Brahms, J. (1967) *Proc. R. Soc. London, Ser. A 297*, 150-162.
Brahms, J., & Brahms, S. (1970) *Biol. Macromol. 4*, 191-270.
Brahms, J., Michelson, A. E., & Van Holde, K. E. (1966) *J. Mol. Biol. 15*, 467-488.
Brahms, J., Maurizot, J. C., & Michelson, A. M. (1967) *J. Mol. Biol. 25*, 465-480.
Bush, C. A. (1974) *Basic Princ. Nucleic Acid Chem. 2*, 91-169.
DeTar, D. F. (1969) *Anal. Chem. 41*, 1406-1408.
Dorman, B. P., & Maestre, M. F. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 255-259.
Dorman, B. P., Hearst, J. E., & Maestre, M. F. (1973) *Methods Enzymol. 27*, 767-796.
Draper, D. E., & von Hippel, P. H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1040-1044.
Fasman, G. D., Lindblow, C., & Grossman, L. (1964) *Biochemistry 3*, 1015-1021.
Greve, J., Maestre, M. F., Moise, H., & Hosoda, J. (1978) *Biochemistry 17*, 887-893.
Grunberg-Manago, M., & Gros, F. (1977) *Prog. Nucleic Acid Res. Mol. Biol. 20*, 209-284.
Hershey, J. W. B., Yanov, J., Johnson, K., & Fakunding, J. L. (1977) *Arch. Biochem. Biophys. 182*, 626-638.
Howe, J. G., Yanov, J., Meyer, L., Johnston, K., & Hershey, J. W. B. (1978) *Arch. Biochem. Biophys. 191*, 813-820.
Kenner, R. A. (1973) *Biochem. Biophys. Res. Commun. 51*, 932-938.
Kolb, A., Hermoso, J. M., Thomas, J. O., & Szer, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2379-2383.
Leffler, S., & Szer, W. (1974) *J. Biol. Chem. 249*, 1458-1464.
Lodish, H. F. (1976) *Annu. Rev. Biochem. 45*, 39-72.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Mets, L. J., & Bogorad, L. (1974) *Anal. Biochem. 57*, 200-210.
Revel, M. (1977) *Mol. Mech. Protein Biosynth.*, 245-321.
Schneider, A. S. (1973) *Methods Enzymol. 27*, 751-767.
Steitz, J. A., Wahba, A. J., Laughrea, M., & Moore, P. B. (1977) *Nucleic Acids Res. 4*, 17-29.
Stevens, C. L., & Felsenfeld, G. (1964) *Biopolymers 2*, 293-314.
Subramanian, A. R. (1974) *Eur. J. Biochem. 45*, 541-546.
Suryanarayana, T., & Subramanian, A. R. (1977) *FEBS Lett. 79*, 264-268.
Szkopinska, A., Zagorski, W., Zagorska, L., & Szafrankski, P. (1975) *Eur. J. Biochem. 60*, 289-294.
Thrierr, J. C., Dourlent, M., & Leng, M. (1971) *J. Mol. Biol. 58*, 815-830.
van Duin, J., Kurland, C. G., Dondon, J., & Grunberg-Manago, M. (1975) *FEBS Lett. 59*, 287-290.
Van Holde, K. E., Brahms, J., & Michelson, A. M. (1965) *J. Mol. Biol. 12*, 726-739.
Wickstrom, E. (1972) Ph.D. Thesis, University of California, Berkeley.
Wickstrom, E. (1974) *Biochim. Biophys. Acta 349*, 125-130.
Wickstrom, E., & Tinoco, I., Jr. (1974) *Biopolymers 13*, 2367-2383.
Wickstrom, E., Tyson, R. W., Newton, G., Obert, R., & Williams, E. E. (1980) *Arch. Biochem. Biophys. 200*, 296-300.
Zipori, P., Bosch, L., & van Duin, J. (1978) *Eur. J. Biochem. 92*, 235-241.

Fluorescence Studies of Platelet Tubulin†

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ABSTRACT: Temperature-related changes in tubulin conformation were investigated by several fluorospectroscopic methods utilizing steady-state fluorescence techniques. Tubulin was isolated from human platelets by three cycles of temperature-dependent polymerization–depolymerization. 8-Anilino-1-naphthalenesulfonate (ANS) was used as a probe of polarity of tryptophanyl fluorescence in tubulin. The microtubule protein displayed definite differences at 4 and 37 °C. While the total number of ligand sites was the same, differences in the binding affinity of ANS were noted. Acrylamide, a discriminating and efficient quencher of intrinsic protein fluorescence, also revealed temperature-dependent changes in the exposure of tryptophanyl residues of tubulin. ANS-induced quenching was associated with a transfer of energy from tryptophanyl residues to this fluorophor which could be used to calculate a critical distance between the acceptor–donor pair. The temperature-related changes were not found to be contingent upon the presence of polymerized tubulin.

The optical properties of 8-anilino-1-naphthalenesulfonate (ANS) make it a useful probe of polarity in many proteins. ANS, which exhibits virtually no fluorescence in water, fluoresces strongly in nonpolar solvents or when absorbed to certain proteins, lipids, or membranes (Weber & Laurence, 1954; Brand & Gohlke, 1972; Gally & Edelman, 1965; Stryer, 1965; Einarsson, 1976). Although the properties of fluorophors in general are determined by time-dependent processes occurring in their immediate microenvironment, inferences can be made concerning certain structural details of the molecules to which they are attached.

Quenching of intrinsic protein fluorophors can also be utilized to gain information on the configuration of the polypeptide chains especially on the exposure of fluorescent residues such as the amino acid tryptophan. Since the fluorophor is contained in proteins usually only in small numbers, its fluorescence makes it well suited for topographical studies using low molecular weight substances as potential quenchers of the fluorescent emission from its excited indole ring.

Tubulin, a protein serving a variety of functions in different cells, maintains the discoid shape of platelets in its polymerized

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form (White, 1971). Changes in the state of polymerization of microtubule protein occur rapidly in response to changes in temperature (Houston et al., 1974). Moreover, the ability of cells to affect rapid changes in the assembly of microtubules appears to be of major importance for the expression of various cellular functions (Olmsted & Borisy, 1973). Thus the interaction of platelets with inducers of aggregation leads to rapid but transient disassembly of microtubules occurring within 15–20 s and lasting for 30–60 s to probe changes in the conformation of tubulin as it polymerizes. The binding characteristics of ANS and the quenching of tryptophanyl fluorescence were studied (Steiner & Ikeda, 1979).

Materials and Methods

Tubulin was extracted from human platelets by three cycles of temperature-dependent polymerization–depolymerization (Ikeda & Steiner, 1976). Tubulin was dissolved in 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer, pH 6.9, containing 2 mM $MgSO_4$, 4 mM EGTA, and 0.1 mM GTP, and its final concentration was adjusted to between 0.03 and 1 mg/mL. Protein concentration was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard. The purity of the extracted platelet tubulin was determined by sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis as previously described (Ikeda & Steiner, 1976). Generally, 90–92% of the protein applied to the gels migrated as a polypeptide of M_r 55 000.

The Mg salt of 8-anilino-1-naphthalenesulfonate was obtained from Sigma Chemical Co. It was recrystallized 4 times from hot water prior to use (Weber & Young, 1964). An 0.2 mM stock solution was prepared fresh for each experiment. The concentration of ANS was measured spectrophotometrically from its molar absorbance of 4.9×10^3 M^{-1} cm^{-1} at 350 nm.

Acrylamide was obtained from Eastman Organic Chemicals. It was recrystallized from ethyl acetate before use and had a molar absorbance of 0.230 at 295 nm.

Spectroscopic Measurements. Absorbance measurements were made with a Cary 219 spectrophotometer. Steady-state fluorescence measurements were made with a Perkin-Elmer MPF-44A fluorescence spectrophotometer. The instrument was operated in the ratio mode. The excitation light source was a high-pressure xenon lamp. Samples were illuminated at either 295 or 380 nm. In general, emission was scanned from 300 to 500 nm. Samples were placed in a temperature-controlled cuvette holder, and the temperature was maintained at 4, 25, or 37 °C. Tubulin solutions having an optical absorbance of ≤0.150 at 295 nm were titrated with ANS or acrylamide by addition of 5-μL aliquots of the respective stock solutions which were for ANS 5 mM and for acrylamide 8 M. Fluorescence readings were corrected for the fluorescence, if any, of the free quencher and of the protein, for dilution, and for the attenuation of the excitation light intensity by the added quenching substance. The latter was calculated (Brand & Witholt, 1967) or measured by allowing exciting light to pass through a double-cell cuvette with tubulin solution in one cell half and quencher dissolved in buffer in the other. The wall of the second cuvette chamber facing the excitation light source was blackened to prevent direct illumination of the quencher. Thus all light passing through this cell half originated at 90° to the excitation beam. Calculated and measured values for this correction agreed within ±7%. Corrections for reduction of exciting light by self-absorption of the chromophors tryptophan and ANS were made. Correction factors were calculated according to McClure & Edelman (1967) by utilizing the equation $F_{true} = F_{obsd}[2.303 \epsilon_{ch}[ch]/(1 - 10^{-\epsilon_{ch}[ch]})]$ where [ch] denotes the concentration of the respective chromophor and ϵ_{ch} its molar absorbance. F_{true} and F_{obsd} are the true and observed fluorescence intensities.

Relative quantum yields were determined according to Parker & Rees (1960) by using the comparative method:

$$Q_1/Q_2 = (F_1/F_2)(A_2/A_1) \quad (1)$$

In this equation Q is the quantum yield, F is the area enclosed by the true emission spectrum, and A is the absorbance at the exciting wavelength. Subscripts 1 and 2 refer to the sample and reference, respectively. True emission spectra were calculated from the recorded ones by utilizing the radiant sensitivity data of the photomultiplier furnished by the manufacturer. Integrated areas of the emission spectra were measured by weighing the paper under each peak. These measurements were referred to a known area of the chart paper adjacent to the curves. Quinine bisulfate was used as a reference. Its quantum yield in 0.1 N sulfuric acid was taken as 0.55 (Melhuish, 1961). Optical absorbances were measured at the wavelengths of excitation for tryptophan, 295 nm, and ANS, 380 nm. For these determinations concentrations for quinine sulfate and ANS were kept between 5×10^{-6} and 15×10^{-6} M. Absorbances at these concentrations were ≤0.07. Within these limits there was no dependence of the quantum yield upon concentration.

Fluorescence polarization measurements were performed with ultraviolet-grade prism polarizers inserted into the excitation and emission light path. Corrections were made for unequal transmission of horizontal and vertical components of polarized light by the emission monochromator grating. Errors due to scattering of polarized exciting light resulting in an artifactual increase of measured polarization and scattering of the fluorescence light reducing the degree of polarization were considered (Shinitzky et al., 1971). Polymerized tubulin solutions at the upper concentration range used in these experiments (see above) showed very slight turbidity. The interposition of interference filters between the light source and the sample eliminated possible errors due to stray light. Cutoff filters were always used in the emission path to minimize the effect of scattered light.

Energy transfer was determined from the quenching of the fluorescence of tryptophanyl residues in tubulin by ANS, the acceptor molecules, and from measurements of the sensitized emission of tubulin-bound ANS.

The binding stoichiometries of tubulin and ANS were determined from fluorometric titrations of platelet tubulin performed at two different protein concentrations. From the fluorescence enhancement of the tubulin·ANS complex, the number of binding sites of the fluorophor was calculated. The reciprocal of the experimental data was plotted according to the equation of Klotz (1946)

$$1/j = (1/n)K_d(1/[ANS_f]) + 1/n \quad (2)$$

where j represents the moles of ANS bound per mole of tubulin, ANS_f the amount of free fluorophor, and K_d the apparent dissociation constant of the tubulin·ANS complex. The fraction of ANS bound is the ratio of F_{obsd}/F_m where F_{obsd} is the observed fluorescence corrected for emission of free ligand, protein, and self-absorption of exciting light, and F_m is the fluorescence intensity when a highly excessive protein concentration is used at a given concentration of ANS. The actual

[1] Abbreviations used: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); ANS, 8-anilino-1-naphthalenesulfonate; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GTP, guanosine 5′-triphosphate.

experimental data were statistically analyzed according to Wilkinson (1961) and Cleland (1961) by using weighted and nonlinear regression methods.

The titration of tubulin with ANS was also analyzed according to the equation

$$F = F_m - K_{app}(F/[ANS]) \quad (3)$$

(McClure & Edelman, 1967) where F_m represents the maximal fluorescence of the ANS·tubulin complex and K_{app} is its apparent dissociation constant.

Evaluation of Quenching Results. Quenching data of the intrinsic tubulin fluorescence obtained with acrylamide as quencher molecule were analyzed by Stern–Volmer plots (Lehrer & Leavis, 1978). The corrected fluorescence emissions at ~330 nm were graphically evaluated according to the equation:

$$F_0/F = 1 + K_{eff}[Q] \quad (4)$$

where F_0 and F are the fluorescence emission intensities in the absence and presence of quencher, K_{eff} is an effective quenching constant, and [Q] is the concentration of the quenching solute. As tubulin is a multitryptophan protein with a heterogeneous system of independent fluorophors, a modified version of the Stern–Volmer equation (Eftink & Ghiron, 1976a) has to be applied

$$F_0/F = \left(\sum_i \frac{f_i}{1 + K_i[Q]e^{V_i[Q]}} \right)^{-1} \quad (5)$$

in which f_i is the initial fractional fluorescence contribution of the ith fluorophor, K_i its collisional quenching, and V_i its static quenching constant. The constant V is related to the probability of finding a quencher molecule in the vicinity of a newly excited fluorophor so that the latter can be immediately quenched. The parameter V, according to Eftink & Ghiron (1976a,b), can be considered either as a volume element surrounding the fluorophor or as an association constant for the formation of the ground-state encounter complex between the excited indole ring in the case of tryptophan and the quencher. The evaluation of a heterogeneous fluorescent system is a difficult and complex undertaking which requires quite detailed structural information about the different fluorophors contributing to the total fluorescence emission.

The effective quenching constant for a system of heterogeneous fluorophors can be measured from the initial slope of the direct Stern–Volmer plot which approximates $\sum f_i k_i$, the weighted average of the individual quenching constants (Eftink & Ghiron, 1976a). The initial slope was obtained from a plot of $[F_0/F - 1]/[A]$ vs. [Q] extrapolating to [Q] = 0.

Quenching data have also been represented by another plot utilizing the graphical properties of the analogous binding equations for the interaction of small ligands with macromolecules as described by Klotz & Hunston (1971). A double-reciprocal plot of $(\Delta F/F_0)[Q]^{-1}$ vs. $\Delta F/F_0$ has been found especially well suited for this purpose.

Evaluation of Energy Transfer. Nonradiative energy transfer between tryptophanyl residues of tubulin and ANS was recognized in these experiments. The estimation of molecular distances by intermolecular energy transfer between the emission transition dipole of a donor molecule and the absorption transition dipole of an appropriate acceptor is based on the theory of Förster (1959, 1965). The rate of nonradiative transfer has been calculated to be equal to

$$k_t = (1/\tau)(R_0/R)^6 \quad (6)$$

where τ is the excited-state lifetime in the absence of acceptor, R the distance between acceptor and donor, and R_0 the so-called "Förster critical distance" at which nonradiative energy transfer equals the sum total of the rate of the other modes of deactivation of the excited state of the donor. R_0, which is dependent on spectral properties and on the relative orientation of the donor–acceptor pair, can be evaluated from

$$R_0 = (9.79 \times 10^3)(J\kappa^2\phi_D n^{-4})^{1/6}\text{Å} \quad (7)$$

where J is the spectral overlap integral of donor fluorescence and acceptor absorbance, ϕ_D the quantum efficiency of the donor, n the refractive index of visible light in the medium, and κ^2 a dipole orientation factor. The overlap integral

$$J = \sum_\lambda F_D(\lambda)\epsilon_A(\lambda)\lambda^4\Delta\lambda / \sum_\lambda F_D(\lambda)\Delta\lambda \quad (8)$$

was approximated by summing the terms over 10-nm intervals. The molar absorbance at λ of the acceptor was $\epsilon_A(\lambda)$ and the corrected fluorescence emission at λ of the donor $F_D(\lambda)$. κ^2, the orientation factor which may vary from 0 to 4 has been set equal to $^2/_3$, a value commonly used for comparative purposes. In adopting this value it is assumed that both donor and acceptor dipoles rotate rapidly compared to the donor fluorescence lifetime when bound to tubulin. The uncertainity about the exact value of κ^2 limits the precision of distance measurements by this method.

Energy transfer results in a decrease of the steady-state fluorescence intensity of the donor in the presence of acceptor. The observed donor quenching can be translated into an efficiency of energy transfer by relating the fluorescence intensity of the donor in the presence or absence of acceptor. This ratio at given concentrations and instrument setting is proportional to the quantum yield:

$$E = 1 - \phi_{D\to A}/\phi_D \text{ or } E = 1 - F_{D\to A}/F_D \quad (9)$$

From eq 6 and 7 it follows that E is related to R, the interluminophor distance, by the equation

$$E = R_0^6/(R_0^6 + R^6) \quad (10)$$

Measurement of energy transfer by steady-state techniques is predicated upon correction of the observed transfer efficiency for acceptor stoichiometry. This represents considerable difficulties, especially for a heterogeneous system of donor fluorophors.

Results

Intrinsic Tubulin Fluorescence. Platelet tubulin isolated by three cycles of temperature-dependent polymerization–depolymerization was used for most of the experiments described below. In general, this protein, analyzed by NaDodSO$_4$–polyacrylamide gel electrophoresis, is >90% microtubule protein of molecular weight 55 000 per subunit of the heterodimer. The protein exhibits characteristic fluorescence with maximal intensity at 330 nm when stimulated in the ultraviolet wavelength range. For the studies reported below the exciting wavelength was 295 nm at which tyrosine and phenylalanine have no absorption and tryptophan is the only fluorophor stimulated. Denaturation of tubulin by addition of 6.7 M guanidine hydrochloride produced the expected red shift of the fluorescence emission with the maximum intensity centered at 348–350 nm. Comparison of the observed fluorescence intensity of denatured platelet tubulin with free tryptophan in 6.7 M guanidine hydrochloride allowed an estimation of the number of tryptophanyl residues per tubulin monomer. The average of four determinations at 25 °C was 3.2 ± 0.15 residues per monomer of 55 000 molecular weight. This estimate was in good agreement with the number of tryptophanyl

Table I: Effect of Temperature on Fluorescence of Selected Proteins

protein	relative fluorescence[a] at 4 °C	25 °C	37 °C
platelet tubulin	100	88.7	83.1
lysozyme	100	65.6	51.6
DNase	100	82.0	68.9
globin	100		68.0
fibrinogen (alkylated and reduced)	100	85.9	75.8

[a] Fluorescence stimulated by 295-nm light was monitored from 310 to 380 nm. The values shown in the table are the maximal fluorescence intensities in this wavelength range adjusted to a reading of 100 at 4 °C. All proteins were suspended in Pipes buffer, pH 6.9, at a concentration of 0.3–0.6 mg/mL.

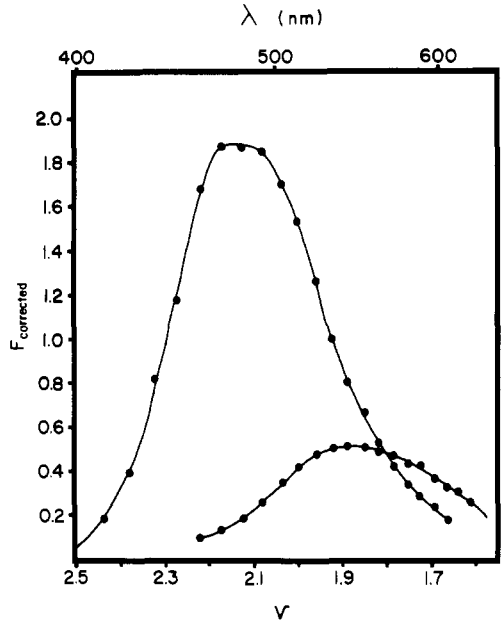

FIGURE 1: True fluorescence emission spectra of free and tubulin-bound ANS. Fluorescence was stimulated by light of wavelength 380 nm. The ordinate gives fluorescence intensities in arbitrary units normalized to read 1.0 at 520 nm. Tubulin, 0.8 mg/mL, and 50 μM ANS (left curve); 50 μM ANS (right curve).

residues measured by absorbance at 295 nm in denatured platelet tubulin. Corrections for the absorbance of tubulin-associated GTP were made. *N*-Acetyltryptophanamide in 6.7 M guanidine hydrochloride, which had a molar abosrbance of 2.3×10^3 M^{-1} cm^{-1}, served as a standard for comparison. The tryptophan content of tubulin was also determined with *N*-bromosuccinimide (Spande & Witkop, 1967). By use of this reagent, 2.9 ± 0.2 tryptophanyl residues were measured per tubulin monomer.

The intrinsic tubulin fluorescence showed the expected inverse relation between the intensity of fluorescence emission and the temperature of the medium. Of a series of proteins tested, tubulin had the lowest temperature-related change in intrinsic fluorescence (Table I). The quantum yield of the tryptophan fluorescence of platelet tubulin in Pipes buffer, pH 6.9, containing 0.1 mM GTP was found to be 0.13 for tubulin at 4 and 37 °C.

Binding of ANS by Platelet Tubulin. Binding of this fluorophor to tubulin is accompanied by a striking enhancement of fluorescence. The quantum yield (see Materials and Methods) increased more than 36-fold over that of the free fluorophor, and the emission maximum showed a marked shift to the blue, from 530 to 460 nm (Figure 1).

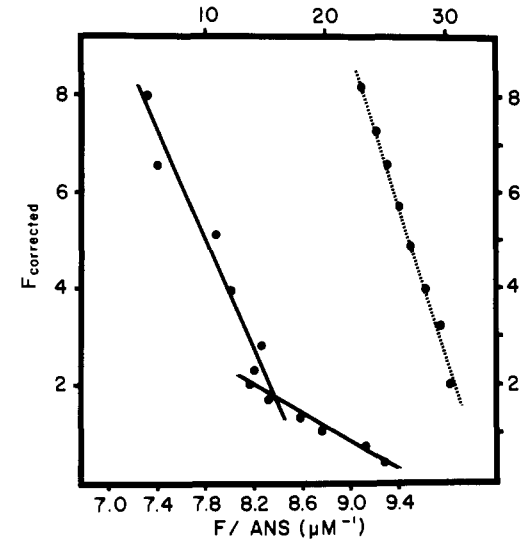

FIGURE 2: Fluorescence titration of platelet tubulin with ANS. The lower abscissa and the left ordinate refer to the titration of tubulin at 4 °C (solid line). The upper abscissa and the right ordinate refer to the titration of tubulin at 37 °C.

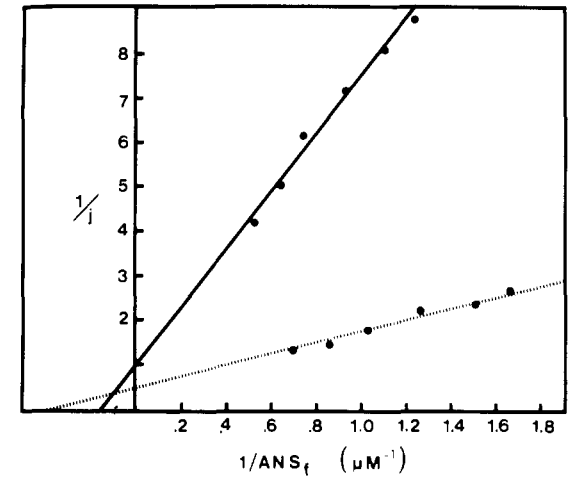

FIGURE 3: Double-reciprocal plot of ANS binding data graphed according to eq 2. To tubulin, <70 μg/mL, were added increasing concentrations of ANS. The same additions were also made to a tubulin solution of ≥4 mg/mL. Calculation of the mole fraction of ANS bound per mole of tubulin dimer was performed as outlined under Materials and Methods. Results obtained with tubulin at 4 °C (solid line) and with tubulin at 37 °C (interrupted line). All fluorescence intensity measurements were corrected for dilutional effects, self-absorption, and inner filter effects.

Within experimental error, titration of tubulin with ANS at 37 °C gave a straight line when plotted according to eq 3. The data presented in Figure 2 allowed calculation of K_{app}, the apparent dissociation constant for the reaction of tubulin with ANS, 0.94×10^{-4} M. At 4 °C a biphasic curve was apparent with K_{app} values of 3.71×10^{-4} and 1.20×10^{-4} M for the two sections of the plot.

Fluorometric titrations of platelet tubulin were also performed at two different protein concentrations, one in large excess over the other. From the fluorescence enhancement of the tubulin·ANS complex the number of binding sites of the fluorophor was calculated as outlined under Materials and Methods. The reciprocal of the experimental data was plotted according to eq 2 (Figure 3). Because of the technical difficulties of obtaining sizable amounts of concentrated tubulin solution from platelets and because of the light scattering effects at high protein concentration, especially of tubulin at 37 °C, the low protein concentration of microtubule protein

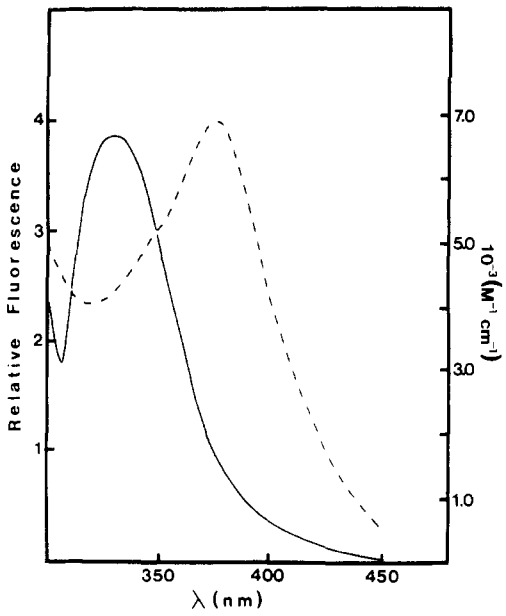

FIGURE 4: Overlap of the tryptophan corrected emission spectrum of platelet tubulin (solid line) excited wavelength 295 nm with the absorption of the tubulin·ANS complex (interrupted line). The left ordinate gives fluorescence intensities measured in arbitrary units; the right ordinate is calibrated in terms of the molar absorbance of the tubulin·ANS complex. The latter was determined by double difference spectroscopy.

was <70 μg/mL. For this reason the concentration of ANS was also kept relatively low, <3 μM. Analysis of the results according to Wilkinson (1961) showed that at 4 °C 1.0 ± 0.08 (SE) molecules of ANS were bound per tubulin dimer. At 37 °C the tubulin dimer of platelets bound 2.1 ± 0.1 (SE) ANS molecules.

Quenching of Tryptophan Fluorescence by ANS. The tryptophan fluorescence of platelet tubulin was quenched by ANS. By exciting the protein solution at 295 nm all fluorescence emission at 330 nm is due to tryptophanyl residues. The absorbance spectrum of ANS bound to tubulin overlaps the emission spectrum of tryptophan (Figure 4). The protein-bound fluorophor has an absorbance maximum which is ~25 nm red shifted compared to that of free ANS and has a substantial overlap region with the fluorescence emission of tryptophan. Tubulin-bound ANS has a molar absorbance of 6.9×10^3 M^{-1} cm^{-1} at 375 nm which was calculated from its absorption spectrum based on an absorbance value of 5.2×10^3 M^{-1} cm^{-1} for free ANS in Pipes buffer, pH 6.9, measured at 350 nm. Sequential increments of ANS added to tubulin solutions produced a progressive blue shift of the emission maximum of the tryptophanyl fluorescence which reached 323 nm at 90 μM ANS.

Quenching by ANS of tubulin excited with light of 285-nm wavelength was initially attempted, but, because of a marked increase in the Stokes component of the normal Raman scatter in polymerized tubulin and the contaminating influence of tyrosine as one of the amino acids absorbing at that wavelength, the excitation light was shifted to 295 nm where tryptophan was the only molecule with significant optical absorbancy. The effect on the Raman scatter was, however, investigated in greater detail. It was clearly the presence of polymerized tubulin which was responsible for the observed increase of the Stokes component of the Raman scatter on addition of ANS since inhibition of microtubule formation by *p*-(chloromercuri)benzoate (Ikeda & Steiner, 1978) or by lowering of the protein concentration to <0.5 mg/mL prevented the increase in intensity of this component of the scatter.

Table II: Depolarization Factor of Raman Line of Platelet Tubulin[a]

[ANS] (μM)	$I_\perp/I_\parallel$[b] at 37 °C	$I_\perp/I_\parallel$[b] at 4 °C
0	0.183	0.308
4	0.150	0.313
12	0.131	0.328
20	0.127	0.339
28	0.123	0.346

[a] Preparation of tubulin, 0.3 mg/μL, and measurement of fluorescence polarization are described under Materials and Methods. [b] $I_\perp$ denotes the fluorescence intensity perpendicular to and $I_\parallel$ is the intensity parallel to the plane of polarization of the excitation beam.

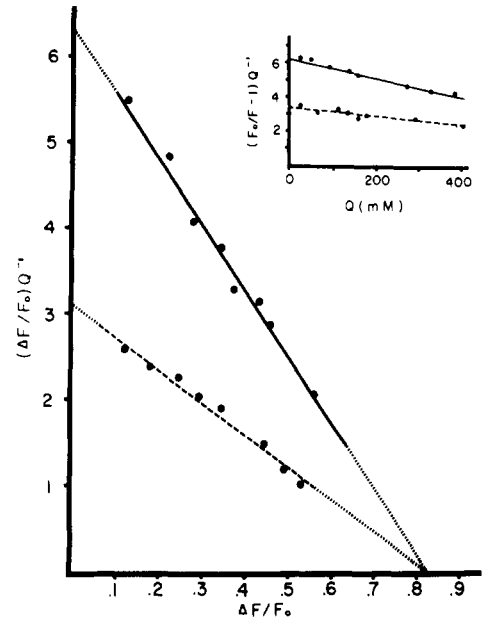

FIGURE 5: Acrylamide-induced quenching of tryptophanyl fluorescence of tubulin. Platelet tubulin, 0.3 mg/mL, was stimulated with light of wavelength 295 nm either at 4 °C (interrupted line) or at 37 °C (solid line). Fluorescence intensities were scanned in the range from 310 to 370 nm. Acrylamide was added in 20–100 mM (final concentration) increments. After each addition fluorescence intensity was scanned in the above wavelength range. All quenching data corrected for self-absorption, inner filter effects, and dilution were plotted according to a modified Stern–Volmer equation (see text). "Corrections" for static quenching were applied by utilizing the $e^{V[Q]}$ factor of Eftink & Ghiron (1976b). The inset represents the estimation of the weighted-average quenching constants for tubulin at 37 °C (solid line) and for tubulin at 4 °C (interrupted line).

The polarization of the scattered light was studied in tubulin at 4 and 37 °C in the presence of various concentrations of ANS (Table II).

Quenching of Intrinsic Tubulin Fluorescence by Acrylamide. Acrylamide quenched the tryptophan fluorescence of tubulin. The direct Stern–Volmer plot of the quenching data corresponded to that seen with other multitryptophan proteins. A downward curving plot was seen with tubulin at both 4 and 37 °C, but different K_{SV}(eff) values characterized the respective curves which were estimated by extrapolation as described under Materials and Methods (Figure 5 and Table III). Evaluation of the acrylamide quenching results by a modified Stern–Volmer plot (Lehrer & Leavis, 1978) (eq 11)

$$(\Delta F/F_0)Q^{-1} = \left(\sum_i \frac{f_i K_i Q}{1 + K_i Q}\right)^{-1} \qquad (11)$$

showed monophasic curves (Figure 5). Both intercepted the abscissa at 0.82, the value of the fractional accessible

Table III: Summary of Data Used in Estimating R_0[a] and Quenching Constants[b] for Acrylamide

parameter	value
J	7.155×10^{-15}
Q_D(4 °C)	0.13
Q_D(37 °C)	0.13
n	1.4
κ^2	$^2/_3$
R_0	22.7

	K_{SV}(eff) (M^{-1})			
	4 °C		37 °C	
quencher	initial slope	mod S-V plot	initial slope	mod S-V plot
acrylamide	3.4	3.8	6.3	7.4

[a] Determination of the parameters for calculation of R_0 has been described under Materials and Methods. [b] The quenching constants were estimated from initial slope approximations of direct Stern-Volmer plots or from the final slopes of the curves graphed according to the modified Stern-Volmer equation shown in Figure 5.

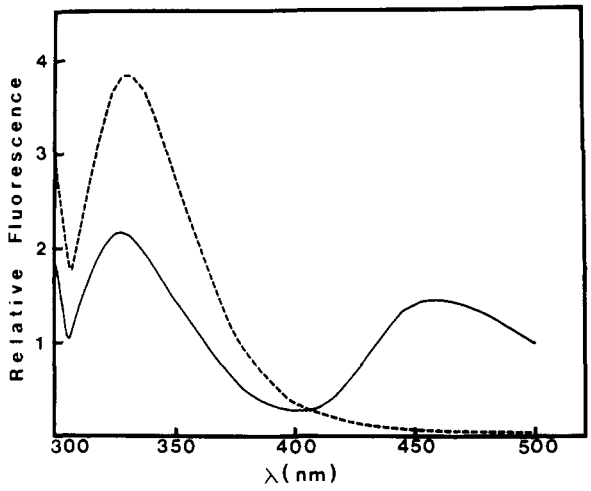

FIGURE 6: Intrinsic energy transfer from tryptophanyl residues of tubulin to ANS. Tubulin, 0.5 mg/mL, was excited with light of wavelength 295 nm and fluorescence scanned from 300 to 500 nm (interrupted line). When ANS was added, fluorescence spectrum indicated by the solid line was obtained.

fluorescence, but had different slopes signifying different effective quenching constants, 3.7 and 7.3 M^{-1} for tubulin quenched at 4 and 37 °C, respectively. These values are in reasonably, good agreement with the constants calculated from the initial slope of the direct Stern–Volmer plot (Table III).

Energy Transfer. Tryptophanyl residues in platelet tubulin functioned as energy donors for ANS added to the system. The resultant tubulin·ANS complex when excited with light of 295 nm was able to emit fluorescence with a maximum intensity at 460 nm, the wavelength at which tubulin·ANS fluoresces when stimulated with 380-nm light (Figure 6). The intrinsic energy transfer was accompanied by quenching of tryptophanyl emission at 330 nm. The corrected transfer data were plotted as $1/F$ vs. 1/[ANS] (Figure 7). This reciprocal plot showed a linear relation between these two variables and had an intercept with the ordinate which gave the maximal value of intrinsic energy transfer measured as fluorescence intensity at 460 nm. For tubulin at 4 °C, the normalized (to 1 mg of protein/mL) F_{max} was found to be 60.2 and for tubulin at 37 °C, 45.5. Assuming a temperature factor, $F_{4°C}/F_{37°C}$, of 1.8 similar to that of the tubulin·ANS complex at ANS concentrations >5 μM, the transfer yields lower fluorescence intensities in tubulin at 4 °C than would be expected were the

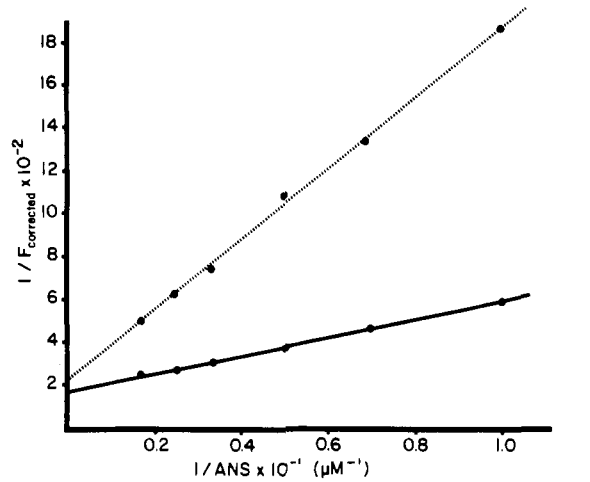

FIGURE 7: Evaluation of the fluorescence intensities obtained by intrinsic energy transfer from tryptophanyl residues to ANS. Tubulin, 0.5 mg/mL, was excited with light of 295-nm wavelength, and fluorescence emission was monitored at 460 nm. All data were corrected for inner filter effects, self-absorption, and dilution. Results obtained with tubulin at 4 °C (solid line) and with tubulin at 37 °C (interrupted line) are presented in a double-reciprocal plot.

interluminophor distance unchanged. Because the acceptor stoichiometry of ANS in relation to the tubulin tryptophanyl residues is unkown, the efficiency of energy transfer could not be calculated. However, the available information was sufficient to calculate the critical transfer distance between donor and acceptor luminophor. The ANS absorbance overlaps the tryptophan fluorescence as shown in Figure 4. The overlap integral was computed in the range from 310 to 450 nm, and, by using a quantum yield of 0.126 for tryptophan fluorescence in platelet tubulin, R_0 was calculated to be 22.7 Å (Table III). In view of the complexity of the system no conclusions could be drawn about actual or relative change in donor–acceptor distance in the course of temperature change from 4 to 37 °C. Temperature itself may affect multiple parameters including excited-state rate constants, orientation factor, and the yields of tryptophan and bound ANS.

Discussion

ANS is absorbed in a rather nonspecific manner on a variety of proteins (Dodd & Radda, 1967; Turner & Brand, 1968). However, these studies have shown that, in platelet tubulin at least, some of the ANS binds quite specifically. The fluorescence properties of the dye change drastically upon binding to tubulin. A marked shift toward lower wavelength in the fluorescence emission is accompanied by a 36-fold increase in quantum yield as ANS is bound to microtubule protein. The large fluorescence enhancement and simultaneous blue shift suggest a decrease in the polarity of its local environment upon complex formation with the macromolecule. The interaction between fluorophor and protein is stoichiometric in polymerized and depolymerized tubulin. Although both forms have the same number of binding sites, depolymerized tubulin (4 °C) displayed a different behavior at low ANS concentrations (<3 μM) at which only one binding site was accessible to the dye molecules. An evaluation of the number of ANS binding sites has been performed on brain tubulin of two animal species by two groups of authors (Bhattacharyya & Wolff, 1975; Lee et al., 1975). Their studies revealed only one ligand site per tubulin dimer for this fluorophor. Species and tissue differences may be responsible for the results obtained in this study with human platelet tubulin.

Titration of tubulin with ANS produced evidence of two

independent binding sites revealed by the different slopes shown by the data of Figure 2. The apparent dissociation constants for ANS in 4 and 37 °C tubulin are in the range reported for other proteins (McClure & Edelman, 1967). The constants also compare favorably with other tubulin ligands, e.g., vinblastine, as reported by Lee et al. (1975).

Determination of the number of tryptophanyl residues in platelet tubulin by three different methods revealed about three residues per monomer. The tryptophanyl residues appear to be located in hydrophobic regions of the protein as indicated by the marked shift of the fluorescence of its residues. The shift of the emission peak to that of free tryptophan on denaturation of tubulin by guanidine hydrochloride is in line with this observation. ANS seems to be bound in the close vicinity of tryptophanyl residues. This is substantiated by the quenching effect of ANS on the fluorescence emission of tryptophan and by the demonstration that ANS is also the recipient of intrinsic energy transfer from tryptophan.

Solute quenching is a dynamic method since the presence of solute alters the fluorescence lifetime and thereby also the steady-state intensity without changing the electronic energy levels that are involved. It reveals considerable information on the exposure and indirectly on the surrounding structure of intrinsic, i.e., protein-bound, fluorophors. Static quenching, on the other hand, is predicated on the fortuitous presence of a quencher molecule in the immediate vicinty of an excited fluorophor or on the formation of a nonfluorescent complex at the instant of excitation. It does not alter the fluorescence lifetime of the fluorophor. Although the static quenching component is of no or very limited value in defining specific interaction between or binding of quenchers to the fluorophor-carrying macromolecule, it does yield information on the accessibility of the chromophor to quenchers (Eftink & Ghiron, 1976a).

An analysis of the quenching results in this study was made difficult by the uncertainty of the relative contribution of the individual tryptophanyl residues to the total fluorescence of tubulin and by the inner-filter effects. The contribution of the latter to the observed reduction of fluorescence intensity was evaluated by two different methods which gave almost identical results. The utilization of a double-cell cuvette as described under Materials and Methods proved to be a convenient way to estimate the necessary correction factors for incremental additions of quenchers. These measured correction factors were almost completely congruent with those calculated (Brand & Witholt, 1967). Since fluorescence lifetime measurements could not be performed, the correction method of Eftink & Ghiron (1976b) was applied to the quenching data. Even though this approximation does not allow a clear separation of static and dynamic or collisional quenching components, information on the difference between tubulin at 4 and 37 °C was obtained from the results. Since these changes can be observed at protein concentrations far below those which are needed to initiate spontaneous polymerization of platelet tubulin, the temperature change appears to effect a profound alteration in the protein structure. Acrylamide, an efficient and discriminating quencher of tryptophanyl fluorescence, was far more effective in reducing fluorescence intensity in tubulin, at 37 °C than at 4 °C. A clear and definite change in the exposure of the fluorophors was thus apparent.

The small blue shift in the fluorescence spectrum of tubulin in the presence of ANS corresponds to the experience of other authors who examined protein quenching (Eftink & Ghiron, 1976a). A plausible explanation for this shift may lie in the heterogeneity of the tryptophanyl residues of a multitryptophan protein as tubulin is. The quenching of tryptophan(s) by ANS may allow other tryptophanyl residues, more deeply buried in the protein and fluorescing at somewhat shorter wavelengths, to contribute a greater share of the emitted light intensity.

The theory of fluorescence energy transfer as promulgated by Förster (1959, 1965) has been repeatedly tested experimentally and shown to be valid for a variety of systems of known geometry. The fact that energy transfer between tryptophanyl residues and ANS exists in tubulin is the most convincing indication of the close proximity of these two luminophors in the protein. While even under the best of circumstances in well-characterized systems some of the determinants of the transfer equation remain subjects of assumption, this has been found to be the case even more so in the protein under study. For this reason only the critical transfer distance, R_0, was calculated by using a value of $^2/_3$ for κ^2, the dipole orientation factor. This value has been taken simply for purposes of comparison. Its use is predicated on the existence of an isotropically random distribution of orientations for donor and acceptor luminophors bound to macromolecules. Although many authors have based their calculations on this assumption, evidence for it is not available as was pointed out by Dale & Eisinger (1974, 1976).

An important property observable in the vibrational Raman effect is the polarization of scattered light. Vibration can be divided into totally symmetrical and nontotally symmetrical vibrations. The evidence of the data presented in this report suggests that platelet microtubules have totally symmetrical vibrations. Progressive addition and binding of ANS to tubulin, whether in the polymerized or depolymerized form, accentuates the existing trend of vibrational behavior in these two forms of protein. In platelet microtubules a tendency toward greater symmetry of the molecule was manifested by the decreasing value of the depolarization factor, while the opposite effect is evident in depolymerized tubulin.

These studies demonstrate the usefulness of fluorophors as probes of the molecular structure of macromolecules. Although limited to measurements of steady-state fluorescence, the methods utilized covered several aspects of this field including binding, quenching, and energy transfer of chromophors. The results give persuasive evidence for a temperature-induced change in tubulin configuration which occurs independent of its polymerization into microtubules.

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References

Bhattacharyya, B., & Wolff, J. (1975) *Arch. Biochem. Biophys. 167*, 264–269.

Brand, L., & Witholt, B. (1967) *Methods Enzymol. 11*, 776–856.

Brand, L., & Gohlke, J. (1972) *Annu. Rev. Biochem. 41*, 843–868.

Cleland, W. W. (1961) *Adv. Enzymol. Relat. Areas Mol. Biol. 29*, 1–32.

Dale, R. E., & Eisinger, J. (1974) *Biopolymers 13*, 1573–1605.

Dale, R. E., & Eisinger, J. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 271–273.

Dodd, G. H., & Radda, G. K. (1967) *Biochem. Biophys. Res. Commun. 27*, 500–504.

Eftink, M. R., & Ghiron, C. A. (1976a) *Biochemistry 15*, 672–680.

Eftink, M. R., & Ghiron, C. A. (1976b) *J. Phys. Chem. 80*, 486–493.
Einarsson, R. (1976) *Biochim. Biophys. Acta 446*, 124–133.
Förster, T. (1959) *Discuss. Faraday Soc. 27*, 7–17.
Förster, T. (1965) in *Modern Quantum Chemistry* (Sinanoglu, O., Ed.) Part III, pp 93–137, Academic Press, New York.
Gally, J. A., & Edelman, G. M. (1965) *Biochim. Biophys. Acta 94*, 175–182.
Houston, L. L., Odell, J., Lee, J. C., & Hines, R. H. (1974) *J. Mol. Biol. 87*, 141–146.
Ikeda, Y., & Steiner, M. (1976) *J. Biol. Chem. 251*, 6135–6141.
Ikeda, Y., & Steiner, M. (1978) *Biochemistry 17*, 3454–3459.
Klotz, I. M. (1946) *Arch. Biochem. Biophys. 9*, 109–117.
Klotz, I. M., & Hunston, D. L. (1971) *Biochemistry 10*, 3065–3069.
Lee, J. C., Harrison, D., & Timasheff, S. N. (1975) *J. Biol. Chem. 250*, 9276–9282.
Lehrer, S. S., & Leavis, P. C. (1978) *Methods Enzymol. 49*, 222–236.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. S. (1951) *J. Biol. Chem. 193*, 265–275.
McClure, W. O., & Edelman, G. M. (1967) *Biochemistry 6*, 559–566.
Melhuish, W. H. (1961) *J. Phys. Chem. 65*, 229–235.
Olmsted, J. B., & Borisy, G. G. (1973) *Annu. Rev. Biochem. 43*, 507–540.
Parker, C. A., & Rees, W. T. (1960) *Analyst (London) 85*, 587–600.
Shinitzky, M., Dianoux, A.-C., Gitler, C., & Weber, G. (1971) *Biochemistry 10*, 2106–2113.
Spande, T. F., & Witkop, B. (1967) *Methods Enzymol. 11*, 498–506.
Steiner, M., & Ikeda, Y. (1979) *J. Clin. Invest. 63*, 443–448.
Stryer, L. (1965) *J. Mol. Biol. 13*, 482–495.
Turner, D. C., & Brand, L. (1968) *Biochemistry 7*, 3381–3390.
Weber, G., & Laurence, D. J. R. (1954) *Biochem. J. 56*, 31.
Weber, G., & Young, L. B. (1964) *J. Biol. Chem. 239*, 1415–1423.
White, J. G. (1971) in *Platelet Aggregation* (Caen, J., Ed.) pp 15–52, Masson et Cie, Paris.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324–332.

Solubilization and Reconstitution of the Adenosine 5′-Triphosphate Dependent Proton Translocase of Bovine Chromaffin Granule Membrane†

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ABSTRACT: The ATP-dependent proton translocase of chromaffin granule membrane has been solubilized with bile salts and nonionic detergents and reconstituted in vesicular structures by removal of the detergent. Solubilization changed the properties of the ATPase which was no longer inhibited by *N,N′*-dicyclohexylcarbodiimide and trisubstituted tin derivatives. Reconstituted vesicles contained a considerable fraction of the solubilized ATPase, but the H^+ pump activity varied with the conditions used for solubilization and reconstitution. Small vesicles (50-nm diameter) possessing an active H^+ pump were reconstituted from deoxycholate extracts of the membrane after dilution of the detergent and from cholate extracts after adsorption of the detergent on Biobeads SM-2. Their ATPase was inhibited by *N,N′*-dicyclohexylcarbodiimide and trisubstituted tin derivatives and was resistant to oligomycin. Upon addition of ATP, a transmembrane potential was generated which was monitored with the extrinsic fluorescent probe OX-V [bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethineoxonol]. Imposition of an artificial proton electrochemical gradient to the vesicle interior reversed the ATPase reaction and induced ATP synthesis. Moreover, the vesicles accumulated *l*-noradrenaline in the presence of ATP, suggesting some reconstitution of the complete system of catecholamine uptake. In contrast, when nonionic detergents were used for solubilization, reconstitution of the H^+ pump either by dilution or by adsorption of the detergent was unsuccessful.

Chromaffin granules are organelles which are involved in the synthesis, storage, and release of adrenaline and noradrenaline by adrenal medulla. In presence of ATP, they accumulate catecholamines by a reserpine-sensitive process (Kirshner, 1962; Carlsson et al., 1963) which is a property of the membrane since ghosts derived from chromaffin granules are also capable of active amine transport (Phillips, 1974a; Ingebretsen & Flatmark, 1979). The uptake mechanism has been shown to involve inward translocation of protons by an electrogenic ATP-dependent proton pump (Pollard et al., 1976; Casey et al., 1977; Phillips & Allison, 1978; Johnson & Scarpa, 1979). The transmembrane potential $\Delta\Psi$ (interior positive) generated in the presence of ATP can be sensed by the extrinsic probe OX-V,[1] the absorption and fluorescence of which are linearly related to potential changes (Scherman & Henry, 1980).

The Mg^{2+}-dependent ATPase of the chromaffin granule membrane has some properties in common with the mitochondrial proton pump but is oligomycin and efrapeptin resistant (Apps & Glover, 1978). It has been solubilized by nonionic detergents such as Lubrol-PX (Trifaro & Warner,

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[1] Abbreviations used: OX-V, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethineoxonol; Hepes, *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid; DTT, dithiothreitol; DOC, deoxycholate; DBH, dopamine β-hydroxylase; MAO, monoamine oxidase; DCCD, *N,N′*-dicyclohexylcarbodiimide; cmc, critical micellar concentration; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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