Mechanism of Atebrin Fluorescence Changes in Energized Submitochondrial Particles†

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With the Technical Assistance of P. Veronese and B. Ziche

ABSTRACT: Atebrin is bound with high affinity to energized submitochondrial particles: number of sites, 43 μmol/g of protein; K_D = 8 μM. At low protein:dye ratios the binding is accompanied by a quenching of fluorescence. During quenching the absorbance spectra indicate dye aggregation rather than acid–base transition. At increasing protein:dye ratios there is first a decrease of the fluorescence quenching and then a fluorescence enhancement. The latter is accompanied by an increase of fluorescence polarization. Kinetically, particle energization at a high protein:dye ratio results first in an enhancement and then in a quenching phase, both of them sensitive to uncouplers. An analysis of atebrin properties shows that the fluorescence quantum yield of the dye increases markedly in media of low polarity and high viscosity. It is proposed that the quenching effect be due to intermolecular interactions following an electrostatic binding of the protonated ligands to nucleophilic sites where the nearest neighbor is a dye-filled site. The decrease of fluorescence quenching is due to destacking of the dye. The enhancement, which occurs when the dye binds to a site where the neighbor is a dye empty site, reflects the polarity or the viscosity of the site microenvironment.

Transition from the deenergized to the energized state in energy transduction membranes, such as chloroplasts, chromatophores, and submitochondrial particles, results in (a) dye uptake and (b) a quenching of fluorescence. However, the interpretation of the mechanism for uptake as well as of the nature of the fluorescence change is controversial.

Kraayenhof (1970) proposed the use of atebrin in chloroplasts as a probe of the energized state. Gromet-Elhanan (1971, 1972) suggested that the quenching be dependent in chromatophores on an active H^+ uptake and not on the energization leading to ATP synthesis directly. Similarly, Schuldiner *et al.* (1972) proposed the use of the acridines as pH probes for the internal pH in chloroplasts. Deamer *et al.* (1972) utilized the atebrin quenching for measuring pH gradients across membranes of liposomes and suggested a self-quenching mechanism. Azzi *et al.* (1971) explained the quenching of atebrin and Acridine Orange fluorescence as due to intermolecular interactions following an accumulation of the dye in the osmotic space of submitochondrial particles. Lee (1971) proposed atebrin as an indicator of the intramembrane pH in submitochondrial particles. Azzone *et al.* (1973) proposed an uptake mechanism based on the binding of the dye to nucleophilic sites in the submitochondrial membranes and a quenching mechanism based on dye–dye interaction. In the present paper a more detailed analysis of the nature of the fluorescence changes of atebrin is presented. It is proposed that the penetration of the dye into the membrane fabric leads either to an increase of fluorescence quantum yield because of the low polarity and or high viscosity of the environment, or to fluorescence quenching because of dye association.

Experimental Section

Flourescence measurements were carried out with the spectrofluorimeter Hitachi Perkin-Elmer Model MPF-2A. All spectra were recorded in the presence of the corrector attachment at room temperature. Furthermore, the spectra were also corrected for the scattering due to membrane turbidity. During kinetic measurements, it was ascertained that no large variations of absorbance of suspension at excitation and emission wavelength did occur. Such variations may cause fluorescence changes because of filtering effects of the suspension. The quantum yields, ϕ, were calculated by the method of Parker and Rees (1960), with a correction for the refractive index of the solvent (Hermans and Levinson, 1951)

$$\phi_x = \phi_q \frac{(1 - T)_q(\text{area})_x n_q^2}{(1 - T)_x(\text{area})_q n_x^2}$$

where x and q refer to sample and reference, respectively, n is the refractive index (Handbook of Chemistry and Physics, 1969), T is the transmittance, and area refers to the area of the emission spectra. This was determined gravimetrically.

Quinine-HCl in 1 N H_2SO_4 was the reference substrate; ϕ_q was taken to be 0.55 (Melhuish, 1961). Absorbance was measured at 340 nm; all absorbance values were <0.05. For these and lower values of absorbance, cells of 4-cm optical path were used. When measured with this method, the fluorescence quantum yield of pure anthracene in absolute ethanol was 0.27, in agreement with the literature values of 0.30 (Weber and Teale, 1957) and 0.25 (Melhuish, 1961).

Measurements of polarization of fluorescence were carried out with the polarization accessory for the Model MPF-2A by obtaining $I_\parallel$ and $I_\perp$ and calculating the polarization p

$$p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$$

In measurement of the fluorescence polarization of atebrin in the presence of a suspension of submitochondrial particles, the corrected formula of Shinitzky *et al.* (1971) was used in order to eliminate the scattering contribution

$$p = \frac{(I_\parallel - I_\parallel^s) - (I_\perp - I_\perp^s)}{(I_\parallel - I_\parallel^s) + (I_\perp - I_\perp^s)}$$

where $I_\parallel^s$ and $I_\perp^s$ were measured with the dye-free particle suspension.

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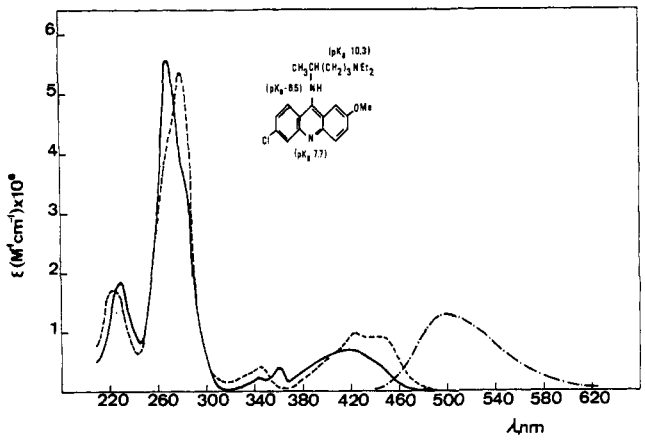

FIGURE 1: Spectra of atebrin. The following spectra are reported: absorbance spectrum in water, pH 8 (—); absorbance spectrum in water, pH 5 (---); emission spectrum in water, pH 5 (-·-), at excitation λ 340 nm, in arbitrary units; 10 μM atebrin. The pK_a values were those reported by Christophers (1967) and Irvin and Irvin (1950).

The absorbance spectra of atebrin in the presence of membrane suspensions were recorded with a split-beam spectrophotometer constructed in the workshop of the Johnson Foundation. In order to avoid any interference from the emitted light, the spectra were recorded in the presence of a Wratten Filter No. 34. All other absorbance experiments were carried out with a Hitachi Perkin-Elmer spectrophotometer Model 124 with the photomultiplier at a distance of 25 cm from the cuvet.

The binding of the dye to the energized submitochondrial particles was measured as described previously (Colonna *et al.*, 1970; Dell'Antone *et al.*, 1972a). The concentration of the dye in the supernatant was measured spectrophotometrically.

Most experiments were carried out with submitochondrial particles prepared from beef heart mitochondria. The sonication of the mitochondria was usually carried out in pyrophosphate. Other procedures were as described in previous papers of the present series. Atebrin was furnished by Sigma and no further purification was made.

Results

The absorbance spectrum of atebrin in water is given in Figure 1. The bands in ultraviolet and near ultraviolet are typical of acridines, while the two maxima (in acid medium) at 425 and 445 nm are typical of atebrin. These two maxima have been attributed to two different ionic forms (Kislyak and Lisenko, 1960[1]). The fluorescence lifetime of the dye was measured by Chen *et al.* (1967) and was found to have a value of 4 nsec (20 μM atebrin in water).

Preliminary experiments were conducted to determine the effect of pH upon atebrin absorbance and fluorescence in water. The spectrum of Figure 2A shows that the acid–base transition resulted in an appearance of a new maximum at 445 nm, with an isosbestic point at 410 nm. The group involved in this transition is the amino group of the ring nitrogen (pK_a = 7.7). This is in accord with the spectrophotometric titration, as seen in Figure 2B. Also, the fluorescence intensity is pH dependent (Lee, 1971). Figure 2B shows that the quantum yield increases with the increase of pH.

Atebrin, like Acridine Orange (Zanker, 1952) and other acridines (Mukerjee and Ghosh, 1970), aggregates in solution

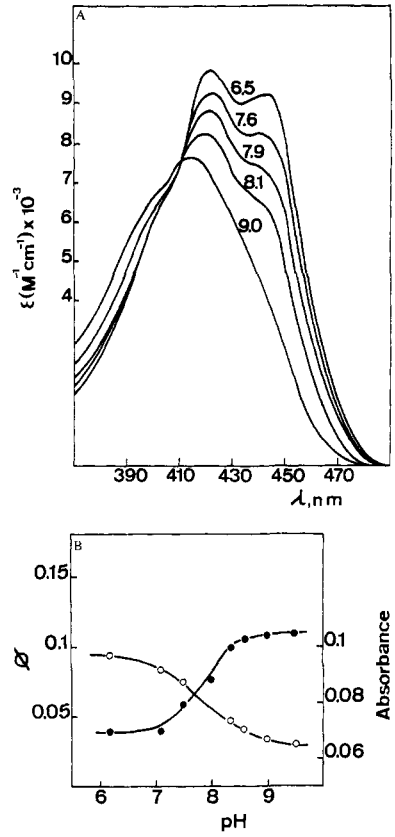

FIGURE 2: Absorbance spectrum and quantum yield of atebrin at various pH values. (A) Absorbance; the medium was 0.1 M KCl–20 mM Tris-Cl at various pH values; 30 μM atebrin. (B) Quantum yield; the dye (10 μm) was added to an aqueous solution containing 2 mM Tris-Cl at various pH values. Quantum yield measurements were calculated as described in the Experimental Section (●). Absorbances at 340 nm were also reported (○).

at high concentrations. The aggregation causes not only a quenching of fluorescence, but also a large change in the absorbance spectra, as shown in Figure 3. While Acridine Orange aggregates in solution at very low concentration (Zanker, 1952) atebrin shows no change in absorption spectra until it reaches a concentration of 100–200 μM. Parallel to the increase of the dye concentration there are decreases of (a) the absorbances of the two maxima and (b) the ratio between the absorbances of the two maxima. The transition monomer–aggregate has two isosbestic points at 456 and 400 nm. That this phenomenon is really aggregation can be verified through the addition of various amounts of ethanol to the concentrated dye in aqueous solution or through a rise in the temperature (not shown). Both effects cause an enhancement of absorbance toward the initial values, with the same isosbestic point. It is known in fact (Bidegaray and Viovy, 1961) that both temperature and ethanol interfere with the aggregation of the dye.

The fluorescence of the dye is also sensitive to the effect of different solvents. In Figures 4A and 4B is shown the dependence of the quantum yield on the polarity and viscosity of the solvent. Both a decrease in polarity (measured by the Z value of Kosower, 1958) and an increase in viscosity increased the quantum efficiency of fluorescence. Fluorescence increase was seen also with other viscous solutions, such as ethylene glycol and glycerin. Figure 4B shows also that the fluorescence polarization increased with the viscosity of the medium. Therefore, both parameters, quantum yield and polarization, are markedly dependent on the degree of immobilization of

[1] Read in *Chemical Abstracts*.

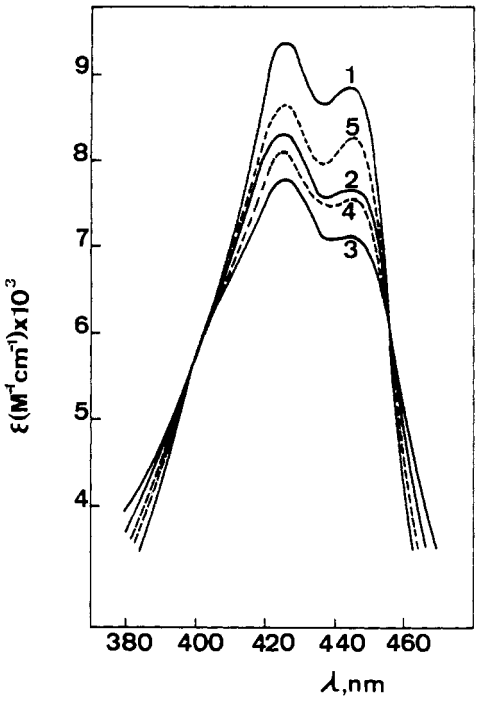

FIGURE 3: Absorbance spectra of atebrin at various concentrations. Atebrin absorbance was measured in cells of variable thickness (1 cm–0.1 mm), so that the product optical path × dye concentration was kept constant; spectra: (1) 100 μM atebrin in water; (2) 5 mM atebrin in water; (3) 10 mM atebrin in water; (4) 10 mM atebrin in 20% ethanol solution; (5) 10 mM atebrin in 40% ethanol solution.

the dye. The enhancement due to viscosity may be dependent on several factors, like rearrangement of excited state, rotation of lateral groups, dynamic quenching, and solvent structuration. At present it is impossible to determine which of these factors is more important. Moreover, by increasing the viscosity of the medium there is a small shift (2–3 nm) to longer wavelengths in the absorbance spectra and to shorter in the emission spectra. The same results were obtained decreasing the polarity of the medium. It is of interest that the values of acetic acid and dioxane do not fit into the general quantum yield *vs.* *Z* relationship, the value being higher in acetic acid and lower in dioxane. This indicates that the hydrogen bonds probably play some role.

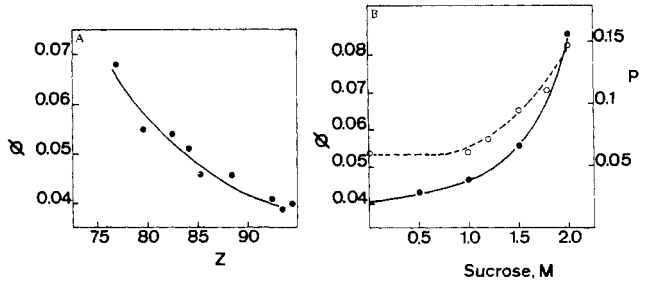

FIGURE 4: Quantum yield and fluorescence polarization of atebrin. (A) Quantum yield in respect to the solvent polarity scale. The solid points designate organic solvent–water mixtures as follows: the first point is isopropyl alcohol, the latter point is water, and the intermediate points are mixtures of ethanol–water and methanol–water. To all mixtures was added 1 μl of HCl (0.1 N). The quantum yield values do not change in the presence of larger amounts of HCl; 10 μM atebrin. (B) Quantum yield and fluorescence polarization in respect to the solvent viscosity. Atebrin (10 μM) was added to a solution containing various sucrose concentrations: (—) quantum yield values; (---) polarization values.

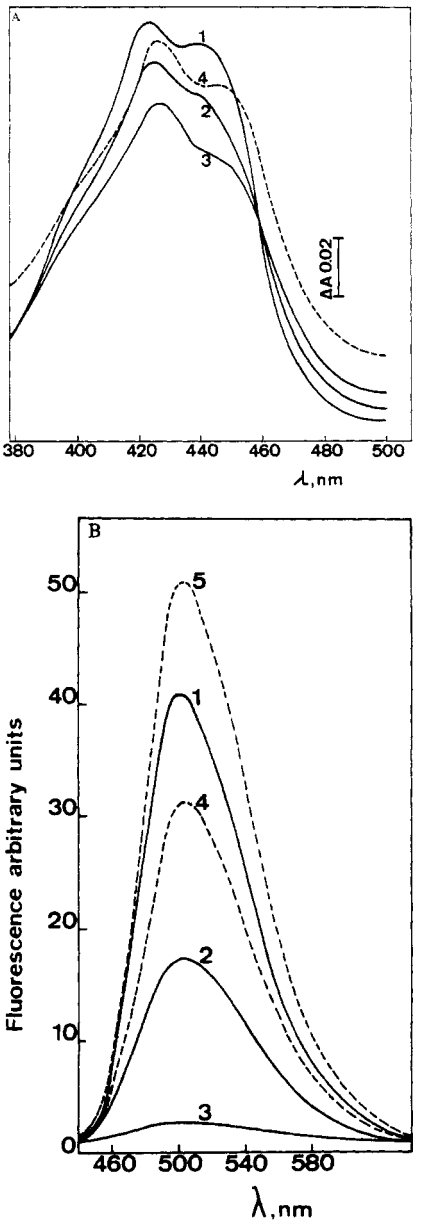

FIGURE 5: Absorbance and fluorescence spectra of atebrin with various amounts of polystyrenesulfonic acid. (A) The medium contained 0.1 M KCl, 5 mM $MgCl_2$, 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid) (pH 5.3), and 20 μM atebrin: (1) 0% polystyrenesulfonic acid; (2) 4×10^{-4} %; (3) 2×10^{-3} %; (4) 3.6×10^{-3} %; final volume, 2.5 ml. (B) The medium contained 10 mM Hepes (pH 5) and 4 μM atebrin: (1) 0% polystyrenesulfonic acid; (2) 8×10^{-5} %; (3) 1.6×10^{-4} %; (4) 1.9×10^{-3} %; (5) 4.3×10^{-3} %.

It is known that polystyrenesulfonic acid causes stacking of metachromatic compounds along the polyanion chain (Mukerjee and Ghosh, 1970; Pal and Schubert, 1963; Vitagliano and Costantino, 1970; Bergmann and O'Konski, 1963). Figures 5A and 5B show the absorbance and fluorescence spectra of atebrin in the presence of increasing concentrations of polystyrenesulfonic acid. The experiments were carried out at pH 5.3, where atebrin is more than 99% protonated. In Figure 5A there was a spectral shift with a more marked decrease at the longer wavelength band, and with an isosbestic point at 458 nm. At higher polystyrenesulfonic acid concentration there was a partial recovery of the initial absorbance. In Figure 5B there was a gradual quenching of fluorescence at low polystyrenesulfonic acid concentrations,

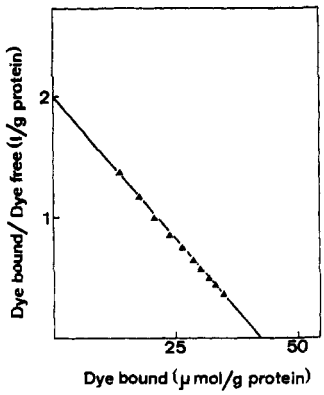

FIGURE 6: Scatchard plot for the binding of atebrin to energized particles. The incubation medium contained: 0.1 M LiCl, 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid) (pH 6.5), 5 mM $MgCl_2$, 2 mM ATP, and 2 mg of protein. Binding measurements are as described in the Experimental Section; final volume 2.5 ml.

while at high sulfonic acid concentration there was a marked fluorescence enhancement. The final fluorescence may also be greater than the initial fluorescence. As will be discussed later, the spectral change induced by the polystyrenesulfonic acid can be interpreted as due to the phenomena of dye–dye interactions promoted by the presence of negative sites in the polymer network.

Energization of submitochondrial particles results in a marked increase of binding of many aminophenazine and acridine dyes. Increased binding of atebrin has already been reported in the literature (Lee, 1971). Figure 6 shows a Scatchard plot for the active binding of atebrin to energized particles. The dissociation constant was 8 μM and the number of sites was ~43 μmol/g of protein at pH 6.5. With another acridine dye, such as Acridine Orange, there was a lower affinity and a similar number of sites (Dell'Antone *et al.*, 1971).

Figure 7 shows the absorbance spectra of atebrin during active binding to the energized particles. The spectral change increased with the amount of particles and was similar to that occurring during binding to polystyrenesulfonic acid. The isosbestic point was at about 450 nm.

Figure 8 shows the quenching of atebrin fluorescence during

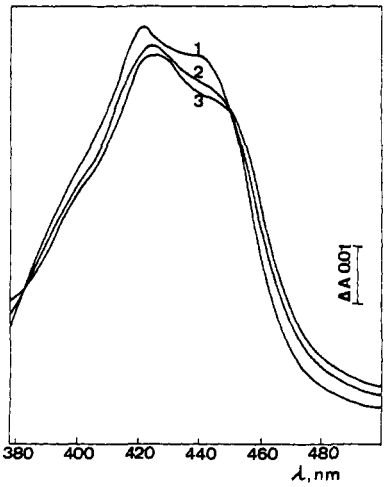

FIGURE 7: Absorbance spectra of atebrin in energized particles. The medium was the same as in Figure 6; 10 μM atebrin: (1) 0.6 mg of protein; (2) 0.6 mg of protein + 2 mM ATP; (3) 1.2 mg of protein + ATP.

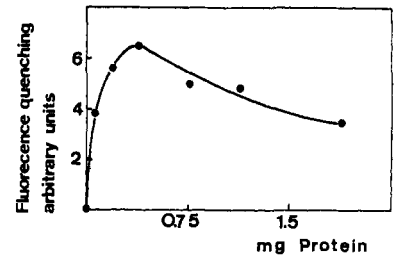

FIGURE 8: Dependence of atebrin fluorescence quenching on protein: dye ratios. The medium was the same as in Figure 6. The ordinate shows the atebrin fluorescence quenching, measured as the difference between the final and initial fluorescence, after particles energization, in arbitrary units; 2 μM atebrin; final volume, 2.5 ml. The excitation wavelength was 420 nm and the emission wavelength was 500 nm.

active binding to submitochondrial particles. The quenching was determined between the initial fluorescence before energization and the final fluorescence after reaching the steady state and is therefore the net of the two phases of enhancement and quenching. As the fluorescence changes of atebrin during interaction with the polystyrenesulfonic acid are markedly dependent on the site: dye ratio, so the changes occurring during active binding to submitochondrial particles are markedly dependent on the protein: dye ratio. At low protein concentration the quenching increased with the increase in the amount of protein. On the other hand, at higher protein concentration there is a decrease of the extent of quenching parallel to the increase of the protein concentration. This corresponds to the range where the amount of the dye bound remains roughly constant.

Figure 9 shows the kinetics of the fluorescence quenching at three different atebrin concentrations. At low dye concentration, energization of the particles resulted in a marked fluorescence enhancement, which was reversed by the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. At intermediate dye concentration there was a smaller phase of fluorescence enhancement, followed by a quenching phase. At high dye concentrations the enhancement phase was abolished and energization resulted only in the well known quenching. Similar effects were observed when the protein: dye ratio was changed by increasing the amount of particles at constant dye concentration (Azzone *et al.*, 1973).

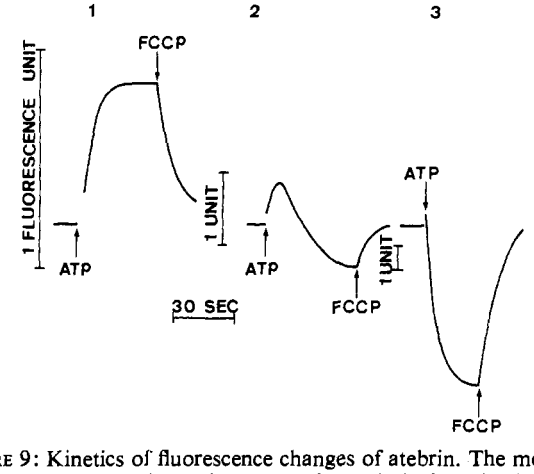

FIGURE 9: Kinetics of fluorescence changes of atebrin. The medium was the same as in Figure 6; 1.5 mg of protein in 2.5 ml. The reaction started with the addition of 1 mM ATP. After reaching the steady state, 2 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added: (1) 1 μM atebrin; (2) 4 μM atebrin; (3) 16 μM atebrin.

Polarization measurements were made at a high protein: dye ratio (1 μM dye in a solution containing 1.6 mg of protein/ml), where only enhancement of fluorescence was observed. In this condition nearly all dye molecules are bound in the energized membrane. Before the ATP addition, the polarization value was 0.08, indicating an enhancement of viscosity of the solution due to the presence of protein ($p = 0.06$ in water). After the ATP addition, the polarization raised to a value of 0.16, indicating a strong immobilization of the dye.

Discussion

The more accepted interpretation of the uptake and fluorescence quenching of atebrin in energy transduction membranes is that the accumulation occurs in the particle osmotic space and is driven by a pH gradient across the membrane. This mechanism is supported by the following observations. (a) Addition of 6 mM NH_4Cl to chloroplasts abolishes the fluorescence quenching of the dye but not the energization (Gromet-Elhanan, 1972); nigericin is a strong inhibitor of uptake and quenching in submitochondrial particles (Lee, 1971). (b) The acridine fluorescence quenching during chloroplast energization runs parallel to the uptake of methylamine, a weak base which is supposed to be distributed across the membrane dependent on a pH gradient (Shuldiner *et al.*, 1972). (c) The role of pH gradient is important also for the response of the dye fluorescence in liposomes (Deamer *et al.*, 1972). (d) Uptake and fluorescence quenching in submitochondrial particles are partially counteracted by weak anions (Lee, 1973). However, the present work suggests that the uptake and fluorescence quenching of atebrin in submitochondrial particles are due to intermolecular interaction of the dye bound to nucleophilic sites in the energized membrane.

As to the atebrin fluorescence quenching, a base–acid transition has been questioned for the following reasons. (1) A base–acid transition may account only for 50% of fluorescence quenching (Bening and Eilerman, 1973). (2) There is quenching also where 99% of the dye is already protonated (Azzone *et al.*, 1973). These arguments, however, do not take into account the possibility that a large quenching may occur during base–acid transition in a particular environment such as that of the membrane fabric. The acid–base mechanism is also incompatible with the following observations. (3) In the case of Acridine Orange, which gives quenching phenomena similar to that of atebrin, it is the deprotonation of the acridine ring which causes quenching (Azzone *et al.*, 1973). (4) Both in the case of atebrin and Acridine Orange (Dell'Antone *et al.*, 1971) the absorbance spectra give no indication of base–acid transitions.

As to the atebrin active uptake the conclusion of a binding to the membrane rather than an accumulation in the osmotic inner space is in accord with (1) the lack of parallel accumulation of counterions (Azzone *et al.*, 1973; Dell'Antone *et al.*, 1973), (2) the absence of an osmotic barrier in the case of liver particles (Azzone and Massari, 1972), (3) the analogy between the spectral changes during the acridine binding to energized particles and to polystyrenesulfonic acid (Dell'Antone *et al.*, 1972b; Azzone *et al.*, 1973), (4) the strong immobilization of the accumulated dye, and (5) the dependence of the fluorescence changes on the particle:dye ratios.

The nature of the binding is not, at the present, a well explained phenomenon. We suggest that the binding is due to both electrostatic and hydrophobic interactions. This suggestion is supported by the analogy between the absorbance changes of the acridines with the sulfonic acid and those with submitochondrial membranes (Dell'Antone *et al.*, 1972b; Azzone *et al.*, 1973). The role of the hydrophobic bond is supported by the phenomenon of the dilution of the dye along the sites (see below) which occurs only with polyanions with negative sites embedded in a hydrophobic environment. Furthermore, the dissociation constants of atebrin, Neutral Red, and Acridine Orange, presumably bound to the same sites, are 8, 2, and 1 μM at pH 7.0, respectively, which is an order of increasing hydrophobicity of the dyes (Dell'Antone *et al.*, 1972a).

The role of the strong anions may be rationalized in terms of changes of exposition of membrane groups (Dell'Antone *et al.*, 1973). The effect of weak anions advocated by Lee (1973) is not yet explained. It should, however, be remembered that the effect is negligible in the presence of strong anions. As to the quenching of acridine fluorescence in liposomes we think that the binding of atebrin to phospholipids plays an important role, and the dye cannot be used simply as a ΔpH indicator. Experiments are in progress in our laboratory on this aspect.

As pointed out previously (Colonna *et al.*, 1973) the uptake of cations in mitochondria and particles depends on the nature of the charge on the ligand. Indeed the active binding of atebrin and other protonated cations is peculiar of submitochondrial particles, while the active binding of nonprotonated cations, such as safranine, pinacyanole, and Ca^{2+}, is peculiar of mitochondrial membranes. These data suggest also that the inhibitory effect of NH_4^+ (Gromet-Elhanan, 1972) may be related to the specific role of the protonated cations in the interaction with membrane sites.

The decrease of fluorescence quenching parallel to the increase of the protein: dye ratios may be tentatively interpreted as follows. At low site: dye ratios, because of an excess of free dye, the increase of number of sites results in an increase of dye binding, with dye–dye interaction and quenching. At high site:dye ratios, where the amount of free dye is negligible, an increase in the number of sites results only in redistribution of the dye along the polyanion chain. The higher the site: dye ratio, the higher is the probability that the nearest neighbor is an empty site. This causes a decrease of dye–dye interaction (destacking effect) and of quenching.

Under conditions where the site: dye ratio is high the energy linked binding of atebrin is accompanied by a fluorescence enhancement. It has been proposed (Azzone *et al.*, 1973) that this enhancement is due to the presence of a certain amount of dimer in solution and to monomerization of the dimer when the dye penetrates into the membrane environment. This interpretation is, however, in contrast with the data of Figure 3 where, at a concentration of 2 μM, atebrin is present in solution almost exclusively in a monomeric form. A proton transfer between the protonated dye and the nucleophilic sites in the membrane is also unlikely in view of the absence of acid–base transitions in the excitation spectra with both energized submitochondrial particles and polystyrenesulfonic acid. Therefore, we consider more likely that the enhancement effect is an expression of the translocation of the dye from the water phase where the dye has a relatively low quantum yield to the membrane phase where the quantum yield is relatively high, since both a low polarity and a high viscosity increase considerably the quantum yield of the dye. In accord with this view are the polarization measurements which indicate that the dye molecules when bound at high site: dye ratios are held in considerable rigidity. The complex kinetics observed at high site: dye ratios may be an expression of two effects. The initial rapid enhancement phase is due to

the increase of quantum yield following the penetration of the dye into the membrane fabric. The second slower quenching phase is due to the interaction of the dye molecules occupying neighboring sites. An interesting difference between atebrin and Acridine Orange is the absence of the fluorescence enhancement in the case of the latter dye. Since the association of the dye molecules is stronger in the case of Acridine Orange (which is more hydrophobic) than with atebrin, it would seem that the stronger destacking effect in the case of atebrin is due to weaker dye–dye interaction forces.

In conclusion, we propose that the interaction of aminophenazine and acridine dyes with the energized particles is a binding of protonated ligands to nucleophilic sites situated in environments of low polarity and close enough to permit dye–dye interaction. Under conditions of dilution of the dye along the sites, the dye becomes a probe of the viscosity or polarity of the site.

References

Azzi, A., Fabbro, A., Santato, M., and Gherardini, P. L. (1971), *Eur. J. Biochem. 21*, 404.

Azzone, G. F., Colonna, R., Dell'Antone, P., Frigeri, L., and Massari, S. (1973), *in* Mechanisms in Bioenergetics, Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E., and Siliprandi, N., Ed., New York, N. Y., Academic Press, p 61.

Azzone, G. F., and Massari, S. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 28*, 61.

Bening, G. J., and Eilerman, L. J. M. (1973), *Biochim. Biophys. Acta 292*, 402.

Bergmann, K., and O'Konski, C. T. (1963), *J. Phys. Chem. 67*, 2169.

Bidegaray, J. P., and Viovy, R. (1964), *J. Chem. Phys. 41*, 1383.

Chen, R. F., Vurek, G. G., and Alexander, N. (1967), *Science 156*, 949.

Christophers, R. (1967), *Ann. Trop. Med. Parasitol. 31*, 43.

Colonna, R., Dell'Antone, P., and Azzone, G. F. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 10*, 13.

Colonna, R., Massari, S., and Azzone, G. F. (1973), *Eur. J. Biochem. 34*, 577.

Deamer, D. W., Prince, R. C., and Crofts, A. R. (1972), *Biochim. Biophys. Acta 274*, 323.

Dell'Antone, P., Colonna, R., and Azzone, G. F. (1971), *Biochim. Biophys. Acta 234*, 541.

Dell'Antone, P., Colonna, R., and Azzone, G. F. (1972a), *Eur. J. Biochem. 24*, 553.

Dell'Antone, P., Colonna, R., and Azzone, G. F. (1972b), *Eur. J. Biochem. 24*, 566.

Dell'Antone, P., Frigeri, L., and Azzone, G. F. (1973), *Eur. J. Biochem. 34*, 448.

Gromet-Elhanan, Z. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 124.

Gromet-Elhanan, Z. (1972), *Eur. J. Biochem. 25*, 84.

Handbook of Chemistry and Physics (1969), Cleveland, Ohio, Chemical Rubber Publishing Co.

Hermans, J. J., and Levinson, S. (1951), *J. Opt. Soc. Amer. 41*, 460.

Irvin, J. M., and Irvin, E. N. (1950), *J. Amer. Chem. Soc. 72*, 2745.

Kislyak, G. M., and Lisenko, G. M. (1960), *Fiz. Probl. Spektrosk., Mater. Soveshch. 13th 1*, 336.

Kosower, E. M. (1958), *J. Amer. Chem. Soc. 80*, 3253.

Kraayenhof, R. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 6*, 161.

Lee, C. P. (1971), *Biochemistry 10*, 4375.

Lee, C. P. (1973), *in* Mechanisms in Bioenergetics, Azzone, G. F., Ernster, L., Papa, S., Quagliariello, E., and Siliprandi, N., Ed., New York, N. Y., Academic Press, p 115.

Melhuish, W. H. (1961), *J. Phys. Chem. 65*, 229.

Mukerjee, P., and Ghosh, A. K. (1970), *J. Amer. Chem. Soc. 92*, 6403.

Pal, M. K., and Schubert, M. (1963), *J. Amer. Chem. Soc. 84*, 4384.

Parker, C. A., and Rees, W. T. (1960), *Analyst 85*, 587.

Schuldiner, S., Rottenberg, H., and Avron, M. (1972), *Eur. J. Biochem. 25*, 64.

Shinitzky, M., Dianoux, A. C., Gitler, C., and Weber, G. (1971), *Biochemistry 10*, 2106.

Vitagliano, V., and Costantino, L. (1970), *J. Phys. Chem. 74*, 197.

Weber, G., and Teale, F. W. J. (1957), *Trans. Faraday Soc. 53*, 646.

Zanker, V. (1952), *Z. Phys. Chem. 199*, 225.