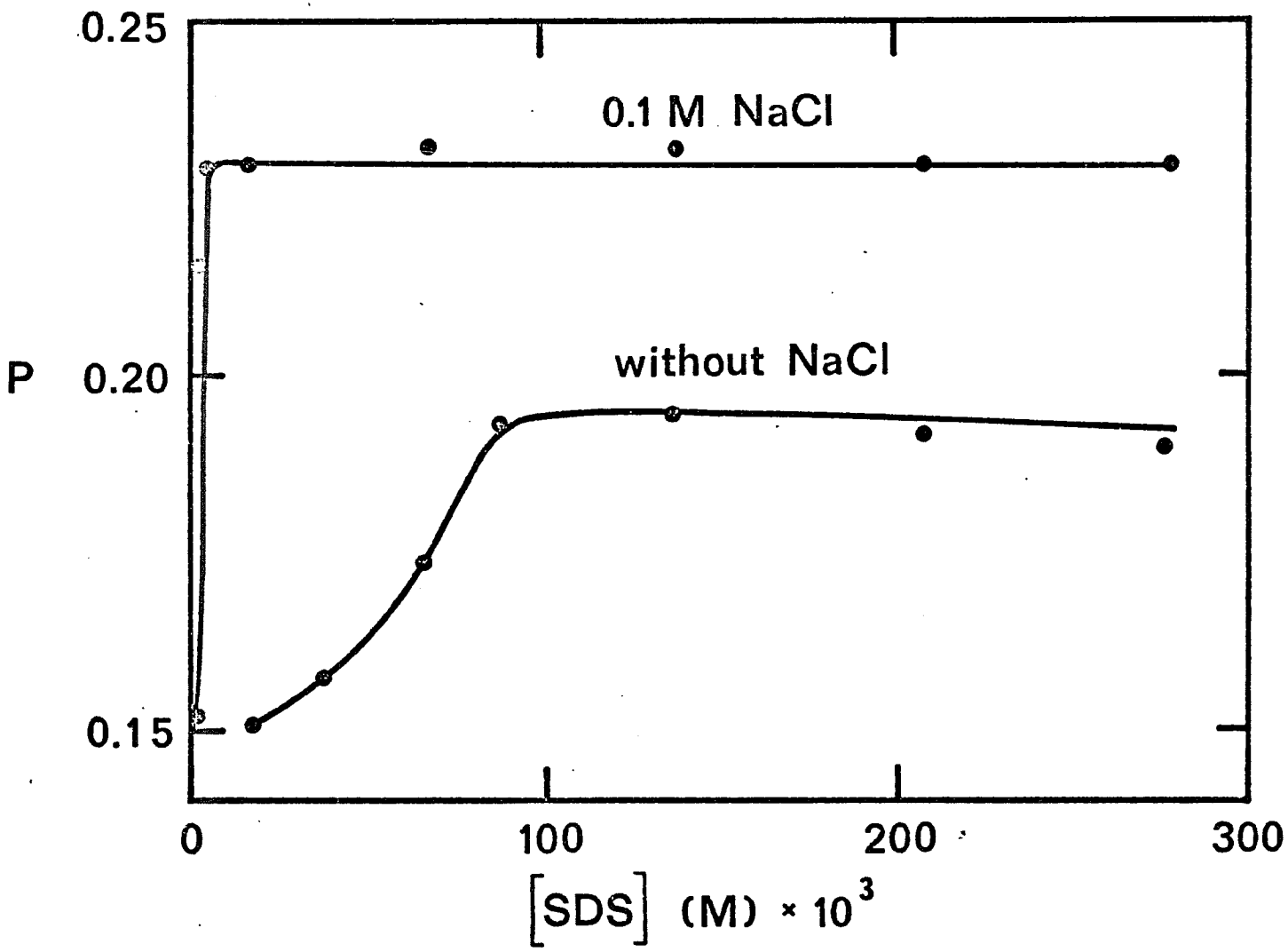

−2.0
−2.5
−3.0
Log [(cmc) (M)]
2.5
−2.0
−1.5
−1.0
−0.5
0
Log [cmc (M) + added salt (M)]

Figure 3. Polarization of fluorescence emission spectrum in water and in 0.1 M NaCl at 25^{o}. [TNS] = 8.61×10^{-5}M.


0.25
0.1 M NaCl
P
0.20
without NaCl
0.15
0
100
200
300
[SDS] (M) × 10³

The binding between TNS and the micelle of SDS. Salt effect and temperature effect.

Figure 4a shows the relative fluorescence enhancement at three different TNS concentrations and varying SDS concentrations in water at 25°C. Figure 4b shows a linear relation for the reciprocal plot of the intensity and SDS values of Fig. 4a at SDS above 0.0694 M (see eq 7). By extrapolating SDS to infinity, and I_{max} value of each TNS concentration in Fig. 4a is obtained. The results in Fig. 4c show that a linear relationship exists between I_{max} and TNS_{total} (eq 7). It should be noted here that one micelle of SDS consists of about 100 monomers[16] and micelle size is roughly independent of SDS concentrations up to 0.25 M[21].

Since a high solution viscosity may enhance TNS fluorescence[1], viscosity measurements at various SDS concentrations were made and the results are shown in Fig. 4a. The viscosity contribution to fluorescence enhancement was determined by measuring the TNS fluorescence enhancement in sucrose solutions. The result in Fig. 4d indicates that in the concentration range of SDS used, the viscosity contributed insignificantly to the fluorescent enhancement of TNS.

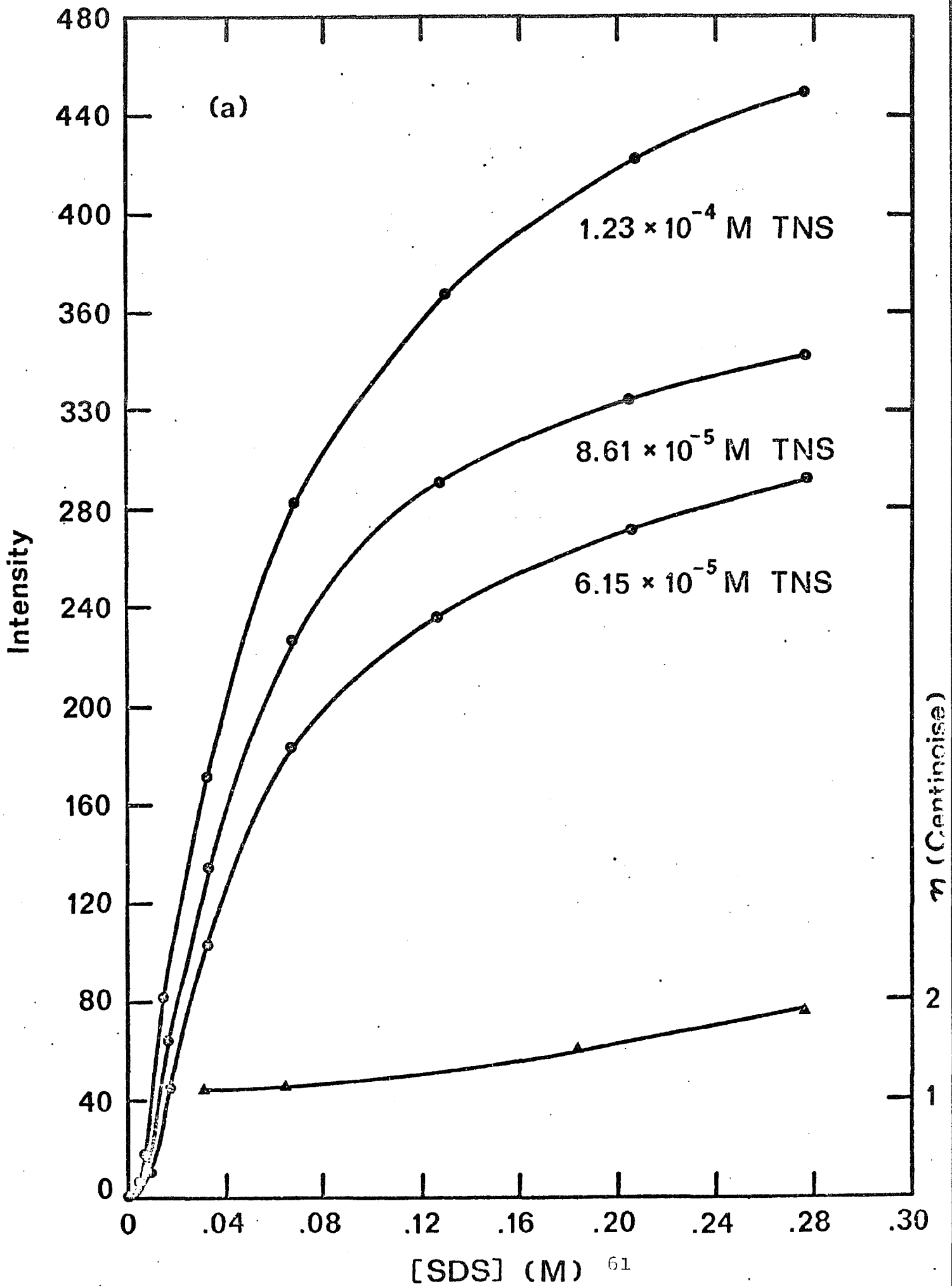
The plots of 1/SDS vs. 1/I at three different salt concentrations 0, 0.05, and 0.1 M are given in Fig. 5. Fig. 5a-c was obtained at 15, 25, and 35°, respectively. The line intercept is independent of salt concentration for each temperature, but the slopes are dependent on both salt concentration and temperature. The α_T values are calculated to be 58.14×10^5 M^{-1}

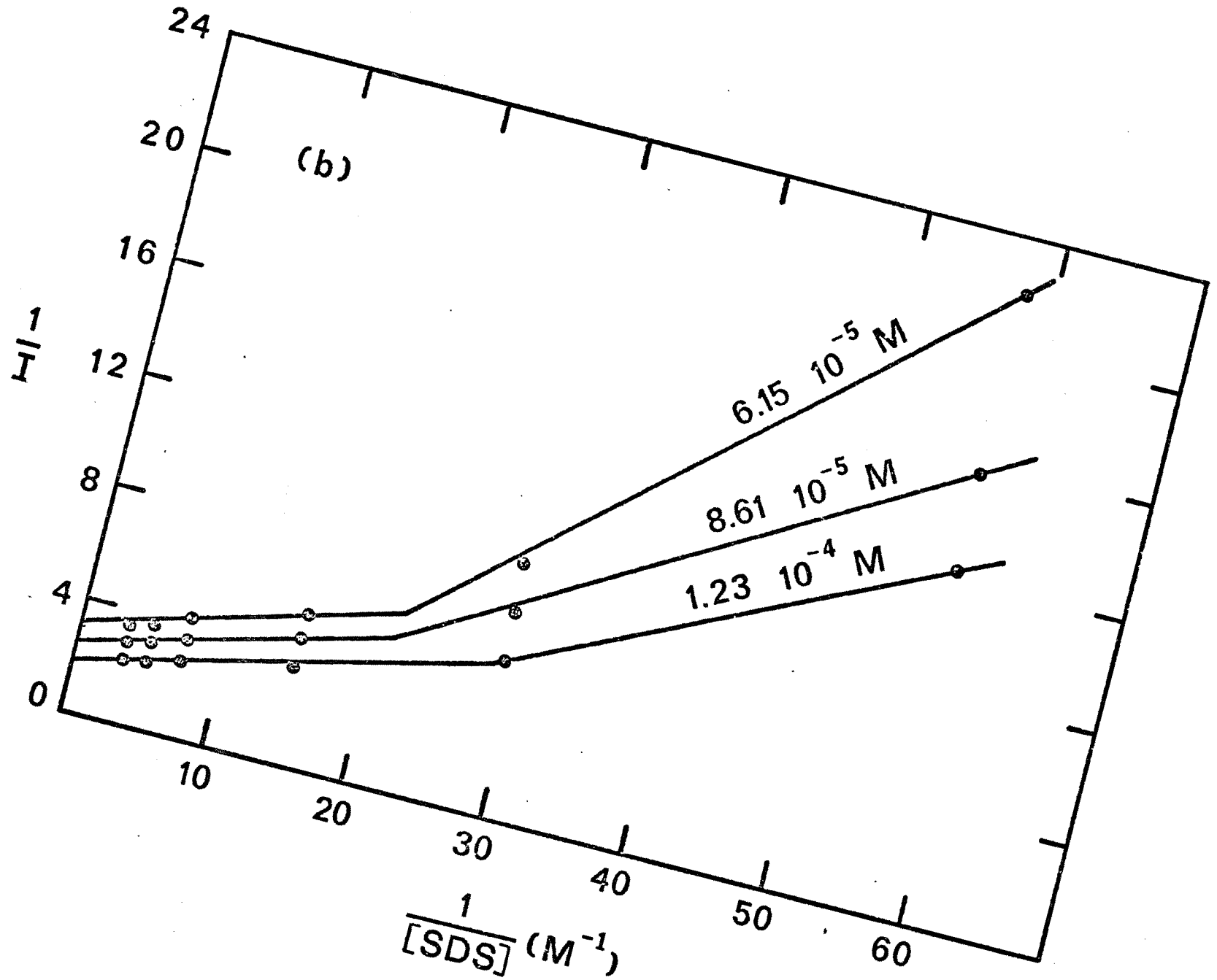
Figure 4. (a) Fluorescence titration (•) and viscosity measurement (▲) at various SDS and TNS concentrations, temperature 25°C.

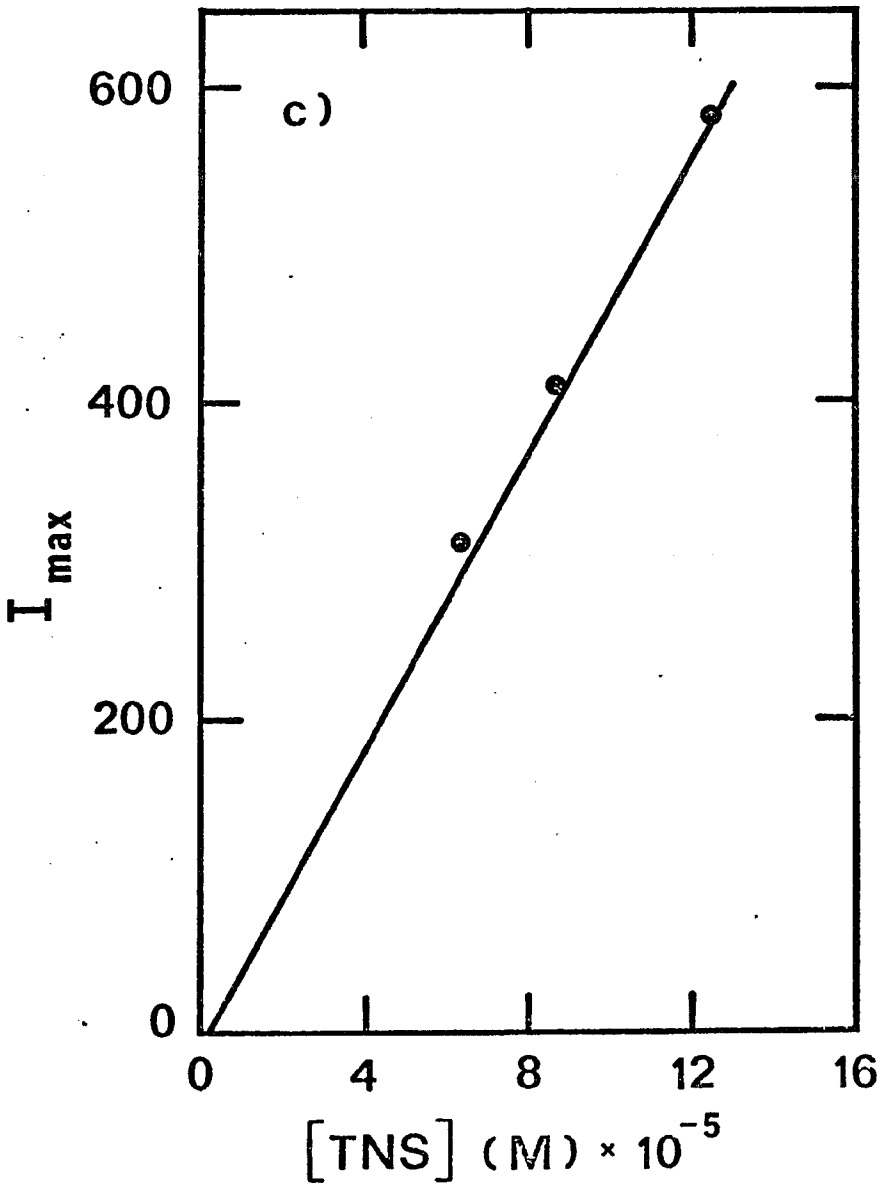
(b) The reciprocal plot of Figure 4a (see eq 7).

(c) The I_{max} vs. TNS concentration. The I_{max}'s at various TNS concentrations were obtained by extrapolating the 1/I vs. 1/[SDS] lines in Figure 4b to infinite SDS concentration.

(d) Fluorescence enhancement vs. viscosity. Sucrose solutions were used. [TNS] = 1.23 10^{-4} M.


480
440
400
360
330
280
240
200
160
120
80
40
0
(a)
1.23 × 10⁻⁴ M TNS
8.61 × 10⁻⁵ M TNS
6.15 × 10⁻⁵ M TNS
Intensity
0
.04
.08
.12
.16
.20
.24
.28
.30
[SDS] (M)
2
1
η (Centipoise)


(b)
24
20
16
12
8
4
0
1/I
6.15 10^-5 M
8.61 10^-5 M
1.23 10^-4 M
10
20
30
40
50
60
1/[SDS] (M^-1)


c)
600
400
200
0
I_{max}
0
4
8
12
16
[TNS] (M) × 10^{-5}

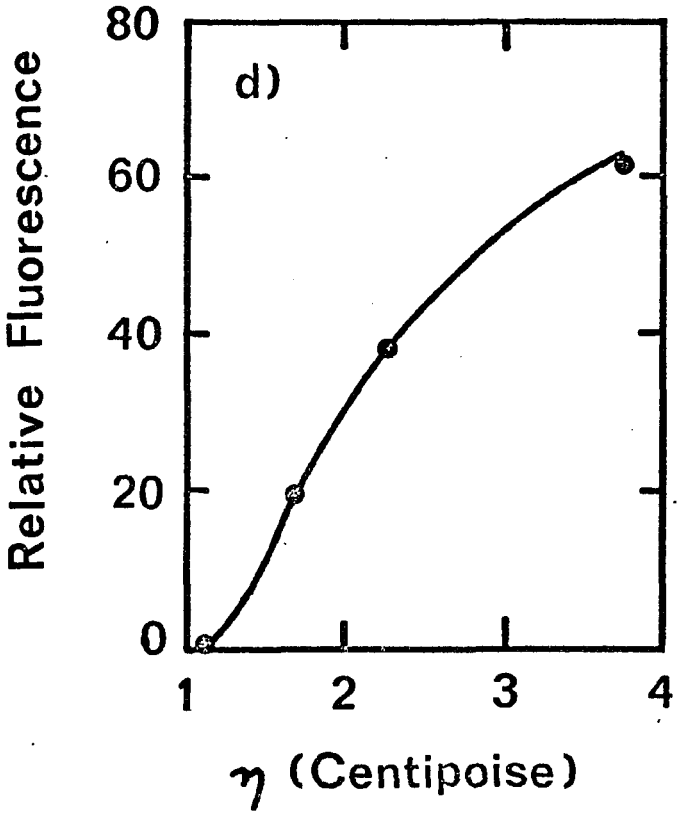
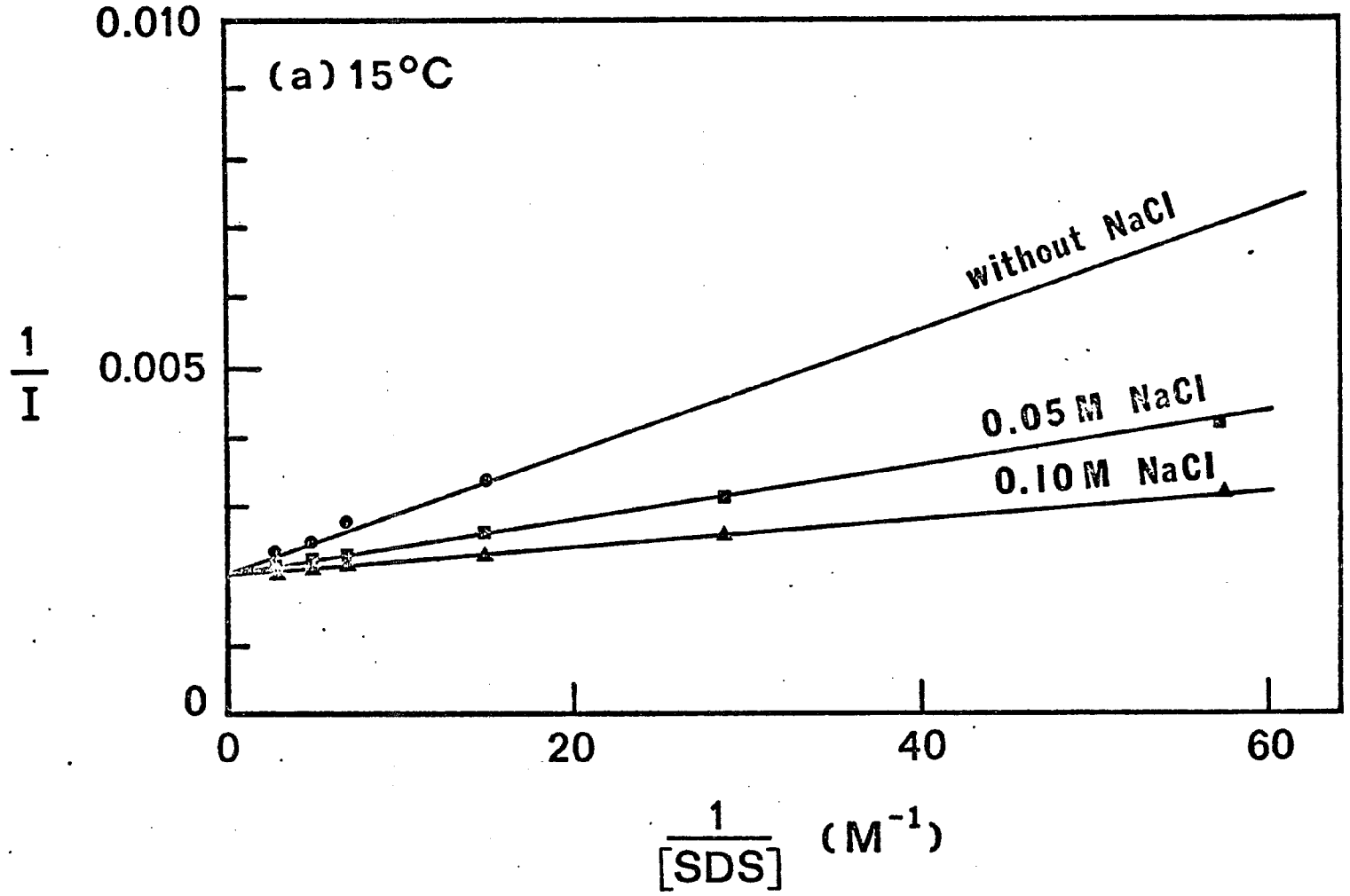
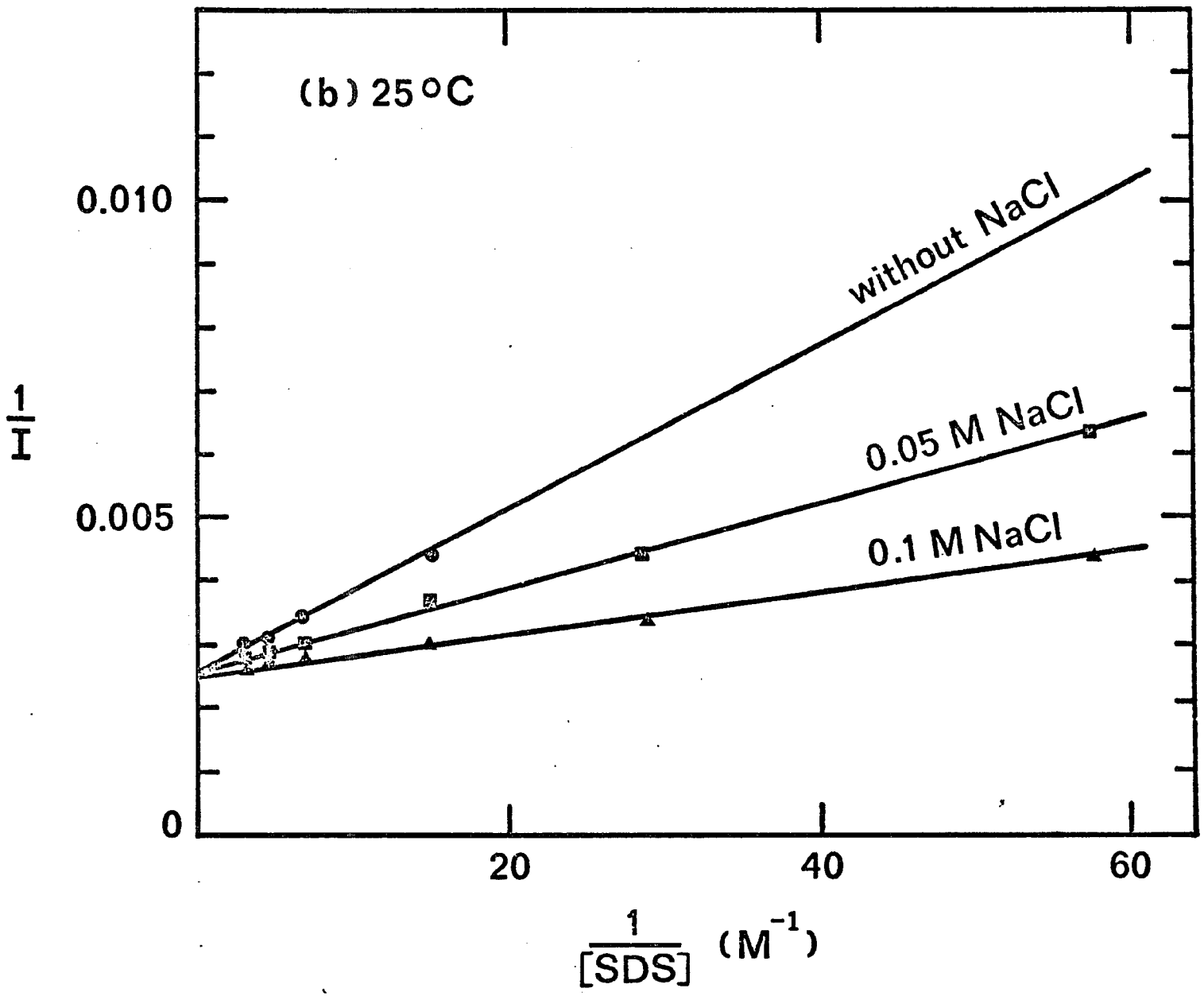
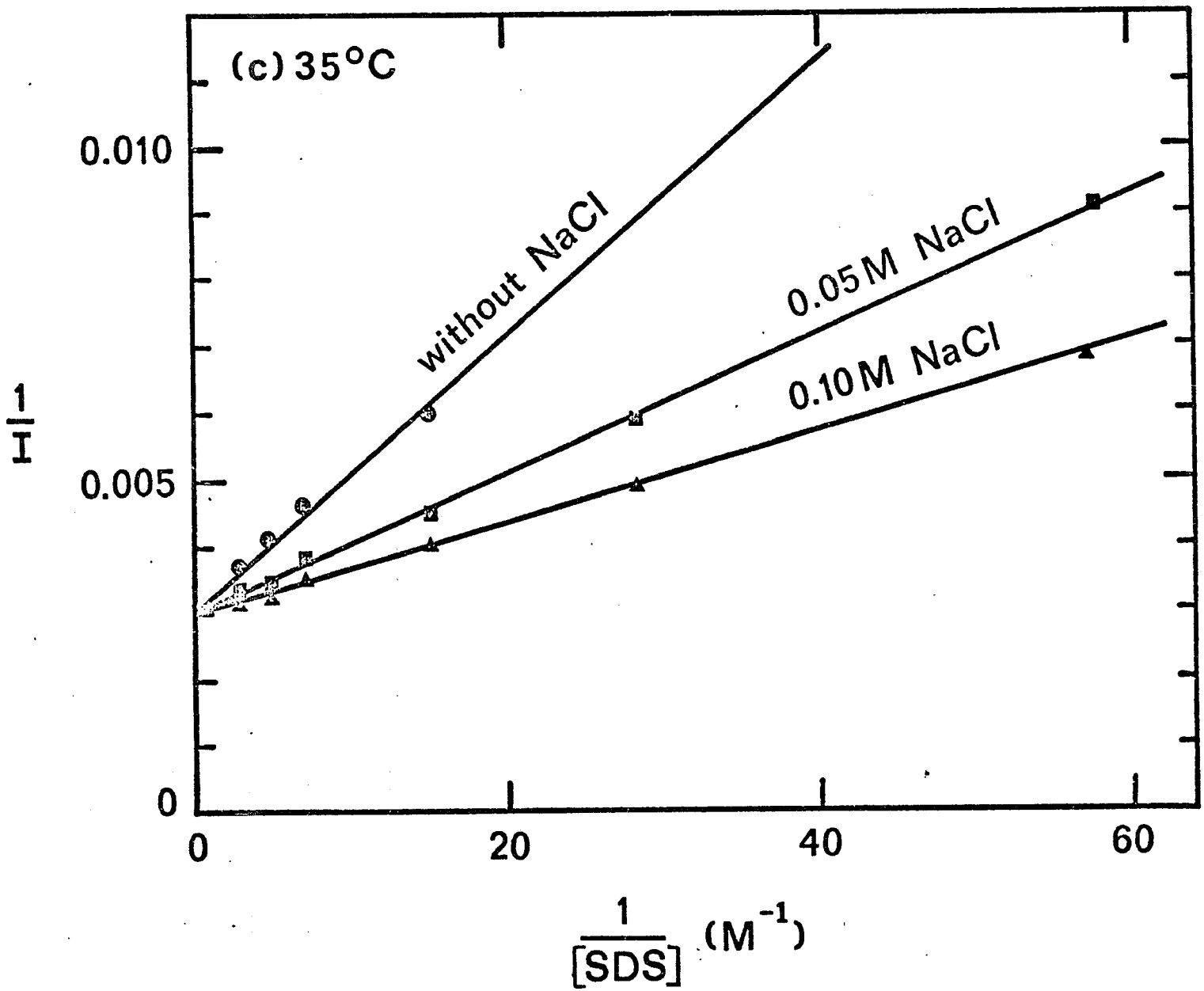

d)
Relative Fluorescence
80
60
40
20
0
1
2
3
4
η (Centipoise)

Figure 5. (a-c) The plot of 1/I vs. 1/[SDS] at 15, 25, and 35^{o}.


0.010
(a) 15°C
0.005
0
1/I
without NaCl
0.05 M NaCl
0.10 M NaCl
0
20
40
60
1/[SDS] (M⁻¹)


(b) 25°C
0.010
0.005
0
1/I
without NaCl
0.05 M NaCl
0.1 M NaCl
20
40
60
1/[SDS] (M⁻¹)


(c) 35°C
0.010
0.005
0
1/I
0
20
40
60
1/[SDS] (M⁻¹)
without NaCl
0.05M NaCl
0.10M NaCl

at 15^{o}, 47.7×10^{5} M^{-1} at 25^{o}, and 41.9×10^{5} M^{-1} at 35^{o} and the plot is shown in Figure 6. Temperature dependent quenching is a general phenomenon which is caused by greater collisional quenching at higher temperature[22]. No significant wavelength shift of the emission spectra can be noted in the test of temperature dependent frequency. The wavelength of maximum emission is about 455 nm.

The association constant K_T can be obtained from the slope of Figure 5. In the calculation of K_T, according to eq 7, n = 100 is assumed and the α_T values at different temperature as obtained from the data in Figure 6 are used. As indicated in Table II, the association constant increases as the salt concentration increases for each temperature. The ΔH of binding was determined from the van't Hoff plot of association constant vs. 1/T (Figure 7). They are listed in Table II and some conclusions can be drawn. First, no significant difference in enthalpy can be seen at different temperatures and salt concentrations. The negative value of enthalpy contributed to the negative free energy of binding between TNS and micelles of SDS. Second, salt increases the association constant by increasing the entropy. The increase in the magnitude of the association constant in salt solution is a general phenomenon if the binding force is hydrophobic[23].

It is well established that micelle formation at the cmc is cooperative[15]. In Figure 8, the Hill plot[24,25] of binding between TNS and the micelle of SDS at 0, 0.05, and 0.1M NaCl

Figure 6. The plot of α_T vs. temperature (see eq 6).

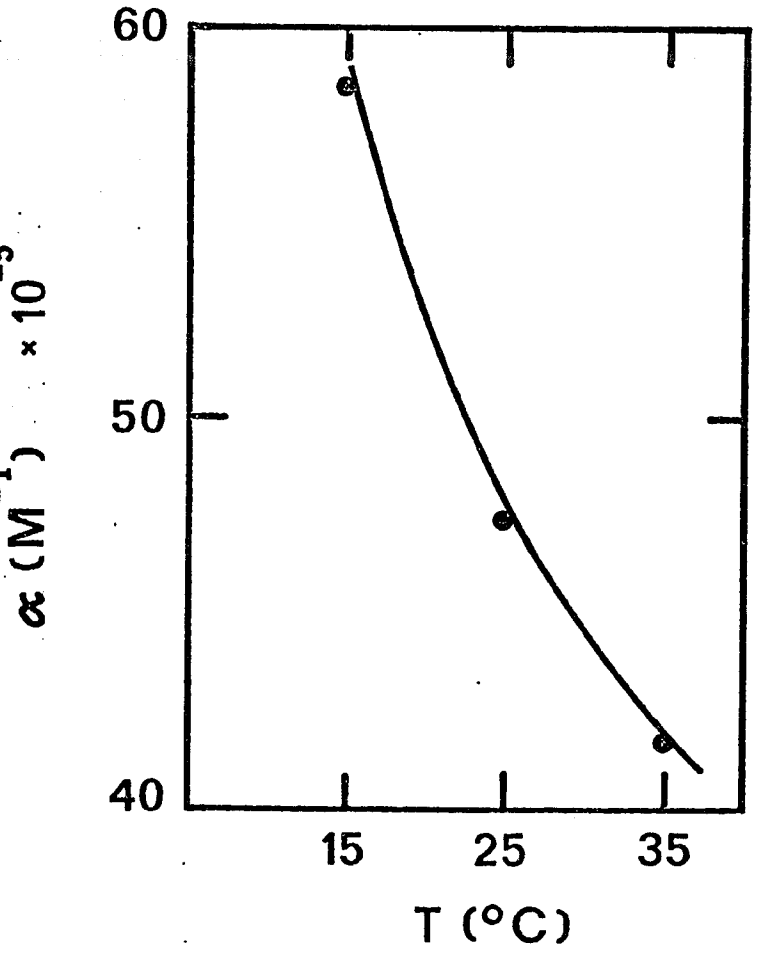

60
50
40
15
25
35
T (°C)
α (M⁻¹) × 10⁻⁵

Figure 7. The plot of log K vs. 1/ T(^{o}K).

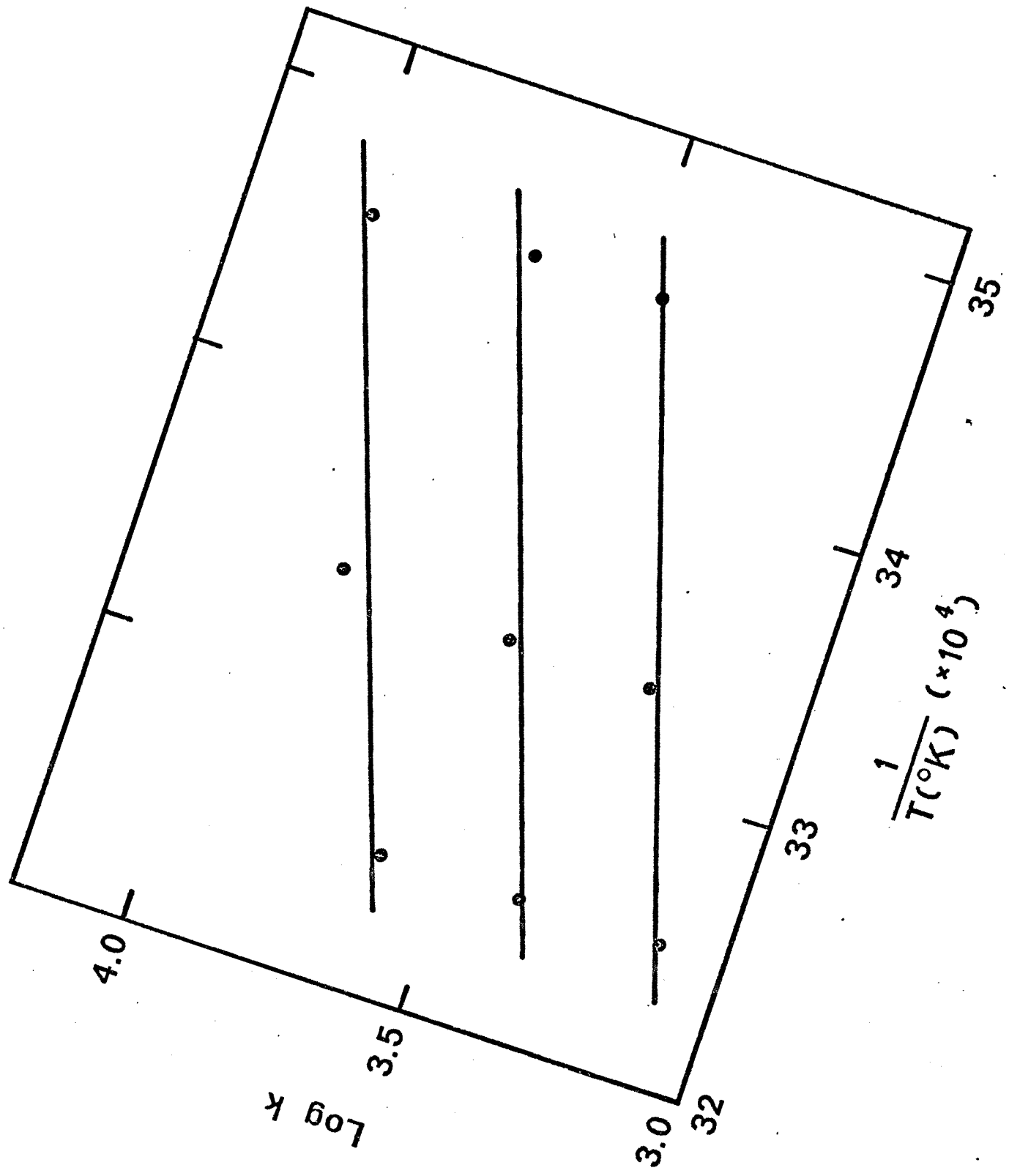
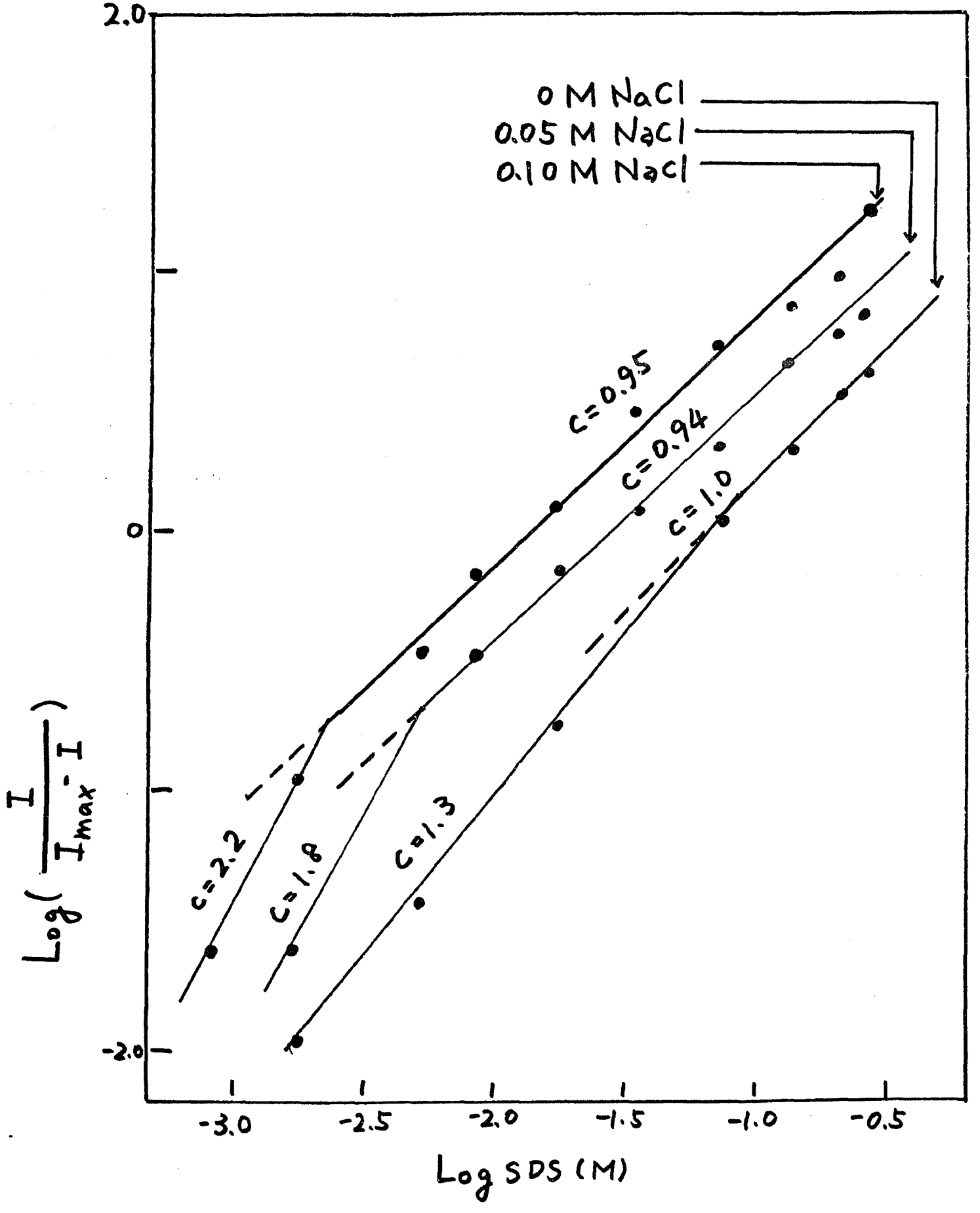

35
34
33
32
3.0
3.5
4.0
Log k
$\frac{1}{T(°K)}$ (×10⁴)

Figure 8. Hill plot of the interaction of TNS with SDS at various salt concentrations, at 25°.


0 M NaCl
0.05 M NaCl
0.10 M NaCl
C=0.95
C=0.94
C=1.0
C=2.2
C=1.8
C=1.3
2.0
0
-1
-2.0
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
Log SDS (M)
Log (I / Imax - I)

is given. In all cases a biphasic property is observed. The Hill coefficients near the critical micelle concentrations are greater than 1; the Hill coefficient at SDS concentration higher than the cmc is about 1. The results suggest that there is cooperative formation of micelles at an SDS concentration near the cmc. Moreover, the higher the concentration of added salt, the greater cooperativity is observed. At SDS concentrations higher than the cmc, the micelle seems to act independently. The salt also increased the polarization of the TNS fluorescence (Figure 3) indicating a salt-induced micelle rigidity.

Discussion

Evidence presented here supports the view that hydrophobic interactions contribute to the binding between TNS and the SDS micelle. It is based on the finding that salt increases the association constant only by decreasing the entropy gain caused by the added NaCl was about 2.5 eu when its concentration was increased from 0 to 0.1 M at each temperature. The entropy increase, being caused by a hydrophobic effect, may results from the freeing of water molecules from the solvent shell of the nonpolar moiety of TNS and their transfer into the bulky water phase which is less ordered than the solvent shell. It is known that hydrophobic bonds are strengthened by addition of the electrolyte[23]. This is supported by the solvent studies which suggest that spectral changes of TNS that accompany binding to a macromolecule result importantly from the loss of the aqueous

solvation characteristics of structural bulky water[26]. The nagative entropy change for the interaction of SDS micelle with TNS may have resulted from the fact that the entropy gain caused by the loss of so-called "icebergs"[27] around the aromatic residue of TNS is not greater than the entropy loss caused by the salt-induced rigidity of the SDS micelle-TNS complex (Figure 3).

The themodynamic data obtained for the interaction of TNS and apomyoglobin were $\Delta F = -7.32$ kcal/mol, $\Delta H = -5.06$ kcal/mol and $\Delta S = 7.71$ eu[28]. The values obtained for the interaction of TNS and collagen were $\Delta G = -5.7$ kcal/mol, $\Delta H = -4.0$ kcal/mol, and $\Delta S = 6.0$ eu[10]. In these two cases, the enthalpy contributes predominately to the binding force. This is in good agreement with the enthalpy data which we have obtained for the interaction of TNS and the SDS micelle. The main contribution of ΔH to the binding force may be the result of London disperson forces for the binding of TNS to the SDS micelle. However, the possibility exists that when the TNS molecule binds to SDS molecule, weak, distorted hydrogen bonds, formed between water molecules in the iceberg and the TNS anilino group, break and become stronger hydrogen bonded H_2O molecules in the bulky water. Camerman and Jensen mentioned the possible existence of hydrogen bonding in the hydrated crystal of TNS[29].

The charge repulsion of a negatively charged fluorescent probe at the binding site of a macromolecule may be critical for the binding if the hydrophobic characteristic of the binding site is not sufficiently large or if steric hindrance, created

by negatively charged groups, exists in the binding site. In the interaction of TNS with the SDS micelle, however, charge repulsion is overcome since there is a suitable hydrophobic binding environment for TNS. Tanford[15] has described the possible formation of these kinds of mixed micelles.

It has been demonstrated that a fluorescent probe-protein complex[10, 30-32] or a fluorescent probe-membrane complex[8,33] shows substantial fluorescence enhancement in acidic solution. Two aspects of this have been given in the literature. First, when proteins are denatured in acidic medium, there is an increase in the value of the dissociation constants and there are a greater number of binding sites and/or there is an increase in fluorescent quantum yield of the bound probe. For example, the ANS-haptoglobin[32] or TNS-chymotrypsin[30] complex show these effects. Second, the decreased charge repulsion between negatively charged fluorescent probes with collagen[10] and with phospholipid[8] resulted in fluorescence enhancement when the pH was changed from pH 6 to 3. Neither observation requires charge neutralization for the binding of negatively charged fluorescence probes with macromolecules.

Salt-induced regidity of a SDS micelle may be due to decreasing the charge repulsion of SDS monomers in the micelle. This may cause more efficient packing of SDS monomers in the micelle.

Flanagan and Ainsworth[14] concluded that anionic ANS could not bind to the SDS micelle because of charge repulsion. We

find that the ANS concentration used in their experiments are too low (1×10^{-6}M) to detect micelle formation. Also, ionic strength is critical variable for the cmc value. We were not able to confirm their observation that 0.05% w/v SDS, in a solution to which no salt had been added, was the critical micelle concentration.

Acknowledgement

The authors thank professor Paul Haberfield for helpful discussions.

References

1. W. C. McClure and G. M. Edelman, Biochemistry, 5, 1908 (1966)
2. L. Brand and J. R. Gohlke, Ann. Rev. Biochem., 41, 843 (1972)
3. L. Stryer, Science, 162, 526 (1968)
4. T. Takagi, Tampakushitsu Kakusan Koso, Bessatzu, 143 (1974)
5. C. F. Beyer, L. C. Craig, and W. A. Gibbons, Biochemistry, 11 , 4920 (1972)
6. J. Lynn and C. D. Fasman, Biochem. Biophys. Res. Commun., 33, 327 (1968)
7. G. Witz and B. L. van Duurren, J. Phys. Chem., 77, 648, (1973)
8. M. T. Flanagan and T. R. Hesketh, Biochim, Biophys. Acta, 298, 535 (1973)
9. G. K. Radda, Biochem. J., 122, 385 (1971)
10. H. -C. Chiang and A. Lukton, Biopolymers, 14, 1655 (1975)
11. J. A. Gally, Doctoral Dissertation, The Rockefeller University, New York, N. Y., p 50
12. S. K. Phillips and W. A. Cramer, Biochemistry, 12, 1170 (1973)
13. L. Brand and J. R. Gohlke, and D. S. Rao, Biochemistry, 6, 3510 (1967)
14. M. T. Flanagan and S. Ainswirth, Biochim. Biophys. Acta 168, 16 (1968)
15. C. Tanford, "The Hydrophobic Effect" Wiley, New York, N. Y., 1973

16. M. F. Emerson and A. Moltzer, J. Phys. Chem., 71, 1898 (1967)

17. A. B. Scott and H. V. Tarter, J. Am. Chem. Soc., 65, 692 (1943)

18. J. N. Phillips, Trans. Faraday Soc., 51, 561 (1955)

19. J. Steinhardt, N. Stocker, D. Carroll and K. S. Birdi, Biochemistry, 13, 4461 (1974)

20. G. Weber, Adv. Protein Chem., 8, 415 (1953)

21. F. Reiss-Husson and V. Luzzati, J. Phys. Chem., 68, 3504 (1964)

22. R. W. Ricci, J. Chem. Educ., 51, 692 (1974)

23. W. Kauzmann, Adv. Protein Chem., 14, 1 (1959)

24. A. V. Hill, J. Physiol. (London), 40, 4 (1910)

25. F. Wold, "Marromolecules: Structure and Function", Prentice-Hall, Englewood Cliffs, N. J. 1971.

26. R. L. Reeves, M. S. Maggio, and L. F. Costa, J. Am. Chem. Soc., 96, 5917 (1974)

27. H. S. Frank and M. W. Evans, J. Chem. Phys., 13, 507 (1945)

28. L. Stryer, J. Mol. Biol., 13, 482 (1965)

29. A. Camerman and L. H. Jensen, J. Am. Chem. Soc., 92, 4200 (1970)

30. W. O. McClure and G. M. Edelman, Biochemistry, 6, 559 (1967)

31. D. P. Bloxham, Biochemistry, 12, 1602 (1973)

32. S. F. Russo and W. W. C. Chen, Biochemistry, 13, 5300 (1974)

33. J. Hawiger and S. Timmons, Biochem. Biophys. Res. Commun., 55, 1278 (1973)

PART III

Salt Effect on the Interaction of the Hydrophobic Fluorescent Probe, 2-p-toluidinylnaphthalene-6-sulfonate with Some Quaternary Ammonium Ions. Hydrophobic Interaction vs. Charge Neutralization*.

* This manuscript is submitted to publication.

Abstract

The salt effect (NaCl) was used to investigate the nature of the interaction of 2-p-toluidinylnaphthalene-6-sulfonate (TNS) with nonyltrimethylammonium bromide (NTAB), decyltrimethylammonium bromide (DTAB), dodecyltrimethylammonium chloride (DoTAC), hexadecyltrimethylammonium chloride (HTAC), and octadecyltrimethylammonium (OTAC) respectively. Three distinguishable kinds of emission spectra corresponding to the DoTAC micelle-TNS complex ($\lambda^{F}_{max} \simeq 443$ nm, depending on the salt concentration), the DoTAC oligomer-TNS complex ($\lambda^{F}_{max} = 437$ nm) and the precipitate of the DoTAC monomers-TNS complex ($\lambda^{F}_{max} = 418$ nm) were observed. Similar results were obtained for the binding of HTAC or OTAC with TNS. Although an NTAB micelle-TNS formed, this was not the case for the NTAB monomer. The conclusion that the toluidinylnaphthalene ring is a potentially hydrophobic bonding contributor in the interaction of TNS with these alkyltrimethylammonium halide ions (ATAH) is based upon (1) the complex formation of TNS with ATAH monomer which is dependent on the alkyl chain length of ATAH; (2) an increase of the association constants of DoTAC micelle-TNS complex with a corresponding blue shift of emission spectrum and an increasing quantum yield at high salt concentration; (3) a salt induced increase in the cooperative binding interaction factor for the DoTAC oligomer-TNS complex. Evidence for both hydrophobic interaction and charge neutralization was found in the DoTAC monomer and DTAB monomer-TNS complex in low ionic

strength. The possible configurations of TNS in various complexes are discusse.

Introduction

The fluorescent probe, 2-p-toluidinylnaphthalene-6-sulfonate (TNS)[1], and related N-substituted aminonaphthalenesulfonate compounds, have been extensively used to monitor protein conformational change and to examine biomembrane structures[1-3]. The usefulness of the probe compound is based upon the fact that its fluorescence quantum yield increases as the solvent polarity decreases with an accompanying blue shift of its emission spectrum[4]. These and other fluorescent properties, such as fluorescent polarization and exited state lifetime, provide information concerning the polarity and the microviscosity of macromolecules at the fluorescent probe binding site.

The binding forces involved in the interaction between these amphiphilic compounds and macromolecules is not well defined[8,12]. The critical question is whether the sulfonate group or hydrophobic residue of TNS plays a decisive role in the binding.

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(1) Abbreviation used: TNS, 2-p-toluidinylnaphthalene-6-sulfonate; NTAB, nonyltrimethylammonium bromide; DTAB, decyltrimethylammonium bromide; DoTAC, dodecyltrimethylammonium chloride; HTAC, hexadecyltrimethylammonium chloride; OTAC, octadecyltrimethylammonium chloride; ATAH, alkyltrimethylammonium halide; I_F, relative fluorescence intensity; λ^{F}_{max}, emission wavelength of maximum intensity; λ^{EX}_{max}, exitation wavelength of maximum intensity.

Recent evidence indicates that no charge interaction occurs in the binding of 0-iodobenzoic acid, o-iodoaniline etc. and the hydrophobic cavity of concanavaline A and that benzene ring binds to the hydrophobic cavity of the protein[5]. In general, the evidence supports the hypothesis that charge neutralization is not an obligatory requirement for the binding of the probe to macromolecules or to micelle structures. Brand et al. showed that 1-anilinonaphthalene-5-sulfonate (1, 5-ANS), but not N, N-dimethyl-1-aminonaphthalene-5-sulfonate (DNS) could bind to alcohol dehydrogenase[6] indicating that the hydrophobic interaction between the aromatic moiety of fluorescent probe and the hydrophobic residue of protein plays an important role in binding. The studies on the interaction of poly-lysine and TNS show that only the β-sheet conformation of poly-lysine, the form which has no charges, results in TNS fluorescence[9,10]. The incorporation of 2-(N-methylanilino)-naphthalene-6-sulfonate (MNS) into the negatively charged phospholipid layers[11], and of TNS into micelles of sodium dodecyl sulfonate (SDS)[12] has been found, regardless of possible charge repulsion which could limit the hydrophobic interaction between these fluorescent probes and the amphiphilic macromolecules. On the other hand, Beyer et al. suggested that, although a hydrophobic environment is essential for enchancing the fluorescence of TNS, charge neutralization is required to provide binding between some peptides and TNS[8].

Solvent effect studies suggest that spectral changes of TNS that accompany binding to a macromolecule result importantly

from the loss of the aqueous solvation characteristics of structural bulky water[13]. Salt increases the association constant between TNS and SDS micelle by increasing the positive entropy change (a hydrophobic effect) which suggests that the loss of bulky water around the aromatic rings of TNS may contribute to the driving force for the TNS binding with SDS micelle[12].

In a further attempt to elucidate the relative contribution of hydrophobic and ionic interactions, we have examined the nature of TNS binding with alkyltrimethylammonium halide (ATAH)[1]. This has been achieved by studying the effect of neutral salt (NaCl) on the interaction. It is known that neutral electrolyte weakens the salt linkages but strengthens the hydrophobic interaction[14]. The results presented here show that the aromatic system of TNS contributes to the hydrophobic interaction with the quaternary ammonium ions.

Experimental Section

Materials: The potassium salt of 2-p-toluidinylnaphthalene-6-sulfonate was a product of Sigma Chemical Co.(lot no. 80c-5130) and it was stored in the dark at 5°C. The compound was found pure on TLC[8]. Dodecyltrimethylammonium chloride (DoTAC) and octadecyltrimethylammonium chloride (OTAC) was the product of Armour Industrial Chemical Co. The nonyltrimethylammonium bromide (NTAB), decyltrimethylammonium bromide (DTAB), and hexadecyltrimethylammonium chloride (HTAC) was purchased from

Eastman Kodak Co..

Fluorescence measurements: Fluorescence measurements were performed on a Perkin-Elmer fluorescence spectrophotometer MPF-2A equipped with a thermostated cell holder. Both emission and excitation slits were set at 4 nm. The excitation wavelength was calibrated with the differential spectrum using a Cary 17 spectrophotometer with TNS in water as reference. The sample was excited at the longest wavelength of maximum intensity of differential spectra. The emission spectrum of anthracene obtained on this instrument is in agreement with known corrected spectrum of anthracene in respect to relative fluorescence intensity and emission maxima which occur at 445, 421, 397 and 376 nm[15].

The ATAH monomers-TNS complex precipitate was isolated by filtration through paper, air dried and then the paper was attached to a triangle mirror supplied with the instrument. This allows for the fluorescence measurement of a small quantity of solid sample ($\sim 10^{-5}$ g).

The quantum yield was calculated from the equation[16]:

$$\Phi_2 = \Phi_1 \left(\frac{\text{Area}_2}{\text{Area}_1}\right) \left(\frac{1 - e^{-2.3A_1}}{1 - e^{-2.3A_2}}\right) \left(\frac{E_1}{E_2}\right)$$

where the quantum yield of anthracene in ethanol 0.30[15] was used as Φ_1, the integrated areas under the emission curves of sample and anthracene solution are Area_2 and Area_1 respectively. A_2 and A_1 are the absorbance corresponding to sample and anthracene respectively. The relative energies of excitation of the

sample and anthracene solution are given as E_2 and E_1.

The calculations of association constants: The association constant for the binding of TNS with micelle of DoTAC was calculated according to equation 1[12]:

$$\frac{1}{I - I'} = \frac{1}{\alpha_T [TNS_{total}]} + \frac{n}{\alpha_T [TNS_{total}] (M_{total} - cmc) K_T} \quad (1)$$

where I is total fluorescence intensiites of sample and I' is the fluorescence intensities caused by the complex of DoTAC monomer and TNS. α_T is temperature dependent coefficient of the following relation:

$$I_F = \alpha_T [M_n - TNS]$$

n is the number of monomer molecules which compose one micelle and is taken as 65[17] in the calculation of K_T. The monomer and micelle of DoTAC was expressed as M and M_n respectively. cmc is the critical micelle concentration.

It was found in this work that the binding between DoTAC monomer with TNS at DoTAC concentrations smaller than cmc is cooperative. The Hill coefficient, C[18,19], is calculated from the following equation 2:

$$\log\left(\frac{I}{I_{max} - I}\right) = C \log[M] + \log K'' \quad (2)$$

K" of eq. 2 can be normalized into two factors[20], one is the intrinsic association constant K_1 which is the first binding of M to TNS, and the other is the average interaction factor F_a for the successive bindings of M to M-TNS complex:

$$C\sqrt{K''} = K_1 \times F_a \qquad (3)$$

K_1 can be obtained at the lower end of the [M] region where the Hill coefficient is equal to 1. The F_a can be obtained by substituting K" and K_1 into eq. 3.

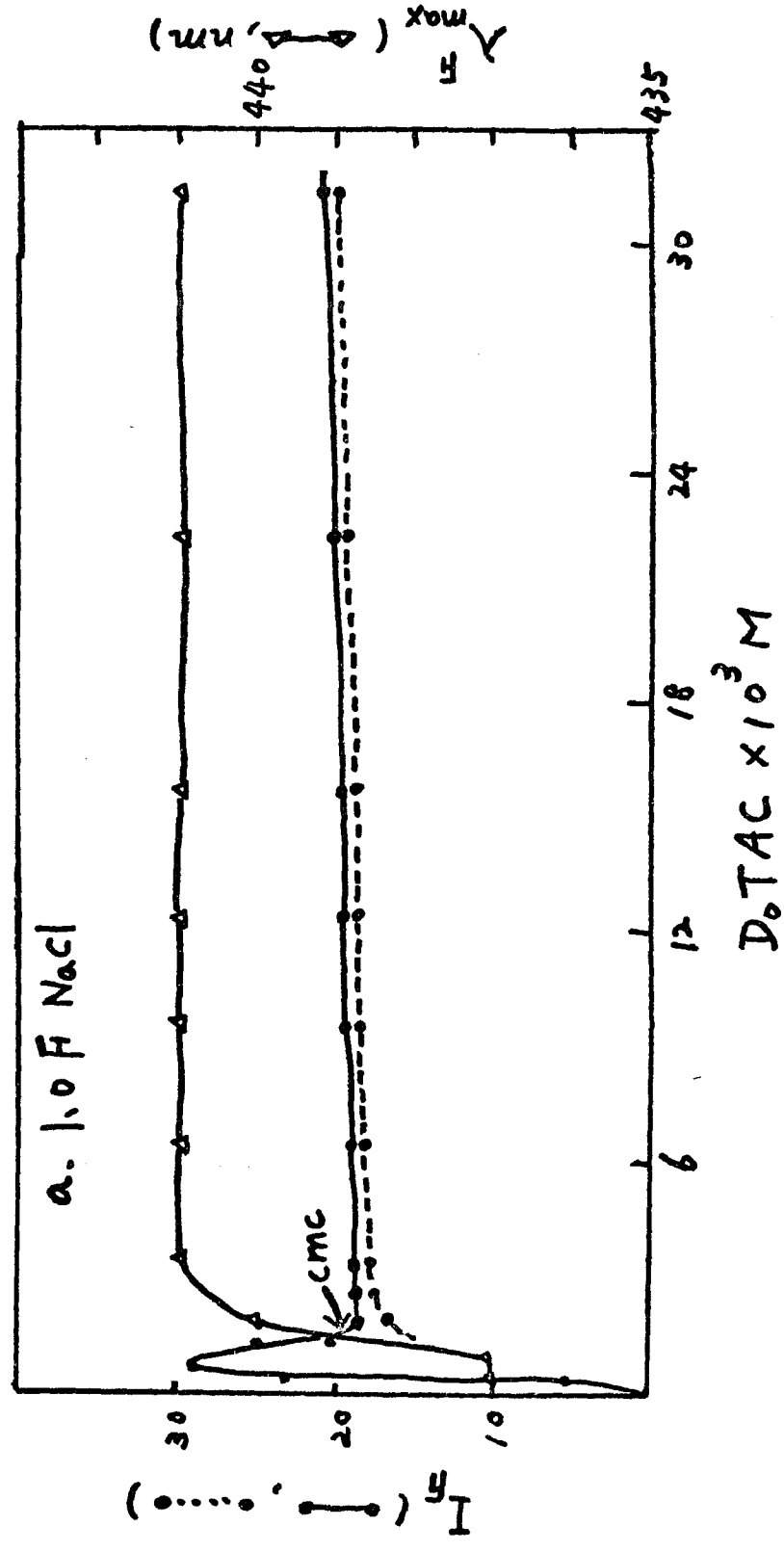
Results

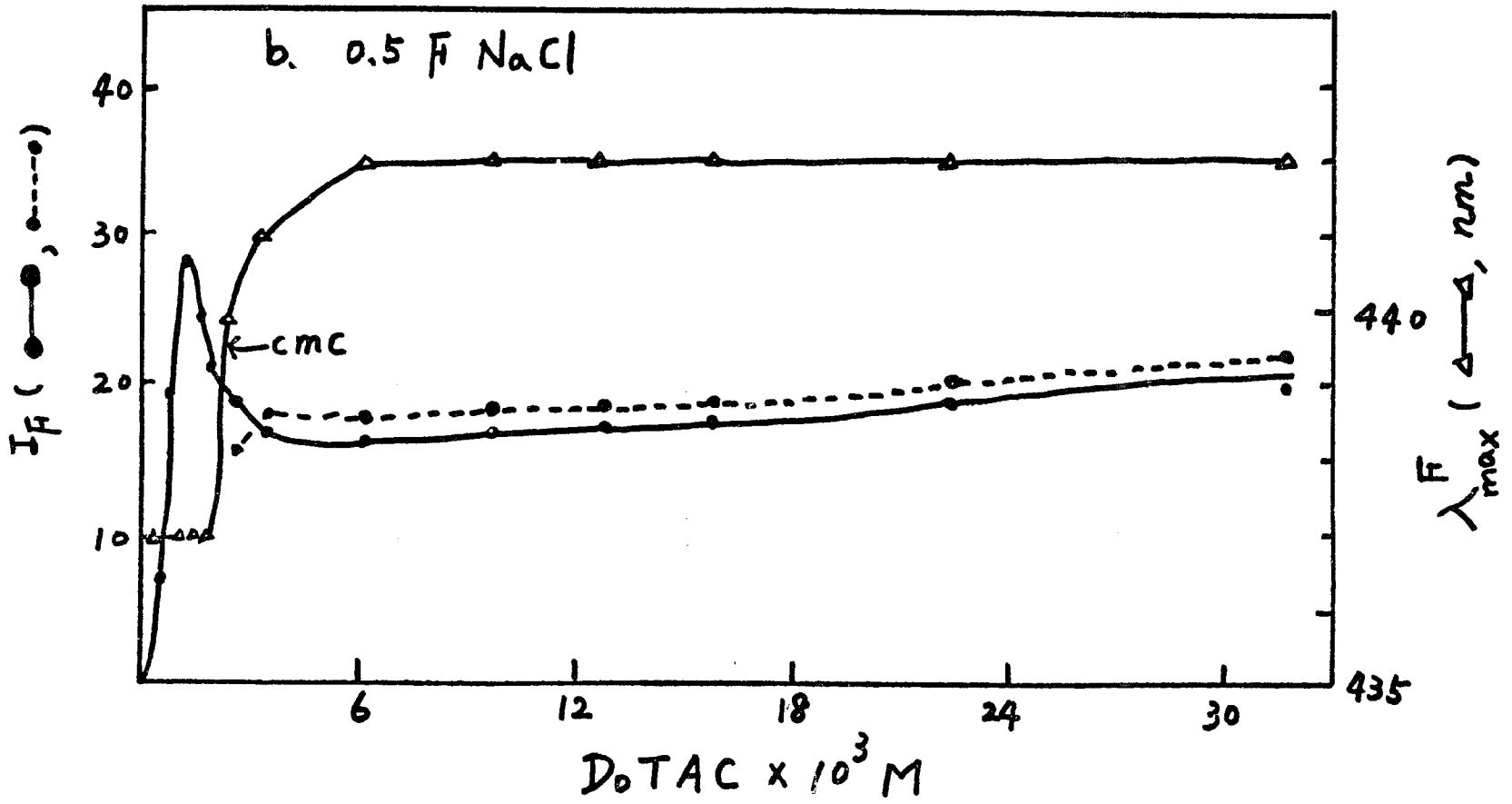
Binding of dodecyltrimethylammonium chloride (DoTAC) with TNS.

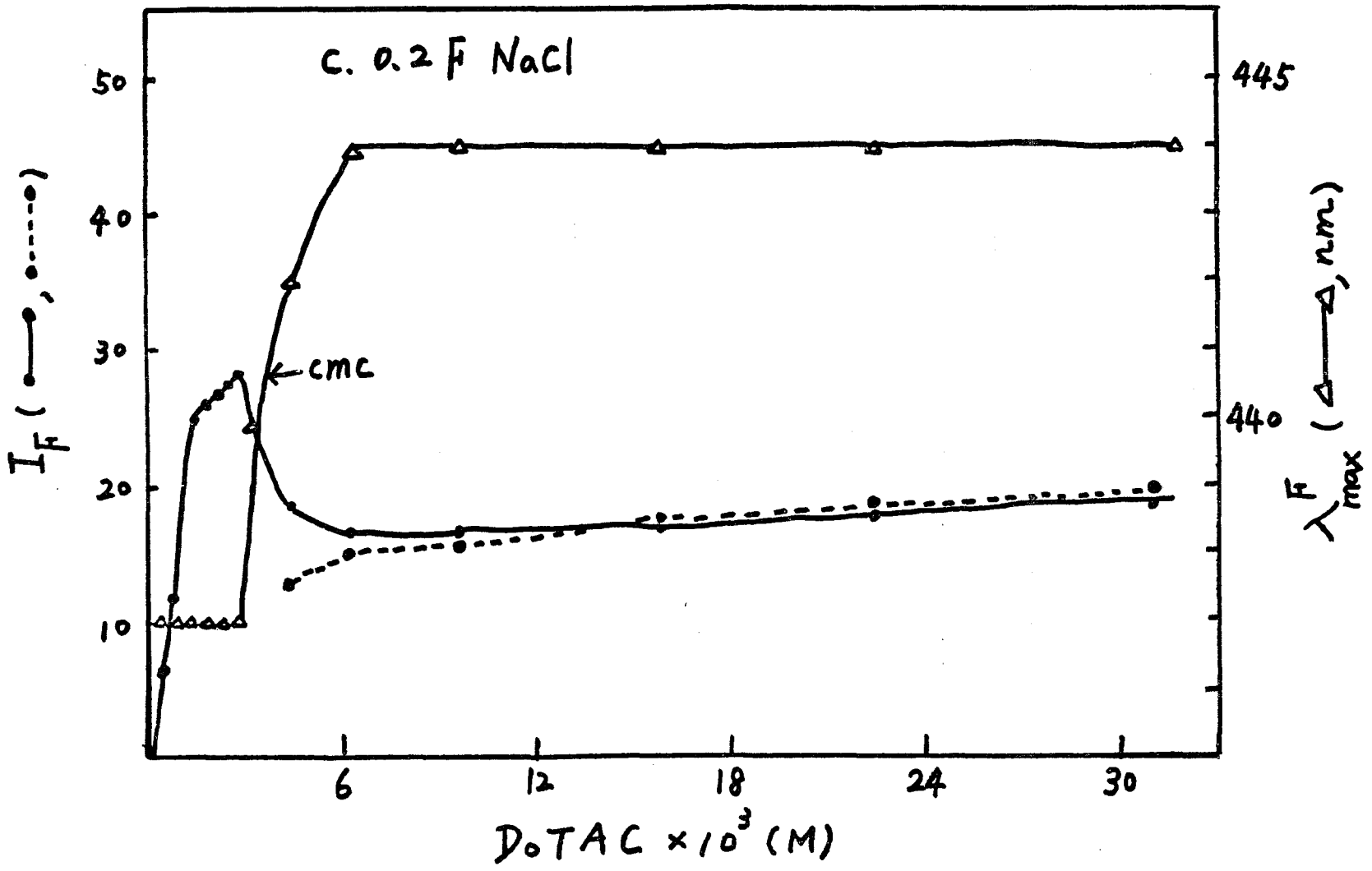
The solid lines of Figure 1(a-e) show the TNS fluorescence intensity and λ^F_{max} change as the DoTAC and the added NaCl concentrations are varied. The fluorescence intensity reached a maximum at low DoTAC concentrations, then it decreased to a minimum with slightly increasing DoTAC concentration. As the fluorescence intensity decreased from the maximum, there was a corresponding red shift in the emission spectrum. A further increase in the DoTAC concentration increased the fluorescence intensity only slightly. The concentration of DoTAC at the transition point of the red shift was assigned as the cmc as shown in Figure 1 (a-e). The cmc values in water, 0.05, 0.20, 0.50 and 1.0 F NaCl were 2.1×10^{-2}M, 0.84×10^{-2}M, 0.40×10^{-2}M, 0.24×10^{-2}M and 0.11×10^{-2}M respectively. The values are in agreement with those obtained by the method of light scattering and conductivity[17].

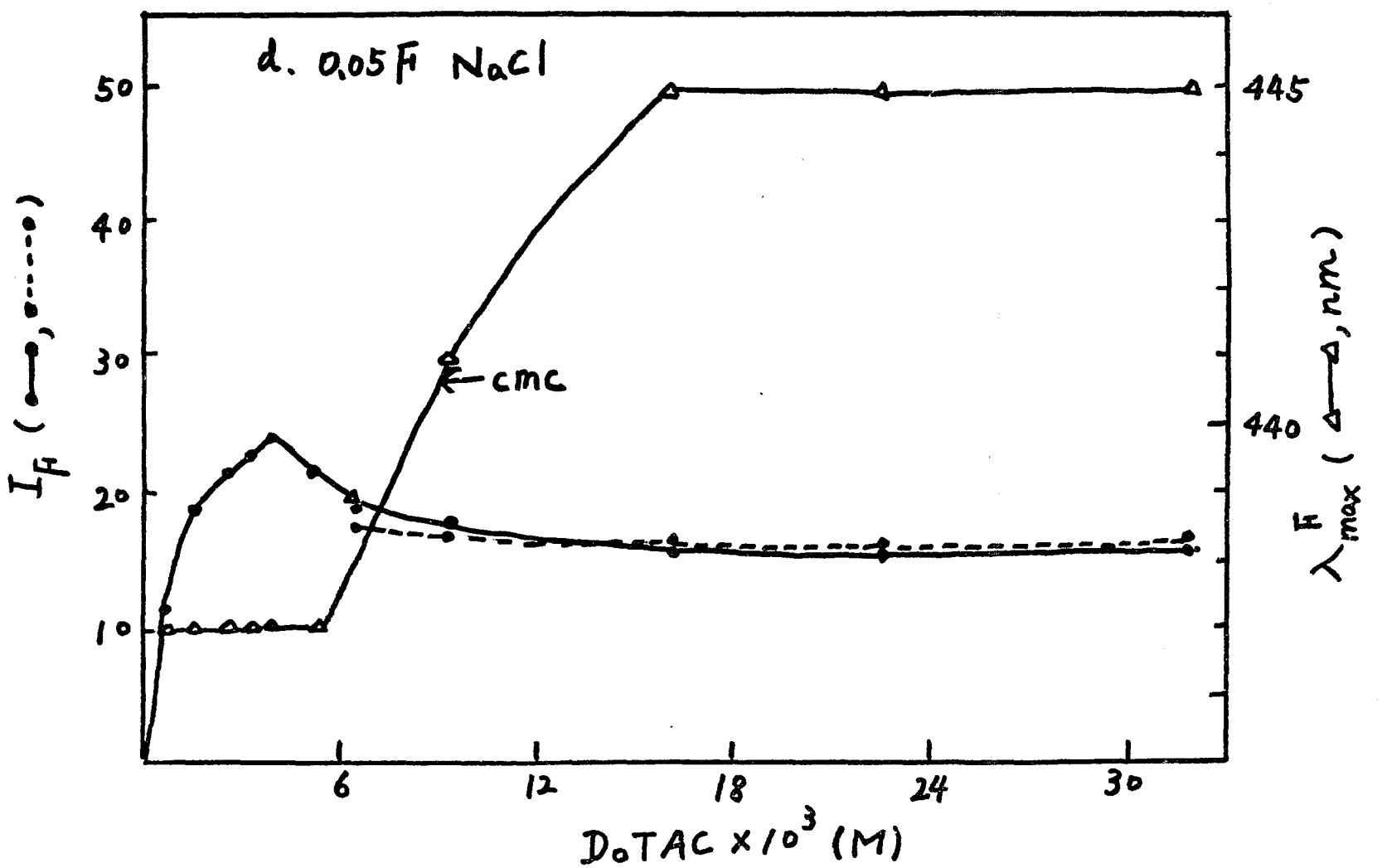
These data show that the degree of red shift is inversely dependent on salt concentration and the effect is asserted on the DoTAC micelle-TNS complex. The association constants at various salt concentrations for the complex are listed on Table I

Figure 1. The relative fluorescence intensity (I_F) and emission wavelength of maximum intensity (λ^F_{max}) of TNS at various n-dodecyltrimethylammonium chloride concentration in different NaCl concentrations. The solid line (•—• and ▵—▵) was obtained by measuring within 1 minute the emission spectra of solution when TNS was mixed with DoTAC. The dotted line (•·····•) was obtained by measuring the TNS-DoTAC solutions after 24 hrs. TNS: 9.6×10^{-6}M. The temperature is 25°C.


a. 1.0 F NaCl
cmc
I_y (•····• , —•—)
30
20
10
6
12
18
24
30
D₀TAC ×10³ M
λ_{max} (◆—◆ , nm)
440
435


b. 0.5 F NaCl
40
30
20
10
cmc
6
12
18
24
30
DoTAC x 10³ M
440
435
I_F (●—●, ●---●)
λ^F_{max} (△—△, nm)


c. 0.2 F NaCl
50
40
30
20
10
445
440
cmc
I_F (●—●, ●----●)
λ^F_{max} (△—△, nm)
6
12
18
24
30
DoTAC $\times 10^3$ (M)


d. 0.05 F NaCl
50
40
30
20
10
445
440
cmc
6
12
18
24
30
I_F (●—●, ●···●)
λ^F_{max} (△—△, nm)
D₀TAC × 10^3 (M)

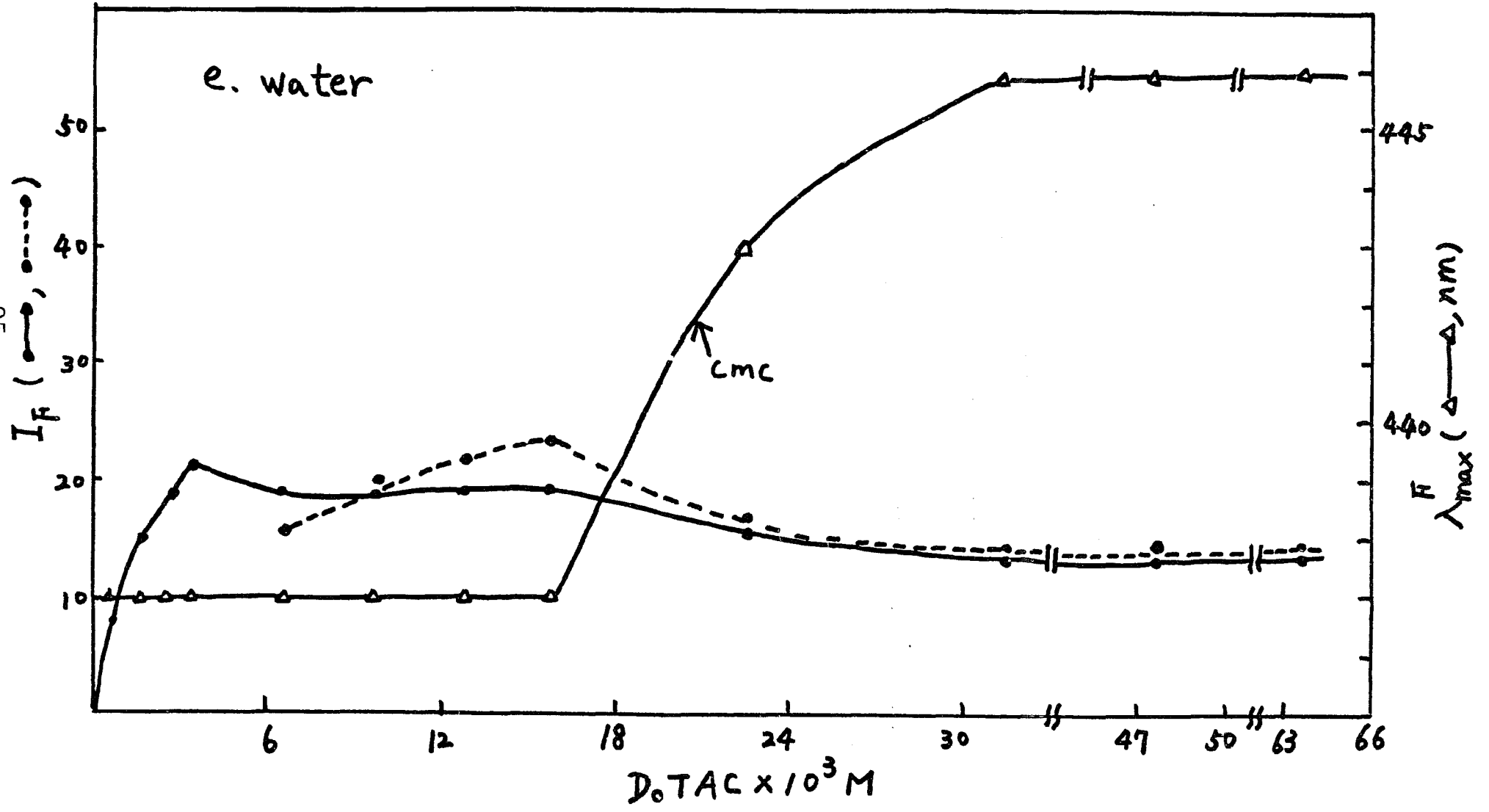

e. water
50
40
30
20
10
I_F (●—●, ●---●)
cmc
445
440
λ_{max}^F (△—△, nm)
6
12
18
24
30
47
50
63
66
$D_oTAC \times 10^3 M$

Table I. Association constants of DoTAC micelle-TNS complex(K) and DoTAC monomer-TNS complex(K_1)

Added NaCl conc. (F)	K (M^{-1}) ($\times 10^{-3}$) 25°C	K_1 (M^{-1}) 14°C
0.05	1.0	18 ± 3
0.20	2.0	10 ± 3
0.50	2.3	15 ± 3
1.00	2.9	

Table II. Emission wavelength of maximum intensity (λ^{F}_{max})*, excitation wavelength of maximum intensity (λ^{EX}_{max})* and quantum yield of ATAH micelle-TNS complex.

Added NaCl conc. (F)	NTAB			DoTAC			HTAC			OTAC		
	λ^{F}_{max}	λ^{EX}_{max}	ϕ	λ^{F}_{max}	λ^{EX}_{max}	ϕ	λ^{F}_{max}	λ^{EX}_{max}	ϕ	λ^{F}_{max}	λ^{EX}_{max}	ϕ
water	446	371	0.140	446	371	0.140	444	371	0.150	442	371	0.185
0.01										440	372	0.262
0.05	446	371	0.140	445	371	0.150	442	371	0.185			
0.20	445	371	0.140	444	371	0.177	440	371	0.233	437	373	0.470
0.50	445	371	0.145	442	372	0.185						
1.00	445	371	0.150	441	372	0.190						

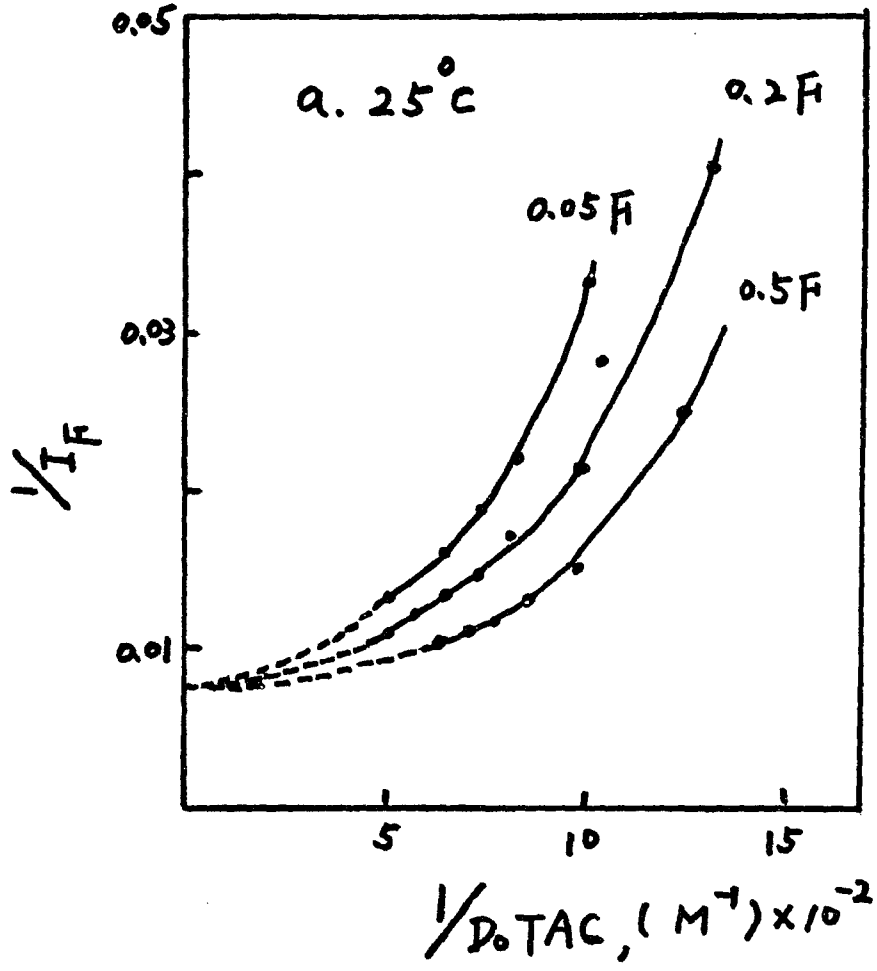
*wavelength unit is nm.

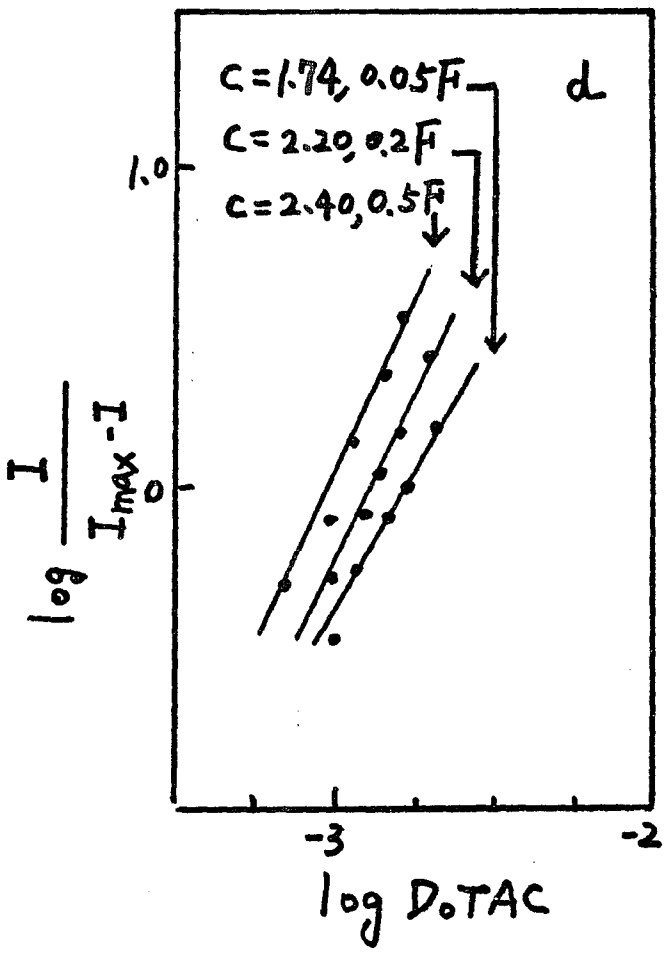
and the wavelength of the emission maximum and quantum yields are shown in Table II. As, expected, the magnitude of the association constants is inversely related to that of the red shift. It is well established that the TNS emission spectrum has a blue shift in the nonpolar solvents[4]. Thus a decreasing red shift with increasing salt concentration suggests that hydrophobic bonding participates in the interaction of DoTAC micelle with TNS and that it becomes stronger with increasing salt concentration. In addition, salt also increased the potential for the micelle formation, since the λ^{F}_{max} curves in the Figure 1 (a-e) shows that the range of DoTAC for micelle formation decreased with increasing salt concentration.

The λ^{F}_{max} = 437 nm was cuased by non-micellular complexing of DoTAC monomer and TNS. This complex λ^{F}_{max} will form a precipitate with its emission spectrum shifting to λ^{F}_{max} = 418 nm. The melting point of the precipitate was 225-230^{o}C(uncorr.). The precipitate, which formed about two minutes after mixing of TNS and DoTAC monomer solution, was not caused by a salting out effect of NaCl on TNS, since the emission spectrum (λ^{F}_{max} = 437 nm) in water (Figure 1e) still existed with λ^{F}_{max} = 418nm in the precipitate.

Double reciprocal plots of fluorescence intensity at the emission maximum λ^{F}_{max} = 437 nm vs. DoTAC concentration in three added NaCl concentration (0.05, 0.2 and 0.5 F) are shown in Figure 2(a-e) at three temperatures 14, 19, 25^{o}C. The plots are nonlinear and appear to be parabolas. The Hill plots of these data given in Figure 2(d-f) (see eq. 2). The Hill

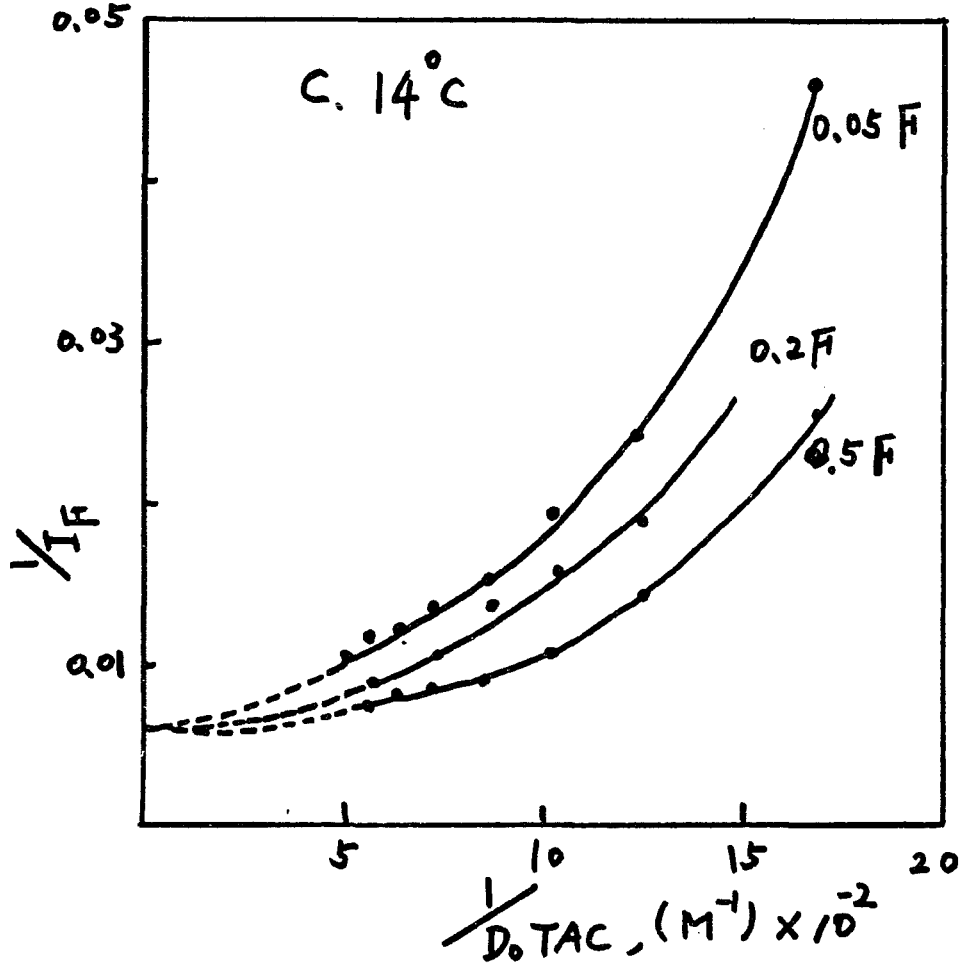
Figure 2. Double reciprocal plots of $1/I_F$ vs. $1/[DoTAC]$ at 25^{o}, 19^{o} and 14^{o} (a-c). And the Hill plots (d-f) of Figure 2(a-c). TNS: 9.6×10^{-6} M.

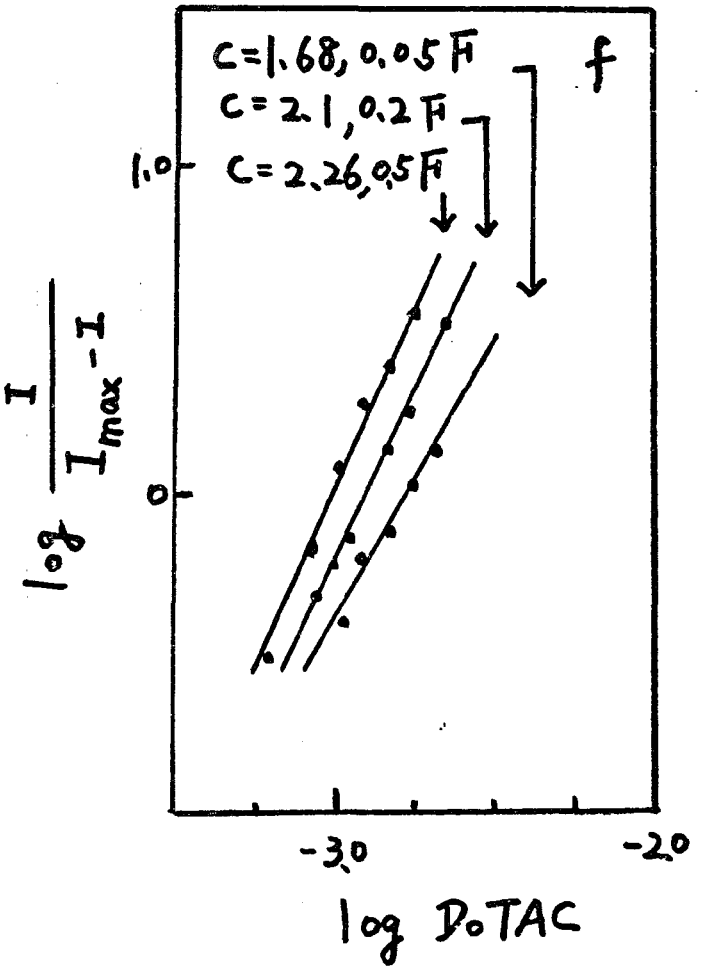

a. 25°C
0.2F
0.05F
0.5F
0.05
0.03
0.01
1/I_F
5
10
15
1/D₀TAC, (M⁻¹) x 10⁻²


C=1.74, 0.05F
C=2.20, 0.2F
C=2.40, 0.5F
d
1.0
0
log I/(I_max - I)
-3
-2
log D₀TAC

b. 19°C
0.05F
0.2F
0.5F
$1/I_F$
0.01
0.03
0.05
$1/D_oTAC, (M^{-1}) \times 10^{-2}$
5
10
15
20

$\log \frac{I}{I_{max} - I}$
-1.0
0
1.0
$\log D_oTAC$
-3
-2
C=1.78, 0.05F
C=2.14, 0.2F
C=2.38, 0.5F
e


C. 14°C
0.05 F
0.2 F
0.5 F
0.05
0.03
0.01
1/I_F
5
10
15
20
1/D₀TAC, (M⁻¹) x 10⁻²


C=1.68, 0.05 F
C=2.1, 0.2 F
C=2.26, 0.5 F
f
1.0
0
log I/(I_max - I)
-3.0
-2.0
log D₀TAC

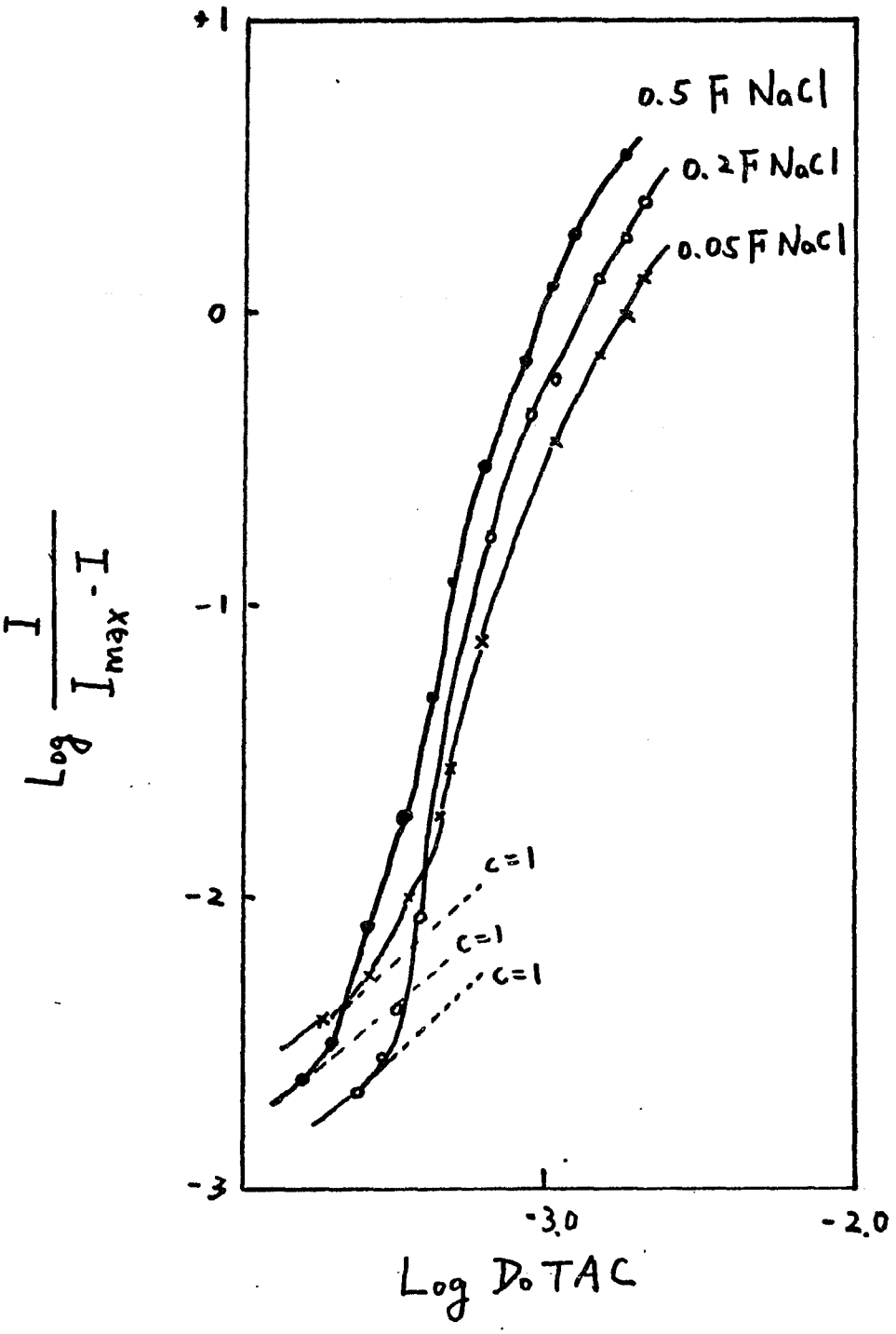
coefficients at the half maximum fluorescence intensity region are about 2.3, 2.1 and 1.7 at these three temperatures for 0.5, 0.2 and 0.05 F NaCl respectively. The Hill coefficients suggest that the binding of DoTAC monomer with TNS is highly cooperative and that at least 3 DoTAC could bind with one TNS. The Hill plot in Figure 3 extends the data seen in Figure 2f to the lower DoTAC concentration range, which is the region where the Hill coefficient is equal to one. The association constants in this region (C = 1), which is assumed to correspond to the DoTAC monomer-TNS complex, was estimated to be $(18 \pm 3)M^{-1}$ in 0.05 F NaCl, $(10 \pm 3)M^{-1}$ in 0.2 F NaCl, and $(15 \pm 3)M^{-1}$ in 0.5 F NaCl (Table I and solid line of Figure 4). The association constant for DoTAC monomer-TNS complex decreases with increasing salt concentration to reach a minimum value. Further increase in the NaCl concentration increased the association constants (solid line of Figure 4).

Assuming that the association constant in zero ionic strength for the DoTAC monomer-TNS complex is $25M^{-1}$, based on the extrapolation of solid line of Figure 4, and that the charge neutralization is the only force to provide the linkage between TNS and DoTAC monomer, the theoretical association constants (K_1) at various ionic strengths can be calculated from eq. 4 which is obtained according to Debye-Huckel limiting law[21]:

$$TNS^- + M^+ \rightleftharpoons \overset{-}{TNS} - \overset{+}{M}$$

$$K_1 = \frac{[\overset{-}{TNS} - \overset{+}{M}]}{[TNS^-][M^+]}, \text{ and } K_1' = \frac{(a_{\overset{-}{TNS} - \overset{+}{M}})}{(a_{TNS^-})(a_{M^+})}$$

Figure 3. Hill plot of Figure 2f at lower DoTAC concentration. The temperature is 14^{o}C.


+1
0
-1
-2
-3
0.5 F NaCl
0.2 F NaCl
0.05 F NaCl
c=1
c=1
c=1
-3.0
-2.0
Log D₀TAC
Log I / (I_max - I)

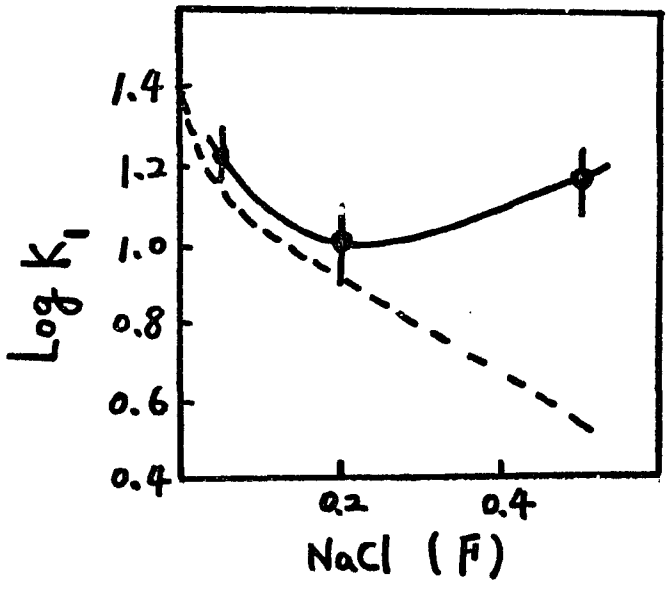
where a is the activity of the ion specie. Then

$$\log K_1^{'} = \log K_1 + 1.08\sqrt{\mu} \quad \text{at } 14^{o}C \qquad (4)$$

As mentioned above, $K_1^{'} = 25M^{-1}$ was assumed at $\mu = 0$. Therefore, the theoretical K_1 values at various ionic strength can be calculated. This result is shown as the dotted line of Figure 4. Since the Debye-Huckel limiting law is useful only at very low ionic strength solution, thus, the line only expresses the minimum theoretical association constants at various ionic strengths. The solid line in Figure 4 shows a minimum K_1 value, this suggests that both charge neutralization and hydrophobic interaction participate in the DoTAC monomer-TNS complex. The greater the ionic strength, the greater contribution of the hydrophobic interaction and the smaller the ionic interaction. At 0.5 F NaCl, the theoretical ionic interaction for the DoTAC monomer-TNS complex should be very small, the complex is formed only by hydrophobic interactions. Even at very low ionic strength, the hydrophobic bond still contributes to the total binding free energy. This was also supported by the finding that the DoTAC monomer-TNS interaction in water still causes fluorescence, indicating that the aromatic rings of TNS should be surrounded by hydrophobic residue of other molecule, namely the dodecyl group of DoTAC.

The interaction factor F_a (eq. 3) is calculated to be 70, 75, 30 for 0.5, 0.2 and 0.05 F NaCl solutions respectively at the half maximum fluorescence intensity area. The F_a values,

Figure 4. Observed association constants (————) and theoretical association constants (– – – –) of DoTAC monomer-TNS complex. Temperature: 14°C.


1.4
1.2
1.0
0.8
0.6
0.4
Log K1
0.2
0.4
NaCl (F)

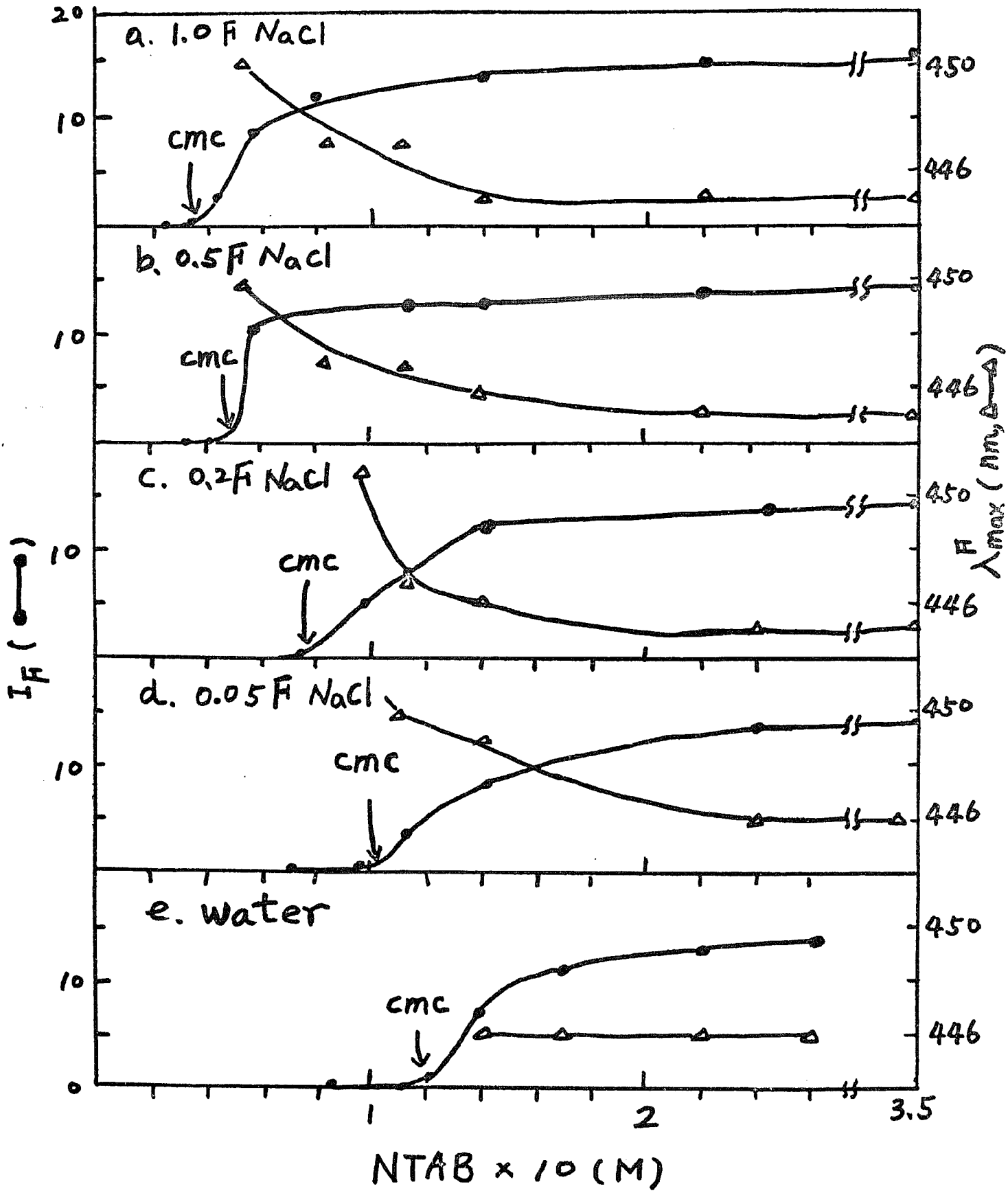
which indicate the relative binding efficiency for cooperative interaction, suggest that NaCl increased the binding constant for the successive binding of the DoTAC monomer to the DoTAC monomer-TNS complex. Although the first DoTAC binding with TNS involves both hydrophobic interactions and charge neutralization, the values of F_a at different ionic strength clearly suggest that the charge neutralization contribution to the polymeric complexes, that is, DoTAC dimer-TNS and DoTAC trimer-TNS etc., should be very small since NaCl increases the interaction factor.

The ratio of I_{max} values (eq. 2) at 25, 19 and 14°C was found to be 1 : 1.17 : 1.33. The same temperature dependent quenching phenomenon for α_T value in the SDS micelle-TNS complex has been noticed[12]. This general phenomenon is caused by more solvent collision quenching with the excited state of TNS at higher temperature.

Binding of nonyltrimethylammonium bromide (NTAB) with TNS.

The fluorescent titration curves of TNS as a function of the NTAB concentration in 1.0, 0.5, 0.05 F NaCl and water are shown in Figure 5 (a-e). The TNS concentration used is 9.6 $1o^{-6}$M, which is the same concentration used in the study of the interaction of DoTAC with TNS. The micelle formation was accompanied with fluorescent enhancement and a blue shift of the emission wavelength. The NTAB concentration at the onset of fluorescence enhancement was assigned as the cmc. The cmc values are 1.2×10^{-1}M, 0.98×10^{-1}M, 0.80×10^{-1}M, 0.46×10^{-1}M and $0.30 \times$

Figure 5. The relationship of I_F and F_{max} vs. NTAB concentration. See legend of Figure 1.


a. 1.0 F NaCl
b. 0.5 F NaCl
c. 0.2 F NaCl
d. 0.05 F NaCl
e. water
cmc
20
10
0
450
446
1
2
3.5
NTAB × 10 (M)
I_F (~)
λ_{max}^F (nm, Δ)

10^{-1}M in water, 0.05, 0.20, 0.50 and 1.0 F NaCl respectively. A strikingly similar fluorescence enhancement phenomenon was previously found for the interaction of TNS with SDS. In that case there was no significant binding between SDS monomer and TNS. The result shows that no sinificant complex formation between NTAB monomer and TNS was found at the TNS concentration used (9.6×10^{-6}M). However, at the same concentration, the complex formation between DoTAC monomer and TNS was observed. It is, then obvious that the complex formation between ATAH[1] monomer and TNS is alkyl chain length dependent.

The salt effect on the quantum yield and wavelength of emission spectrum of the NTAB micelle-TNS complex is shown in Table II. The salt effect on the NTAB micelle-TNS complex is similar to that on the DoTAC micelle-TNS complex, i.e., salt increased the quantum yield and caused a blue shift of TNS fluorescence. These results suggest that the environment surronding TNS in the NTAB micelle-TNS should be hydrophobic in nature.

The possible contribution of viscosity and salt (NaCl) to fluorescence enhancement and emission wavelength was examined by two controlling experiments. First, various concentrations of sucrose solution were prepared to match the viscosity of NTAB solution which has ρ = 1.43 cp at 0.43 M. The fluorescence enhancement of TNS in those sucrose solutions was found less than 2% of the total fluorescence enhancement caused by the NTAB solution. Second, the emission spectra in 40% ethanol solution with no added NaCl and 1 F NaCl was measured separately.

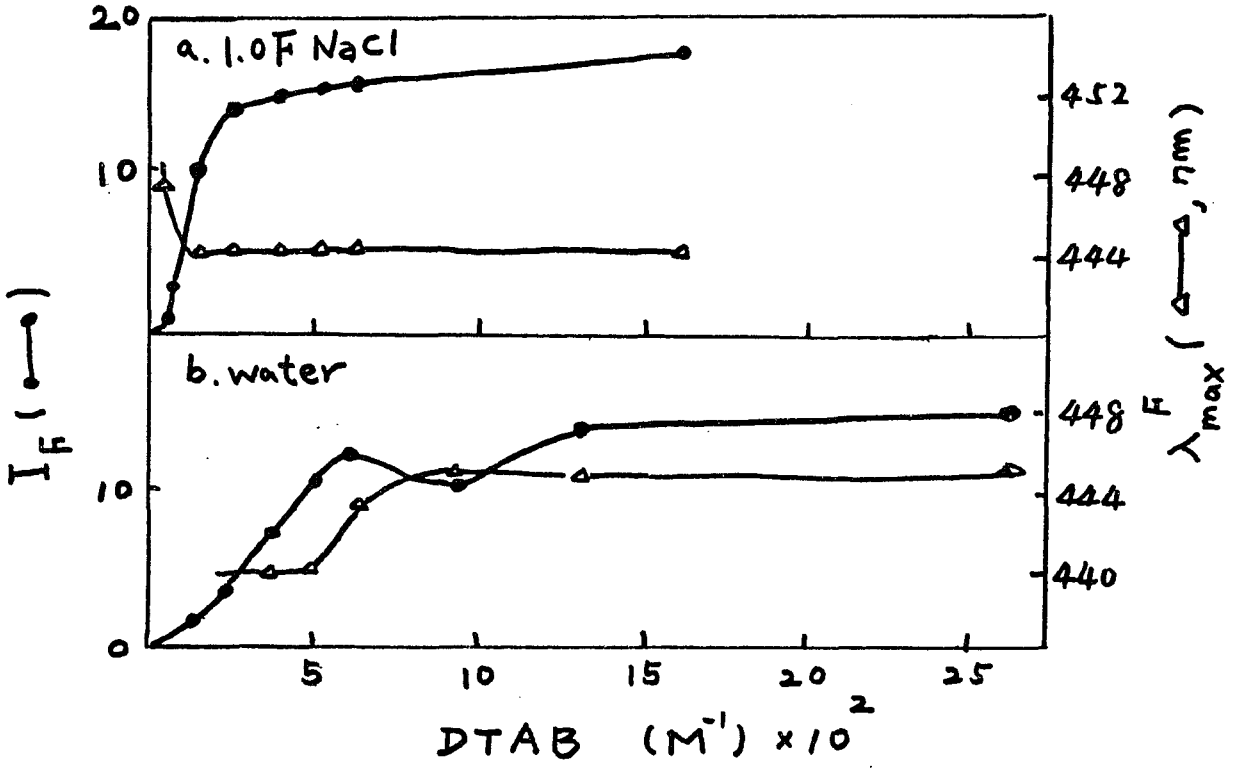
It was found that no significant fluorescence enhancement and emission wavelength shift (λ^{F}_{max} = 454 nm) caused by added NaCl occurred.

Finally, it should be noted here that TNS forms an insoluble complex (m.p. 202-203°C, uncorr.; λ^{F}_{max} = 425 nm) with NTAB monomer at high TNS concentrations. However, no complex formation between TNS and NTAB monomer is observed in the TNS concentration of 9.6×10^{-6}M which we used in the experiment.

Binding of decyltrimethylammonium bromide (DTAB) with TNS.

The fluorescent titrations were performed at TNS concentration of 9.6×10^{-6}M in 1.0 F NaCl (Figure 6a) and water (Figure 6b) respectively. The result shown in Figure 6a indicates that there is no detectable complex formation between TNS and DTAB monomer. This phenomenon is similar to that of the interaction of TNS with NTAB monomer (Figure 5). On the contrary, the titration curve of Figure 6b is similar to that of the interaction of TNS with DoTAC (Figure 1(a-e)), i.e. there is a complex formation between TNS with DoTAC monomer in water. These results suggest that both hydrophobic bonding and ionic interaction participate in the formation of the DTAB monomers-TNS complex in water which is prevented in 1.0 F NaCl. It may be that the ionic interaction of the complex is completely eliminated in high NaCl concentration and that the hydrophobic interaction is not sufficiently increased to maintain the complex in high NaCl concentration. It is interesting to note that decyl group of DTAB monomer is shorter by two methylene groups than the dodecyl group of DoTAC.

Figure 6. The relationship of I_F and $^{F}_{max}$ vs. DTAB concentration. See legend of Figure 1.

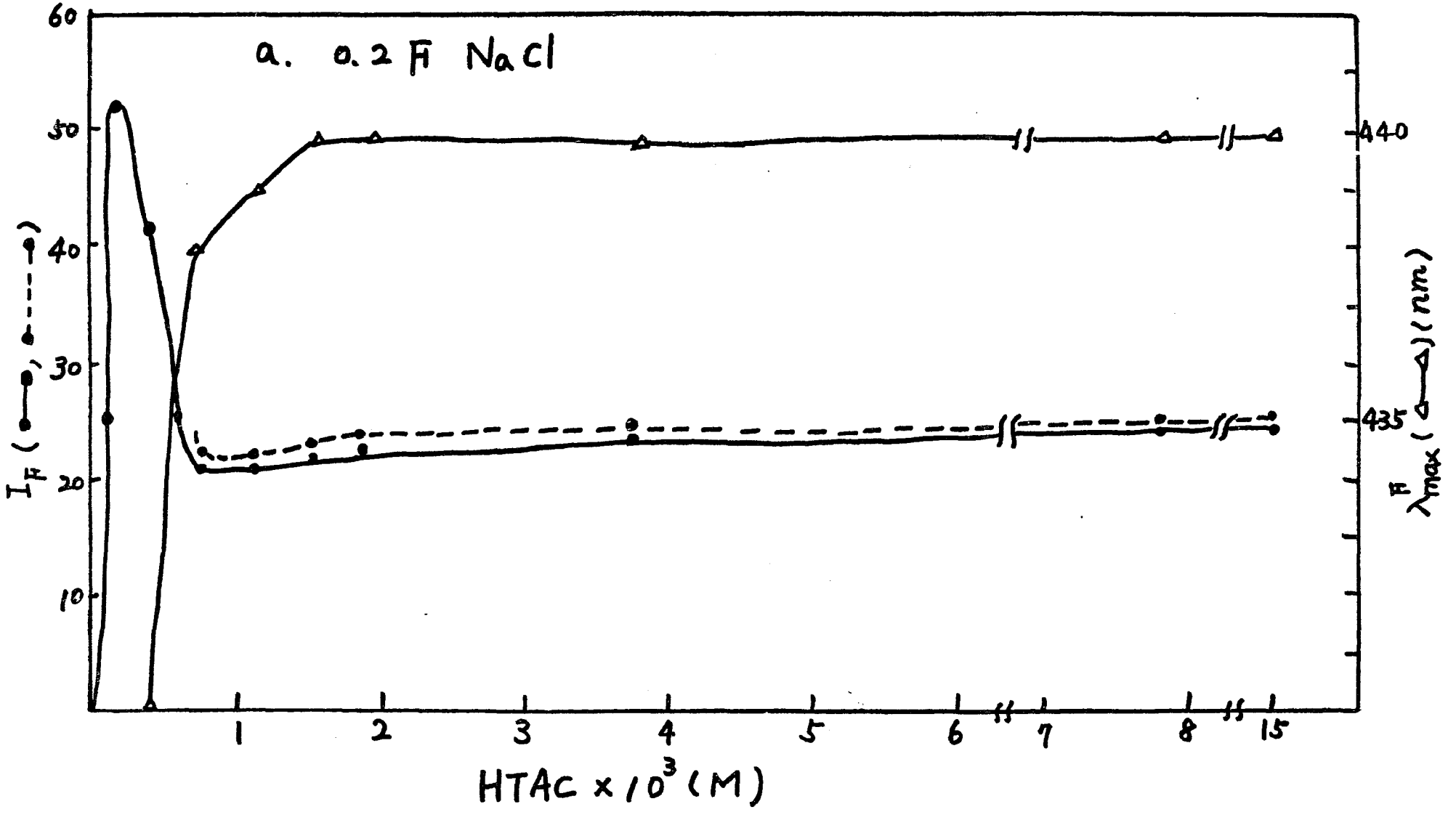

a. 1.0F NaCl
b. water
20
10
0
452
448
444
448
444
440
5
10
15
20
25
DTAB (M⁻¹) x10²
I_F (—)
λ_max F (mμ, ——)

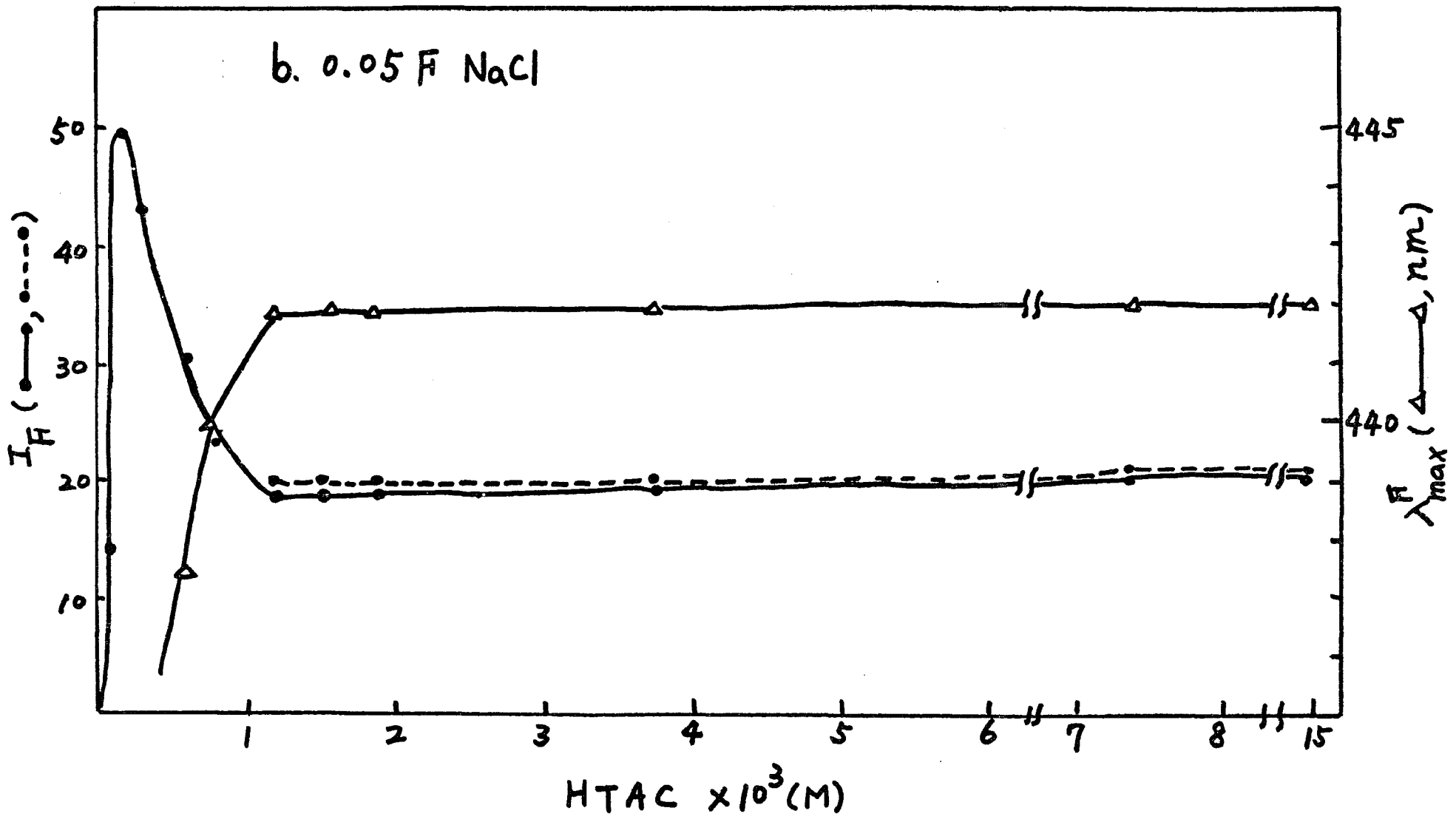
This will decrease the hydrophobic bonding between TNS and DTAB monomer. Thus, the added salt can only cause a small incremental hydrophobic bonding increase in the complex formation of DTAB monomers with TNS. In contrast, the added salt effectively increased hydrophobic bonding in the DoTAC monomer-TNS complex such that the limiting effect of added salt on charge neutralization can be completely compensated for. The results shown in Figure 6 also indicate that there is a DTAB micelle-TNS complex in water or in 1.0 F NaCl solution. The m.p. of the isolated precipitate crystal of DTAB monomers-TNS complex is 209 - 210°C and has a λ^{F}_{max} = 425 nm.

Binding of hexadecyltrimethylammonium chloride (HTAC) and octadecyltrimethylammonium chloride (OTAC) with TNS.

The plots of fluorescence enhancement vs. the quaternary ammonium ion concentration (solid lines of Figure 7 (a-c) for HTAC and Figure 8 (a-c) for OTAC) are essentially similar to those of the DoTAC-TNS interaction (Figure 1 (a-e)). They differ from the NTAB-TNS interaction in that the monomers of DoTAC, HTAC and OTAC can bind with TNS respectively, but there is no detectable complex formation between TNS and monomer of NTAB at the TNS concentration of 9.6×10^{-6}M. The HTAC monomer-TNS interaction and OTAC monomer-TNS interaction resulted in the rapid precipitation of the complex. Thus the measured wavelength of maximum intensity of fluorescence (Figure 7(a,b), Fig. 8(a-c) showed no difinite λ^{F}_{max} for the HTAC monomers-TNS or OTAC monomers-TNS complexes.

Figure 7. The relationship of I_F and λ^F_{max} vs. HTAC concentration. See legend of Figure 1.


a. 0.2 F NaCl
60
50
40
30
20
10
I_F (—●—, - - -●- - -)
440
435
λ^F_{max} (—△—) (nm)
1
2
3
4
5
6
7
8
15
HTAc x 10^3 (M)


b. 0.05 F NaCl
50
40
30
20
10
I_H (—●—, ---●---)
445
440
λ^F_{max} (—△—, nm)
1
2
3
4
5
6
7
8
15
HTAC $\times 10^3$ (M)

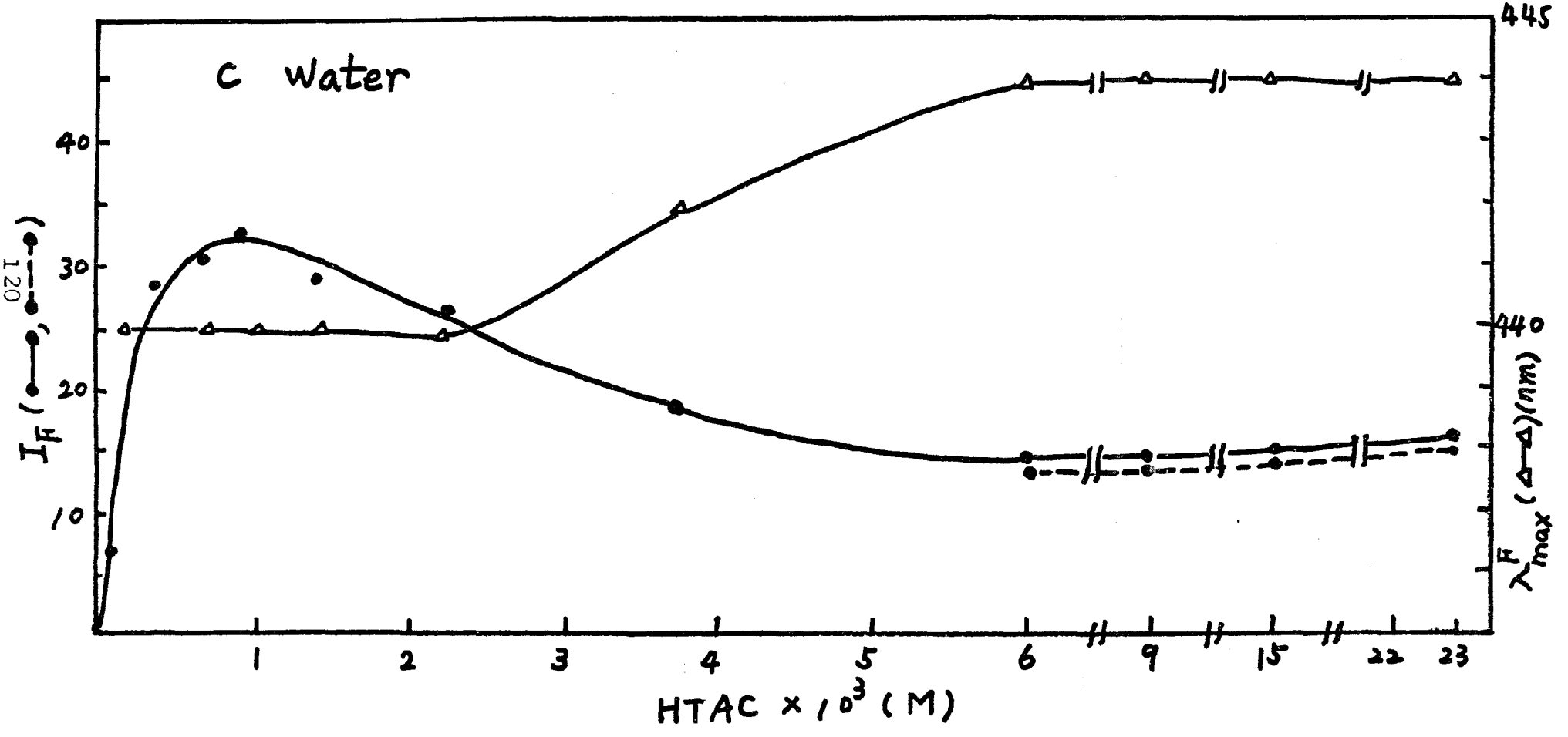
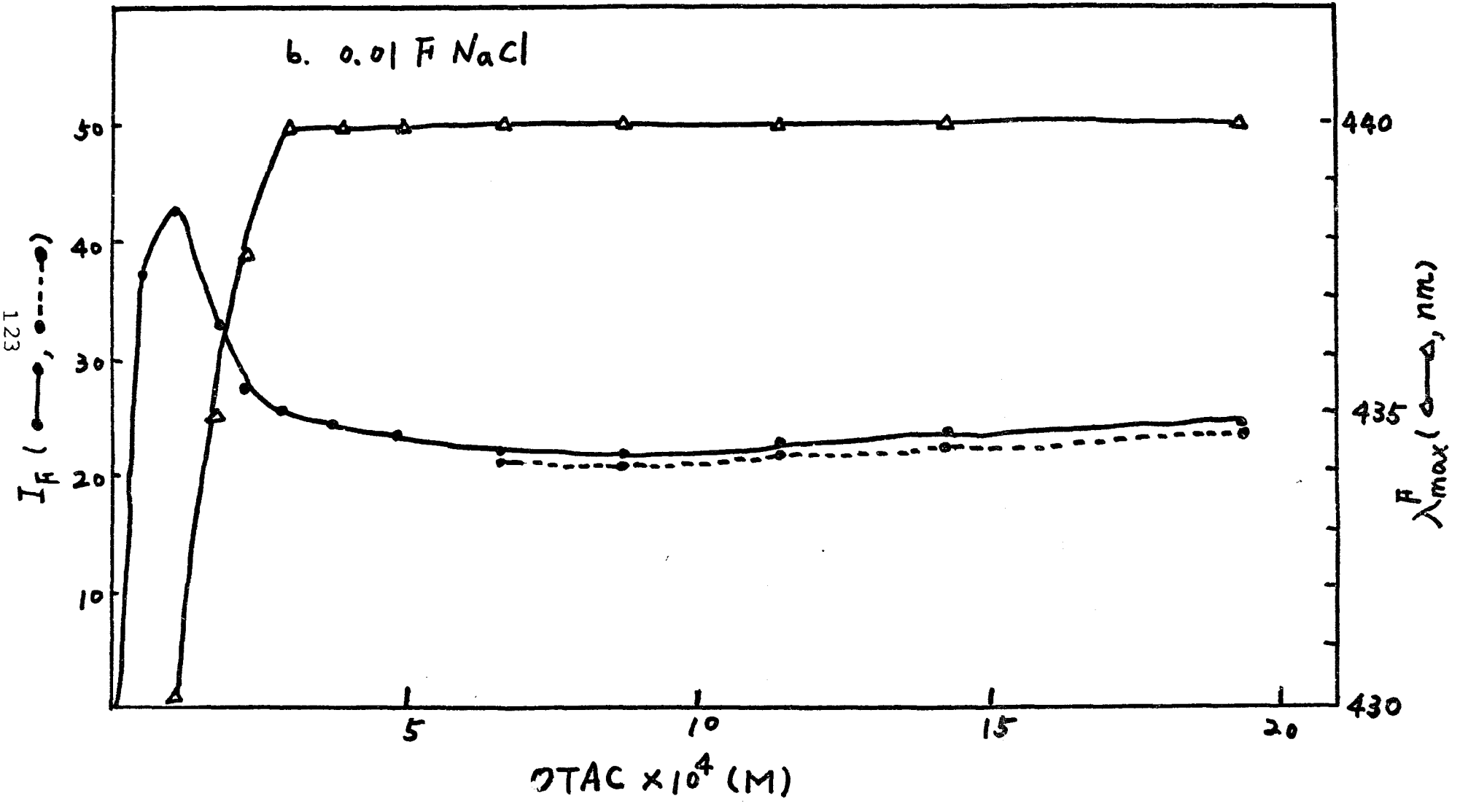
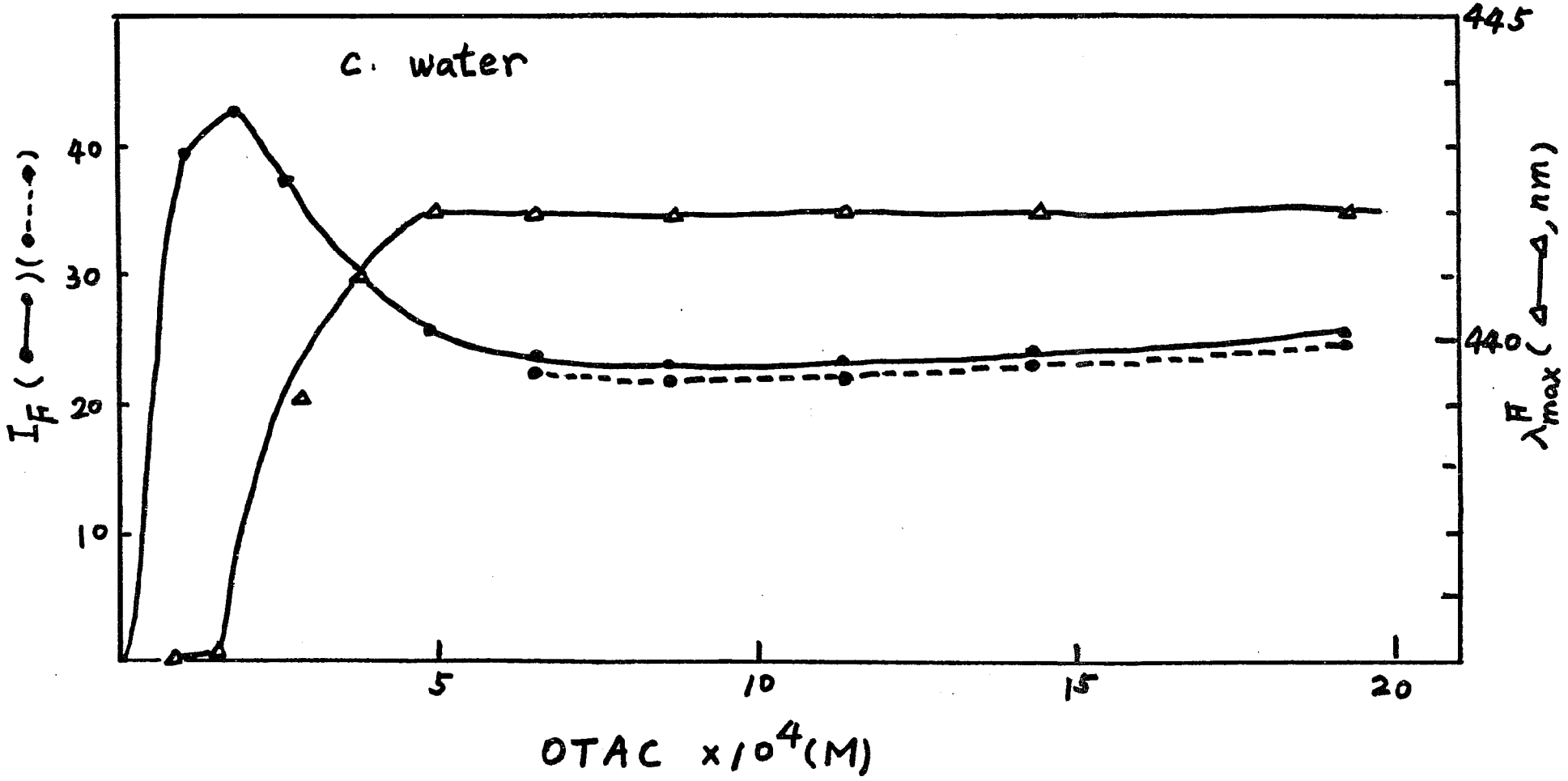

c Water
445
440
40
30
20
10
120
I_F (●—●, ●--●)
λ^F_{max} (△—△) (nm)
1
2
3
4
5
6
9
15
22
23
HTAC × 10^3 (M)

Figure 8. The relationship of I_F and λ^F_{max} vs. OTAC concentration. See legend of Figure 1.

I_F (●—●, ●----●)
10
20
30
40
50
60
OTAC × 10^4 (M)
5
10
15
20
a 0.2 F NaCl
λ_{max}^F (△—△, nm)
435
440


b. 0.01 F NaCl
50
40
30
20
10
I_H (●----●, ●—●)
440
435
430
λ^F_{max} (○—○, nm)
5
10
15
20
DTAC $\times 10^4$ (M)


c. water
I_F (●—●)(●---●)
40
30
20
10
5
10
15
20
OTAC $\times 10^4$(M)
445
440
λ^F_{max} (△—△, nm)

The melting points of the precipitates of HTAC monomers-TNS complex and OTAC monomers-TNS complex are 210-225°C and 175-185°C respectively. Both precipitates show λ^{F}_{max} = 398 nm.

The λ^{F}_{max} values of the HTAC micelle-TNS complex (Figure 7) and the OTAC micelle-TNS complex (Figure 8) are also salt concentration dependent. The higher the salt concentration, the more blue shift of the emission spectrum and the higher quantum yield (Table II).

The environment of bound TNS in its complexes with micelle or monomer of ATAH(1).

The λ^{F}_{max} and λ^{EX}_{max} for ATAH micelle-TNS complexes are shown in Table II. It can be concluded, in general, the smaller the λ^{F}_{max} value (blue shifting), the larger the λ^{EX}_{max} value (red shifting). Also the magnitude of λ^{F}_{max} change is greater than the corresponding change of λ^{EX}_{max}. It has been shown that the emission and excitation spectra of TNS in non-polar solvents will undergo blue shift[4] and red shift[22] respectively. The results suggest that the polarity of TNS in the ATAH micelle-TNS complex of shorter ATAH alkyl group is larger than that of TNS in the complex of longer ATAH alkyl group. Because the emission spectrum of the ATAH micelle-TNS complex, the hydrophobicity of TNS in the former complex should be smaller than that of TNS in the latter complex.

Discussions

Hydrophobic interaction vs. charge neutralization.

The length of the ATAH alkyl group seems to be important for the interaction of ATAH monomer with TNS since no significant complex formation was found between NTAB monomer and TNS. A complex did form between TNS and the longer chain monomers, DoTAC, HTAC or OTAC. Salt increased the interaction of DoTAC oligomer-TNS complex as reflected by the fact that both the interaction factor (F_a) and Hill coefficient (C) were larger at higher salt concentrations. Salt also increased the association constant for the DoTAC micelle-TNS complex (Table I). The same results were seen for the SDS micelle-TNS complex[12]. Thus, hydrophobic bonding is of primary important in the complex formation. That is, the aromatic residues of TNS are probably surrounded by hydrophobic alkyl group of ATAH.

Evidence indicating that charge neutralization exists in the DoTAC monomer-TNS complex at low ionic strength is provided in the association constant decrease from $(18 \pm 3)M^{-1}$ to $(10 \pm 3)\ M^{-1}$ for the ionic strength range of 0.05 to 0.2 F. However, hydrophobic bonding still exists in the DoTAC monomer-TNS complex because a further increase of ionic strength to 0.5F increased the association constant to $(15 \pm 3)\ M^{-1}$. Similarly, both hydrophobic interactions and ionic interaction also participate in the complex formation between TNS and DTAB monomer.

The fluorescent characteristics of the TNS in the ATAH

micelle-TNS complex (Table II) also indicates that aromatic rings of TNS should bind with the hydrophobic alkyl group. Here, the blue shift of the TNS emission spectra and the red shift of the TNS excitation spectra occurred at the higher NaCl concentrations.

An examination of CPK molecular moles of TNS and of ATAH molecules shows that the length of the TNS aromatic rings is almost equal to that of the extended dodecyl group of DoTAC (Fig. 9). The nonyl group of NTAB then, is too short to cover the aromatic rings of TNS with the consequence that there is no complex formation of NTAB monomer at low TNS concentrations. The pairing between aromatic rings of TNS with the ATAH alkyl group may be indicated by the melting points of the ATAH monomers-TNS complex precipates. The melting points of the complexes for the NTAB monomer, DTAB monomer, DoTAC monomer, HTAC monomer, OTAC monomer were 202-203, 209-210, 225-230, 210-227, 175-185^{o}C respectively. This suggests that the crystal lattice of the DoTAC monomers-TNS complex is the most compactly packed. And that, in general those alkyl groups of ATAH which are longer or shorter, will decrease the m.p. of the crystal lattice formed with TNS. This is in agreement with CPK model measurement, which shown that the length of aromatic rings of TNS is almost equal to that of dodecyl group of DoTAC.

In conclusion, the configuration of ATAH monomer-TNS complex should consist of both ionic interaction and hydrophobic interaction. Such a configuration of the DoTAC monomer-TNS complex is complex I of Figure 10. Because salt increases the

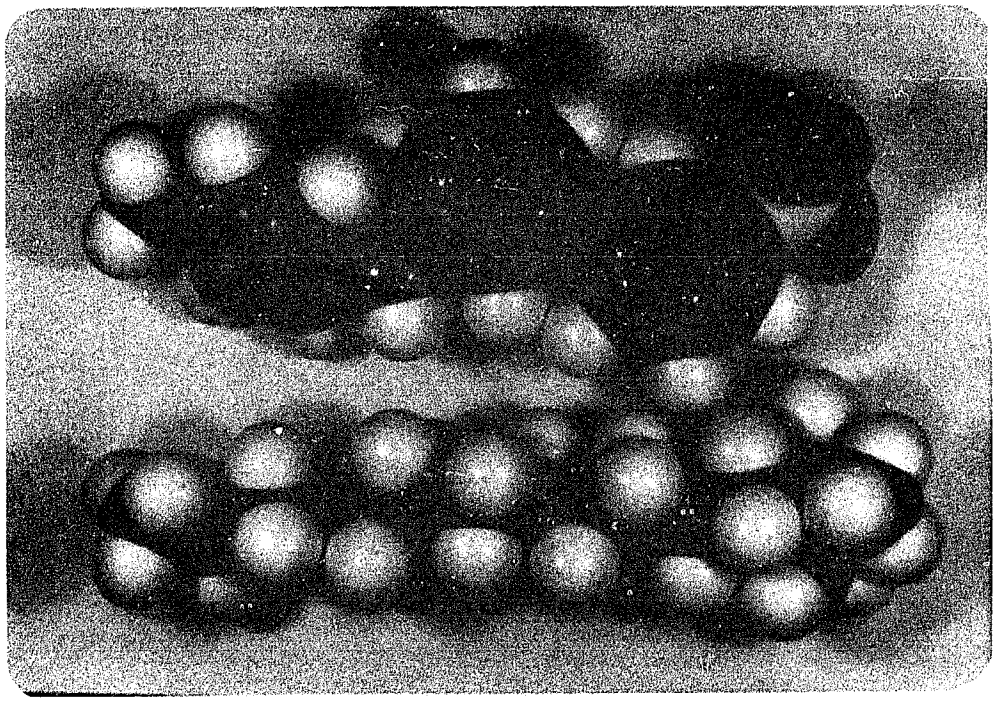


Fig. 9. The CPK molecular models of TNS and DoTAC molecules.

association constant of DoTAC micelle-TNS complex and the interaction factor (F_a) of DoTAC oligomer-TNS complex, the hydrophobic interaction should participate in these two complex formation and the charge neutralization should be a minor force in the complex.

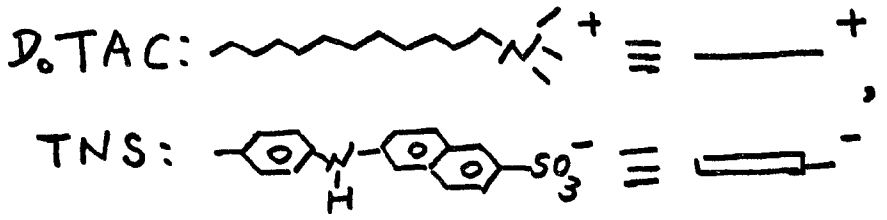
The cooperative binding of DoTAC monomer to TNS.

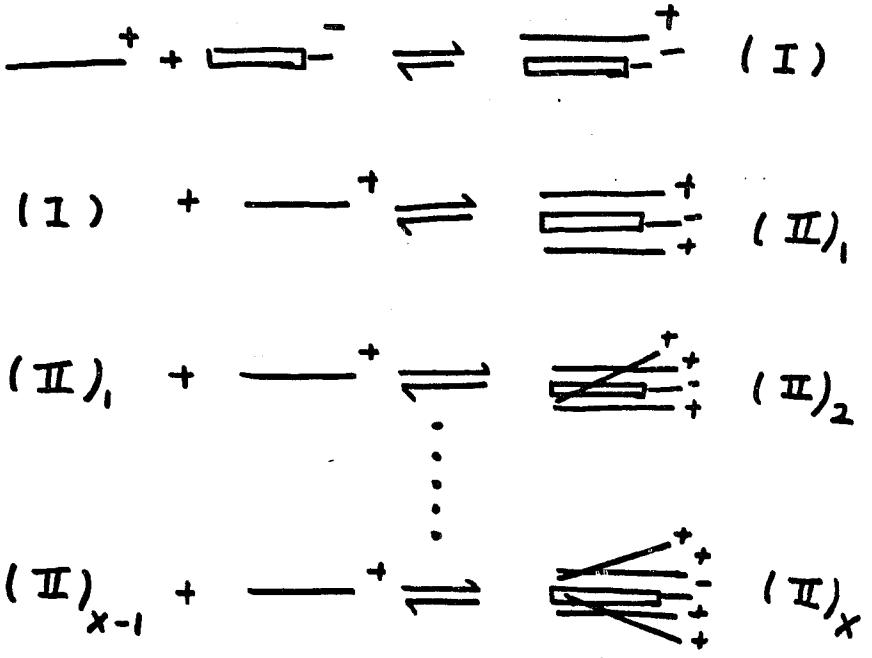
Figure 10 summaries the results of the cooperative binding between DoTAC monomer with TNS to form the DoTAC oligomer-TNS complex. The first binding of DoTAC monomer to TNS to form DoTAC monomer-TNS complex (complex I of Figure 10) involves both hydrophobic interactions and charge neutralization. The subsequent binding of DoTAC monomer to complex I was highly cooperative and the complex formed is DoTAC oligomer-TNS complex (complex II of Figure 10). The cooperativity was increased by salt.

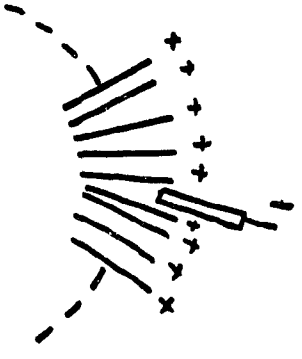
Comparison of the hydrophobicity of TNS in the environment of the ATAH micelle-TNS complex and the ATAH oligomer-TNS or the ATAH monomer-TNS complex.

It was found that the emission spectrum of the ATAH micelle-TNS complex is red shifted compared to that of the complex of TNS with the ATAH monomer. This seems to be opposite to what is expected. It is generally accepted that the hydrophobicity of the core of the micelle of quaternary ammonium ions should be greater than that of the monomers, and that the emission spectra in the lower polar solvents are blue shifted. A

Figure 10. The configurations of DoTAC monomer-TNS complex (I), DoTAC oligomer-TNS complex (II_1, II_2, II_x) and DoTAC micelle-TNS complex (III) at the binding site of TNS in micelle.







(III)

possible explanation of this anomoly has been provided by Burton and Minch's investigation of the interaction of aromatic compounds with quaternary ammonium ions[23]. Their results suggest that the π system of the benzene ring of benzoate or phenylacetate, etc. interacts with the partially positive N-methyl proton. The entire aromatic ring does not locate in the core of the micelle but rather it locates in the positively charged head region of the quaternary ammonium ion. This suggests that, in the case of ATAH micelle-TNS complex, part of the aromatic rings of TNS may be in the location of the Stern layer of the micelle with some part of the aromatic rings interacting with the positively charged head of the quaternary ammonium ion (complex III of Figure 10). This model could explain the observation that the hydrophobic environment of TNS in the micelle is more polar than that of TNS in the complex of TNS with ATAH monomers which has a parallel arrangement of alkyl group and the toluidinylnaphthalene ring with a charge interaction between the sulfonate group and the quaternary ammonium ion to provide a more hydrophobic environment for TNS (complex I of Figure 10).

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References

1. L. Stryer, Science, 162, 526 (1968)
2. G. M. Edelman and W. C. McClure, Accounts Chem. Res., 1, 65 (1968)
3. L. Brand and J. R. Gohlke, Ann. Rev. Biochem., 41, 834 (1972)
4. W. C. McClure and G. M. Edelman, Biochemistry, 5, 1908 (1966)
5. K. D. Hartman and C. F. Ainsworth, Biochemistry, 12, 4442 (1973)
6. L. Brand, J. R. Gohlke and D. S. Rao, Biochemistry, 6, 3510 (1967)
7. L. Stryer, J. Mol Chem., 13, 482 (1965)
8. C. F. Beyer, L. C. Craig and W. A. Gibbons, Biochemistry, 11, 4920 (1972)
9. J. Lynn and C. D. Fasman, Biochem. Biophys. Res. Commun., 33, 327 (1968)
10. G. Witz and B. L. van Duurren, J. Phys. Chem., 77, 648 (1973)
11. G. K. Radda, in "Biological Membranes" ed. by D. S. Parsons, Clarenden Press, Oxford, 1975, pp. 81-95.
12. H.-C. Chiang and A. Lukton, J. Phys. Chem., 79, 1935 (1975)
13. R. L. Reeves, M. S. Maggio, and L. F. Costa, J. Am. Chem. Soc., 96, 5917 (1974)
14. W. Kauzmann, Adv. Protein Chem., 14, 1 (1959)
15. T. Y. Porro, R. E. Anacreon, P. S. Flandreau and I. S. Fageson, Journal of the AOAC, 56, 607 (1973)
16. C. A. Parker and W. T. Rees, Analyst, 85, 587 (1960)

17. M. F. Emerson and A. Holtzer, J. Phys. Chem., 71, 1898 (1967)

18. A. V. Hill, J. Physiol. (London), 40, 4 (1910)

19. F. Wold, "Macromolecules: Structure and Function", Prentice-Hall, Englewood Cliffs, N. J. 1961

20. L. H. Segel, "Enzyme Kinetics", Wiley, New York, N. Y., 1975

21. W. J. Moore, "Physical Chemistry", Prentice-Hall, Englewood Cliffs, N. J. 4ed., 1972

22. F. C. Greene, Biochemistry, 14, 747 (1975)

23. C. A. Bunton and M. Minch, J. Phys. Chem., 78, 1490 (1974)