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# TERTIARY AND QUATERNARY STRUCTURE OF TOBACCO MOSAIC VIRUS AND PROTEIN. II. EMISSION, EXCITATION, POLARIZATION AND POSITION OF TNS BINDING

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# TERTIARY AND QUATERNARY STRUCTURE OF TOBACCO MOSAIC VIRUS AND PROTEIN. II. EMISSION, EXCITATION, POLARIZATION AND POSITION OF TNS BINDING

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SUMMARY

The emission, excitation and polarization spectra of tobacco mosaic virus protein, TMVP, were identical for the aggregated protein, Agg Pro, and disaggregated protein, A Pro, with the exception that the quantum yield of fluorescence decreased 25% on aggregation. This decrease in the absence of other spectral changes suggests a quenching interaction between adjacent subunits. The aggregation apparently produces, at most, minor conformational changes within subunits. The polarization of fluorescence is low but does not decrease on aggregation, indicating that energy migrates efficiently within subunits but not between subunits. Intersubunit space must be large, relative to intrasubunit space.

Fluorescence from the tyrosine residues in TMVP is not observable, and evidence from the excitation spectrum of TMVP is given which suggests that tyrosine energy is efficiently transferred to the tryptophanes in TMVP. These results and the low polarization indicate that TMVP subunits are rather compact.

2-p-Toluidinylnaphthalene-6-sulfonate, TNS, a hydrophobic probe, bound more strongly to Agg Pro than to A Pro. This was shown to be

due to the charge change associated with the aggregation as TNS sulfonamide, TNSA, a neutral probe, bound with equal efficiency to A Pro and Agg Pro. As exposure of the intersubunit regions led to no increase in hydrophobic binding of TNS or TNSA, the hydrophobicity of the intersubunit surface is weak.

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The secondary and tertiary structure of a number of proteins has been well characterized by X-ray diffraction studies.[1,2,3] Hydrogen bonding between suitably situated peptide linkages plays a dominant role in determining secondary structure of proteins, whereas hydrophobic bonding predominates in the determination of tertiary structure.

The nature of quaternary structure, however, is less clear, as only limited data from X-ray diffraction studies has been brought to bear on the problem. In the absence of such X-ray data, chemical and physical methods of investigation have been used to obtain information about quaternary structure.

Tobacco mosaic virus, TMV, and tobacco mosaic virus protein, TMVP, have proved to be a useful system for such a study because the individual protein subunits are relatively small and have been sequenced[4,5] and the conditions leading to their aggregation and disaggregation have been well investigated.[7,8,9,10]

It has been shown that in solutions of TMVP in the mg/ml concentration range at pH 7, 20°C, and ionic strength $\sim$0.1, the trimer is the most stable species. On lowering the pH to 6.5, aggregation occurs and one proton per subunit is taken up by the protein.[8] Water is released

upon aggregation, and it is believed that the free energy decrease for aggregation is derived largely from the entropy increase associated with this release of water.[8]

It has been suggested, then, that hydrophobic bonding provides the major interaction between subunits.[8] Other types of bonding have also been implicated. Evidence from hydrogen exchange experiments indicates that a substantial portion of peptide hydrogens are involved in intersubunit bonding in TMV and TMVP.[11] There is also evidence that the tyrosyl groups play a role in intersubunit bonding.[12] A number of studies have implicated electrostatic bonding.[8,9,14,15,16] In particular, the pH induced aggregation of TMVP around pH 6.5 may involve the formation of an ionic bond between adjacent subunits.

Both carboxyl[9,15] and amino groups[16] have been suggested as titrating near neutral pH in TMVP. As there are no histidine groups in TMVP, an amino acid residue which titrates near neutral pH must be in an unusual environment. This group has been ascribed to a carboxylate anion, either buried in a region of low dielectric constant[15] or hydrogen bonded to a carboxyl group.[9] Another alternative is that an ε-amino group of lysine is stabilized in its neutral state in a hydrogen bonded complex to a tyrosyl group.[16]

The purpose of the present work is to obtain some information about the quaternary structure of TMVP and TMV using fluorescence spectroscopy of TMV and TMVP and several of their dye conjugates.

## Materials and Methods

Virus and protein. Preparation of TMVP from TMV has been described

in the previous paper.[6] The monomer of TMVP is formed spontaneously at 4°C in 0.1 mg/ml solutions of TMVP at or above pH 6.5.[17] Protein solutions at pH > 7 were buffered in 0.1 μ Tris/HCl/KCl and those at pH < 7 were buffered in 0.1 μ phosphate or acetate buffers. Protein solutions were stored cold at pH 7-8, filtered through 0.3 μ millipore filters and diluted when needed.

Reagents. 2-p-toluidinylnaphthalene-6-sulfonate, TNS, was previously described.[6] The sulfonamide of TNS, TNSA, was prepared by addition of a solution of TNS sulfonyl chloride (kindly supplied by Dr. E. Holler) in acetone to ammonium hydroxide. TNSA was precipitated by addition of ice and was twice recrystallized from aqueous ethanol, m.p. 211-213°. Other reagents, auramine O (Allied Chemicals), N-acetyl-L-tryptophan amide, N-acetyl-L-tyrosine amide, N-acetyl-L-phenylalanine ethyl ester (all Cyclo) and 3-methylindole (Aldrich) were used as received.

Stock solutions of 1-2.5 x $10^{-3}$ M TNS and auramine O were prepared by weight and later diluted to desired concentrations. Ultracentrifuge pictures of 3 mg/ml TMVP at pH 7 in the presence or absence of $10^{-4}$ M TNS were identical. TNSA was nearly insoluble in water and was therefore dissolved in ethanol to yield a solution of $\sim 10^{-3}$ M. Aliquots of this solution were then diluted 1/100 with a $10^{-2}$ M NaCl solution in water. This solution was then passed through a 0.3 μ millipore filter and the resulting solution (or fine suspension) was diluted 1:1 with the protein solution to be analyzed. Reproducible results were obtained in this manner and enhancement of the fluorescence of TNSA was of the order of 10X. The spectrum of the millipore filtrate showed a very weak absorption at 320 nm, indicating a concentration of TNSA of $\sim 10^{-6}$ M based on $\varepsilon$ of TNS in water of 1.93 x $10^4$ $M^{-1}$ $cm^{-1}$.

Instrumental. The fluorimeter has been described in the previous paper.[6] For measurements of fluorescence of TMVP or TMV, the fluorescence intensity was so great relative to scattered excitation light that filters were not needed to absorb scattered excitation light. Narrow slit widths, entrance and exit slit widths < 4 nm, were employed. The fluorescence of TMV and TMVP decreased slowly with increasing irradiation time and the narrow excitation slit moderated this effect.

In measurements where fluorescence of the protein was quenched or where emission from a dye was monitored, wider slit widths, exit annd entrance = 6 nm, were used, and the fluorescence light was passed through a suitable cutoff filter before it entered the analyzer. In cases where emission from a dye conjugated to a protein was weak, the excitation beam was passed through a Corning 7-54 filter. In polarization measurements of TMVP and TMV, using excitation light below 270 nm, interference filters corresponding to the excitation wavelength were placed in the excitation beam as the polarizer transmitted substantially more light at longer wavelengths (where fluorescence was measured) than at the shorter wavelengths of excitation, and the excitation light was contaminated by higher wavelength light. The spectra presented here are uncorrected for instrumental properties unless otherwise indicated.

In measurements of the fluorescence spectra of TMVP and TMVP-dye conjugates, it was necessary to subtract a background fluorescence which originated from a contaminant or an unusually complexed or ionized group in the protein. This background was weak in intensity with a maximum around 400 nm and a broad excitation maximum in the range 320-360 nm. All virus and protein preparations showed this emission, which was more

intense in older preparations. Dialysis or differential ultracentrifugation failed to substantially reduce the intensity of this emission. A curious observation was that reconstituted TMV showed a weaker background than did a mixture of components before reconstitution. A background from unbound dye was also subtracted from the spectrum of the conjugate. The dye background was of the order of several percent.

The optical density of solutions used for binding studies or excitation spectra was kept below 0.05 at the excitation wavelength, either by appropriate dilution and/or use of microcells of 1 x 3 or 3 x 3 nm pathlengthhs. A linear relationship between intensity of fluorescence and absorbance was thus assured.

Polarization of fluorescence, P, was measured with a Perkin Elmer polarization attachment with linearly polarized excitation light. P was calculated from the expression:[18]

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

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.

The fluorescence quantum yield of TMVP was calculated relative to indole in ethanol, which has a corrected quantum yield of 0.42.[19] Both TMVP and indole in ethanol were excited with light of 290 nm, where the tryptophane groups in TMVP absorb ~95% of the incident radiation. Both solutions had identical optical densities. As the fluorescence spectra of the two solutions were very similar, no correction for instrumental

response was applied. The quantum yields of the TNS-TMVP conjugates were similarly determined, except TNS in 90% dioxane was used as a secondary standard (see Results section). Even at high concentrations of protein, i.e., 12 mg/ml, only ~50% of the TNS was bound, so that values of $\emptyset_{TNS-TMVP}$ had to be extrapolated to infinite protein concentration from plots of 1/[TMVP] vs. 1/Intensity.

In calculating the overlap integral, $J_\nu$, which was used to calculate the critical distance for energy transfer between TMVP and TNS, the absorption spectrum of TNS was replotted in terms of wave number and the fluorescence spectrum of TMVP was replotted to display the curve as relative quanta per wave number, $dQ/d_\nu$.[20] It was, however, first necessary to correct the fluorescence spectrum of TMVP for instrumental response. This correction was small and was most easily accomplished by normalization of the spectra of standard substances obtained on our fluorimeter, such as indole, to published corrected spectra.[19] The normalization factors were then applied to the spectrum of TMVP measured under the same instrumental conditions.

Unless otherwise specified, all measurements were performed at 20°C in 0.1 μ buffer. The presence of buffers at the concentrations employed in this study did not alter the fluorescence spectra. The order of addition of reagents also had no effect on the fluorescence spectra. Measurements were then repeated at least once to reproducibility, as occasionally irreproducible effects were obtained. Older preparations tended to give slightly different intensities of fluorescence of TMVP, TMV, and their dye conjugates than did fresh preparations. Preparations less than one month old were used. Variations in binding constants of dyes to TMVP and TMV

from different preparations were also observed, although relative values were fairly constant.

Determination of concentrations of TMV and TMVP has been described.[6] The fractional absorption of each of the aromatic acid residues of TMVP was estimated from the absorption spectrum of N-acetyl-L-phenylalanine ethyl ester, N-acetyl-L-tryptophanamide, and N-acetyl-L-tyrosine amide.

## Results

Fluorescence of TMVP and TMV. The fluorescence spectra of TMV at pH 7, TMVP at pH 7, TMVP at pH 6, and monomeric TMV are shown in Fig. 1A. TMVP at pH 7, 20°C, is composed of mixture of monomer, trimer and a small contribution of higher aggregates.[8] This mixture, in which the trimer is the predominant species is generally referred to as A Protein, A Pro. Each protein subunit contains three tryptophane residues, four tyrosyl residues, and eight phenylalanine residues,[8] all of which show fluorescence as the free amino acids. In proteins containing tryptophane, however, fluorescence from phenylalanine is not observed,[21,22,23] and fluorescence from tyrosine, if observed, is manifested by a weak short wavelength emission superimposed on the major emission, which originates from the tryptophane residues.[21,22,23,24] The wavelength of maximum intensity of the fluorescence, $\lambda_{max,p}$, of TMVP, 320 nm, is somewhat shorter than of most tryptophane containing proteins,[23,24] which generally fall in the range of 328-342 nm. As longer wavelength fluorescence from tryptophane is correlated with increased polarity of the environment around the indole ring,[21,22] the tryptophane residues (at least those which emit) are probably in a less polar environment in TMVP than in most other proteins examined. This

interpretation is best taken only as an indication of exposure, as exceptions are known.[24]

A spectrum of 3-methylindole in 2:1 v/v cyclohexane/dioxane is included in Fig. 1 for comparison. As it contains the same fluorophore as TMVP and a very similar spectrum and bandwidth, it appears that the fluorescence of TMVP is composed mainly of emission from one tryptophane, although a composite of two or three tryptophanes emitting at similar $\lambda_{max,p}$ is also possible.

The spectrum of TMVP at pH 6 corresponds to that of the aggregated protein,[8] Agg Pro. It is about 75% as intense as that of A Pro (which is present at pH 7). A Pro has a quantum yield of 0.23. When the two spectra are normalized at 360 nm they are coincident at all wavelengths. They are also coincident when excited at other wavelengths. In a polypeptide chain with environmental sensitive fluorophores and a number of possible participants in an energy transfer chain it would not be likely that a change in conformation of the chain, or environment of the tryptophanes, could occur without some concomitant changes in the shape of the fluorescence spectrum. As no such changes occur here, we think that decreased intensity of Agg Pro relative to A Pro results from a quenching interaction, most likely between tryptophanes in adjacent subunits, which is made possible by aggregation. It is known that the aggregation of TMVP leads to inaccessibility of two tryptophanes per subunit towards N-bromosuccinimide oxidation.[12] These tryptophanes are presumably situated in regions of interaction between subunits and might be involved in this fluorescence quenching. No such decrease in intensity of fluorescence is observed in the same pH increment with TMV.[6] The decrease in intensity, then, is probably associated with the aggregation of the protein.

The fluorescence spectrum of TMV is identical to that of Agg Pro if one assumes that light absorbed by TMV-RNA is radiationlessly degraded. As will be shown subsequently in this paper, this assumption is justified. The coincidence of the fluorescence of TMV and Agg Pro is consistent with X-ray diffraction[25] and electron microscopic[8] data that Agg Pro and TMV are very similar in structure.

The fluorescence spectrum of monomeric TMVP cannot be measured under the same conditions as those given in Fig. 1 (see Experimental section). However, when the fluorescence spectra of Agg Pro and monomeric proteiin are measured under identical conditions, the two spectra are coincident at all wavelengths when normalized at 360 nm. The ratio of intensities of Agg Pro to monomeric protein is the same as that for Agg Pro to A Pro, indicating that the fluorescence spectrum of the monomer is identical to that of A Pro.

The spectra of TMVP obtained with 275 and 295 nm excitation are also shown in Fig. 1B. In some proteins a noticeable difference in the fluorescence spectra is observed when the excitation wavelength is changed.[21,24] This difference is attributed to a contribution to the fluorescence spectrum by tyrosine, present at 275 excitation but absent at 295 nm excitation. Using model compounds to estimate the fractional absorption of tyrosine, we estimate that at 275 nm the tyrosine groups absorb 34% of the incident light but <5% at 295 nm. The coincidence of the fluorescence spectra of TMVP on 275 and 295 nm excitation show that the contribution from tyrosine fluorescence to that of TMVP is negligible. Apparently very efficient energy transfer from and/or quenching of tyrosine occurs in Agg Pro and A Pro. As intertyrosyl and tyrosyl-tryptophane energy transfer are dependent

on the distances between the energy donor and acceptor,[21] it appears that these distances are similar in A Pro and Agg Pro.

Excitation Spectra. The excitation spectra of TMV, A Pro, Agg Pro, and monomer are shown in Fig. 2. Like the fluorescence spectra, these spectra are coincident at all wavelengths when normalized at one wavelength. As the excitation spectra of TMV and Agg Pro were coincident, energy transfer from RNA to the protein coat in TMV does not occur, although the RNA absorbs a significant portion of the incident radiation.[26] The coincidence of all the excitation spectra again indicates conformational similarity between the subunits in A Pro, Agg Pro, monomeric Pro and TMV. The excitation spectra of A Pro and Agg Pro for emission at 310 and 360 nm were also coincident, further indicating that fluorescence of TMVP is not a composite of emissions.

Also shown in Fig. 2A is the excitation spectrum of 3-methylindole in 2:1 v/v cyclohexane/dioxane. The spectrum has been normalized to that of A Pro above 292 nm, where tyrosyl absorption in A Pro is negligible. It is seen that A Pro possesses a higher fluorescence intensity than 3-methylindole, when excited in the range where tyrosine absorbs significantly, <290 nm. At 275 nm, for instance, the fluorescence intensity of A Pro is 16% higher than that of 3-methylindole. As tyrosine absorbs ~35% of the incident light at 275 nm, energy transfer from tyrosine to tryptophane is ~50% efficient. This is somewhat more efficient than in most other proteins examined[21] and might, together with the absence of tyrosine emission in A Pro, be taken as an indication of a relatively close proximity of tyrosyl and tryptophanyl groups within a subunit of TMVP. Some caution in this conclusion is necessary, however, since the ratio

and number of tyrosines and tryptophanes in a particular protein could also affect its efficiency of energy transfer.

Fluorescence polarization. The fluorescence absorption polarization spectra of TMVP is shown in Fig. 2A. The spectra of A Pro, Agg Pro and monomeric protein were coincident as was that of TMV. The polarization of TMVP is considerably less than that of tryptophane in a viscous medium.[22] As no increase in polarization of TMVP and TMV was observed on transfer from water to a viscous medium, 60% sucrose, depolarization due to molecular rotation is not the cause of the low observed polarization. Energy migration has been shown to play a major role in depolarization of fluorescence.[22] The polarization of TMVP and TMV is quite low, even for proteins[22] and indicates a large degree of energy migration within each subunit. With the exceptions of tyrosines 70 and 72, all of the possible partners in the energy migration chain (tyrosine → tyrosine, tyrosine → tryptophane, tryptophane → tryptophane) are separated by at least twelve residues. As the critical radii for energy transfer between the components are 8-20 Å,[22] it is apparent that energy migration is possible because of the three-dimensional folding of the protein and should be a sensitive indicator of conformational differences between subunits in the different aggregates of TMVP and TMV. The similarity of the polarization spectra of all the aggregates is taken then as another indication that the subunits in the different aggregates of TMVP and TMV are nearly identical in conformation. The identity of the polarization of TMV and TMVP indicates again that energy transfer from RNA to coat protein does not occur in TMV. A significant result is that energy transfer does not occur between subunits, as the polarization of Agg Pro or TMV is not less than that of A Pro.

This finding might seem at variance with the earlier discussed indication that quenching between tryptophane groups in adjacent subunits may occur. It has, however, been demonstrated for tryptophane that energy migration between tryptophanes occurs in rigid medium, but quenching occurs in more fluid medium.[22] The region between subunits may be large, allowing some motion of pendant tryptophane residues in this region.

Binding of dyes to TMV and TMVP. The binding of TNS to TMV and TMVP has been used to monitor changes in aggregation, conformation and charge of TMV and TMVP.[6] The binding of dyes to proteins is believed to result from a combination of hydrophobic, electrostatic and hydrogen bonding interactions between dye and protein.[27,28] The wavelength of maximum intensity of emission, $\lambda_{max}$, of TNS is responsive to the polarity of its environment. Dyes such as TNS are weakly fluorescent in water but strongly fluorescent when bound to proteins.[28] The fluorescence spectra of the TNS-TMVP and TNS-TMV conjugates are shown in Fig. 3. The wavelength of maximum emission from the TNS-TMVP complexes, $\lambda_{max,T}$, were similar, 423 for A Pro and 426 for Agg Pro, while that for TMV was at 437 nm. The dissociation constants of the TNS conjugates were determined from Benesi-Hildebrand Plots[29] and are given in Table I along with the extrapolated quantum yields for the conjugates. All concentrations of TMVP and TMV are expressed in moles/liter of subunit. At a particular pH, TNS always bound more strongly to TMVP than to TMV. The stoichiometry of the complexes was also determined[30] and in all cases was 1:1, indicating that new binding sites for TNS were not exposed on disaggregation or that these sites bound TNS only weakly. The quantum yields were determined relative to TNS in 90% dioxane which has $\phi = 0.29$ and $\lambda_{max,T} = 434$ nm,[31]

similar to that for the TNS-TMVP and TMV complexes.

As the pH induced aggregation of TMVP is accompanied by a net increase in positive charge on the protein,[8] it was of interest to determine whether the smaller dissociation constant of TNS to Agg Pro than to A Pro was the result of electrostatic or hydrophobic bonding. For this purpose, an uncharged derivative of TNS was prepared, 2-p-toluidinyl-naphthalene-6-sulfonamide, TNSA. TNSA showed significant fluorescence enhancement, ~10X, when added to 5 mg/ml A Pro or Agg Pro. It bound to both A Pro and Agg Pro with equal values of $\lambda_{max}$ of 433 nm and equal efficiency, $K_d = 3 \times 10^{-5}$ M. Apparently A Pro and Agg Pro are equally hydrophobic towards TNSA. The fact that TNSA binds more strongly to TMVP than TNS does probably reflects the more unfavorable interaction of the uncharged TNSA with water. An attempt was made to determine $K_d$ for TMV-TNSA complexes. The fluorescence enhancement was so low, ~2X, that estimates of $K_d$ were not possible. Presumably the binding of TNSA to TMV was weaker than to TMVP, as was the case with TNS.

The binding of the positively charged dye, auramine O, bis-(p-dimethylaminophenyl)-methyleneimine hydrochloride was also investigated. This dye showed no affinity for TMVP over TMV as did TNS and TNSA, indicating perhaps that auramine O binds to a different site than does TNS or TNSA. This site is presumably favorable to positively charged ligands. In fact, at pH 7, auramine O is bound more strongly to TMV than to TMVP, perhaps reflecting the greater negative charge of TMV due to its nucleic acid. Auramine O bound with equal efficiency to A and Agg Pro, and in all cases with 1:1 stoichiometry.

Energy transfer. The excitation spectra of the TNS-TMVP conjugates,

shown in Fig. 4, show a large peak at 282 nm which is not nearly as prominent in the excitation spectrum of unconjugated TNS, which is also shown in Fig. 4. The excitation spectrum of TNS taken for comparison is that of TNS in 90% dioxane, which has $\lambda_{max,T}$ = 434 nm, similar to that of TNS-TMVP, indicating an environment around TNS of similar polarity in 90% dioxane as when bound to the protein, and probably a similar absorption spectrum to TNS bound to TMVP. At 285 nm excitation (where TNS has a minimum in its absorption spectrum) the fluorescence intensity of the TNS-Agg Pro complex measured at 440 nm is 1.02X that of the excitation maximum at 325 nm, which corresponds to direct excitation of TNS since TMVP does not absorb at 325 nm. The corresponding ratio in the excitation spectrum of TNS in 90% dioxane is only 0.20. In the TNS-Agg Pro conjugate, then, the ratio of fluorescence obtained with direct excitation of TNS at 285 nm, to that obtained by irradiation into the protein absorption at 285 nm, is 0.20/0.82, or at 285 nm excitation, 80% of the fluorescence results from energy transfered from Agg Pro to TNS. At 285 nm, in a 1:1 TNS-TMVP complex, TMVP would absorb 71% of the incident radiation, assuming the absorption spectrum of TNS in 90% dioxane is similar to that of TNS conjugated with TMVP. This is probably a reasonable assumption since the absorption spectrum of TNS is not very sensitive to the environment of TNS.[31] Direct measurement of the absorption spectrum of the TNS-TMVP complex is not possible, as the fraction of bound components is considerably below unity, even at high concentrations of TMVP and TNS. The close correspondence between the fraction of light absorbed by TMVP and the fraction of energy transferred from TMVP to TNS could indicate that energy transfer is nearly 100% efficient in the TNS-TMVP complex,

i.e., every photon absorbed by TMVP is transferred to TNS. Alternatively, it might be thought that a long range energy transfer could extend over several subunits, with non-bound subunits contributing to the fluorescence of TNS. A calculation of $R_0$, the distance at which the probability of energy transfer from TMVP to TNS equals that for spontaneous decay, indicates energy transfer from unbound subunits to TNS is a distinct possibility. $R_0$ was calculated from the equation:[32,33]

$$R_0 = \frac{1.66 \times 10^{-33} \times \tau J_\nu}{n^2 \times \nu_0^2} \qquad (1)$$

where $\tau$ is decay time of donor fluorescence, $\bar{\nu}_0$ the mean of the peak positions in wave numbers, of the donor emission and lowest energy absorption bands of the acceptor, $J_\nu$ the overlap integral and n the refractive index. A value for $\tau$ of 3 nsecs was assumed, as this value is close to that obtained for other proteins with similar quantum yields.[24] $J_\nu$ was obtained graphically (see Experimental section), = $1.4 \times 10^{12}$ $cm^3$ $mM^{-2}$, $\bar{\nu}_0 = 3.13 \times 10^4$ $cm^{-1}$, n was taken as 1.6 and $R_0$ was calculated to be 38 Å. This calculation is only approximate, as an orientation factor of 2/3 is assumed; $\tau$ has not been measured and the refractive index within the subunit is not certain. Also useful for our purposes is $r_{95\%}$, the distance at which the efficiency of energy transfer, E, is 95%. This distance was obtained by the equation:[32,33]

$$E = \frac{r^{-6}}{r^{-6} + R_0^{-6}} \qquad (2)$$

$r_{95\%}$ = 24 Å.

A consideration of a schematic model of TMVP, shown in Fig. 6, shows that TNS, if suitably situated, could easily quench the fluorescence of more than one subunit, if $R_0$ = 38 Å and $r_{95\%}$ = 24 Å.

In the TNS-monomeric TMVP conjugate, where there are no neighboring subunits, appreciable energy transfer from TMVP to TNS still occurs, although not as efficiently as from Agg Pro to TNS. If one normalizes at 325 nm (direct excitation of TNS) the excitation spectra of TNS-monomeric TMVP and TNS-Agg Pro, both measured at 440 emission, and compares the ratio of fluorescence efficiencies at 285 nm excitation, for TNS-monomeric TMVP and TNS-Agg Pro, one observes a somewhat greater efficiency for the TNS-Agg Pro conjugate. To quantitatively interpret this difference, the contribution to the 285 excitation from direct absorption by TNS must be subtracted. This contribution is 20% of the intensity of the 325 peak (see preceding discussion). One then obtains a ratio of 1.22, for intensity of fluorescence on 285 excitation, of Agg Pro-TNS to monomeric TMVP-TNS. It is possible, then, that 22% of TNS excitation in Agg Pro-TNS originates from unbound subunits. A similar calculation for A Pro-TNS indicates that 14% of TNS excitation may originate from unbound subunits. Inherent in this type of calculation is the assumption that TNS is bound in a similar location of the subunit of Agg Pro, A Pro and monomer. The similarity of $\lambda_{max,p}$ and $K_d$ of TNS and TNSA for their respective conjugates indicates this may be so. TMV, on the other hand, exhibited much different binding properties to TNS and TNSA. It also showed a lower efficiency of energy transfer to TNS, ~80% that of monomeric TMVP to TNS.

It would be desirable also to have some direct quenching measurements of TMVP fluorescence. However, because of the relatively weak binding constants of TMVP and TMV to TNS, rather large concentrations of TNS are needed to quench a substantial portion of TMVP fluorescence. At these large concentrations, i.e., $5 \times 10^{-4}$ M, corrections for screening by unbound TNS

and TMVP, reabsorption by TNS and non-linearity of fluorescence with concentration of TNS-TMVP become too great to allow accurate determination of quenching. Rough calculations made at $2.5 \times 10^{-5}$ M TNS and TMVP indicated that binding of ∿5% of the subunits led to ∿8% quenching of Agg Pro fluorescence, and binding of ∿3% of the subunits led to 4% quenching of A Pro fluorescence. The agreement of the quenching values with those obtained from the excitation spectra is as good as can be expected, considering the large percentage errors at such low fractions of binding and quenching.

Energy transfer from TMVP to TNS via diffusion of TNS and TMVP together during the lifetime of excited TMVP is precluded, even for a long range energy transfer because the concentration of TNS is so low, $2.5 \times 10^{-5}$ M, that the probability of collision within the normal lifetime of an excited protein is negligible. It has been shown[34,35] that concentrations of acceptor in the $10^{-2}$ to $10^{-3}$ M range are required to effect substantial quenching of excited singlets which have the same order of lifetime as that for proteins, even when $R_0 > 50$ Å.

If TNS is able to capture every quantum of light absorbed by the subunit to which it is bound, it must do so much more rapidly than the time interval between light absorption and emission, since it must quench not only the fluorescence, but must compete with the more rapid radiationless pathways of decay which compose 4/5 of the energy degradation of excited TMVP. Apparently the tryptophanes in TMVP sit within 24 Å of the TNS, or intertryptophane energy migration to a particular tryptophane occurs with nearly 100% efficiency, and this tryptophane sits within 24 Å of TNS.

On further examination of Fig. 6 it is somewhat surprising that TNS can so efficiently scavenge energy from the subunit to which it is bound, but can only capture energy rather inefficiently from neighboring subunits when it is bound to Agg Pro or A Pro. In view of the large value of $R_0$ and the size and shape of an individual subunit, which is believed to be ellipsoid of revolution with major and minor semiaxes of 35 and 12.25 Å, resp.,[8,25] TNS is most likely bound in a crevice within the subunit, near one of the ends of the major semiaxis of the ellipsoid so that overlap of the TNS quenching sphere with adjacent subunits is at a minimum. The volume of one subunit equals $5.9 \times 10^4$ Å$^3$ and is nearly identical to that of the quenching sphere of TNS which equals $5.8 \times 10^4$ Å$^3$. It may also be seen that if TNS is bound near the long axis of a subunit its ability to quench adjacent subunits would be substantially reduced if the intersubunit region is sparsely packed. Alternative possibilities are that random orientation of TNS and the tryptophanes is not achieved and $R_0$ is actually smaller than calculated, or orientation of TNS is unfavorable with that of the tryptophanes in adjacent subunits.

Fluorescence polarization of TNS. The fluorescence absorption polarization spectrum of TNS-TMVP shown in Fig. 4 is in agreement with that expected for a large percent energy transfer from TMVP to TNS. The ratio of fluorescence polarization (measured at 440 nm), at 285 excitation to that obtained with 325 excitation is ∿0.1 in TMVP-TNS compared with 0.60 for the corresponding ratio of TNS in glycerol, indicating that ∿83% of the TNS-TMVP fluorescence obtained by 285 excitation originates from TMVP (assuming every transfer leads to depolarization of resulting emission).

## Discussion

Tertiary structure of TMVP subunits and some relationships to quaternary structure. One of the most striking features of this study is the great similarity of the spectra of monomeric TMVP, A Pro, Agg Pro, and TMV. The similarities are observed in the fluorescence, excitation, and polarization spectra of TMV and the various allosteric forms of the protein, and also in the spectra of many of their dye conjugates. Apparently the conformational integrity of the subunits is quite strictly preserved in TMVP and TMV on aggregation and disaggregation. While it might be thought that fluorescence techniques are insensitive to conformational changes of proteins, numerous studies have shown that this is not the case,[21,22,23,24] and our preceding paper has shown that for TMVP and TMV, in particular, fluorescence changes parallel conformational changes.[6] One of the results which emerged from that study and others[8,12,13] was that extremes of pH were necessary to alter the tertiary structure of the subunits, whereas only one group need be titrated to alter quaternary structure.

There are other indications discussed later that the bonding within subunits is stronger than that between subunits, and the corollary that alteration of quaternary structure need not affect secondary and tertiary structure, as indicated in the first paragraph of the Discussion.

High efficiency of energy migration within a subunit, as indicated by the low fluorescence polarization of TMVP and TMV, the efficient energy transfer from tyrosine to tryptophane, and perhaps the failure to observe tyrosine emission and the highly efficient energy transfer from TMVP to TNS, result from proximity of groups in the energy migration chain. As

discussed earlier in this work, such proximity must result from three-dimensional folding of the polypeptide chain. The lack of energy migration between adjacent subunits, as indicated by the lack of further depolarization of fluorescence on aggregation, and the rather inefficient energy transfer from adjacent subunits to TNS, indicates that the distance between the members of the energy migration chain in adjacent subunits is greater than that within subunits, and/or the intersubunit energy migration chain has ruptured link, i.e., a fluid bond at which point quenching instead of transfer occurs. Apparently no interchain folding occurs, and the intersubunit space is very sparse in protein material. This model seems to be in agreement with pictures obtained by electron microscopy of the closely related aggregates of TMVP, double disks, and stacked double disks.[36,37]

The intrasubunit fluorescence properties of TMVP and TMV are revealing when compared with those of other proteins. TMVP monomer, A Pro, Agg Pro, and TMV show a relatively short wavelength fluorescence, indicating that the fluorescing tryptophane(s) is quite unavailable for efficient solvation.[21] The polarization of fluorescence of TMVP and TMV is lower than most proteins in solution which have been investigated,[22] as is the contribution of tyrosine fluorescence to the total fluorescence.[21] The contribution of energy transfer from tyrosines is somewhat high.[21] All of these results taken together suggest that TMVP subunits may be more compact and tightly bound internally than most proteins. As TMVP is somewhat unique in its ability to rapidly aggregate and disaggregate, it is tempting to correlate this functional property of the protein with its physical and chemical properties. A compact, relatively inflexible subunit would probably represent an efficient building block in a rapidly aggregating or disaggregating system, as the free energy change necessary to drive

the aggregation or disaggregation could then be totally manifested in the formation and breakage of intersubunit bonds and not be needlessly expended in the formation and breakage of intrasubunit bonds. This model implies that the intersubunit bonds are much weaker than the intrasubunit bonds, otherwise the formation or breakage of bonds on the surface of the subunit and the packing or unpacking of the subunits into a large lattice would tend to distort the structure and organization in the interior of the subunit.

Nature of intersubunit bonds. A significant result in this study was the failure to detect any increase in hydrophobic bonding sites, either in number or hydrophobicity, by the use of hydrophobic dye probes. The transition from Agg Pro to A Pro to monomeric Pro results in successive exposure of all intersubunit binding sites with no concomitant increase in hydrophobic binding observed. In sharp contrast to this behavior is the effect, on binding of TNS, of exposure of the binding sites which determine the tertiary structure of TMVP. When these sites are successively exposed by raising or lowering pH or heating TMVP,[6] a sharp increase in binding of TNS is observed, corresponding to the degree of exposure of the hydrophobic binding sites. Similar behavior has been observed with other proteins.[38,39] Hydrophobic bonding is known to be a major determinant of tertiary structure[1,2] and exposure of tertiary binding sites results in the binding of hydrophobic dyes. The question is, then, is the converse true for quaternary structure? Does the failure to detect any increase in hydrophobic binding of dyes on exposure of quaternary binding sites indicate that hydrophobic bonding is not a major determinant of quaternary structure of TMVP. We think not, for the following reason. Hydrophobic dyes may detect new binding sites, but only

if the new binding sites have a similar or greater binding power than those already present. Thus, TNS, TNSA and auramine O may not bind to intersubunit regions because there is another region on the subunit surface of greater binding power. Studies of excitation transfer from TMVP to TNS (discussed earlier) have also indicated that TNS is probably bound near one end of the major semiaxis of the subunit and not between subunits or along the sides of the subunits.

We envision the surface of each subunit, then, as being rather homogenous in its hydrophobicity, with the exception of the binding site for TNS, and rather weak in its hydrophobic binding power, as none of the sites exposed on disaggregation can compete effectively for TNS already bound, and the binding of TNS by TMVP is itself not strong compared with the binding of dyes by other proteins.[28] As proteins fold with their most polar groups on the exterior, it is not surprising that hydrophobic forces at the surface of a protein are relatively weak compared with those on the interior, as the pH study cited above indicates.

A relatively weak and uniform hydrophobic bonding surface would allow each subunit to maximize other types of bonding interactions with another subunit, while maintaining a relatively constant hydrophobic environment. Other types of bonding interactions might include hydrogen bonding, and ionic bonding. Hydrogen exchange studies of Fraenkel-Conrat and Budzynski[11] have indicated that about 30% of the peptide hydrogens of TMVP subunits of TMV and Agg Pro are engaged in intersubunit hydrogen bonding. Ionic bonding is also indicated, as the aggregation of subunits is dependent on pH,[8] and presumably the charge on particular groups in

the subunits. Both hydrogen and ionic bonding between subunits would be favored by a non-polar environment, so that the solvent would not compete for the binding sites. Such a model of intersubunit bonding is a cooperative one in the sense that one type of bonding cannot occur in the absence of another. Quaternary structure may not be dominated by any one type of bonding as secondary and tertiary structure are. Such a model then allows for recognition of neighboring subunits, since another subunit in order to bind, would have to contain a complementary distribution of binding sites. It is of interest that TMVP subunits have been prepared with unusually large net positive charge, and such subunits would not bind to each other, but would copolymerize with normal TMVP subunits. They would not, however, copolymerize with other basic proteins such as egg white lysozyme or pancreatic ribonuclease, indicating that TMVP has specific binding sites. If, for instance, hydrophobic bonding was the sole driving force for aggregation, any hydrophobic surface should have sufficed for aggregation.

Identification of the group associated with the pH induced aggregation of TMVP. The dye binding studies have indicated that the cause of enhanced binding of TNS to Agg Pro relative to A Pro is the increase in net positive charge on the Agg Pro. *A priori*, one might think that the increased attraction of the negatively charged TNS for Agg Pro could be the result of increased electrostatic attraction between TNS and a positively charged group in the protein or a decreased electrostatic repulsion between TNS and a negatively charged group in the protein. We feel that it is unlikely that TNS when binding to A Pro would orient itself with its negative charge near another negative charge on the protein. As charged side chains in a protein

are nearly always on the protein surface where they may be solvated, the negative charge on TNS should behave similarly and if the negative charge is attracted towards the protein surface there must be an energetically favorable ground for this attraction, i.e., a complementary positive charge. We therefore favor the suggestion (discussed in the introduction) of Lauffer[16] that the group titrating near pH 7 is a lysine group, hydrogen bonded to a tyrosyl hydroxyl.

Agg Pro and TMV. Although TMV and Agg Pro have identical fluorescence, excitation, and fluorescence polarization spectra, their dye binding properties are different. As the fluorescence from TMV and Agg Pro appears to be mainly that of the individual subunits (see earlier portion of the Discussion), the differences in their dye binding properties apparently result from differences in their quaternary structure. At a given pH, TMV binds TNS or TNSA more weakly than Agg Pro does. From the longer wavelength of the fluorescence of TNS bound to TMV than to Agg Pro, it appears that TNS is in more polar environment when bound to TMV than when bound to Agg Pro. These results might suggest that the subunits are held more tightly in TMV than in Agg Pro, thus allowing greater access of TNS with Agg Pro than TMV. Results from titration studies[14] and hydrogen exchange studies[11] show that TMV titrates less readily and exchanges hydrogens more slowly than Agg Pro. Both results support this explanation. However, it is also possible that TNS binds to Agg Pro (and also A Pro and monomeric TMVP) in the crevice left vacant by RNA. TNS must then bind elsewhere to TMV. This explanation would be in better agreement with the evidence obtained from the energy transfer studies, that TNS binds near

one of the ends of the major axis of the subunit, and not along the side, i.e., in the cracks between subunits. It would also explain the similarities in binding of TNS and TNSA to the various aggregates of the protein, and the much weaker binding to the native virus. It also implies that the positive charge associated with the aggregation is close enough to the binding site of TNS to interact with TNS, as TNS binds more strongly to Agg Pro than to A Pro. The fact that the positively charged auramine O appears to bind to a different site on TMVP than TNS and TNSA do, also supports this suggestion. It is tempting to speculate that one function of TMV RNA in inducing the assembly of TMV from RNA and TMVP subunits is to promote protonation of the (lysine) group associated with the aggregation by stabilization of the resulting positive charge with a phosphate negative charge. It should be noted that TMVP and RNA reconstitute most efficiently near neutral pH,[10] where the A Pro and not the Agg Pro is the more stable aggregate. As TMVP is negatively charged at neutral pH,[14] interaction with the negatively charged RNA would not seem to represent an effective mode for inducing aggregation in the abse ce of any compensating effect. The protonization of lysine could be such a compensating effect, as it has been shown that aggregation of TMVP will not occur if lysine 53 cannot attain a positive charge,[41] and TMV will not reconstitute from TMVP and TMV RNA if lysine 53 is acetylated.[42] The suggestion has indeed been made that lysine 53 is associated with a phosphate negative charge in TMV.[41]

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Figure Captions

Fig. 1A. Fluorescence spectra of: A Pro, Agg Pro, and TMV, 2.0 x $10^{-5}$ M subunit at 20°C. The spectrum of monomeric Pro was taken at 0.5 M subunit at 4°C (see Experimental section). Superimposed on the spectrum of A Pro is that of Agg Pro at higher sensitivity. The spectra of TMV and monomeric Pro are also coincident with that of A Pro when recorded at higher sensitivities. The dashed line indicates the spectrum of 3-methylindole in 2:1 v/v, cyclohexane/dioxane. Excitation light of 285 nm was used for all spectra.

Figure 1B. Fluorescence spectra of A Pro (more intense spectra) employing 275 and 295 nm excitation. The intensities of the spectra have been normalized at 360 nm. The less intense spectra are those of Agg Pro obtained with 275 and 292 nm excitation, normalized at 360 nm. Due to the intense scattered excitation peak by Agg Pro, its spectrum is only recorded above 300 nm using 292 nm excitation instead of the 295 nm excitation used for A Pro. Both A Pro and Agg Pro were 6.2 x $10^{-5}$ M subunit.

Figure 2A. Excitation spectra recorded at 320 nm of: A Pro, Agg Pro, TMV and monomer. The conditions are the same as those given in Fig. 1. The dashed line shows the spectrum of 3-methylindole in 2:1 v/v, cyclohexane/dioxane normalized to that of A Pro above 292 nm. Superimposed on the spectrum of A Pro is that of Agg Pro as higher sensitivity. The spectra of TMV and monomeric TMVP were also coincident at all wavelengths with that of A Pro when normalized at one wavelength. The polarization of fluorescence, recorded at 340 nm, was identical for Agg Pro, A Pro, monomeric TMVP and TMV.

Figure 2B. Excitation spectra of $2.0 \times 10^{-5}$ M subunit A Pro recorded at 310 and 360 nm, normalized at 280 nm. The less intense spectra are those of Agg Pro recorded at 310 and 360 nm, normalized at 280 nm.

Figure 3A. Fluorescence spectra of $1.0 \times 10^{-4}$ M TNS +:
$2.5 \times 10^{-5}$ M subunit Agg Pro, pH 6.0 - upper curve
$2.5 \times 10^{-5}$ M subunit A Pro, pH 7.0 - middle curve
$2.5 \times 10^{-5}$ M subunit TMV, pH 5.0 - lower curve
Excitation was at 330 nm. The spectrum of TNS-Agg Pro has been attenuated 20% to put in on scale.

Figure 3B. Benesi-Hildebrand Plot[29] for the determination of the dissociation constants for conjugates of TNS with A Pro, O, at pH 7, TMV, ⊖, at pH 5, and Agg Pro, ◐, at pH 5.5. The concentration of TNS was $2 \times 10^{-5}$ M and that of TMVP subunit was in the range $0.7 - 2.2 \times 10^{-4}$ M. $K_d$ was calculated from the expression: $I_\infty = I - K_d \frac{I}{[\text{TMVP subunit}]}$, where I is the fluorescence intensity, corrected for background, at the concentration [TMVP subunit], $I_\infty$ is the extrapolated value of I at infinite [TMVP subunit], and $K_d$ is the dissociation constant of the complex. The excitation light was 330 nm and the fluorescence was analyzed at 440 nm. The stoichiometry of the binding of TNS was determined from the plot in the inset by the method of Job.[30] Excitation was at 370 nm and the fluorescence was analyzed at 440 nm. The total concentration of TNS and TMVP subunit was $1.0 \times 10^{-4}$ M.

Figure 4A. Excitation spectra of TNS-Agg Pro, ●, pH 6.0, TNS-A Pro, O, pH 7.0, TNS-monomeric TMVP, □, pH 7.0, TNS-TMV, ⊖, pH 5.0, and TNS in 90% dioxane/water △. All spectra have been normalized to the same intensity at their maxima near 325 nm. The concentrations of TMVP subunit and TNS were $2.5 \times 10^{-5}$ M. The concentration of TNS in 90% dioxane/water was $10^{-5}$ M. Fluorescence was analyzed at 440 nm.

Figure 4B. The polarization of the fluorescence of TNS: in glycerol, X, bound to Agg Pro, ●, and bound to A Pro, O. The dashed line shows the percent of the polarization of TNS in glycerol for TNS-Agg Pro and the dotted line shows that percent for TNS-A Pro. Fluorescence was analyzed at 440 nm and the concentrations of TNS and TMVP subunit were $2.5 \times 10^{-5}$ M.

Figure 5. Absorption spectrum of $2.5 \times 10^{-5}$ M TNS in 90% dioxane/water, and fluorescence spectrum of A Pro.The maximum intensity of fluorescence has been normalized to the maximum intensity of the absorption spectrum of TNS.

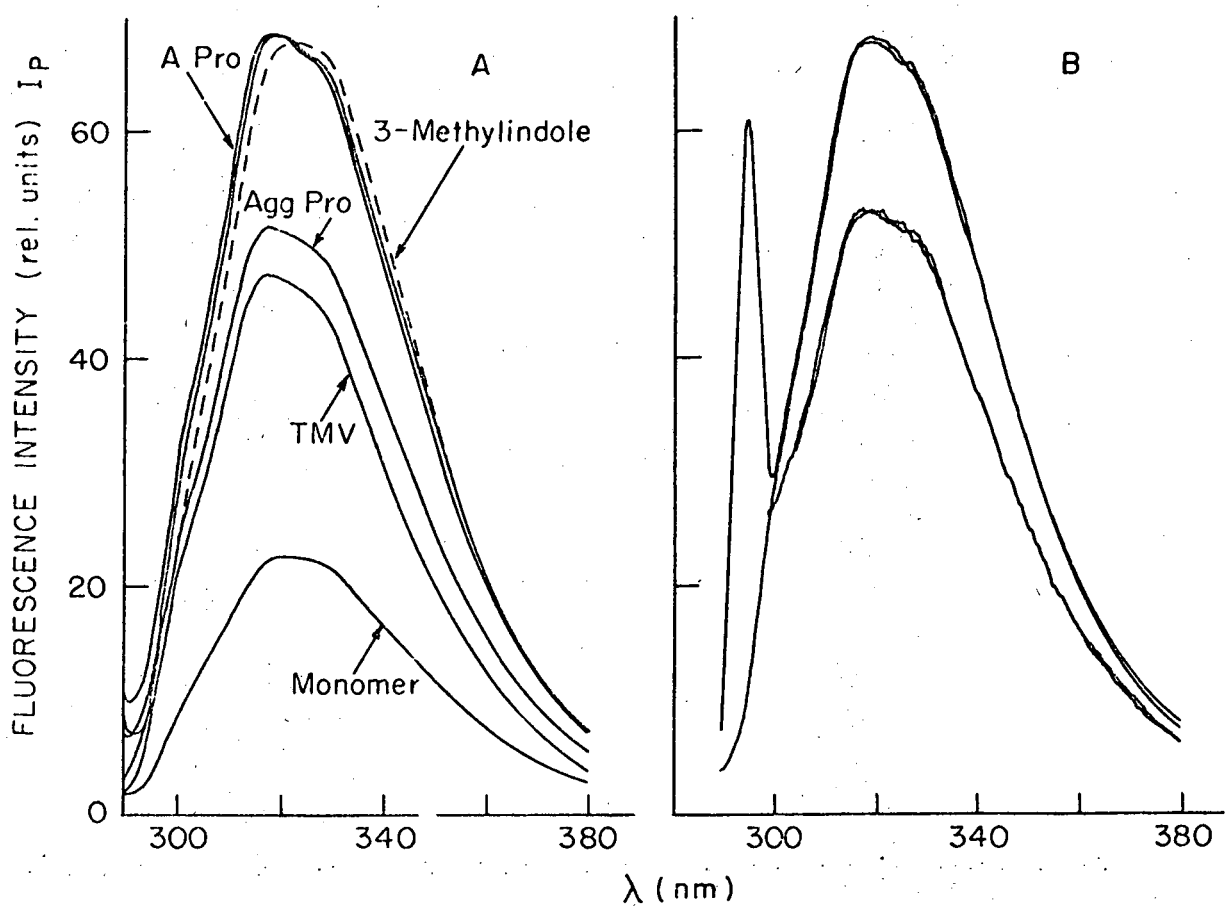
Figure 6. A schematic model[7,8,25] of TMVP helix and a cross section of a 95% quenching sphere, indicated by solid circles, of TNS (shown in several arbitrary locations) in relation to it. The 50% quenching sphere is shown with dotted lines. For simplicity, 16 subunits per turn of helix are shown, although the actual number is 16.34 per turn of virus helix. The dots indicate the center of a quenching sphere.

Table I - BINDING OF DYES TO TMVP AND TMV

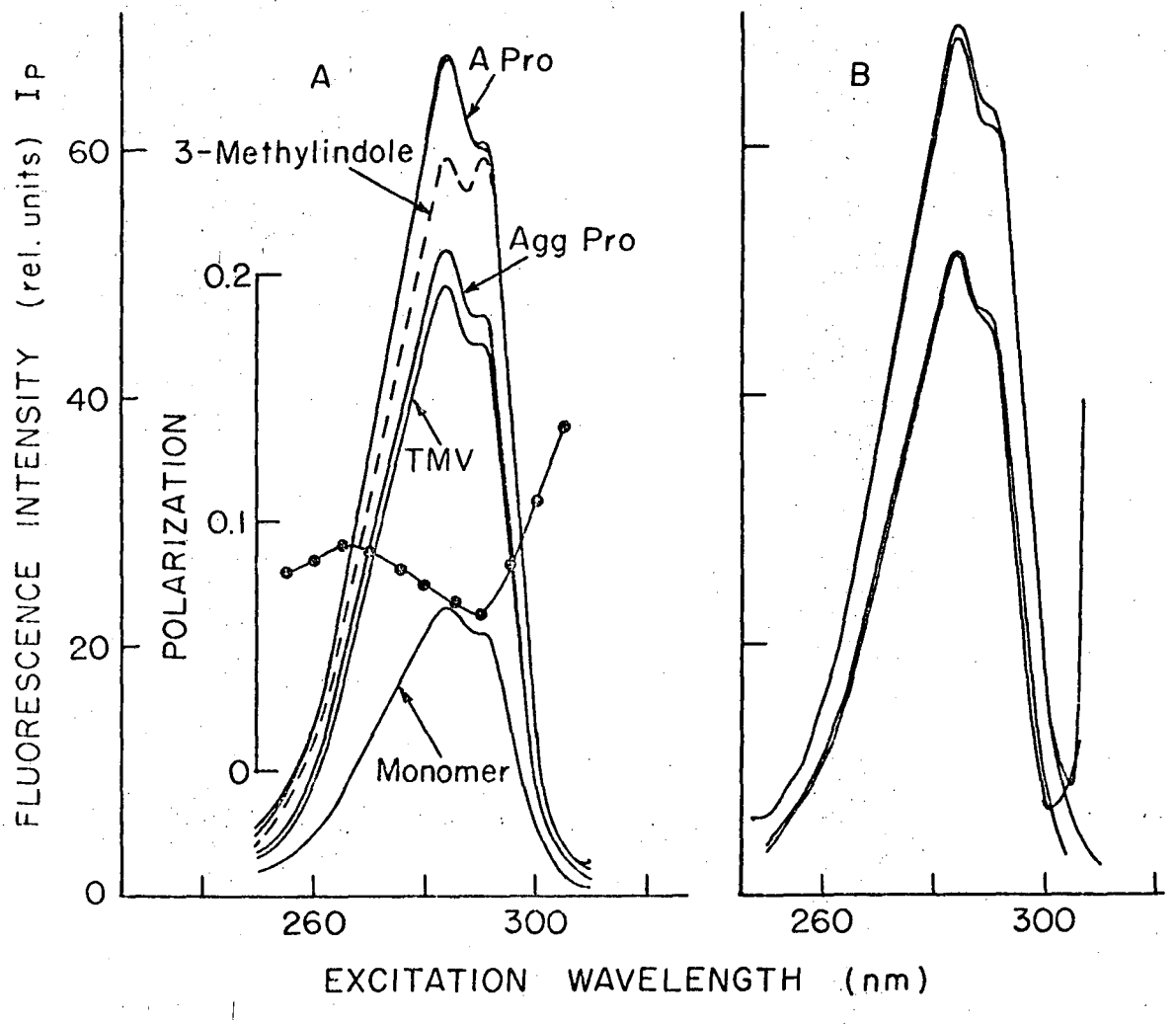
| Species | | Dye | $K_d$** (M) | $\phi_{inf.}$ | $\lambda_{max}$ (nm) |
|---|---|---|---|---|---|
| Agg Pro | pH 6.0 | TNS | $2 \times 10^{-4}$ | 0.31 | 426 |
| A Pro | pH 7.0 | TNS | $3.3 \times 10^{-4}$ | 0.24 | 423 |
| Monomer | pH 7.0 | TNS | * | * | 423 |
| TMV | pH 7.0 | TNS | * | * | * |
| TMV | pH 5.0 | TNS | $5 \times 10^{-4}$ | 0.28 | 437 |
| Agg Pro | pH 6.0 | TNSA | $3 \times 10^{-5}$ | - | 433 |
| A Pro | pH 7.0 | TNSA | $3 \times 10^{-5}$ | - | 433 |
| TMV | pH 5.0 | TNSA | * | - | * |
| TMV | pH 7.0 | TNSA | * | - | * |
| Agg Pro | pH 6.0 | Aur. 0 | $\sim 5 \times 10^{-4}$ | - | 500 |
| A Pro | pH 7.0 | Aur. 0 | $\sim 5 \times 10^{-4}$ | - | 500 |
| TMV | pH 5.5 | Aur. 0 | $\sim 5 \times 10^{-4}$ | - | 500 |
| TMV | pH 7.0 | Aur. 0 | $\sim 3 \times 10^{-4}$ | - | 500 |

*The fluorescence was too weak to allow accurate determination.

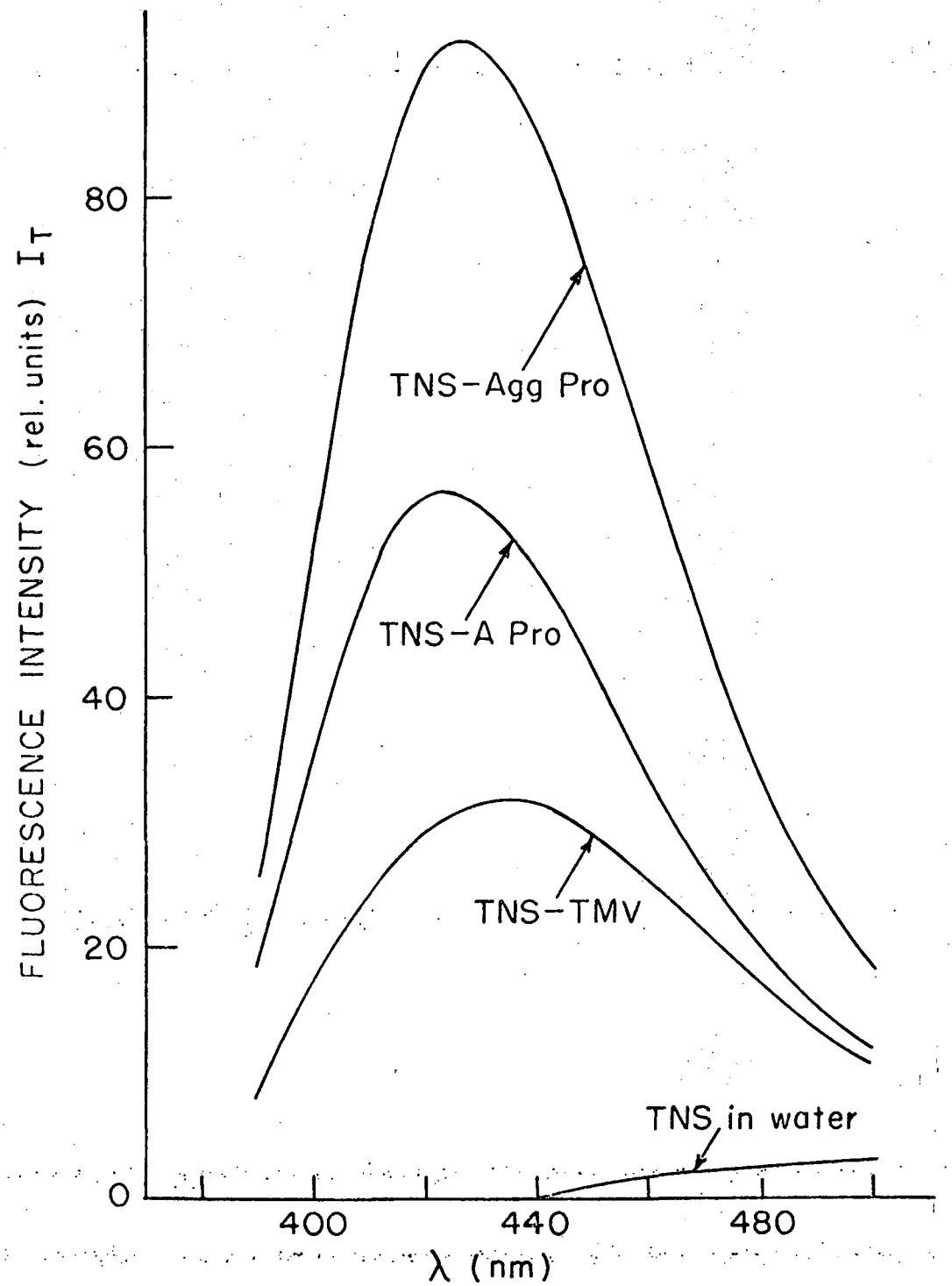
**Average value of several protein or virus preparations.


A Pro
A
B
3-Methylindole
Agg Pro
TMV
Monomer
FLUORESCENCE INTENSITY (rel. units) $I_P$
0
20
40
60
300
340
380
300
340
380
λ (nm)

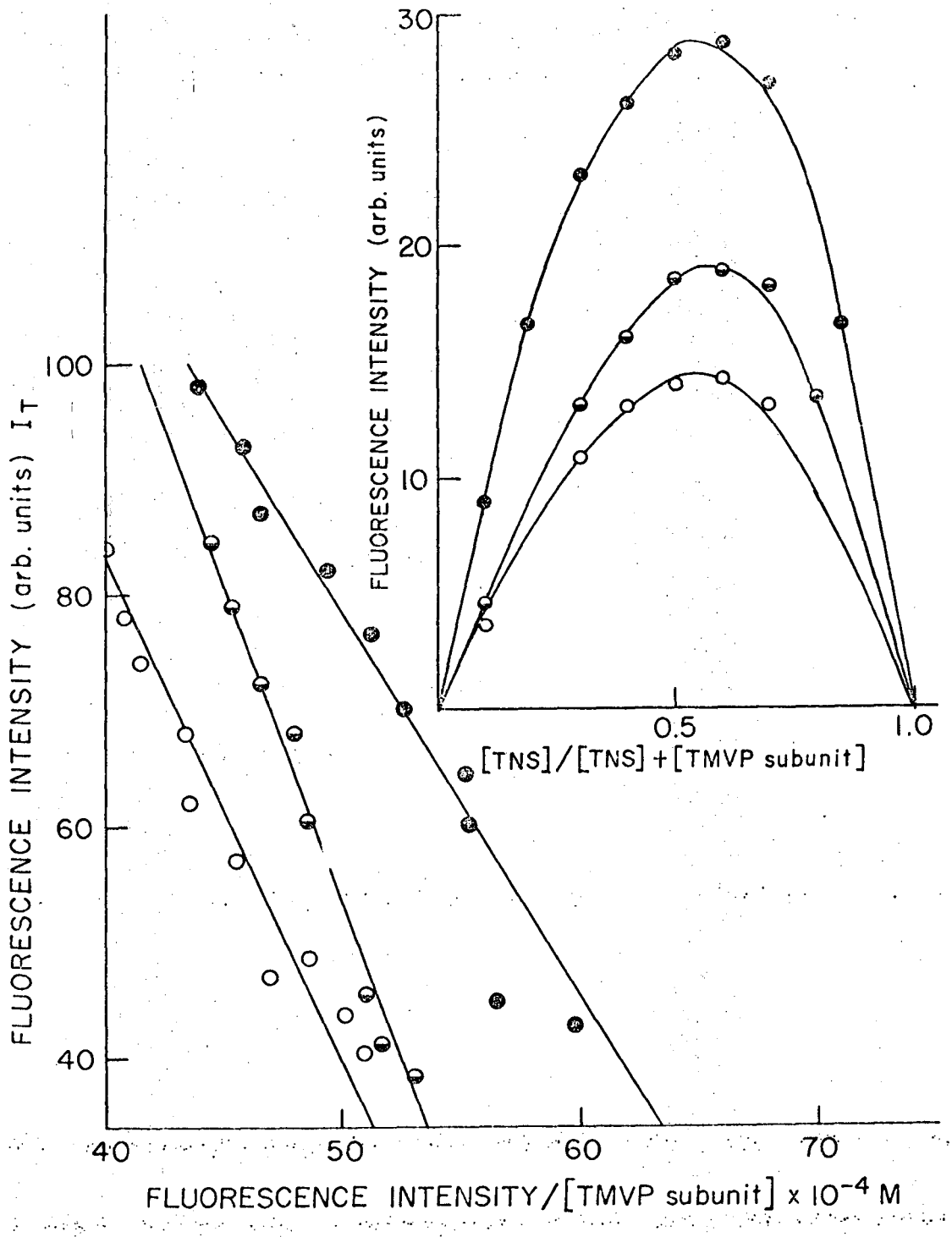
XBL731-4624


A
B
A Pro
3-Methylindole
Agg Pro
TMV
Monomer
FLUORESCENCE INTENSITY (rel. units) Ip
POLARIZATION
0
20
40
60
0
0.1
0.2
260
300
260
300
EXCITATION WAVELENGTH (nm)

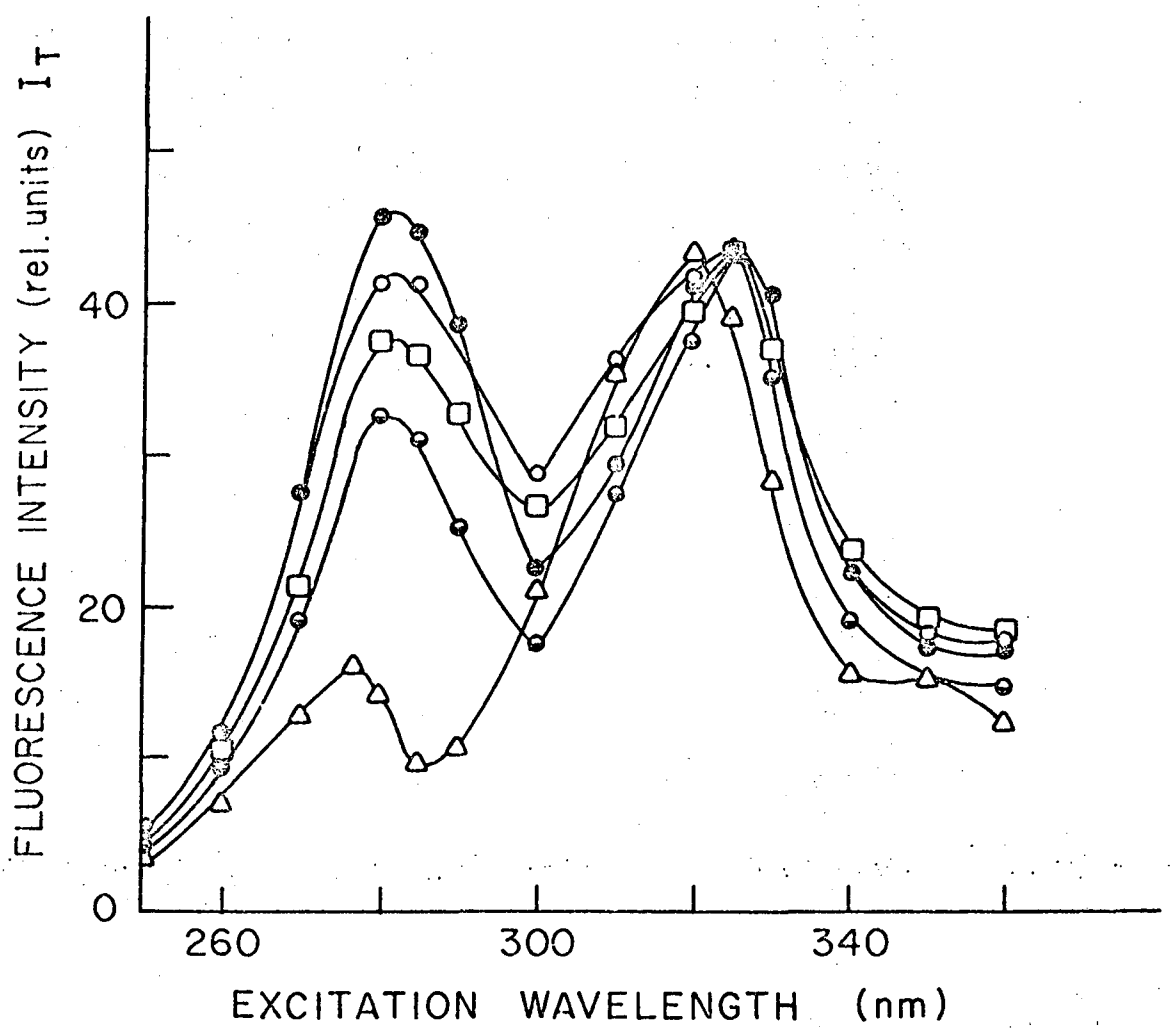
XBL731-4631


FLUORESCENCE INTENSITY (rel. units) $I_T$
80
60
40
20
0
TNS-Agg Pro
TNS-A Pro
TNS-TMV
TNS in water
400
440
480
λ (nm)

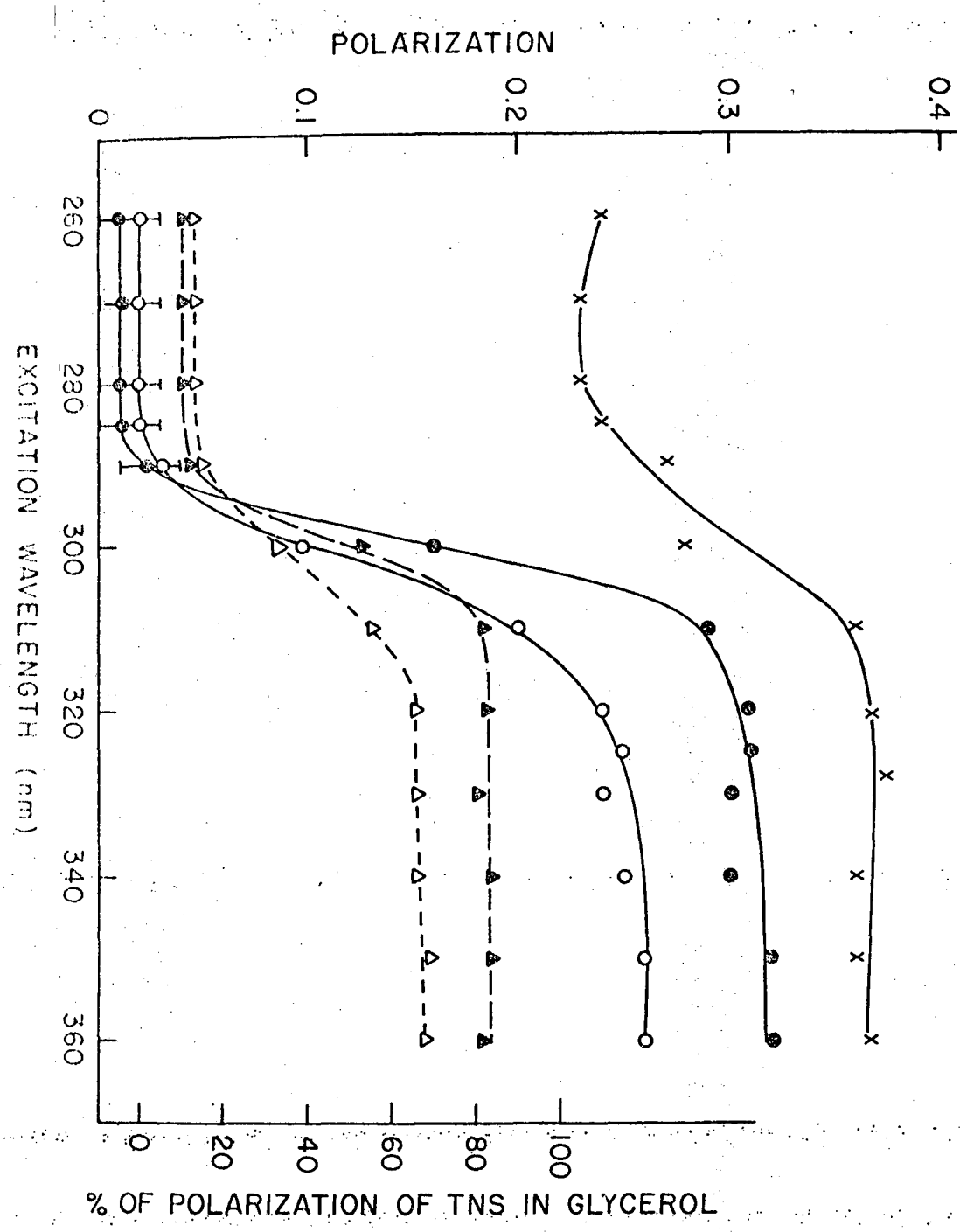
XBL731-4623

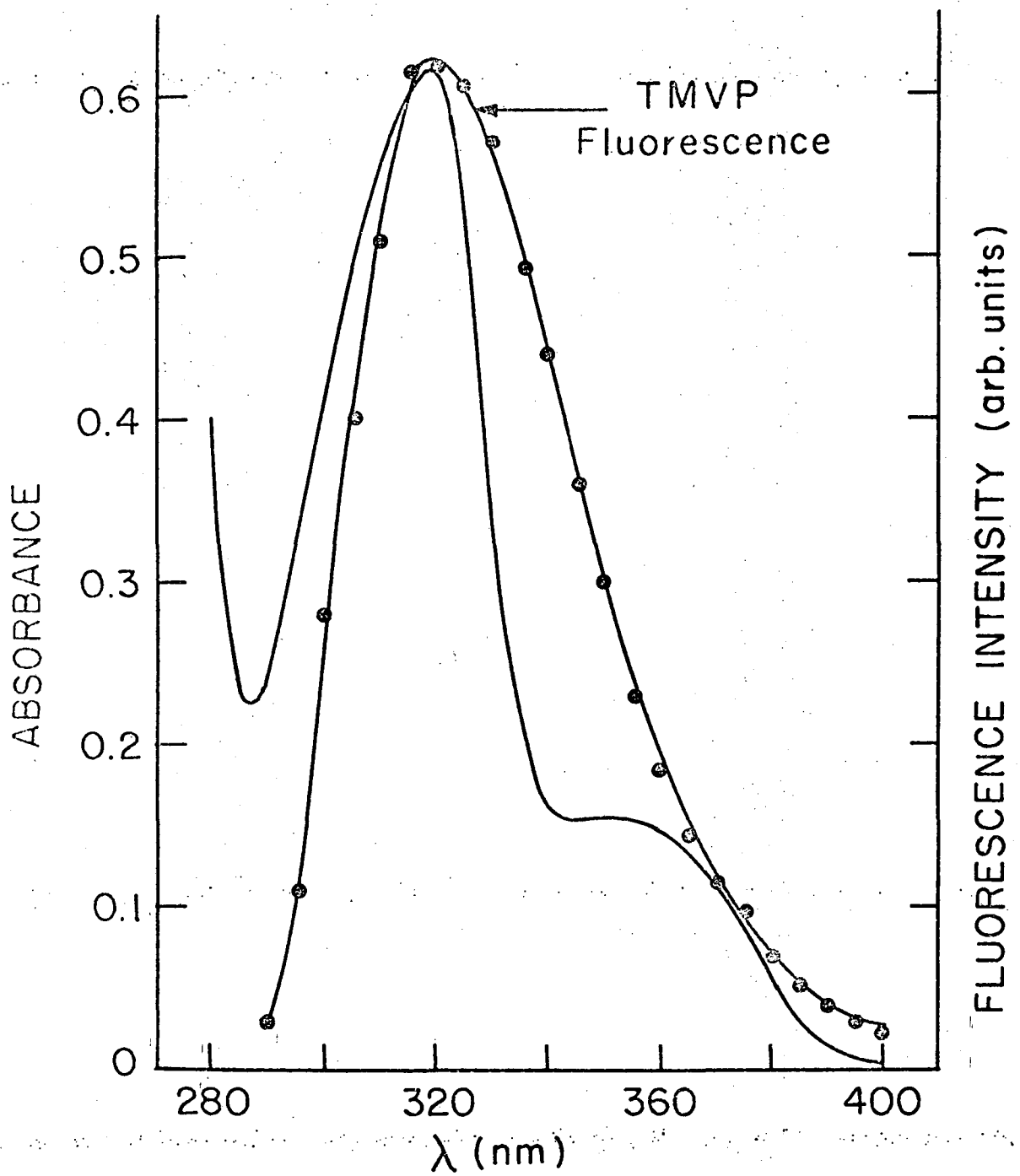

FLUORESCENCE INTENSITY (arb. units) $I_T$
100
80
60
40
40
50
60
70
FLUORESCENCE INTENSITY/[TMVP subunit] x $10^{-4}$ M
FLUORESCENCE INTENSITY (arb. units)
30
20
10
0.5
1.0
[TNS]/[TNS]+[TMVP subunit]

XBL731-4625

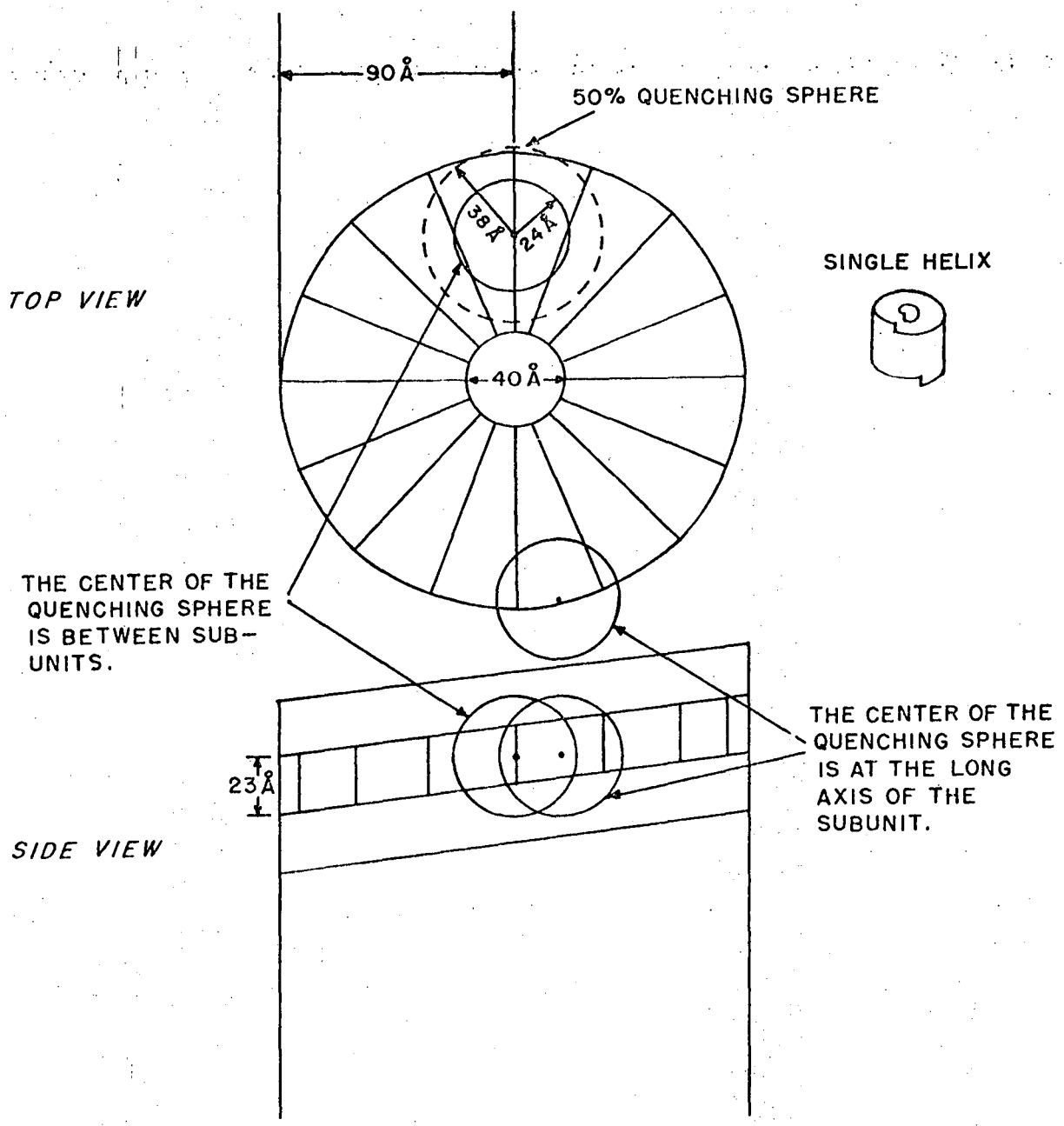

FLUORESCENCE INTENSITY (rel. units) $I_T$
40
20
0
260
300
340
EXCITATION WAVELENGTH (nm)

XBL731-4622


POLARIZATION
0.4
0.3
0.2
0.1
0
260
280
300
320
340
360
EXCITATION WAVELENGTH (nm)
0
20
40
60
80
100
% OF POLARIZATION OF TNS IN GLYCEROL


TMVP
Fluorescence
ABSORBANCE
FLUORESCENCE INTENSITY (arb. units)
0.6
0.5
0.4
0.3
0.2
0.1
0
280
320
360
400
λ (nm)

XBL731-4630


90 Å
50% QUENCHING SPHERE
38 Å
24 Å
SINGLE HELIX
TOP VIEW
40 Å
THE CENTER OF THE QUENCHING SPHERE IS BETWEEN SUB-UNITS.
THE CENTER OF THE QUENCHING SPHERE IS AT THE LONG AXIS OF THE SUBUNIT.
23 Å
SIDE VIEW

XBL731-4626

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