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ON THE STIMULATION OF THE LIGHT-INDUCED PROTON UPTAKE BY UNCOUPLING AMINOACRIDINE DERIVATIVES IN SPINACH CHLOROPLASTS

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SUMMARY

1. Light-induced proton uptake by spinach chloroplasts is enhanced several-fold by 9-(4-diethylamino-1-methylbutylamino)-6-chloro-2-methoxyacridine (atebrin). This stimulation does not depend on the chlorophyll concentration. The amount of extra protons taken up in the presence of atebrin is determined by the pK_a values of atebrin and the pH of the incubation medium.

2. Both the stimulation of the proton uptake and the maximal binding capacity for atebrin is sensitive to uncouplers. However, the ratio of bound to free atebrin does not depend on the presence of uncoupler up to the saturating atebrin concentration.

3. From simultaneous kinetic measurements of atebrin fluorescence and proton movement it seems that after binding of the completely protonated atebrin the dye and the protons can move separately. This can also be inferred from the spectral behaviour of atebrin in illuminated chloroplasts.

4. The stimulation of the proton uptake by atebrin does not depend on the presence of salts in the incubation medium. However, the 'saturating' atebrin concentration increases strongly with increasing salt concentration in the medium.

5. It is concluded that the interaction of atebrin and other acridines with energized chloroplasts most likely occurs at the level of the membrane proper.

6. It is proposed that uncoupling by atebrin is a consequence of the creation of a high proton activity at the periphery of the thylakoid membrane, which opposes a proton gradient across the membrane. The uncoupling by atebrin is not of the protonophoric type according to this mechanism.

Abbreviations: Atebrin, 9-(4-diethylamino-1-methylbutylamino)-6-chloro-2-methoxyacridine; ACMA, 9-amino-6-chloro-2-methoxyacridine; PEA, 9-(propylaminoethylamino)-6-chloro-2-methoxyacridine; DMPA, 9-(3-methylaminopropylamino)-6-chloro-2-methoxyacridine; MBPA, methylamino-bis-(9-propylamino-6-chloro-2-methoxyacridine); S_{13}, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide.

INTRODUCTION

The integrity of energy-conserving membranes is a strict requirement for their proper functioning. This condition together with the very complicated nature of the energy-conserving apparatus makes its resolution extremely difficult. The introduction of fluorescent [1] and paramagnetic [2] probes as a new approach to this problem, soon led to the idea that physicochemical changes in uncoupler molecules, which directly interact with the energy-conserving system, may give additional information [3].

Based upon this idea the fluorescence behaviour of 9-(4-diethylamino-1-methylbutylamino)-6-chloro-2-methoxyacridine (atebrin) in chloroplasts was investigated and it was found that its fluorescence is quenched upon energization [3]. The usefulness of this fluorochrome as a sensitive energy probe has since been well established for other organelles, including coupled membrane particles of *Azotobacter vinelandii* [4], beef-heart mitochondria [5–7], chromatophores [8, 9] and subchloroplast particles [10]. Energization of all these organelles leads to a more or less complete quenching of the atebrin fluorescence, with a concomitant binding of the dye to the organelles. Similar fluorescence changes have been observed with other acridine dyes in both mitochondrial and chloroplast preparations [5, 11–13].

As to the mechanism of the uptake and fluorescence quenching of acridines two different interpretations exist: On the one hand an accumulation of the dye inside the osmotic space driven by a proton gradient across the membrane [9, 13, 14], on the other hand an electrostatic binding of the dye onto or inside the energized membrane [15–17]. These two interpretations have one feature in common; the acridine molecule resides in the organelle in the charged form, i.e. in the protonated form. This implies that the binding or uptake of acridines with pK_a values around the pH value of the incubation medium is accompanied by an extra uptake of protons. This extra proton uptake will be the subject of this paper. Preliminary results of these experiments have been reported elsewhere [18].

MATERIALS AND METHODS

Chloroplasts were prepared as previously described [19]. The total chlorophyll content was determined according to Whatley and Arnon [20]. The incubation medium contained 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM phosphate and *N*-tris (hydroxymethyl) methylglycine (tricine) buffer of pH 8.0. In proton uptake experiments the concentration of tricine was 1 mM, while under phosphorylating conditions 5 mM tricine was present.

The fluorescence and pH changes were measured simultaneously under continuous stirring in a cuvette with a total volume of 2.5 ml, thermostated at 20 °C and recorded on a multi-channel recorder (Rikadenki Kogyo). Side illumination was provided by a quartz-iodine lamp, the light of which was passed through a 5-cm water filter and a red cut-off filter (Schott RG 1).

The fluorescence was measured front-face. Monochromatic exciting light (420 nm) was provided by a Zeiss monochromator. The fluorescence emission was detected with an RCA photomultiplier (IP 28) screened by a Corning 9782 glass-filter plus a Wratten 57 filter.

The pH changes were measured with a micro-glass electrode (EIL GM 23/B) and a calomel reference electrode and amplified with a sensitive and rapid pH meter as described elsewhere [21]. The response time of the total pH-measuring system was less than 100 ms.

Fluorescence excitation spectra were measured on a fluorescence spectrophotometer (Perkin-Elmer MPF 2A). Absorption spectra were measured on a dual-wavelength spectrophotometer (American Instrument Co.).

Atebrin was purchased from Sigma, 9-aminoacridine from the British Drug Houses Ltd, while the other acridine derivatives mentioned below were synthesized by R.K.

5-Chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S_{13}) was kindly donated by Dr P. C. Hamm, Monsanto Comp., St. Louis, Mo. U.S.A.

RESULTS

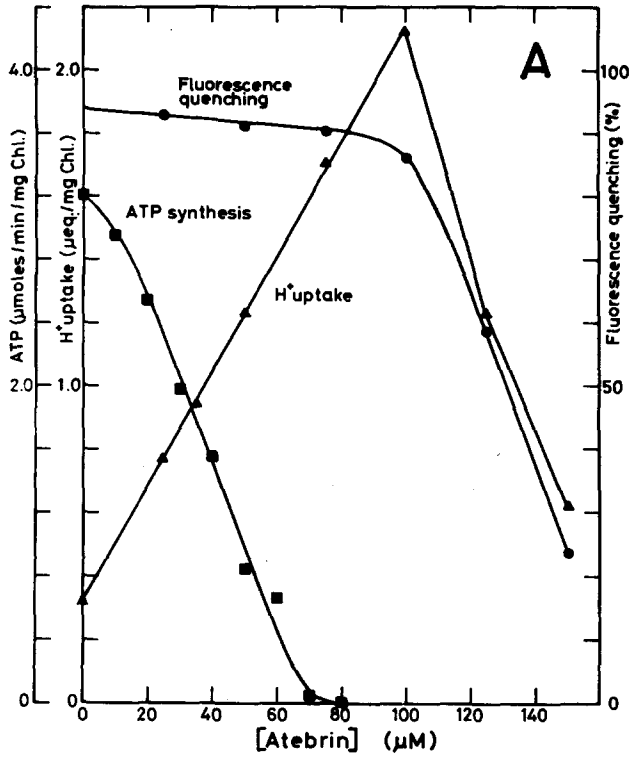
Fig. 1 shows the effect of atebrin on several energy-linked processes in illuminated chloroplasts. Atebrin, being an uncoupler of photophosphorylation, inhibits ATP synthesis (A) and stimulates State 4 electron transport (B). Upon illumination the fluorescence of atebrin is quenched almost completely up to the concentration that completely inhibits phosphorylation. Above this 'saturating' concentration the percentage of fluorescence quenching gradually decreases. The light-induced proton uptake is considerably enhanced with increasing concentrations of atebrin. This enhancement is linearly dependent on the concentration of atebrin. Maximal proton uptake occurs at the 'saturating' concentration. It may be noted that both maximal proton uptake and maximal inhibition of ATP synthesis coincide with the 'saturating' atebrin concentration under any condition tested.

Concomitant with these phenomena a substantial increase in light scattering at 545 nm is observed (not shown), which is in accordance with earlier observations [22, 23]. The light scattering changes have the same dependence on the atebrin concentration as the other energy-linked processes.

Fig. 2 shows that the percentage of the light-induced fluorescence quenching of 9-aminoacridine, when present at low concentrations, is increased by the addition of atebrin as well, concomitant with the stimulation of the proton uptake. The fluorescence emission of 9-aminoacridine in this experiment was detected at 430 nm and excited at 400 nm so that no contribution of atebrin fluorescence was present. Although the increase in the percentage of fluorescence quenching of 9-aminoacridine is only about 10 % it is highly reproducible. The addition of atebrin had no effects on either the fluorescence intensity or shape and position of the fluorescence excitation and emission spectra of 9-aminoacridine. Apparently neither 'inner-filter' effect nor energy transfer occurs under these conditions.

Fig. 3 shows that the enhancement of the proton uptake does not depend on the chlorophyll concentration; the slope of the curves representing proton uptake is similar for all atebrin concentrations, provided that the atebrin concentration is not yet 'saturating' at the particular chlorophyll concentration. The independence of the chlorophyll concentration suggests that the enhancement of the light-induced proton uptake is a basic property of the atebrin molecule itself.

Fig. 4 shows the pH dependence of the extra proton uptake induced by



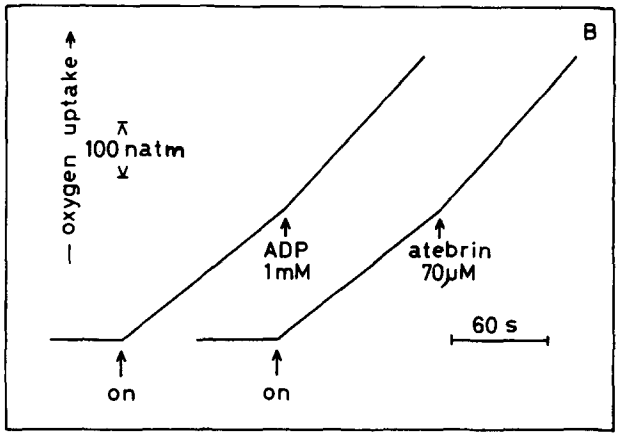


Fig. 1. (A) Inhibition of ATP synthesis and stimulation of the light-induced proton uptake by atebrin. Conditions as described in Materials and Methods. Under phosphorylating conditions 1 mM ADP was present. The chlorophyll content was 40 μg/ml. Pyocyanine (10 μM) was present as a cofactor for cyclic electron transport. (B) Stimulation of electron transport by atebrin. The incubation medium was as described in Materials and Methods. Electron transport was measured as oxygen uptake in the presence of 50 μM diquat plus 2 mM azide. The chlorophyll content was 30 μg per ml. The saturating atebrin concentration was about 70 μM under these conditions.

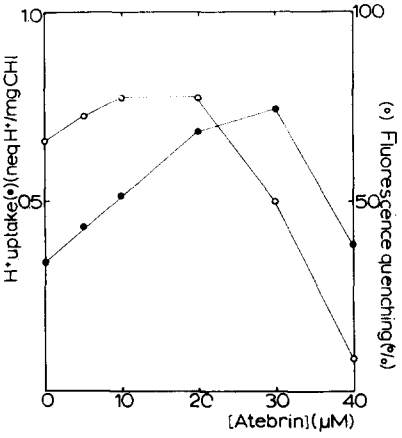


Fig. 2. Effect of atebrin on the light-induced fluorescence quenching of 9-aminoacridine and the proton uptake. Conditions as described in Fig. 1A. The 9-aminoacridine concentration was 5 μM. The chlorophyll content was 25 μg per ml.

atebrin. Fig. 4A demonstrates that this dependence decreases with decreasing pH of the incubation medium, whereas the binding of atebrin determined from the percentage of fluorescence quenching is hardly pH dependent. The results suggest that the extra proton uptake in the presence of atebrin is related to the pK_a values of the dye. This has been worked out in Fig. 4B, which shows the pH dependence of the proton uptake in the absence and presence of 20 μM atebrin. At pH values higher than 7.2 the proton uptake is stimulated by atebrin, whereas at the lower pH values the proton uptake is inhibited. Below pH 7.2 the percentage of fluorescence quenching

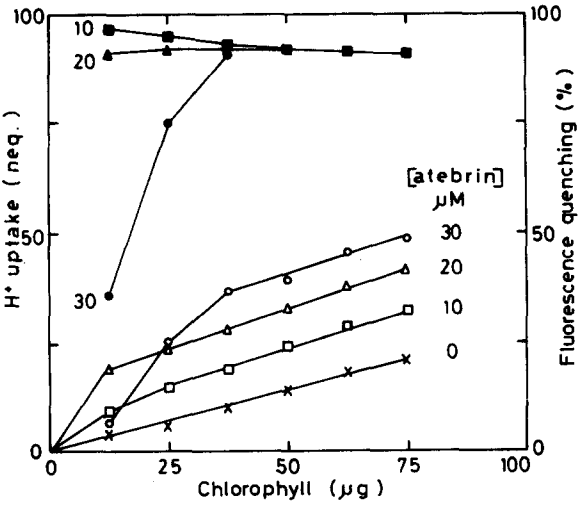


Fig. 3. The stimulatory effect of atebrin on the light-induced proton uptake as a function of the chlorophyll concentration. Conditions as described in Fig. 1A. The chlorophyll concentration is expressed as μg per 2.5 ml. Proton uptake is represented by the open symbols and fluorescence quenching by the closed symbols. Atebrin concentrations are as indicated in the figure.

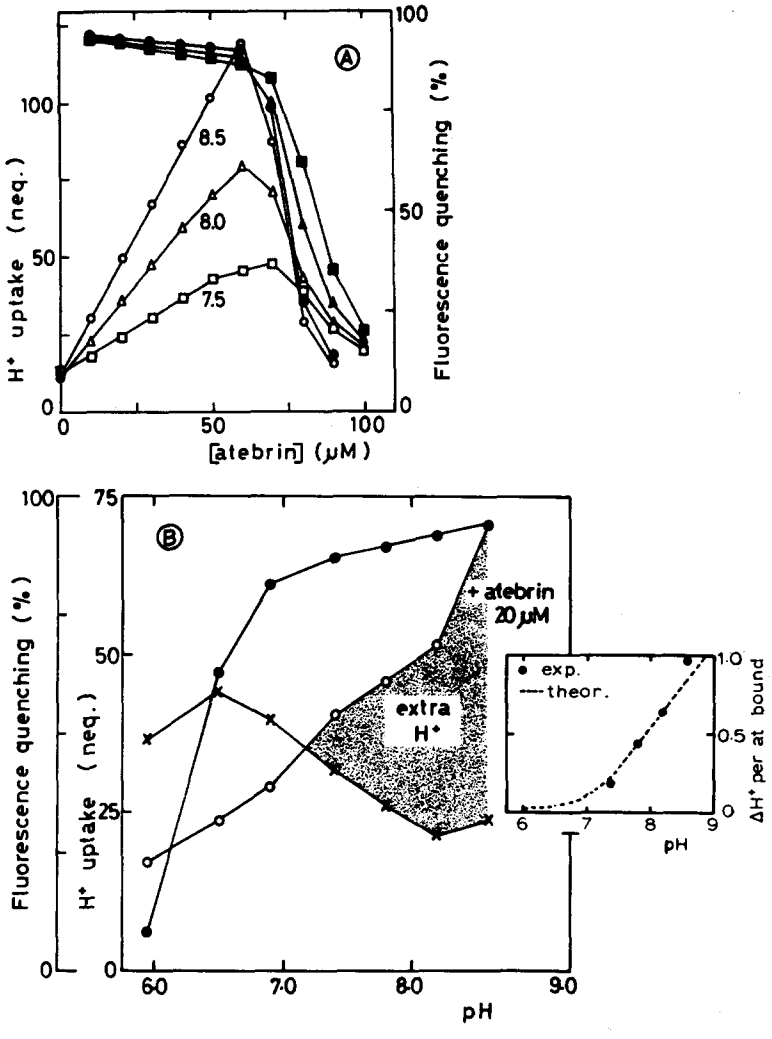


Fig. 4. pH dependence of the atebrin-induced extra proton uptake. (A) Titration with atebrin at the indicated pH values. Further conditions as described in Fig. 1A. The chlorophyll content was 20 μg per ml. The open symbols represent proton uptake, the closed symbols the fluorescence quenching. (B) The pH dependence of extra proton uptake in the presence of 20 μM atebrin. Conditions as described in Fig. 1A. Chlorophyll content, 35 μg/ml. Proton uptake, control ($\times - \times$) and in the presence of atebrin ($\circ - \circ$); fluorescence quenching ($\bullet - \bullet$). The closed symbols in the inset represent the experimentally determined H^+ over atebrin ratios.

decreases sharply as well, indicating that 20 μM atebrin under these conditions is over-saturating the system. Using the known pK_a values of 10.45 and 7.9, respectively [24, 25], the number of protons required for complete protonation per molecule of atebrin can be calculated for any pH value. This calculation yields the dashed curve in the inset of Fig. 4B. Assuming now that the fluorescence of every molecule of atebrin, bound to illuminated chloroplasts, is quenched completely, it is possible to determine the ratio of atebrin bound to the extra proton uptake (the shaded area

in Fig. 4B) at any pH. The closed circles in the inset of Fig. 4B give the results of this calculation. It is clear that the correlation between theoretical and experimentally determined values is quite good above pH 7.2.

Further evidence that the stimulation of the proton uptake by atebrin is governed by the pK_a values, is given by the effect of a series of other acridine derivatives on the light-induced proton uptake. It was found, that neither 9-aminoacridine nor 9-amino-6-chloro-2-methoxyacridine (ACMA), which are both monoamines with nucleus pK_a values of 10.0 and 8.9, respectively [25], enhanced the light-induced proton uptake. However, the diamines 9-(propylaminoethylamino)-6-chloro-2-methoxyacridine (PEA) and 9-(3-methylaminopropylamino)-6-chloro-2-methoxyacridine (DMPA), which have nucleus pK_a values of 7.3 and 7.8, respectively [25], and also methylamino-bis-(9-propylamino-6-chloro-2-methoxyacridine) (MBPA), which has two dissociable groups with pK_a values of 7.8, did stimulate the light-induced proton uptake in a pH-dependent way and to the expected extent. The stimulation by MBPA was about twice as effective as that by atebrin, which is expected on the basis of the number of 'protonizable' groups per molecule.

Fig. 5A shows that the extra proton uptake induced by atebrin is sensitive to uncoupling of photophosphorylation. The extra proton uptake is decreased in the presence of S_{13}; moreover the saturating atebrin concentration is lower under these conditions. However, the percentage of fluorescence quenching below the saturating atebrin concentration is similar in the absence and presence of S_{13}, indicating that the number of atebrin molecules bound to the chloroplasts is hardly affected by the presence of the uncoupler. Fig. 5B shows the dependence of the light-induced proton uptake in the presence and absence of 20 μM atebrin and the fluorescence quenching on the concentration of S_{13}. It appears that the proton uptake in the presence of atebrin decreases with increasing S_{13} concentration. However, it is enhanced with

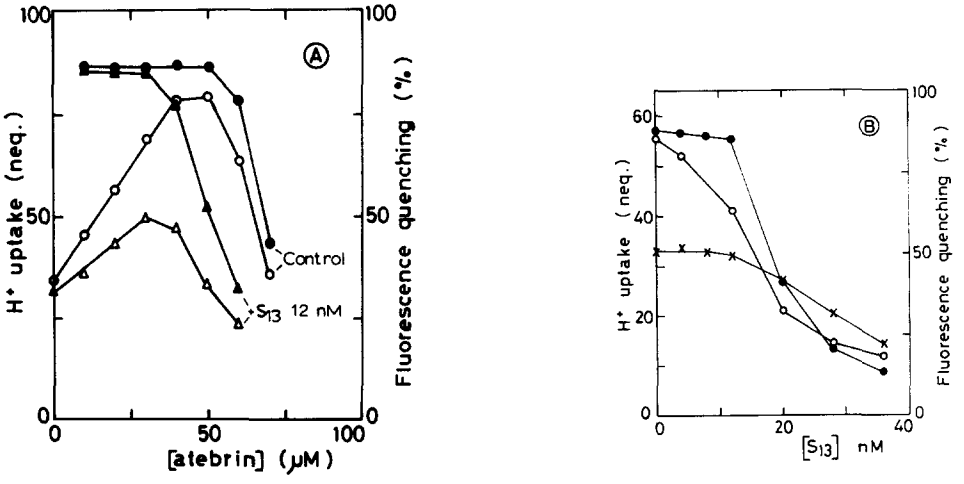


Fig. 5. Sensitivity of the atebrin-induced stimulation of the proton uptake to S_{13}. (A) Conditions as in Fig. 1A. Chlorophyll content, 30 μg per ml. The concentration of S_{13} was 12 nM. The open symbols represent proton uptake and the closed symbols represent fluorescence quenching. (B) Conditions as in A. $\times - \times$, proton uptake in the absence of atebrin; $\circ - \circ$, proton uptake in the presence of 20 μM atebrin; $\bullet - \bullet$, fluorescence quenching in the presence of 20 μM atebrin.

respect to the control (no atebrin present) up to about 18 nM S_{13}. The percentage of fluorescence quenching of atebrin is hardly affected up to this concentration of S_{13}.

It has been found earlier, that atebrin uncouples photophosphorylation completely at concentrations of about 10–20 μM. These observations were made in sucrose media at relatively low salt concentrations. However, in the experiments shown above it was found, that much higher concentrations of atebrin were required to 'saturate' illuminated chloroplasts in media with higher salt concentrations. Because of the drastic effect of the medium composition on the 'saturating' atebrin concentration, it was of interest to see whether the stimulation of the proton uptake was affected as well.

Fig. 6 shows an experiment in which the proton uptake is measured together with the fluorescence quenching as a function of the atebrin concentration in media of variable composition. The concentration of NaCl was varied, while the osmolarity of the medium was kept constant by the addition of sucrose. Apparently the enhancement of the proton uptake by atebrin, notably the slope of the initial part of the curves, is independent of the medium composition. The saturating atebrin concentration increases with increasing salt concentration in a linear way, as can be seen from the inset in the figure.

From the results in Fig. 5 it seems, that under steady-state conditions the enhancement of the proton uptake by atebrin is not obligatorily coupled (at least

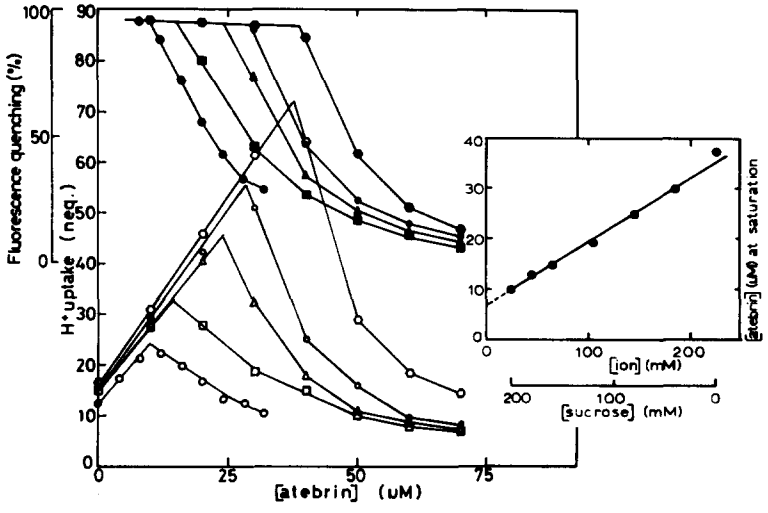


Fig. 6. Effect of the salt concentration in the medium on the stimulation of the proton uptake by atebrin and the saturating atebrin concentration. Fluorescence quenching is represented by the closed symbols and proton uptake by the open symbols. The incubation medium contained 5 mM $MgCl_2$, 2.5 mM potassium phosphate, 2 mM tricine (pH 8.0) and in addition 50 mM NaCl plus 50 mM KCl (◇—◇), 40 mM NaCl plus 40 mM KCl plus 40 mM sucrose (○—○), 30 mM NaCl plus 30 mM KCl plus 80 mM sucrose (△—△), 10 mM NaCl plus 10 mM KCl plus 160 mM sucrose (□—□) and 200 mM sucrose (○—○). The chlorophyll content was 30 μg per ml. 10 μM pyocyanin was present. The inset in the figure represents the saturating atebrin concentration as a function of the medium composition.

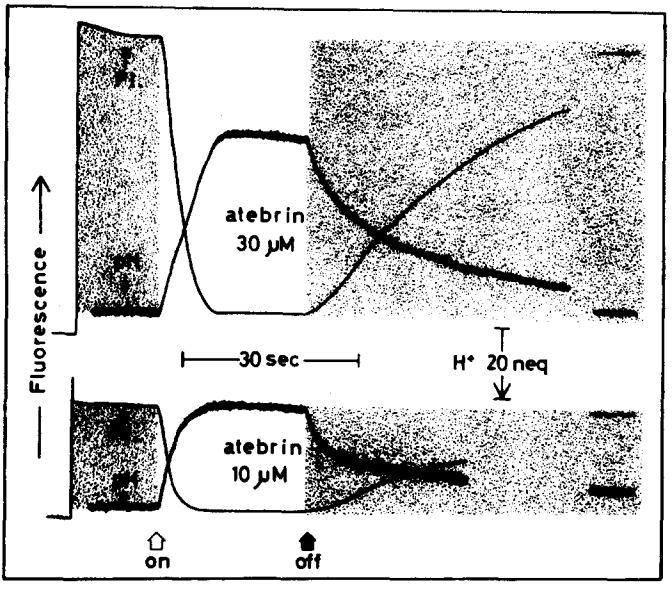


Fig. 7. The kinetics of the atebrin-induced stimulation of proton uptake. Conditions as in Fig. 1A. The chlorophyll content was 25 μg per ml.

quantitatively) to the binding of atebrin. Kinetic measurements of the fluorescence and the proton uptake might provide more information on this.

Fig. 7 shows the kinetics of the proton uptake and the fluorescence quenching at two different atebrin concentrations (10 and 30 μM). The time required to reach half maximal binding of atebrin as well as the time required for half maximal proton uptake increases with increasing atebrin concentration.

From the steady-state measurements of binding and proton uptake (cf. Figs 1 and 4) it follows that the amount of protons taken up, corrected for the proton uptake in the absence of atebrin, is proportional to the amount of atebrin molecules bound. During the entire time course of the 'on'-reaction in the kinetic experiments it is found, that the fluorescence quenching of atebrin is roughly proportional to the overall proton uptake. Minor deviations from this proportionality are found at the higher atebrin concentrations, which can be completely accounted for by assuming retardation of the basal proton uptake with respect to the binding of the protonated atebrin species.

During the 'off'-reaction proportionality between fluorescence quenching and proton uptake is no longer found. Upon turning off the light an immediate rapid release of protons is observed, whereas the fluorescence returns much slower; especially at low concentrations of atebrin (10 μM) the fluorescence remains quenched maximally, when the protons already have been released for about 50 %. At lower temperatures these kinetic differences become even more pronounced (this will be published elsewhere).

The kinetic discrepancy of the proton uptake and the fluorescence quenching between the 'on'- and the 'off'-reaction seems to indicate that the atebrin, although presumably binding in its protonated form, not necessarily remains protonated once

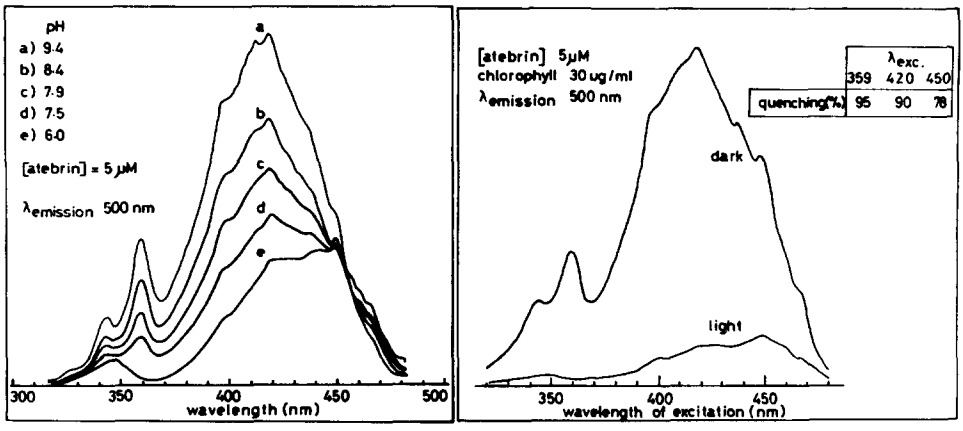


Fig. 8. Fluorescence excitation spectra of atebrin in aqueous solution at variable pH (left-hand part) and in illuminated chloroplasts at low intensity of the illuminating light (right-hand part). Conditions were as described in Fig. 1A. The illuminating light intensity was about 10 % of the saturating light intensity.

it is bound to the membrane. More information on this point could be obtained from spectral properties of atebrin in illuminated chloroplasts.

Fig. 8 shows the fluorescence excitation spectra of atebrin in aqueous solution at different pH values (left-hand part of the figure) and in a chloroplast suspension which is illuminated at a low intensity of actinic light (right-hand part of the figure).

In aqueous solutions it is found, that upon decreasing the pH the fluorescence is quenched almost completely using an excitation wavelength of 360 nm. Quenching of the fluorescence is less the higher the excitation wavelength used and at 450 nm the fluorescence of atebrin is hardly affected upon acidification. The excitation spectrum of atebrin in a chloroplast suspension in the dark at pH 8.0 is similar to that in aqueous solution at pH 8.0. When the suspension is illuminated at a low light intensity a change in the excitation spectrum is observed. At saturating light intensity the remaining fluorescence at all exciting wavelengths is too low to obtain reliable spectra. The characteristics of the spectral change observed resemble those of atebrin in aqueous solution at low pH.

More conclusive information is obtained from the absorption spectra shown in Fig. 9. The top figure shows an absorption difference spectrum of atebrin (50 μM) in aqueous solution at various pH values. The reference cuvette was at pH 9.5. The most significant spectral change that occurs upon acidification is the appearance of an absorption band centered around 450 nm, apart from minor changes in other regions of the spectrum. The bottom figure shows a light minus dark difference spectrum of a chloroplast suspension in the presence of atebrin (5 μM). The spectral change which takes place upon illumination does not show any characteristic band of atebrin; neither an absorption decrease which can be attributed to the disappearance of atebrin caused by a shielding effect, nor an absorption increase around 450 nm, which could be attributed to protonation of atebrin inside the organelle.
The actual change found upon illumination is an absorption decrease, which has the

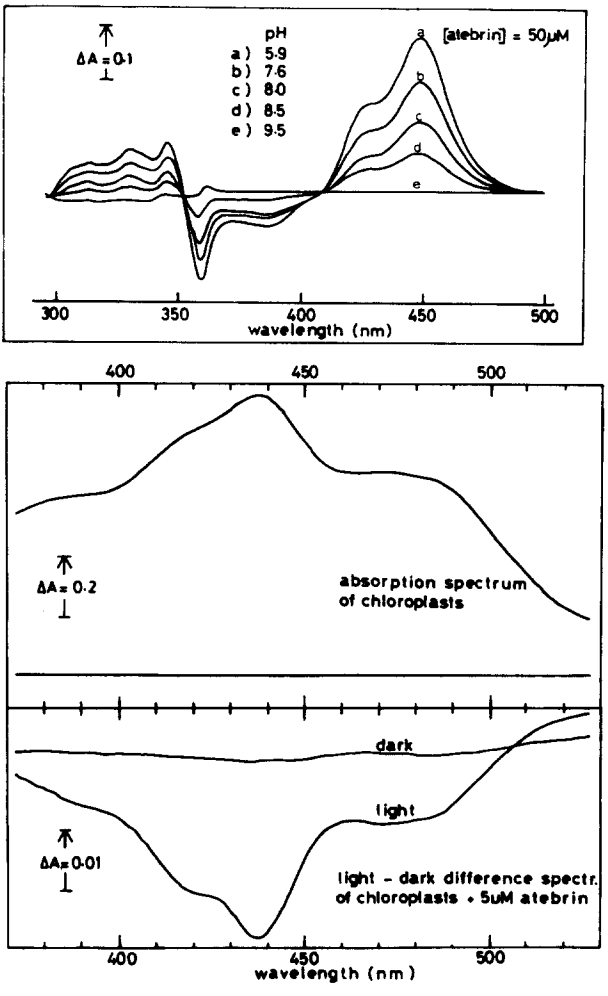


Fig. 9. Absorption difference spectra of atebrin. Upper part: absorption difference spectra of atebrin (50 μM) in aqueous solution as a function of pH. The reference cuvette was at pH 9.5. Middle part: absorption spectrum of chloroplasts (20 μg chlorophyll per ml) against water. Lower part: light minus dark difference spectrum of illuminated chloroplasts (20 μg chlorophyll per ml) in the presence of 5 μM atebrin. Further conditions as described in Materials and Methods.

form of a chlorophyll spectrum; this may be seen by comparison with the spectrum of a chloroplast suspension against water (middle figure).

It should be noted, however, that the apparent chlorophyll bleaching in the presence of atebrin is a much slower process, than the light-induced fluorescence quenching of atebrin. Nevertheless, it is dependent on the presence of atebrin and it is sensitive to other uncouplers such as S_{13}.

DISCUSSION

Enhancement of the light-induced proton uptake by uncoupling compounds is a phenomenon, which is not found for uncouplers like S_{13}, FCCP, gramicidin etc.,

which act, as is generally accepted, by a protonophoric mechanism. Enhancement of the light-induced proton uptake has been reported before for tetramethylethylenediamine [26] and more recently for aniline [27].

The stimulatory effect of atebrin on the proton uptake reported in this paper resembles that of these two compounds by the fact that it seems to be determined quantitatively by the pK_a values of the amine and the pH of the incubation medium (cf. Figs 1, 3 and 4). The pH dependence of the proton uptake by atebrin (cf. Fig. 4B) shows an increased uncoupling activity at pH values below 7.0. This is unlike what is found for the more simple amines [28]. This observation suggests that the charged atebrin species plays an important role in the mechanism of uncoupling by atebrin.

The explanation of the stimulatory effect of tetramethylethylenediamine and aniline was based on the assumption that the cationic form of the amine is redistributed across the thylakoid membrane in equilibrium with a pH differential across this membrane established upon illumination. Uncoupling in this mechanism might occur by back leakage of the charged amine [26, 28]. In the case of atebrin this explanation is not satisfactory.

It has been demonstrated previously that binding of atebrin to illuminated chloroplasts results in complete quenching of its fluorescence [17, 21]. It can be calculated according to Schuldiner et al. [13] from the fluorescence data that, going from low to saturating concentrations of atebrin, the internal pH is raised by 0.3 unit maximally (cf. Figs 1 and 4A). If the enhancement of the proton uptake in the presence of atebrin were to be explained in terms of a ΔpH-driven mechanism, the observed enhancement of the proton uptake could be quantitatively accounted for only, if it were concluded that the ΔpH does not decrease up to the saturating atebrin concentration. At the saturating atebrin concentration phosphorylation is completely inhibited (cf. Fig. 1). If the ΔpH is the main driving force of ATP synthesis [29, 30] complete inhibition of phosphorylation is not expected under conditions where the ΔpH remains essentially constant.

From these and other (see below) considerations it must be concluded that atebrin is not taken up inside the chloroplasts by a ΔpH-driven mechanism.

The validity of the use of 9-aminoacridine as a ΔpH probe [13] has been challenged previously [32]. The observed enhancement of the light-induced fluorescence quenching of 9-aminoacridine in the presence of atebrin (cf. Fig. 2) cannot be attributed to an "inner-filter" effect or to an increase of the osmotic volume (see below). This presents another condition where the calculation of the ΔpH from the fluorescence quenching of 9-aminoacridine is rather meaningless. It rather seems that the presence of atebrin facilitates the binding of 9-aminoacridine molecules onto the membrane by a cooperative interaction between atebrin and 9-aminoacridine.

Another argument against the uptake of atebrin into the osmotic volume is the osmotic behaviour of illuminated chloroplasts in the presence of atebrin (in contrast to the case of simpler amines [28]). It can be calculated that accumulation of atebrin inside the osmotic space may result in internal atebrin concentrations as high as 0.5 M at the higher atebrin concentrations added, assuming the osmotic volume to be in the order of 10 μl per mg chlorophyll [30]. Extensive swelling of the organelle would be expected under these conditions. Experimentally the opposite is found from absorption and scattering measurements; light scattering increases in the presence of atebrin have been reported before [22, 23]. Light scattering changes

might reflect structural changes in the membrane itself [31] apart from volume changes. This would be indicative of a direct interaction between atebrin and the membrane.

The fact, that the ratio of bound to free atebrin up to the saturating concentration is not affected by the presence of the uncoupler S_{13}, while the enhancement of the proton uptake is sensitive to S_{13} (Fig. 5), is incompatible with a mechanism in which the binding of atebrin is driven by a transmembrane pH differential. The observed proton uptake in the presence of S_{13} indicates a considerably higher inside pH, compared with the value calculated from the fluorescence quenching data. S_{13} and atebrin, both being uncouplers of photophosphorylation compete in discharging the energized state. This competition is revealed by the decrease in the binding capacity for atebrin by S_{13} and other high-affinity uncouplers, as reported previously [17, 21].

Another way in which the extra proton uptake can be explained is based on the assumption that only protonated atebrin is bound to the chloroplasts, presumably by an electrostatic mechanism. In such a mechanism a serious difficulty is presented by the total amount of atebrin that can be bound to the membrane. It can be calculated that at the saturating atebrin concentration about one elementary charge per 100 $Å^2$ would be needed on the thylakoid membrane, to account for the maximal binding of protonated atebrin. This is in the order of the maximal surface charge that can be present on an artificial phospholipid membrane [33, 34]. One could possibly take this value as a maximum for natural membranes as well. A change in surface charge of this magnitude upon illumination of chloroplasts might be not realistic. More probably a turnover of surface charges occurs, which would also account for the catalytic nature of the uncoupling by atebrin.

The involvement of electrostatic interactions in the binding of atebrin to chloroplasts is also suggested by the capability of acridines in general to bind strongly to polyanionic substances [16, 35–38] and to heat-denatured chloroplast membranes [17].

The exposure of charges on the membrane upon energization may result from conformational rearrangements of the protein components in the membrane as proposed earlier [37]. As to the question what cation is displaced by the charged atebrin or what anion is co-transported to the membrane no definite answer can be given as yet. However, in this respect further investigation of the effects of salt on the binding capacity for atebrin (cf. Fig. 6) to illuminated chloroplasts may provide a more conclusive answer. The apparent stabilizing effect of salts on the acridine-membrane interaction has been reported before [32].

From the kinetic experiments (cf. Fig. 6) it seems that the binding of atebrin occurs indeed simultaneously with the binding of protons. However, the kinetics of the fluorescence and the proton movement upon turning off the light are quite different, the release of protons being faster than the release of atebrin. This observation suggests a separation of the atebrin and its protons within the energized membrane, leaving the atebrin in the membrane in the uncharged form. The presence of protonated atebrin could indeed not be demonstrated in light minus dark difference absorption spectra. Although the change in the fluorescence excitation spectra upon illumination could be interpreted in terms of protonation of the atebrin, concentrational effects can account for the change as well. Concentrational effects on the absorption spectrum of atebrin have been reported by Massari et al. [16]. An increase in the

atebrin concentration above 200 μM results in a decrease of the absorbance at 420 and 450 nm and the concentration-dependent change of the absorption spectrum has isosbestic points at 400 and 456 nm. It is possible that such a spectral change occurs in illuminated chloroplasts as well, but that it is masked by the chlorophyll bleaching. In energized sub-mitochondrial particles this kind of spectral change has been observed [16].

From the experiments presented in this paper we conclude that the fluorescence response of acridines in illuminated chloroplasts is the result of binding of the protonated dye to the membrane by electrostatic interaction, rather than of a transmembrane transport of this compound in its uncharged form. This conclusion has been drawn earlier for the interaction of atebrin with energized sub-mitochondrial particles and illuminated chloroplasts [7, 16–18, 32].

To explain the uncoupling activity of atebrin it is proposed that the binding of atebrin results in the creation of a high proton activity at the periphery of the membrane. Under these conditions the membrane-bound ATPase will sense a decreased proton gradient across the membrane, resulting in lower rates of ATP synthesis.

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