ANTHROYL STEARATE AS A FLUORESCENT PROBE OF CHLOROPLAST MEMBRANES

D. L. VANDERMEULEN and GOVINDJEE

Departments of Physiology and Biophysics and Botany, University of Illinois, Urbana, Illinois 61801 (U.S.A.)

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SUMMARY

1. A reversible light-induced enhancement of the fluorescence of a "hydrophobic fluorophore", 12-(9-anthroyl)-stearic acid (anthroyl stearate), is observed with chloroplasts supporting phenazine methosulfate, cyclic or 1,1'-ethylene-2,2'-dipyridylium dibromide (Diquat) pseudo-cyclic electron flow; no fluorescence change is observed when methyl viologen or ferricyanide are used as electron acceptors. The stearic acid moiety of anthroyl stearate is important for its localization and fluorescence response in the thylakoid membrane, since structural analogs of anthroyl stearate lacking this group do not show the same response.

2. This effect is decreased under phosphorylating conditions (presence of ADP, P_i, Mg^{2+}), and completely inhibited by the uncoupler of phosphorylation NH_4Cl (5–10 mM), as well as the ionophores nigericin and gramicidin-D (both at $5 \cdot 10^{-8}M$). The $MgCl_2$ concentration dependence of the anthroyl stearate enhancement effect is identical to that previously observed for cyclic photophosphorylation, as well as for the formation of a "high energy intermediate". The anthroyl stearate fluorescence enhancement is inhibited by increasing concentrations of ionophores in parallel with the decrease in ATP synthesis, but is essentially unaffected by specific inhibitors (Dio-9 and phlorizin) of photophosphorylation; thus, it appears that anthroyl stearate monitors a component of the "high energy state" of the thylakoid membrane rather than a terminal phosphorylation step.

3. The light-induced anthroyl stearate fluorescence enhancement is suggested to monitor a proton gradient in the energized chloroplast because (a) similar enhancement can be produced by sudden injection of hydrogen ions in a solution of anthroyl stearate; (b) when the proton gradient is dissipated by gramicidin or nigericin light-induced anthroyl stearate fluorescence is eliminated; (c) when the proton gradient is dissipated by tetraphenylboron, light-induced anthroyl stearate fluorescence decreases, and (d) light-induced anthroyl stearate fluorescence change as a function of pH is qualitatively similar to that observed with other probes for a proton gradient (e.g. 9-aminoacridine). Furthermore, anthroyl stearate does not monitor H^+ uptake per se

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Abbreviations: Anthroyl stearate: 12-(9-anthroyl)-stearic acid; Diquat: 1,1'-ethylene-2,2'-dipyridylium dibromide.

because (a) the pH dependence of H^+ transport is different from that of the anthroyl stearate fluorescence change, and (b) tetraphenylboron, which does not inhibit H^+ uptake, reduces anthroyl stearate fluorescence.

Thus, anthroyl stearate appears to be a useful probe of a proton gradient supported by phenazine methosulfate or Diquat catalyzed electron flow and is the first "non-amine" fluorescence probe utilized for this purpose in chloroplasts.

INTRODUCTION

The fluorescence probe anthroyl stearate was originally synthesized and partially characterized by Waggoner and Stryer [1], and has been used in a fluorescence energy transfer study of the association of the internal membrane protein with the lipid bilayer in influenza virus [2], and as a probe of the ubiquinone region of the mitochondrial membrane, possibly indicating a structural transition [3]. Based on a consideration of the structure of anthroyl stearate, X-ray analysis [4], spectroscopic studies [1, 5], and work done in other systems [2, 3], anthroyl stearate appeared to have a high probability of being located in a hydrophobic, relatively interior portion of the thylakoid membrane, and so might be useful in monitoring the events which are postulated to be involved in the mechanism of coupling in the energy conservation steps of photosynthesis. Lenard et al. [2] have pointed out in their study of viral lipid bilayers that the anthroyl group is sufficiently large that "precise localization within the hydrophobic region . . . is not possible."

The fluorescence changes of anthroyl stearate were recently associated with an energetic state of the membrane presumed to be involved in the coupling of phosphorylation to photosynthetic electron flow [6]. The light-induced anthroyl stearate fluorescence increase is inhibited by uncouplers of phosphorylation (e.g., NH_4Cl), suppressed by phosphorylating conditions (about 2/3 inhibition by 2 mM ADP+5 mM KH_2PO_4 in the presence of $MgCl_2$ at pH 8), and shown to be different from the response of the probe atebrin, another indicator of a membrane energized state [7].

A correlation [6] between anthroyl stearate fluorescence changes and a "diffusion-potential" stimulation of ATP synthesis [8] (by valinomycin in the presence of KCl) is probably not valid, since valinomycin with KCl was subsequently found to enhance anthroyl stearate fluorescence in the absence of chloroplasts (unpublished data of the authors). In this study, we present new evidence that changes in the fluorescence of anthroyl stearate in chloroplast suspensions are related to light-induced changes in membrane energization coupled to cyclic phenazine methosulfate or pseudocyclic (Diquat) electron flow. We conclude that these fluorescence changes are unrelated to the terminal phosphorylation step since Dio-9 and phlorizin are without effect on anthroyl stearate fluorescence. However, anthroyl stearate fluorescence enhancement decreases in parallel with phosphorylation when increasing concentrations of "uncouplers" (gramicidin and nigericin) are added to chloroplasts. Comparisons between anthroyl stearate and analogs suggest that the stearate moiety is necessary for the response. We also report new data on differences between responses of anthroyl stearate and other fluorescent probes to energization. Finally, our experiments show that anthroyl stearate mainly monitors the proton gradient in the phenazine methosulfate/Diquat system.

METHODS AND MATERIALS

Class II chloroplasts were isolated from spinach (*Spinacea oleracea*), oats (*Avena sativa*) or lettuce (*Lactuca sativa*) and resuspended in the homogenizing medium: 50 mM phosphate buffer, pH 7.8, containing 10 mM NaCl and 400 mM sucrose (see ref. 9). Chlorophyll concentration was determined by the method of MacKinney [10], with a Cary 14 spectrophotometer.

Unless otherwise stated, the reaction mixture contained 3.3 mM NaH_2PO_4, 3.3 mM $MgCl_2$, 16.7 mM KCl, 10 μM anthroyl stearate and chloroplasts equivalent to 15–20 μg chlorophyll/ml, adjusted to pH 8 with NaOH. Anthroyl stearate was incubated with chloroplasts for about 1 min with thorough mixing; the exact incubation time was not critical. Electron flow was catalyzed by phenazine methosulfate (5–10 μM), unless otherwise indicated. Spectrofluorimetric measurements of probes were performed as described previously [6, 9]; excitation was at 365 nm, and emission monitored at 460 nm (anthroyl stearate, anthroyl stearate analogs, 9-aminoacridine) or 510 nm (atebrin). Saturating actinic light was passed through several filters (Corning C.S. 2-59, C.S. 3-73, a No. 7440 heat glass and 5 cm water) and was blocked from the measuring monochromator with a Corning C.S. 4-96 filter. Fluorescence data are presented in arbitrary units and the scales of different figures are not directly comparable.

Phenazine methosulfate shows some fluorescence* under these conditions and undergoes a small (10–15 %) light-induced fluorescence quenching, but this introduces only a small contribution to the total observed fluorescence measured at the given wavelengths; moreover, this phenazine methosulfate fluorescence change was found to be pH independent in contrast to the fluorescence of the probes.

Thorough mixing (complete in about 2–3 s) of added chemicals was insured by use of a 1-cm plastic cell stirrer, explaining the loss of some information observed in some figures. High speed centrifugation was accomplished with a Beckman model L3-50 ultracentrifuge. Photophosphorylation and light-induced proton flux were measured essentially as described by Dilley [11].

The chemicals anthroyl stearate ADP, ATP, phenazine methosulfate, dithioerythritol, 9-aminoacridine and atebrin (quinacrine) were purchased from Sigma Chemical Co., St. Louis; 9-methylanthracene and 9-anthroic acid were from Eastman Organic Chemicals, Rochester, N.Y.; nigericin was from Eli Lilly Laboratories, Indianapolis; gramicidin was from Calbiochem, Los Angeles; and phlorizin was from Aldrich Chemical Co., Milwaukee.

RESULTS

General characterization of anthroyl stearate response in chloroplasts

The light-induced enhancement of anthroyl stearate fluorescence in lettuce chloroplasts supporting phenazine methosulfate cyclic electron flow is shown to be

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* When an excess (1000 times) of the reducing compound dithioerythritol is added, the intensity of phenazine methosulfate fluorescence increases (approx. 30 %) and the fluorescence emission maximum undergoes a blue shift (20–25 nm) in solution at pH 8; thus, the small fluorescence quenching seen with phenazine methosulfate and chloroplasts may be due to an oxidation of reduced phenazine methosulfate.
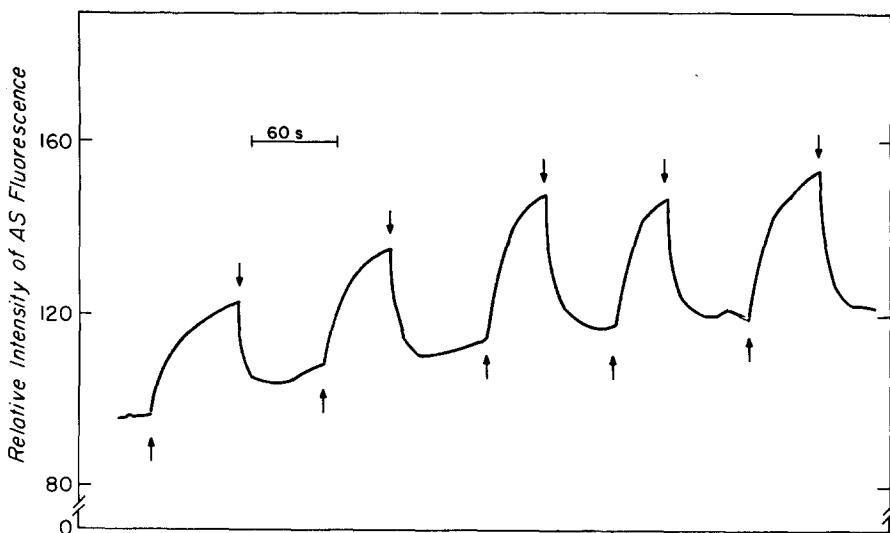
Fig. 1. Reversible light-induced anthroyl stearate (AS) fluorescence change with phenazine metho-sulfate (PMS) catalyzed cyclic electron flow in lettuce chloroplasts. [AS] $= 30\,\mu M$; [PMS] $= 10\,\mu M$; [chlorophyll] $= 9\,\mu M$. In this and following figures, unlabeled upward arrows: red actinic light on; downward arrows: light off.

reversible (Fig. 1), confirming similar results with spinach chloroplasts [6]. The small slow increase (which does not affect the anthroyl stearate enhancement effect, and levels off in a couple of minutes) in the "dark" anthroyl stearate fluorescence could conceivably reflect a slow movement of the probe in, onto, or off the thylakoid membrane; however, a light-induced response remains intact for a long time; clearly, no extensive migration occurs to place the predominant population of anthroyl stearate molecules in a different "site".

A similar light-induced enhancement of anthroyl stearate fluorescence was also observed when Diquat (5 μM) was employed in place of phenazine methosulfate; on the other hand, when ferricyanide (0.4 mM) or methyl viologen (0.1 mM) was used, little or no fluorescence change was measured (not shown).

In order to better determine how the anthroyl stearate molecule is interacting with the thylakoid membrane, and in particular which moieties of the compound are most significant in determining its placement or response in the membrane, we examined the response of structural analogs of anthroyl stearate to red light in chloroplasts. Neither 9-methyl anthracene nor 9-anthroic acid (anthracene-9-carboxylic acid), which possess methyl and carboxyl groups, respectively, instead of stearic acid, showed any light-induced change in fluorescence in samples that demonstrated the usual light-induced change in fluorescence with anthroyl stearate. The negative data obtained with the structural analogs of anthroyl stearate further minimizes the possibility that the observed light-induced enhancement of anthroyl stearate fluorescence is due to optical artifacts, as the two analogs used here have absorption and emission spectra similar to that of anthroyl stearate.

To determine whether or not formation of micelles of anthroyl stearate in aqueous solution is a complicating factor in our work, we measured the polarization
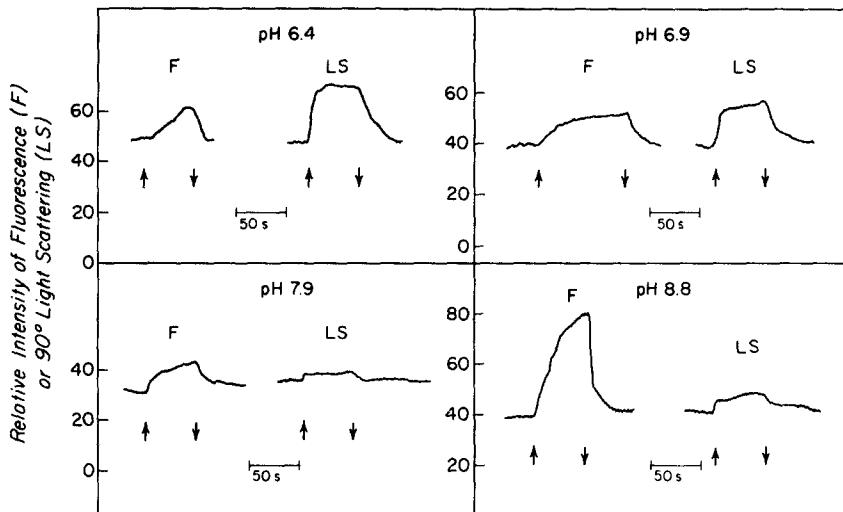
Fig. 2. Comparison of pH dependence of anthroyl stearate (AS) fluorescence enhancement and 90° light scattering with phenazine methosulfate (PMS) electron flow in lettuce chloroplasts. [AS] = 10 μM; [PMS] = 7.5 μM; [chlorophyll] = 15 μM. Fluorescence and light scattering measurements were made on the same sample and with the same instrument; light scattering changes were monitored both at 365 and 460 nm (at 460 nm in this Fig.), the wavelengths for excitation and measurement of anthroyl stearate fluorescence, respectively, yielding similar kinetics. Rise-times for fluorescence changes are 25 s (pH 6.4), 15 s (pH 6.9), 9 s (7.9), and 15 s (pH 8.8); rise-times for scattering changes were approx. 2–3 s for all pH values.

of anthroyl stearate fluorescence in three media: (1) 25 mM Tricine at pH 8; (2) 3.3 mM NaH_2PO_4, 3.3 mM $MgCl_2$, 15 mM KCl at pH 8; and (3) unbuffered water. In all three cases, we measured low values of polarization (approx. 3 %), quite constant over the concentration range 0.1 μM to 0.1 mM anthoryl stearate. Therefore, we judge that under the conditions of our experimentation (10 μM anthroyl stearate in aqueous buffer) no large aggregation of anthroyl stearate occurs (i.e., micelles are not formed) since one expects a substantial increase in polarization if the critical micelle concentration were exceeded and thereby restrict the rate of "rotational depolarization". One also expects the large anthracene ring to hinder substantially any tendency of stearic acid to aggregate as a micelle.

Since the light-induced enhancement of anthroyl stearate fluorescence observed here occurs in a fairly slow (seconds) time range, the possibility of an artifactual contribution by light scattering must be considered. A comparison of the kinetic traces from the anthroyl stearate fluorescence and light scattering changes at four different pH values is presented in Fig. 2. The half-rise time for enhancement in anthroyl stearate fluorescence varies between about 9 (pH 8) and 25 s (pH 6 and 9), whereas the half-time for the increase in scattered light intensity remains at about 2–3 s over the pH range measured. Also, no quantitative correlation is observed between the magnitude of the light scattering and fluorescence changes. For example, a low light scattering change may be accompanied by a high or low fluorescence change (see bottom curves, Fig. 2). It seems evident that the anthroyl stearate fluorescence in energized chloroplasts are not simply a trivial reflection of the structural changes responsible for the light scattering effects.

Anthroyl stearate fluorescence and "late" events in photophosphorylation

In order to better characterize what aspect of the high energy state the anthroyl stearate probe is reflecting, comparative experiments were performed with atebrin; the quenching of atebrin fluorescence has been suggested to reflect the rate of energy generation in chloroplasts [7]. As shown by Kraayenhof [7], the light-induced quenching of atebrin fluorescence is maintained in the dark by adding 3 mM ATP after preillumination in presence of magnesium and dithioerythritol (Fig. 3, top curves). This post-illumination quenching is reversed by Dio-9, a specific inhibitor of terminal steps in photophosphorylation, at concentrations inhibitory to the ATPase. In contrast, the addition of 3 mM ATP does not maintain the light-induced enhancement of anthroyl stearate fluorescence (Fig. 3, bottom curves)*. It appears that any high energy state produced by ATP hydrolysis and accessible to anthroyl stearate is not identical to that reported by atebrin quenching. The differences observed in kinetics and in the previously observed effects of valinomycin on the fluorescence response of these two probes support this conclusion [6].

Phlorizin and Dio-9 have been shown to be specific inhibitors of a terminal step in photophosphorylation [12, 13]. At concentrations of these compounds that substantially suppress the rate of phosphorylation, the light-induced enhancement of anthroyl stearate fluorescence is affected to only a small extent (Fig. 4). Therefore, the light-induced enhancement of anthroyl stearate fluorescence is not directly correlated with the late steps in the overall process of photophosphorylation; the following sections, however, show that the anthroyl stearate fluorescence effect is correlated with an earlier step(s).

Anthroyl stearate fluorescence and ion transport in thylakoid membranes

Hind et al. [14], using cyclic electron flow, found that most of the charge transferred as H^+ in the light was compensated by Mg^{2+} and Cl^- fluxes. The light-induced enhancement of anthroyl stearate fluorescence in chloroplasts is an increasing function of $MgCl_2$ concentration (Fig. 5). The small effect of increasing sucrose concentration in the medium (no salt present) shows that only a small portion of the effect could be osmotic. (It is important to note that with the buffer used in the experiments of Fig. 5, the fluorescence intensity of anthroyl stearate, minus chloroplasts, showed relatively little change with $MgCl_2$ concentration up to 30 mM.) The light-induced enhancement of anthroyl stearate fluorescence saturates at 5–10 mM $MgCl_2$ (Fig. 5), just as for the rate of photophosphorylation catalyzed by phenazine methosulfate electron flow [15]. Similarly, the formation of a "non-phosphorylated intermediate" or "high energy intermediate", as determined in two-stage (light-dark) photophosphorylation experiments, was found to increase with $MgCl_2$ added in the light state, again saturating at 5–10 mM [15].

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* Further exposure of chloroplasts to light produced a smaller increase, consistent with an ADP (produced by ATPase action) inhibition of the light-induced enhancement of anthroyl stearate fluorescence; however, the estimated amount of ADP produced in this time period (approx. 0.01 mM) probably cannot account for all of the inhibition seen here, as judged from a comparison with the ADP concentration curve. But large amounts of ATP were shown to produce some inhibition of the anthroyl stearate enhancement, accounting for the remainder of the reduction in enhancement. This ATP effect is relatively "non-specific", however, since the same percent inhibition is seen with ADP at a 50 times lower concentration (not shown).
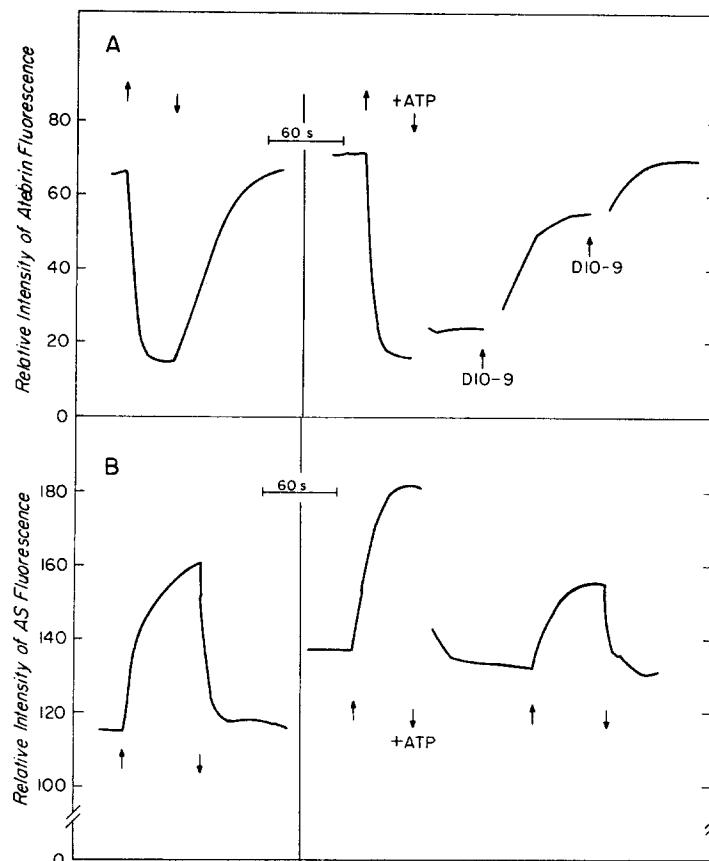
Fig. 3. The effect of an ATP-induced high energy state on anthroyl stearate and atebrin fluorescence in oat chloroplasts. [ATP] = 3 mM was added at the moment the actinic light was turned off. [Dio-9] = 3 μg/ml; [dithioerythritol] = 3 mM. Atebrin (10 μM) was present in (A) and anthroyl stearate (15 μM) in (B).

High levels of proton uptake by energized thylakoid membranes are known to occur in the time range of the observed anthroyl stearate fluorescence enhancement, and so we measured this change in chloroplasts as a function of pH (Fig. 6). The percent change in the fluorescence of anthroyl stearate increases with pH in the 6.0–8.5 range. The "dark" fluorescence of anthroyl stearate in the presence of chloroplasts is virtually constant from pH 7.0–9.5 and increases somewhat below 7.0 (Fig. 7), which could slightly affect the curve for anthroyl stearate fluorescence in the lowest pH range of Fig. 6. With the measurement conditions of Fig. 6, the pH dependence of net light-induced proton transport (Fig. 8) is distinct from the pH curve for anthroyl stearate fluorescence (Fig. 6).

The data of Fig. 6 confirms the known changes in two other fluorescence probes, atebrin and 9-aminoacridine (see Schuldiner et al., ref. 16, for the pH dependence of atebrin fluorescence quenching). Quenching of the fluorescence of 9-amino-acridine and atebrin has been correlated with proton gradients in membrane systems,
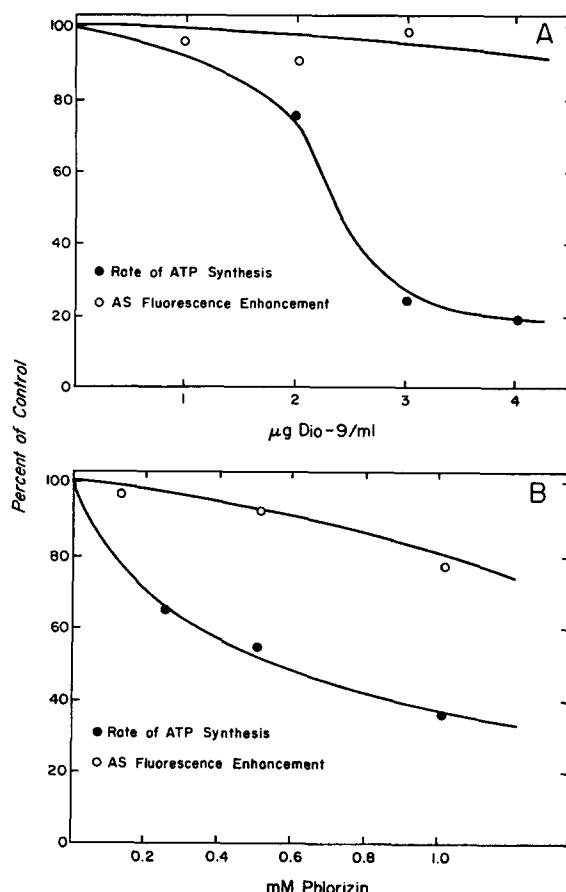
Fig. 4. Effects of the photophosphorylation inhibitors Dio-9 (A) and phlorizin (B) on the rate of ATP synthesis and on anthroyl stearate fluorescence change in spinach chloroplasts supporting phenazine methosulfate cyclic electron flow. Phlorizin was dissolved in ethanol stock solution, but the final ethanol concentration in the chloroplast suspension at the highest concentration used amounted to only 1 %, which we found in control experiments to produce an inhibition of ATP synthesis or anthroyl stearate fluorescence change of 10 % or less. [Chlorophyll] = 20 μM.
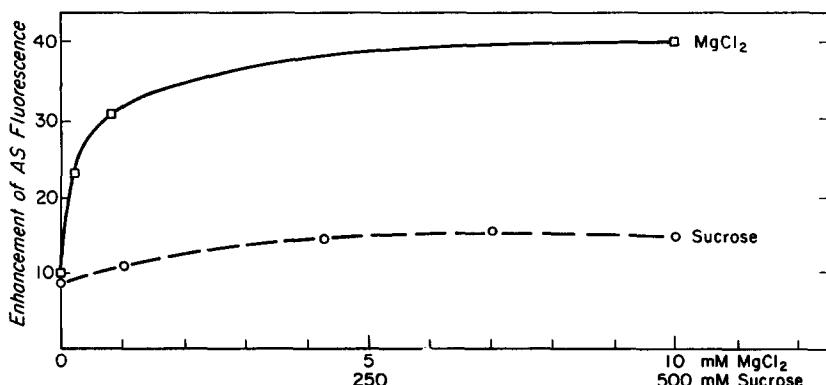

Fig. 5. Light-induced enhancement of anthroyl stearate fluorescence in lettuce chloroplasts as a function of [$MgCl_2$] ($\square-\square$), and sucrose ($O-O$). Suspending medium consisted of 25 mM Tricine, 7.5 μM phenazine methosulfate, adjusted to pH 8 with NaOH.
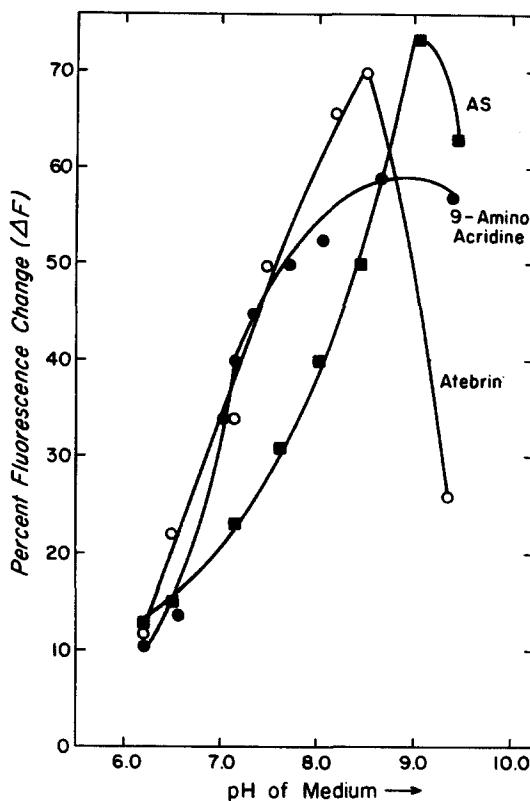
Fig. 6. pH dependence of the light-induced change in fluorescence of the probes anthroyl stearate (+ΔF), 9-aminoacridine (−ΔF) and atebrin (−ΔF), as indicated, in spinach chloroplasts. Probe concentration was 5 μM in all cases; other conditions as in Methods and Materials.
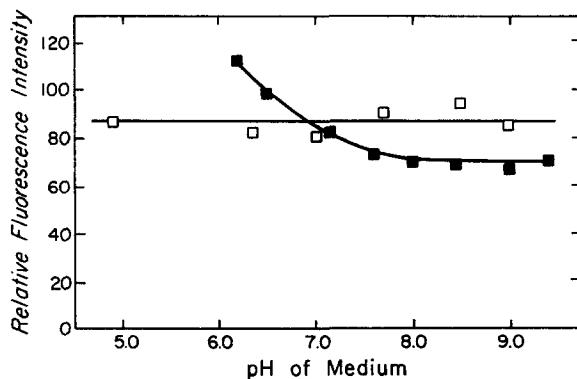

Fig. 7. pH dependence of the fluorescence of anthroyl stearate (10 μM) in 0.1 M phosphate-pyrophosphate buffer (□−□) and of anthroyl stearate (5 μM) in the presence of spinach chloroplasts, with the buffer medium given in Methods and Materials (■−■). Fluorescence values are in arbitrary units and not directly comparable for the two curves.
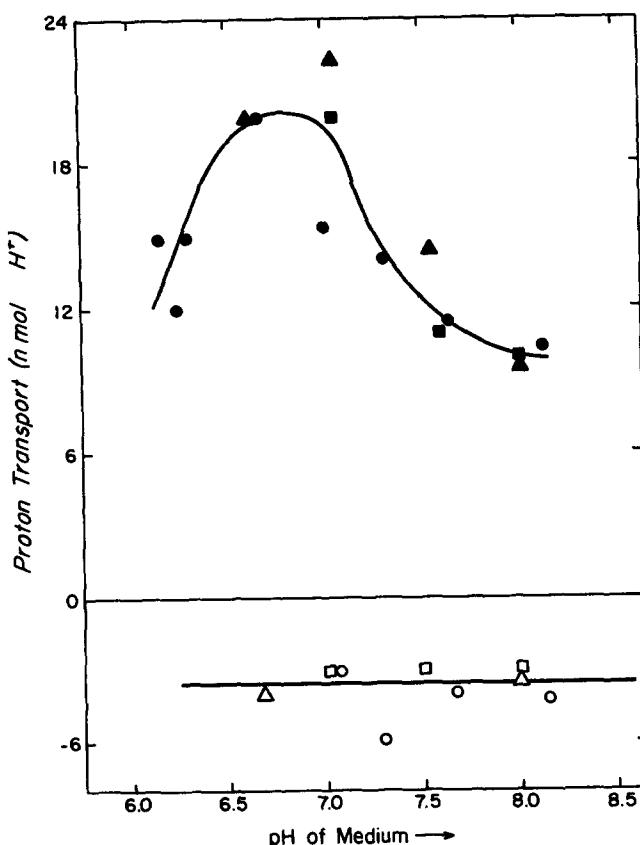
Fig. 8. pH dependence of the extent of light-induced proton transport in spinach chloroplasts catalyzing phenazine methosulfate cyclic electron flow. The curve represents an average of normalized data from three experiments. Closed symbols: H^+ uptake measured with 22 (▲), 21 (●) or 15 (■) μg chlorophyll/ml; open symbols: H^+ efflux in corresponding samples with nigericin added. If anthroyl stearate was present, the same results were obtained.

with 9-aminoacridine being a more quantitative probe [17]. Thus, it seems that anthroyl stearate may also be monitoring proton gradient.

In summary, the evidence presented so far indicates that the anthroyl stearate fluorescence change does not reflect transmembrane proton transport per se or a "late", ATPase-related step, but rather an energized state of the chloroplast membrane related to a proton gradient; this is investigated more fully in the next section.

Anthroyl stearate fluorescence and proton gradients

Tetraphenylboron introduces an inhibition of the light-induced enhancement of anthroyl stearate fluorescence which is transient to a degree dependent on added concentration (Fig. 9). If the light-induced enhancement of anthroyl stearate fluorescence monitors a proton gradient, rather than proton transport as such (cf. Figs. 6
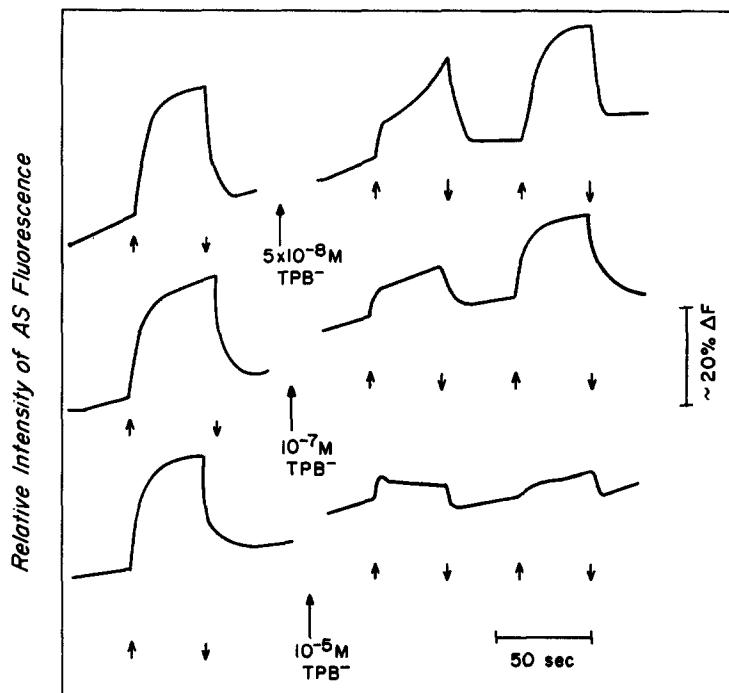
Fig. 9. Effects of the lipid-soluble anion tetraphenylboron on the anthroyl stearate (AS) fluorescence change in spinach chloroplasts supporting phenazine methosulfate cyclic electron flow. Additions of tetraphenylboron were as indicated. [chlorophyll] = 22 μM; [AS] = 10 μM.

and 8), then one would expect this to be reflected in the effects of tetraphenylboron. Fiolet and Van Dam [18] found that 12 μM tetraphenylboron did not suppress the light-induced proton uptake, but considerably inhibited the proton gradient as measured by 9-aminoacridine fluorescence quenching. Fig. 9 shows that with the addition of 10 μM tetraphenylboron the light-induced enhancement of anthroyl stearate fluorescence is less than 25 % of control. However, there is some difference in the response of anthroyl stearate and 9-aminoacridine to tetraphenylboron. The inhibition by 10 μM of the 9-aminoacridine quenching was more "transient" in nature, since 1 min after addition of tetraphenylboron the fluorescence level was nearly restored to the control level, whereas under similar conditions the light-induced enhancement of anthroyl stearate fluorescence remained low (Fig. 9, bottom curve).

The ionophores gramicidin-D and nigericin are also known to dissipate the pH component of the "protonmotive force" [19–21]. The data of Fig. 10 further strengthens the correlation between anthroyl stearate fluorescence changes and a proton gradient; the concentration curves for the two ionophores for the inhibition of net ATP production and the light-induced enhancement are parallel.

To aid in evaluating the possibility that a proton gradient itself is producing the observed light-induced change in anthroyl stearate fluorescence, a sudden change in a proton concentration was produced in a solution of anthroyl stearate; a rapid
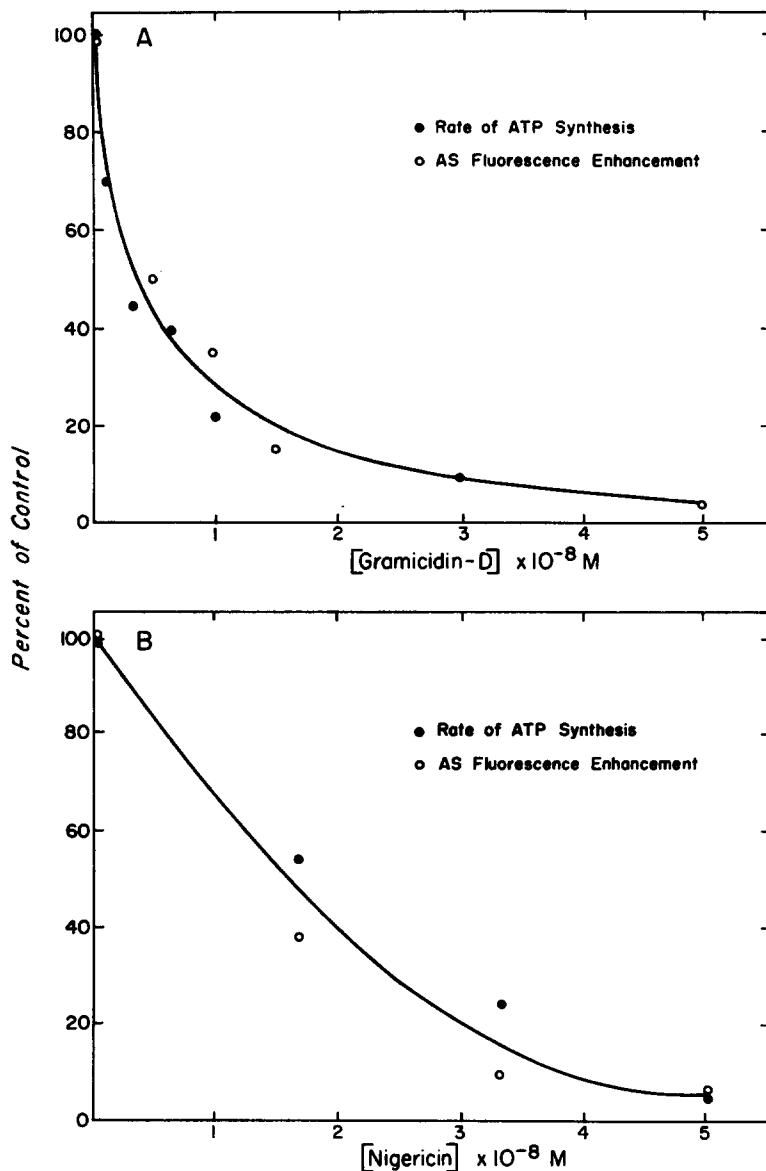
Fig. 10. Effects of the ionophores gramicidin-D (A) and nigericin (B) on the rate of ATP synthesis and anthroyl stearate fluorescence change in spinach chloroplasts supporting phenazine methosulfate cyclic electron flow. [chlorophyll] = 20 μM.

change in the intensity of anthroyl stearate fluorescence was observed (Fig. 11)*. On the other hand, if anthroyl stearate is suspended in well-buffered solutions (minus chloroplasts) covering the pH range 5–9, no variation in fluorescence is seen (Fig. 7, open symbols). The "baseline" fluorescence of anthroyl stearate in the presence of chloroplasts is also constant in the pH range 7.0–9.5; there is a fluorescence increase below neutral pH (Fig. 7, closed symbols), but it is small on the scale of Fig. 11. Thus, anthroyl stearate monitors a sudden change in pH.

In view of the acid-base experiment of Fig. 11, a rapid change in the ionic composition was applied to a solution of free anthroyl stearate by rapidly injecting 0.5 mmol NaCl into the cuvette (injection of buffer produced no fluorescence change). With this procedure, some enhancement in anthroyl stearate fluorescence was observed, but was much smaller (about 10 times under these conditions) and more transient than that induced by a pH jump. Also, the fluorescence intensity of anthroyl stearate (minus chloroplasts) was found to be unaffected by varying the NaCl concentration between 0 and 1.0 M when in a buffer solution (25 mM Tricine, pH 8; not shown). Thus, it appears that the major effect of the acid-base change on anthroyl stearate fluorescence is due to pH specifically, though other effects are not conclusively ruled out.

DISCUSSION

The absence of light-induced fluorescence change observed with the structural analogs of anthroyl stearate (9-methyl anthracene and anthroic acid) minimizes the possibility that the observed anthroyl stearate fluorescence changes are due to optical artifacts since there should be no chemical specificity for some of these effects when comparing compounds of similar spectroscopic properties.

Our "structural analog" data further suggest: (1) the carboxyl group alone (also incorporated in the stearate) is not the crucial factor; (2) hydrophobicity alone is not a sufficient requirement (9-methyl anthracene is less water soluble than anthroyl stearate); and (3) the stearate moiety is significant in determining the fluorescence response to the light-induced perturbation (e.g., proton gradient).

Anthroyl stearate probes the phenazine methosulfate/Diquat system

In this study we have demonstrated that the anthroyl stearate fluorescence enhancement is observed when phenazine methosulfate or Diquat, but not ferricyanide or methyl viologen, are used to support light-induced energization. Based on an extensive study of the photoreactions supported by Diquat in isolated chloroplasts [22], it was shown that under the aerobic conditions of our experiments, Diquat competively inhibits photophosphorylation catalyzed by phenazine methosulfate. This data, when combined with the interpretation of results from the interaction of Diquat and inhibitors and cofactors of photophosphorylation placed Diquat at the

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* We suggest that high local concentrations of positive charge, in becoming bound to the negatively charged moiety of the anthroyl stearate molecule, may overcome an "activation energy barrier" and thereby encourage formation of aggregates (micelles?), and an enhancement in the quantum yield of fluorescence as the local hydrophobicity increases. This interpretation for in vitro experiments does not establish or necessitate that the same effects are responsible for changes in anthroyl stearate fluorescence observed in the membrane-mediated case.

same site as phenazine methosulfate in the electron transfer path of chloroplasts [22]. Thus, it appears that phenazine methosulfate rather selectively monitors a particular aspect (proton gradient, see below) of the high energy state in the phenazine methosulfate or Diquat system. The possibility that anthroyl stearate is able to penetrate only the stroma lamellae but does not enter the grana region cannot be eliminated here.

What is inherent about the cyclic systems tested here that make possible the anthroyl stearate fluorescence response? This probably cannot be deduced on the basis of the present evidence, but the type of specificity observed in the results of this paper is consistent with the concept of phosphorylation coupling sites of differing characteristics that has been established [23–25]. Cyclic photophosphorylation has previously been thought to be associated with a separate coupling site, distinct from those in the main electron transport chain [25]. Studies of Photosystem I reactions using diaminodurene, diaminotoluene, and reduced 2,6-dichlorophenolindophenol as donors with methylviologen as acceptor suggested to Gould [26] that the site of cyclic photophosphorylation and coupling Site 1 of the non-cyclic electron transport system are identical; however, since cofactors such as phenazine methosulfate, Diquat, or pyocyanine were not tested and since an added acceptor was utilized, one probably cannot extrapolate to a "pure" cyclic system. These points clearly deserve further study.

Anthroyl stearate monitors an early step of energy transduction

In this work the fluorescence of anthroyl stearate has been shown to monitor an early high energy state of the membrane, rather than a late step of the energy transduction process (Figs. 3, 4, 9 and 10), because terminal inhibitors (Dio-9 and phlorizin), in contrast to uncouplers of phosphorylation (NH_4Cl, nigericin and gramicidin), do not affect the anthroyl stearate fluorescence response. The fact that the various fluorescence probes (anthroyl stearate, atebrin, 9-aminoacridine) of an "energized state" are different in certain respects (Figs. 3, 6, 9; ref. 6) may, upon further investigation, prove to be useful as a tool in better understanding the different steps of the energy transduction process. The differences in the pH profiles may be related to their specific localization in the membrane (e.g., anthroyl stearate responds only in the phenazine methosulfate or Diquat system), as well as their chemically inherent responses to the perturbation (proton gradient) induced by light.

Anthroyl stearate as a probe of a proton gradient

Now we turn to possible physical causes of the observed light-induced changes of anthroyl stearate fluorescence. The possibility that weak acids as well as amines (such as 9-aminoacridine or atebrin) could serve as fluorescent probes of a proton gradient across membranes has been suggested [17]. Though we cannot rule it out, on the basis of the evidence presented in this paper it seems unlikely that the mechanism for the light-induced fluorescence change of anthroyl stearate fluorescence is the same as that postulated for the amines, i.e., transfer of the molecules across the membrane to the inner (locular) volume, since: (1) anthracene-9-carboxylic acid, lacking the bulky stearate hydrocarbon chain of anthroyl stearate does not show the fluorescent response to light, and (2) the change in anthroyl stearate fluorescence is an enhancement, rather than quenching as expected either by screening, self-quenching or loss of energy by interaction with other molecules if the probe accumulated in the inner spaces.
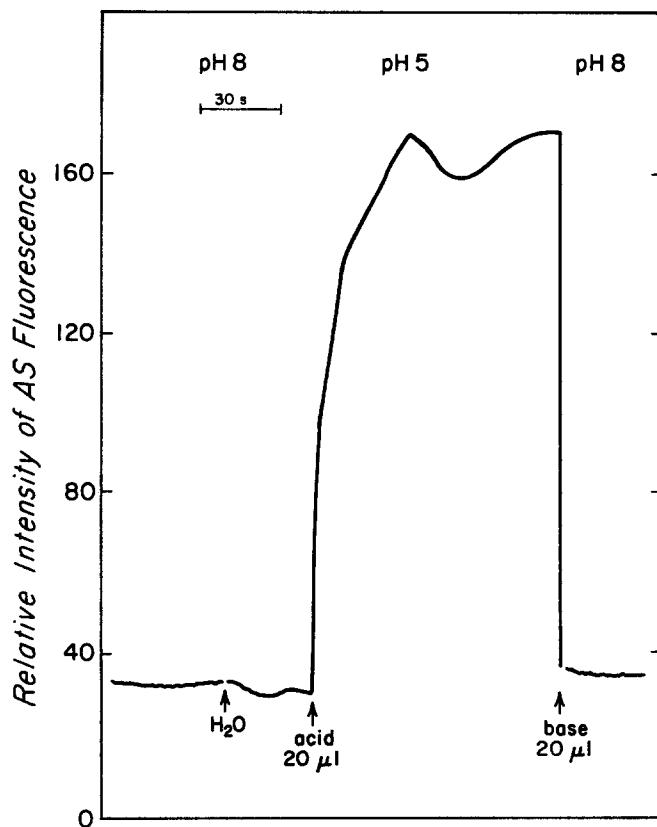
Fig. 11. Reversible hydrogen ion induced changes in the fluorescence of anthroyl stearate in vitro. The pH of the medium (see Methods and Materials for buffer, without chloroplasts) was rapidly shifted by injection of the acid (0.2 M succinic) or base (1 M Tris) in the specified volumes; 1-ml sample was used. The final medium pH after each addition (determined in control measurements) is indicated.

The in vitro experiments (Fig. 11) indicate that anthroyl stearate fluorescence responds to a rapid, sizable change in the concentration of protons of the molecules' immediate environment; in contrast, anthroyl stearate in solution is not affected by the $[H^+]$ per se (Fig. 7). Experiments with chloroplasts (Figs. 6–10) are consistent with these data. The inhibition of light-induced enhancement of anthroyl stearate fluorescence by ionophores suggests that the fluorescence change is dependent on a light-induced proton gradient (Fig. 10, ref. 6): the off kinetics (2–3 s at pH 8, Fig. 1) are consistent with a proton transport (necessary for creation and maintenance of gradient) related step (cf. data of Dilley, Fig. 2 in ref. 11).

The difference in the pH curves of Figs. 6 and 8 and the sensitivity of light-induced enhancement of anthroyl stearate fluorescence to tetraphenylboron show that proton uptake per se (which is insensitive to tetraphenylboron) is not being monitored. A fairly large pH change (2–3 units) is needed to produce an anthroyl stearate fluorescence change comparable to light-induced enhancement of anthroyl stearate fluorescence: this fact practically rules out the possibility that the light-induced enhancement

of anthroyl stearate fluorescence is due to a change in stromal [H$^+$] induced by light-stimulated proton transport, since these effects involve changes we measured to be 0.1 pH unit or less (not shown). In other words, if any changes in anthroyl stearate fluorescence are produced by such small changes in proton concentration, they would be too small to observe.

Tetraphenylboron is a highly penetrating, lipid-soluble anion, which has been shown to appreciably increase the electric conductance of phospholipid membranes [27]. A transient inhibition by tetraphenylboron of photophosphorylation and the light-induced quenching of 9-aminoacridine fluorescence in spinach chloroplasts has been reported [18]. A very low concentration of tetraphenylboron (50 nM) inhibits the "fast" phase of the anthroyl stearate enhancement trace. This suggests that this anthroyl stearate change is sensitive to the state of the membrane electric conductance, which in turn is reflected in ion transfer events. Such a change in membrane charge distribution can be correlated with a "proton gradient". It is possible that a more localized intramembrane effect is the immediate cause of the effects of tetraphenylboron on anthroyl stearate enhancement, since net transport of protons across the membrane remains unaffected [18]. One can thereby suggest that it is intramembrane changes in ionic distribution which are an essential feature of the role of ions in energy coupling. In this view, then, establishment of a transmembrane proton gradient does result in phosphorylation, but only as the internal proton activities in coupling regions are thereby changed. The energy from the gradient could be coupled to phosphorylation by the interaction of ions with specific enzyme complexes (e.g., Weber's concept of proteins as "free energy adders", ref. (28)). As a penetrating anion, tetraphenylboron could rapidly diffuse to the groups that become protonated upon illumination and thus interfere with their action. These suggestions are of course tentative and not exclusive of other interpretations.

In conclusion, based on the control experiments discussed above, it appears that it is a sizable change in the concentration of protons in the environment of the anthroyl stearate molecule, resulting from or concurrent with the gradient produced by light, which is important for the observed fluorescence change in chloroplasts. In energized chloroplasts the ionic composition of the suspending medium (e.g., concentration of protons or salt), the state of proton and ion transport in the thylakoid, and the electric conductance of the membrane are significant in determining the anthroyl stearate fluorescence response. As a working hypothesis, we suggest that anthroyl stearate fluorescence monitors light-induced reversible changes in the membrane's hydrogen ion distribution (not directly measurable by the glass electrode) localized in the phenazine methosulfate or Diquat system.

In terms of the actual molecular mechanism, one cannot yet distinguish whether the change in anthroyl stearate fluorescence results from (1) direct interaction of protons with anthroyl stearate molecule(s) producing the observed photochemical response (cf. the acid-base changes in vitro), or (2) an alteration in the membrane environment of the probe, for example, due to a change in the surrounding conformation or migration to another site. We expect that further research will provide information regarding this point and thereby enhance the usefulness of anthroyl stearate as a probe of thylakoid membranes.

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