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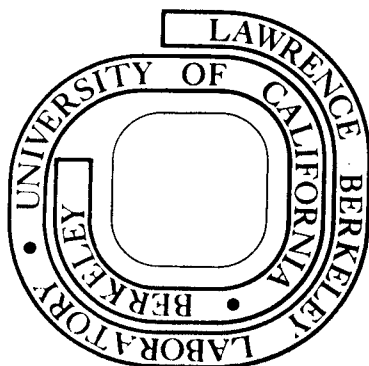
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BINDING OF 2-p-TOLUIDINO-NAPHTHALENE-6-SULFONATE TO 3T3 and SV3T3 CELLS

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SUMMARY

2-p-Toluidino-naphthalene-6-sulfonate (TNS), a fluorescent probe, bound to suspensions of 3T3 and SV3T3 cells. The emission spectra, excitation spectra, emission polarization and protein to TNS energy transfer were similar for both cell types, regardless of cell density. TNS is probably bound to the plasma membrane of the cells. The uncharged probe, TNS sulfonamide, showed properties similar to those of TNS when bound to 3T3 cells.

The binding was time dependent. The fluorescence properties of bound TNS changed slightly with time. Calculations are given which indicate that random distribution of TNS throughout the plasma membrane could account for the observed protein to TNS energy transfer. The similar emission polarizations of TNS bound to 3T3 and SV3T3 cells indicate similar plasma membrane fluidities for these cells. However, this fluidity represents an average of values which may vary within the membrane.

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INTRUDUCTION

TNS (2-p-toluidino-naphthalene-6-sulfonate) is a fluorescent probe that
has been widely used to study molecular movement of proteins and membrane
components [1-8]. TNS fluoresces weakly in water, with a maximum intensity
near 500 nm. In a non-polar environment, the intensity of fluorescence of
TNS generally increases two to three orders of magnitude and the emission
maximum shifts to lower wavelengths [8]. Increasing viscosity of the medium
produces similar effects [8]. When TNS or related probes bind to proteins or
membranous particles a strong blue shift in the emission maximum occurs con-
comitant with large increases in the emission intensity [1-4,8]. These results
suggest that the bound TNS is in a relatively rigid non-polar environment.
Presumably, both hydrophobic and electrostatic forces contribute to the
binding [2-4,8].

We have been interested in using this fluorescent probe to investigate
biological systems. There have been several reports on the use of such probes
in the study of mammalian cells [9-12], but very little has been done with
cells grown in culture. The importance of understanding the properties of
membranes of mammalian cells grown in culture is becoming increasingly apparent.
For example, there is evidence that membrane changes do occur upon viral trans-
formation of mammalian cells [13,14]. It has been suggested that membranes
of transformed cells are less rigid than their normal parent cell membranes [15],
and that the mobility of membrane components of normal cells vary as a function
of growth [16].

The 3T3 mouse fibroblast cells when seeded in a dish at a low density
will actively grow until they reach their saturation density. At this saturation
density the majority of the population becomes quiescent [17,18]. The SV40

virus transformed 3T3(SV3T3), on the other hand, continues to grow even at high cell density [19]. In the experiments reported here we used TNS to compare the fluidity of the membranes of growing and quiescent 3T3 cells with that of SV3T3 membranes from cells growing at low and high cell density.

MATERIALS AND METHODS

Reagents

TNS was obtained from Sigma and twice recrystallized from aqueous ethanol. It was kept as a 2.5 x 10^{-3} M stock solution in water. TNS sulfonamide was prepared as previously described [20] and was stored as a 2.5 x 10^{-3} M stock solution in ethanol. Although insoluble in water, it was readily dispersed and adsorbed by the cells. Measurements with TNS sulfonamide were reproducible and fluorescence enhancement in the presence of the cells was greater than 10X.

Cells

The cells used in this study were carried in 100 mm plastic dishes (Falcon) and incubated in a CO_2 incubator at 37°. They were maintained in Vogt and Dulbecco's modification of Eagles' medium [21], containing 10% calf serum (GIBCO). Both cell lines were transferred twice weekly by removal from the dishes with 0.05% trypsin (Difco, 1:250) in 25 mM Tris buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, and 0.7 mM Na_2HPO_4 (isotonic Tris buffer). The seeding density was 1/10 of the saturation density. The 3T3 cl 4A cells were obtained from Robert Holley, Salk Institute, and cloned prior to use. SV3T3 cl 56 was obtained from Renato Dulbecco, Salk Institute. Both cell lines were shown to be free of mycoplasma by ^{3}H-thymidine incorporation followed by autoradiography.

To harvest the cells the medium was removed and 5 ml of isotonic Tris buffer was added to each dish. The dish was gently swirled, the buffer removed and another portion added. The cells were then scraped from the dish with a rubber policeman and then transferred by pipet to a centrifuge tube. They were passed through the pipet tip several times to assure dispersion and were centrifuged for 10 min at 720 x G in a IEC model HN-S centrifuge. The buffer was aspirated and the procedure repeated. The cells were taken up in isotonic Tris buffer for counting and final dilution. The protein content of the cells was determined by the method of Lowry et al. [22], using bovine serum albumin as a standard.

Measurements

Measurements were performed immediately after harvesting. In order to avoid reabsorption errors and to conserve cells, cuvettes with a 1 x 3 mm cross section were used for fluorescence measurements, and 3 x 3 mm cross section for absorption measurements. Background measurements of the cells were first taken, then TNS from the stock solution was added to yield the desired concentration of TNS. The cells were passed through a pipet tip several times to assure mixing and dispersion. An aliquot was taken for measurements. Precision in the fluorescence intensity of different aliquots from the same cell suspension was 10%. All measurements were taken at 37°.

Instrumental

Fluorescence measurements were carried out using a Perkin Elmer MPF-2A Hitachi spectrofluorimeter with a thermostated cell holder. Excitation light was filtered through a Corning 7-54 filter (ultraviolet transparent, visible opaque) and emitted light was filtered through a 350 mM cutoff filter. Excitation spectra are corrected for the filter absorption. The spectra are

otherwise uncorrected. The background emission from cells alone and from a TNS blank were subtracted from the spectra of cells plus TNS. The background was not more than several percent of the intensity of cells plus TNS. Absorption measurements were performed on a Cary Model 118 spectrophotometer. Polarization of fluorescence, P, was measured with a Perkin-Elmer polarization attachment with linearly polarized excitation light. P was calculated from the expression [23]:

$$P = \frac{I_{VV} - \frac{I_{HV}}{I_{HH}} \cdot I_{VH}}{I_{VV} + \frac{I_{HV}}{I_{HH}} \cdot I_{VH}}$$

where I is the observed fluorescence and the subscripts V and H indicate the orientation, vertical or horizontal, of the excitation and analyzer polarizers, respectively.

RESULTS AND DISCUSSION

When TNS was added to a suspension of 3T3 and SV3T3 harvested from low density cultures the emission intensity increased with time. This effect has been reported by other workers [7,12,24]. The major portion of this increase occurred within 15 min of mixing but some further increase could be detected for at least an hour after mixing. We waited until 2 h after mixing before taking final measurements. It was not possible to take the initial measurements at exactly the same time after mixing, due to differences in mixing efficiencies and thermal convections which led to erratic signals until the sample equilibrated. The initial measurements, then, were taken roughly 2 min after mixing. Table I lists a number of fluorescence parameters which were monitored for TNS bound to 3T3 and SV3T3 cells. The values in this table were taken directly after mixing TNS with cells and 2 h after mixing. The intensities of emission

were similar for 3T3 and SV3T3, suggesting that equal amounts of fluorescent probe were taken up by cells harvested from different growth conditions.

The initial value of the emission maximum was also identical for all the samples tested. This 429 nm maximum indicates that the probe is bound in a non-polar and/or rigid environment [8]. As this value changed with time, some attempt was made to determine how great this change was. By syringing TNS and cells simultaneously into the cuvette, a spectrum of the emission maximum could be scanned forwards and backwards within a minute of mixing. Scanning in both directions was necessary, to assure that the value obtained for the emission maximum was not influenced by the rising signal intensity. A value of 430 nm was obtained, very similar to initial values routinely found. After 2 h the wavelength decreased 3-4 nm (see Fig. 1). This decrease was associated with the binding of about 30% more TNS (see later discussion), indicating that the fluorescence associated with this later bound TNS was at significantly shorter wavelength than that of the early bound TNS. It is not possible to say whether this latter fluorescence emanates from later bound TNS, or earlier bound TNS which has slowly migrated to a different site.

Studies by other workers have indicated that the related probe, ANS, binds to the plasma membrane of intact cells and does not penetrate the cytoplasm [9,12]. As most of the TNS binding occurs within minutes of mixing, it too is probably membrane bound. In addition, we have examined cells with bound TNS, by fluorescence microscopy, and have found no indication that TNS concentrates in any of the organelles. The entire cell appeared luminous, with enhanced intensity at the periphery, consistent with TNS bound to the plasma membrane.

The excitation spectrum of the cells plus TNS, measured at 430 nm, is shown in Fig. 1. It shows a prominent peak near 280 nm which is absent in the spectrum of unbound TNS in non-polar solvent. As tryptophan containing proteins usually

have an absorption maximum near 280 nm, this peak indicates that protein to TNS energy transfer occurs. The critical radius, defined as the donor-acceptor distance where the probability of energy transfer is 50% for energy transfer from protein tryptophan to TNS, is 28 Å [20]. This suggests that proteins containing tryptophan which have TNS bound to them will probably transfer most of their excitation energy to TNS.

To compare the efficiencies of energy transfer in different samples, we have used the ratio of the emission intensity at 285 nm excitation, which is an excitation minimum for unbound TNS to the intensity at the excitation maximum of TNS, near 320 nm. Increases in this ratio, over that for unbound TNS then, represent increases in energy transfer resulting from proximity of TNS and protein, and are useful in determining whether TNS binds to protein. These values are reported in Table I for 3T3 and SV3T3 grown under different conditions, and suggest that equal fractions of protein to TNS energy transfer is occurring in all samples.

Since in this study it is not possible to distinguish the native fluorescence of membrane proteins from that of intracellular proteins, we could not ascertain what fraction of plasma membrane proteins transfer energy to TNS. It is possible, however, to estimate the contribution of transferred excitation to the fluorescence of TNS. Protein tryptophan does not absorb at 320 nm, and so excitation at this wavelength is direct excitation of TNS. Unbound TNS in 90% dioxane gives a fluorescence spectrum similar to that of TNS when bound to cells, and exhibits a ratio of emission intensity on 285 nm excitation in comparison with 320 nm excitation of 0.2 (see Fig. 1). Assuming that the excitation spectrum of bound TNS and TNS in 90% dioxane are similar, then any increase in this ratio is due to energy transfer from tryptophan. This assumption is probably valid since the absorption spectrum of TNS is not very sensitive to

environment [8]. When TNS is bound to intact cells, this ratio is 0.5, indicating that the ratio of transferred excitation to direct excitation at 285 nm is 1.5.

Protein to TNS energy transfer occurs, as has been reported for the related probe ANS, when bound to other membranes. An obvious question arises: is TNS protein bound or simply distributed throughout the membrane so that its average distance from a protein tryptophan is close enough to permit protein to TNS energy transfer, or some combination of the two? In the limiting case, where all of the TNS is protein bound and all of the protein excitation is transferred to TNS, the ratio of transferred excitation to direct excitation at 285 nm will reflect the corresponding absorbances at 285 nm. By protein bound we mean here that the aromatic portion of TNS is in such close proximity to the protein that no other molecules, i.e., solvent or phospholipid, interrupt their common inter-face. This definition distinguishes between electrostatic binding between a protein positive charge and the TNS sulfonate group which could allow greater tryptophan-TNS separation. For one near-limiting case, the binding of TNS to Tobacco Mosaic Virus Protein, this ratio is ca. 3 [20]. Tobacco Mosaic Virus Protein does not have an unusually high number of tryptophans [25] (nor therefore an unusually high tryptophan absorbance). If the plasma membrane proteins of 3T3 and SV3T3 cells do not have unusually low numbers of tryptophans per protein, then one might expect a ratio of at least 3 for transferred to direct 285 nm excitation, if TNS were solely protein bound. A substantial portion and perhaps nearly all of the TNS is not protein bound in the 3T3 and SV3T3 cell membranes, as this 285/320 excitation ratio is only 1.5. Polarization measurements (see subsequent discussion) are in agreement with this conclusion, as are studies of other membranous systems [26-28].

It is possible that random binding of TNS in the phospholipid portions of the membrane might account for the energy transfer which does occur. This con-clusion is based on the following calculations. If the cells are taken to be

spheres with a radius of 10 μ, and the plasma membranes taken to be 85 Å thick, then the volume occupied by one plasma membrane is 1.1×10^{13} Å^3. The amount of protein per 10^7 cells was measured as 1.5 mg, and the cell membrane was taken to contain 5.5% of the total cell protein [29]. If the average protein has a molecular weight of 40,000 [30], the number of protein molecules per cell membrane is 1.1×10^8. If these are randomly distributed throughout the membrane, an average center-to-center interprotein distance of 47 Å is obtained. As there are probably several tryptophans per protein, and the protein size is appreciable in comparison to the interprotein distance, then there is a substantial possibility that a TNS molecule randomly situated in the phospholipid of the membrane will have at least one tryptophan per protein within its critical radius. While this calculation with its many approximations does not prove that TNS is randomly distributed throughout the phospholipid membrane, it indicates that substantial protein to TNS energy transfer may be occurring in the absence of protein-TNS binding. Further evidence will be presented later in this paper.

There was a slight increase in the 285/320 ratio after 2 h. As will be discussed later, this increase may result from some protein-TNS binding. The fraction of transferred energy was similar for 3T3 and SV3T3 cells, both below and at confluency, regardless of when measured.

The polarization of TNS emission, with 320 nm excitation, was also similar for 3T3 and SV3T3 cells below and at confluency. When the viscosity of a solution of a TNS increases, its polarization value increases to a limiting value of 0.4 [8]. TNS, when protein bound in an aqueous solution, has a polarization near 0.25 [20]. When the solvent viscosity is increased, the polarization of protein bound probes also increases. As membrane viscosity is much greater than that of water [15], we would expect that TNS bound to protein in the membrane would have an emission polarization approaching the limiting value of 0.4. The observed polarization of 0.25 would be consistent with that of TNS in a viscous medium, but not with that

of TNS bound to protein in a viscous medium. The excitation spectral data also indicated that TNS was not extensively protein bound. The possibility of TNS to TNS energy transfer in the membrane, which would lead to depolarization, will be considered later.

The polarization 2 h after mixing was slightly higher than immediately after mixing. This increase in polarization was only 0.02, but it did parallel the increased energy transfer observed 2 h after mixing. Both results are consistent with a somewhat greater fraction of protein bound TNS in the later binding sites. In fact, since about 30% more TNS is bound 2 h after mixing (see subsequent discussion), and the limiting polarization of TNS is 0.4, about 1/3 of the later bound TNS would have to be protein bound to produce an increase in 0.02 in the polarization. However, some of this increase might result from binding to more viscous portions of the membrane, not binding to protein.

The emission polarization of TNS bound to 3T3 and SV3T3 cells is much lower using 285 nm excitation instead of 320 nm excitation. The polarization on 285 nm excitation of 0.08 is 70% less than with 320 nm excitation. In the absence of any protein to TNS energy transfer, polarization of TNS using 285 nm excitation is ca. 25% less than that obtained with 320 nm excitation [8]. This suggests that the remaining 45% of the decrease is ascribable to protein to TNS energy transfer. If it is correct that each energy transfer leads to complete depolarization, then the ratio of transferred to direct excitation equals the decrease in polarization due to energy transfer (45%) divided by the actual polarization at 285 nm as a percentage of the 320 nm polarization (30%). The value obtained is 1.5. The same value was obtained using the data from the excitation spectra as calculated above.

In several experiments a sample of cells plus TNS was centrifuged immediately after mixing and another sample was centrifuged 2 h after mixing. The amount of TNS taken up by the cells was calculated by the decrease in the absorbance of TNS

at 318 nm. A blank with no TNS was also run, to assure that no cellular material contributed to the absorbance at 318 nm. Of the initial 2.5×10^{-5} M TNS, $6 \pm 1\%$ was immediately taken up by the cells and another $2 \pm 1\%$ was taken up after 2 h. No differences between 3T3 and SV3T3 cells could be observed, although these could have been masked by the large experimental error in observing such small changes in absorbance. As the concentration of TNS in 3T3 and SV3T3 cells was similar and the fluorescence intensity of bound TNS was also similar, the quantum yield of TNS bound to 3T3 and SV3T3 cells was similar.

Using the total value of 8% of the TNS absorbed to the cells, a value of 2.4×10^8 molecules of TNS per cell was obtained. This value is 40 times less than that obtained for binding of the related probe ANS, to rabbit pulmonary macrophages [12], when both measurements are normalized to the same probe concentration. It is unlikely that this difference results from the different probes, as we have carried out preliminary experiments using ANS with 3T3 and SV3T3 cells. ANS appeared to bind no more efficiently than TNS and may even have bound less efficiently. The difference in probe binding efficiencies between 3T3 or SV3T3 cells and rabbit pulmonary macrophages may result from plasma membrane differences between them. However, different methods of determining the fraction of bound probe were used in this study and the other. Our method may be of limited accuracy due to the low absorbance difference measured, whereas that employed for the macrophage study depended on an extrapolated value.

A possible complication in interpreting the polarization data may now be considered using the above data. If TNS to TNS energy transfer occurred within the plasma membrane, this would lower the emission polarization, and this decrease would be unrelated to the membrane viscosity. Calculations based on the membrane volume (see above) and the fraction of TNS absorbed by the cells, after initial uptake, yield a concentration of TNS in the plasma membrane of ca. 3×10^{-2} M. Using the initial value of 1.8×10^8 molecules of TNS bound per cell, and a

membrane volume of 1.1×10^{13} $\mathring{A}^3$, an average center-to-center TNS distance of 39 $\mathring{A}$ was calculated. To estimate the efficiency of TNS to TNS energy transfer, the critical radius, R_0, was required. It was calculated from the following expression [31,32]:

$$ R_0 = \sqrt[6]{\frac{(1.66 \times 10^{-33})_\tau \, J_z}{n^2 \times \bar{\nu}_0^{-2}}} $$

where τ is the decay time of the donor fluorescence, $\bar{\nu}_0$ the mean of the peak positions in wave numbers of the donor emission and lowest energy absorption bands, J_z the overlap integral, and n the refractive index. An orientation factor of two-thirds is assumed. J_z was obtained graphically from the emission spectrum of initially bound TNS, and from the absorption spectrum of TNS in 90% dioxane, a solvent in which the emission spectrum of TNS is similar to that of TNS bound to the cells. Using this calculation, $J_z = 2.3 \times 10^9$ $cm^3 \cdot mM^{-2}$. An average value of 7 ns was taken for τ [33], as τ is probably a composite of lifetimes. The value for n was taken as 1.4, which is the refractive index of the viscous hydrocarbon dodecane [34], and $\bar{\nu}_0 = 2.73 \times 10^4$. Some leeway in the accuracy of τ and n can be tolerated, as they appear in a sixth root term. R_0 was calculated to be 16 $\mathring{A}$. The energy transfer efficiency, E, for a TNS-TNS separation of 39 $\mathring{A}$ may then be calculated from the following expression [31,32]:

$$ E = \frac{r^{-6}}{r^{-6} + R_0^{-6}} $$

Where r = 39 $\mathring{A}$, E is <1%. The actual efficiency could be somewhat higher if TNS packed so that it had, on the average, one nearest neighbor at a distance of less than 39 $\mathring{A}$. However, the fraction of bound TNS undergoing depolarization due to energy transfer is probably still small. In addition, the further binding of ca. 30% more TNS, after 2 h, led to an increase in emission polarization, which is opposite to what would be expected if the depolarization due to energy transfer

was occurring. Preliminary studies at different concentrations of TNS and cells also gave similar values of polarization, a result consistent with a lack of TNS to TNS energy transfer.

One experiment was carried out with an uncharged probe, the sulfonamide of TNS, TNSA. This probe bound immediately to 3T3 cells and showed fluorescence properties similar to those of TNS when bound to 3T3 cells. Energy transfer from membrane protein to TNSA, as indicated by the ratio of emission intensities on 285 nm and 320 nm excitation, and the emission polarization using 285 nm excitation, is similar for TNSA and TNS when membrane bound. TNSA, having no charged groups, probably binds to the most hydrophobic portions of the membrane, the phospholipid portion. There is no apparent reason for the uncharged TNSA to bind to membrane proteins. As TNS shows an efficiency as an energy acceptor from protein similar to that of TNSA, this is further evidence that TNS is lipid bound.

It would have been preferable to have performed measurements on plated cells, as the disruptive forces in removing the cells may have overwhelmed small differences between the normal and transformed cells. It is unlikely, however, that simply removing the cells from the plates would affect their phospholipid or protein contents. However, if the membrane is stressed due to adhesive forces with the dish, removal might affect membrane fluidity.

We have carried out preliminary experiments with plated cells. The plastic of the dishes and also that of plastic microscope slides fluoresced strongly, masking the TNS fluorescence. We therefore grew the cells on quartz cuvettes or glass slides, carefully withdrew the medium, and washed the cells with isotonic Tris buffer. The cells grew and exhibited normal morphology on glass or quartz. Pretreatment of the glass or quartz with acid or base seemed to facilitate growth. Fluorescence was measured using front face illumination. The binding of TNS to plated cells was much slower than to suspended cells, but the fluorescence spectra obtained were similar to those of cells in suspension. However, within the time

required to perform the measurements (about an hour for plated cells), the cells "rounded up", and we cannot be certain that these measurements represented cultured cells in their usual morphology.

This study has shown that under the conditions described, using the fluorescence probe TNS, the plasma membranes of a normal and transformed cell are similar in their probe binding efficiencies, kinetics, their ability to transfer energy to TNS and, most significantly, their phospholipid fluidities. It should be noted, however, that the fluorescence properties of TNS reported here are average properties and there are probably areas within the plasma membrane where the fluorescence of TNS differs from the average. This is indicated by the change in fluorescence when additional TNS is taken up by the cells; i.e., 2 h after mixing. With different probes it might be possible to probe specific portions of the membranes where differences between normal and transformed cells could exist.

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TABLE I

FLUORESCENCE OF TNS BOUND TO 3T3 AND SV3T3 CELLS TWO MINUTES AND TWO HOURS AFTER MIXING

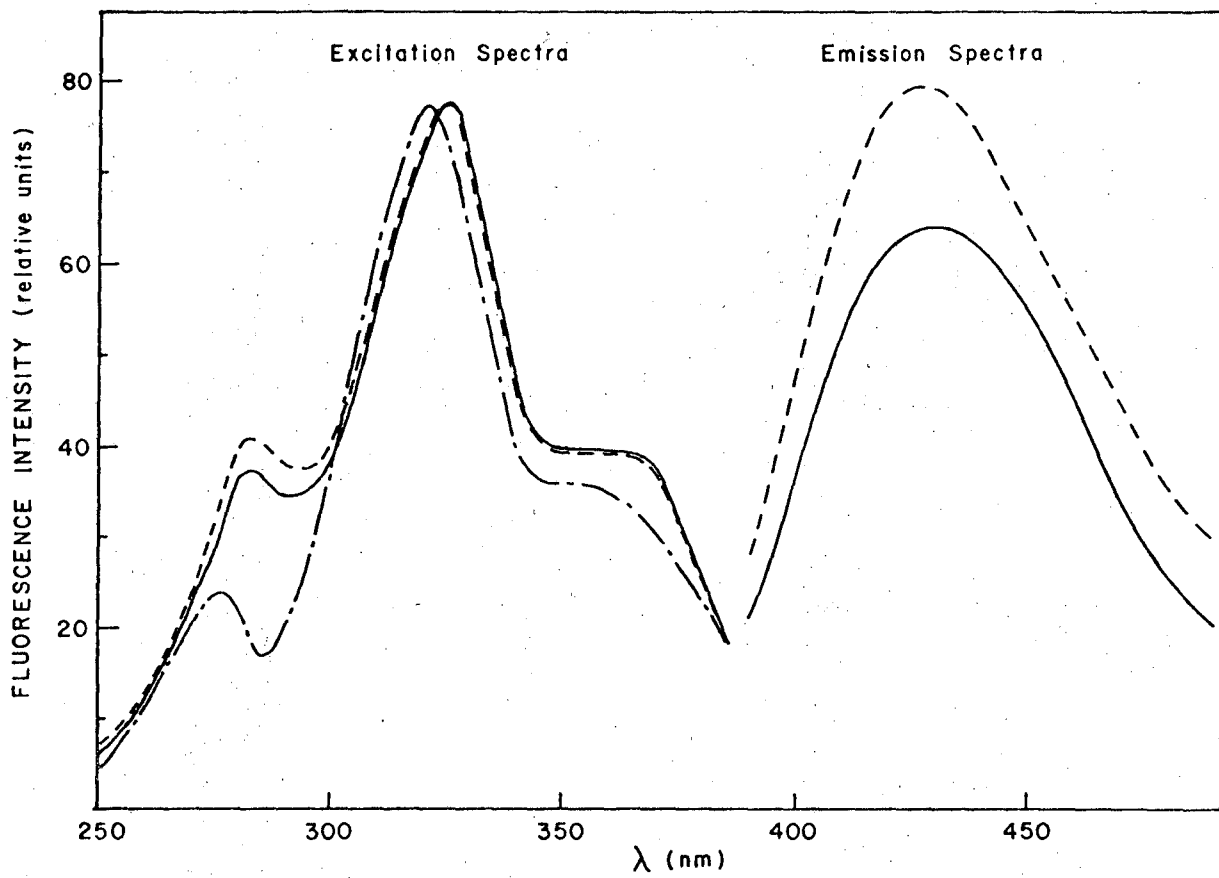The concentration of cells was 5×10^6 /ml, and that of TNS was 2.5×10^{-5} M.

		3T3 subconfluent	3T3 confluent	SV3T3 subconfluent	SV3T3 confluent
Emission maximum[a] ±1 nm	2 min	429	429	429	428
	2 h	427	426	425	425
Maximum emission intensity[a,b] ±10%	2 min	37	43	42	49
	2 h	69	56	62	63
Ratio of emission intensities[a,b] ±0.02 $\frac{285 \text{ excitation}}{320 \text{ excitation}}$	2 min	0.48	0.45	0.49	0.47
	2 h	0.50	0.50	0.55	0.48
Emission polarization ±0.02 320 excitation	2 min	0.24	0.26	0.26	0.27
	2 h	0.27	0.27	0.28	0.29
Emission polarization ±0.01 285 excitation	2 min	0.07	0.08	0.08	0.07
	2 h	0.07	0.08	0.08	0.07

[a] 320 nm excitation

[b] Arbitrary units

FIGURE CAPTION

Fig. 1. Emission and excitation spectra of TNS bound to SV3T3 cells. Several minutes after mixing, ——— ; 2 h after mixing, --------. Also shown is the excitation spectrum of TNS in 90% dioxane, — — —. The intensities of the excitation spectra have been normalized at their maxima, near 320 nm. The concentration of TNS was 2.5×10^{-5} M and the concentration of cells was 5×10^{6} per ml. Emission spectra were obtained using 320 nm excitation and excitation spectra were measured at 430 nm.

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Fig. 1