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THE FLUORESCENT PROPERTIES OF ACRIDINES IN THE PRESENCE OF CHLOROPLASTS OR LIPOSOMES

ON THE QUANTITATIVE RELATIONSHIP BETWEEN THE FLUORESCENCE QUENCHING AND THE TRANSMEMBRANE PROTON GRADIENT

JAN W T FIOLET, EVERT P BAKKER* and KAREL VAN DAM

Laboratory of Biochemistry, B C P Jansen Institute University of Amsterdam, Plantage Muidergracht 12 Amsterdam (The Netherlands)

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SUMMARY

The fluorescent properties of 9-aminoacridine were studied in chloroplasts and phospholipid liposomes

In energized chloroplasts it was found that the percentage of fluorescence quenching was dependent on both the 9-aminoacridine concentration and the chlorophyll concentration On the other hand, it was independent of the osmolarity of the medium

In phospholipid liposomes the dependence of the fluorescence quenching on the concentration of 9-aminoacridine was similar to that in chloroplasts Moreover, the fluorescence quenching depended on the presence of charged compounds in the membrane being larger in negatively charged than in positively charged liposomes

The fluorescence of both the monoamine 9-amino-6-chloro-2-methoxyacridine and the diamine atebrin is quenched more extensively than that of 9-aminoacridine Although the percentage of fluorescence quenching of both atebrin and 9-aminoacridine is dependent on the outside pH, the relationship between the fluorescence quenching of the two probes under similar conditions is not pH-dependent

It is concluded that calculation of ΔpH from the percentage of fluorescence quenching of fluorescent amines is not meaningful, that the osmotic volume of chloroplasts is not involved in the quenching process and, consequently, that the interaction between the acridines and energized membranes is more likely to occur at the level of the membrane proper

INTRODUCTION

When chloroplasts, chromatophores, submitochondrial particles or bacterial respiratory particles are energized in the presence of 9-amino-substituted acridines,

Abbreviations ACMA, 9-amino-6-chloro-2-methoxyacridine, HDTAB, hexadecyltrimethylammoniumbromide, S_{13}, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide

* Present address Weizmann Institute, Rehovot, Israel

quenching of the fluorescence of these compounds is observed [1–7] The binding of these compounds, which is associated with the fluorescence changes, shows typical saturation characteristics In chloroplasts a stoicheiometric relationship between saturating probe concentration and the degree of energization has been found [8]

As to the interpretation of these energy-dependent optical changes, several proposals have been made which differ, neglecting differences in detail, in one essential feature the necessity of permeation of the bulk of the probe through the membrane

Permeation of the acridine probe is not necessary if it is assumed that electrostatic binding of the positively charged aminoacridines occurs to negative sites on or in the membrane, created upon energization The changes in optical properties of the bound aminoacridine molecules may result from local changes in the environment of the probe, e g a more acidic environment [4], changes in the probe molecule itself, e g an upward shift in the nucleus pK_a caused by the negative binding sites [7, 9] or to the interaction between the probe molecules, e g concentration effects [5, 7]

Permeation through the membrane is required in the model proposed by Azzi [5], which involves an electric potential-driven influx of permeant anions, followed by the uptake of the cationic acridine dyes The optical changes then are the result of an aggregation of the dye molecules caused by the high internal concentration

According to the model of Rottenberg et al [10] and Schuldiner et al [6], the acridine molecules will accumulate in the internal volume in response to a proton gradient across the osmotically active membrane The interesting feature of this model is that it allows a quantitative calculation of the proton gradient if the following further assumptions are made (a) the only freely permeant species is the uncharged acridine molecule, (b) no binding of the acridine to the organelle membrane occurs, (c) the optical changes are the result of a condition in the internal osmotic space and (d) the fluorescence of the accumulated dye molecules is completely quenched Based on these assumptions formulae were derived to calculate the transmembrane proton gradient (ΔpH) from the fluorescence quenching for mono- and diaminoacridines

Recent work in artificial membrane systems gave support for this model, especially when 9-aminoacridine was used as the fluorescent probe [11]

In this paper we will scrutinize the quantitative aspects of the model proposed by Rottenberg et al [10] and conclude that the validity of the model may very well be questioned as far as the fluorescent acridines are concerned

MATERIALS AND METHODS

Chloroplasts were prepared in a short-time procedure as described previously [12] The chlorophyll content was determined according to Whatley and Arnon [13]

Liposomes were prepared by evaporating a chloroform solution containing 40 mg of egg lecithin with or without 5 or 10 % dicetylphosphate, or 2 or 4 % hexadecyltrimethylammoniumbromide (HDTAB) The phospholipid was then sonicated under an argon atmosphere for 30 s in a MSE sonifier at maximal output in 5 ml of a medium containing 100 mM phosphate buffer at pH 4 0 plus 1 mM Tris–EDTA The resulting suspension was dialysed overnight against 250 mM sucrose, 10 mM phosphate buffer at pH 4 0 plus 1mM Tris–EDTA

Lecithin was prepared either according to the method of Pangborn [14], or to a shortened Pangborn procedure followed by purification on an aluminium oxide

column (Merck, aktiv neutral, aktivitatsstufe I) according to the method of Singleton et al [15] Results with the former 95 % pure Pangborn preparation [16] and the latter chromatographically pure preparation were essentially the same

The liposome experiments were carried out in a medium containing 100 mM phosphate buffer of which the pH was varied between 6 0 and 9 0 The experiments with chloroplasts were performed in a medium as described in the legends to the figures

The experiments with chloroplasts were carried out under continuous stirring in a cuvette with a volume of 2 5 ml, thermostatted at 20 °C, provided with a micro-glass electrode (Electronic Instruments Ltd GM 23/B) and a calomel reference electrode 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 at an angle of about 30° Monochromatic exciting light was provided by a Zeiss monochromator The wavelength of the exciting light was 400 nm for 9-aminoacridine and 420 nm for atebrin and 9-amino-6-chloro-2-methoxyacridine (ACMA) The bandwidth of the exciting beam was 5 nm The fluorescence emission was detected with an RCA photomultiplier (1P28) screened by a Corning 9782 glass filter for 9-aminoacridine and in addition a Wratten 57 filter for atebrin and ACMA The output signal was amplified and fed to a multichannel recorder (Rikadenki, Kogyo) If necessary, photophosphorylation was measured simultaneously by measuring the alkalinization of the incubation medium, the electrode responses were calibrated with standard solutions of oxalic acid In the experiments with liposomes, the fluorescence was measured in a spectrofluorometer (Perkin-Elmer, MPF-2A) at excitation and emission wavelengths of 400 and 430 nm, respectively

Direct binding of 9-aminoacridine to chloroplasts was determined by measuring the remaining fluorescence after spinning down the chloroplasts in a Coleman centrifuge in the dark or in the light Illumination was provided by a 250 Watt projector lamp, the light of which was passed through a plexiglass deck which was continuously cooled by running water

Atebrin (9-(4-diethylamino-1-methylbutylamino)-6-chloro-2-methoxyacridine) was purchased from Sigma, 9-aminoacridine from The British Drug Houses Ltd and ACMA was synthesized by Dr R Kraayenhof Atebrin and 9-aminoacridine were dissolved in distilled water and ACMA in alcohol at a concentration of 1 mM

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

Dicetylphosphate and HDTAB were obtained from Sigma and Koch Light respectively

RESULTS

The model proposed by Rottenberg and coworkers [6, 10], quantitatively relating the percentage of fluorescence quenching of 9-aminoacridine and the proton gradient (ΔpH) in chloroplasts, is described by the following equations for fluorescent monoamines (equation 1) and for diamines (equation 2) respectively

$$\Delta\text{pH} = \log \frac{Q}{100-Q} + \log \frac{v}{i} \qquad (1)$$

$$\Delta pH = \tfrac{1}{2} \log \frac{Q}{100-Q} + \tfrac{1}{2} \log \frac{V}{v} \qquad (2)$$

in which Q represents the percentage of fluorescence quenching, V is the total volume of the incubation mixture and v is the total osmotically active volume These equations should be valid for amines for which the pK_a value(s) is high with respect to both the pH inside the particles and the pH outside In the case of 9-aminoacridine ($pK_a = 9\,99$ [17]) and ACMA ($pK_a = 8\,99$ [17]) this condition is sufficiently fulfilled In the case of atebrin, which has a pK_a for the ring nitrogen in the order of magnitude of the outside pH ($pK_a = 7\,92$ [18]) and a high pK_a for the side chain ($pK_a = 10\,48$ [18]), a correction term must be added

Fluorescence quenching dependent upon probe concentration and osmotic volume

According to the model, one would expect that, at least in principle, the percentage of fluorescence quenching upon energization of chloroplasts should be independent of the probe concentration This prediction has been tested and the results are shown in Fig 1 As can be seen the fluorescence quenching is not constant After an increase in fluorescence quenching with 9-aminoacridine concentration a maximum is reached above which the fluorescence quenching diminishes again with higher 9-aminoacridine concentration In the case of atebrin, used as a probe in submitochondrial particles, this has been reported earlier [19] Moreover, the fluorescence quenching of 9-aminoacridine is dependent on the composition of the incubation medium In low-salt media the maximum is reached at about 10 μM 9-aminoacridine, while in media with higher salt concentrations maximal fluorescence quenching occurs at higher probe concentrations Also the percentage of fluorescence quenching in dependence of the probe concentration is more pronounced in high-salt media

A second prediction of the model is the existence of a linear relationship between

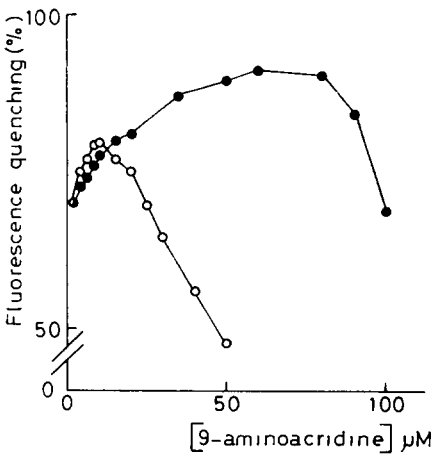

Fig 1 Dependence of the light-induced fluorescence quenching on the concentration of 9-aminoacridine The incubation medium contained 5 mM $MgCl_2$, 2 5 mM P_i and 2 mM tricine (pH 8 0), 10 μM pyocyanine, 30 μg chlorophyll per ml and in addition 200 mM sucrose (○—○) or 50 mM NaCl plus 50 mM KCl (●—●), 9-aminoacridine in the concentrations indicated The final volume was 2 5 ml

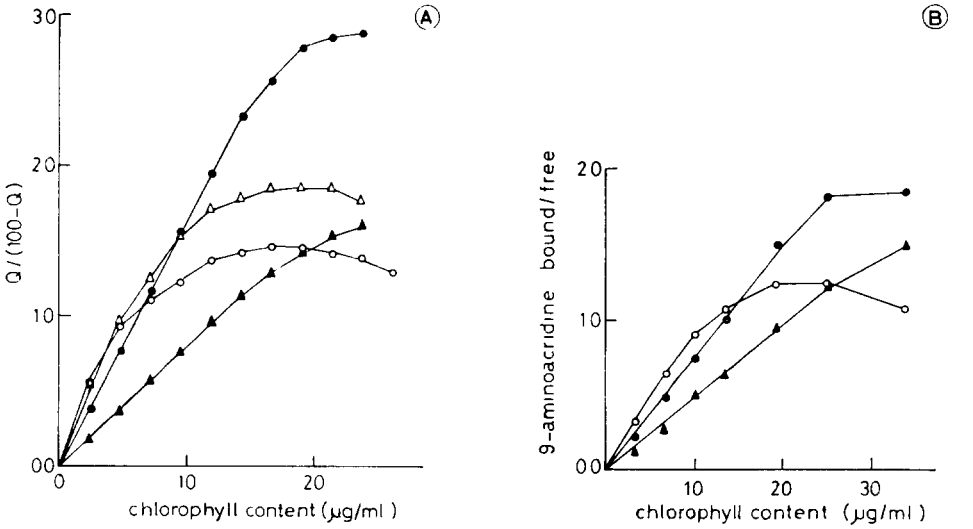

Fig 2 A Fluorescence quenching of 9-aminoacridine as a function of chlorophyll concentration Incubations were carried out in a medium containing 100 mM sucrose, 10 mM KCl, 10 mM NaCl, 3 mM $MgCl_2$, 3 mM P_i, 10 μM pyocyanine and 10 mM tricine (pH 8 0) Chlorophyll concentrations as indicated in the figure The final volume was 2 5 ml 9-aminoacridine concentrations were 1 5 μM (○ – ○), 3 μM (△ – △) 6 μM (● – ●) and 12 μM (▲ – ▲) respectively B Light-induced binding of 9-aminoacridine The binding was determined directly as described under Materials and Methods The incubation medium was as in Fig 2A 9-aminoacridine concentrations were 3 75 μM (○ – ○), 7 5 μM (● – ●) and 15 0 μM (▲ – ▲) respectively

the fluorescence function and the osmotic volume at a constant ΔpH as expressed by equation 1 The osmotic volume may be changed in two ways, by increasing the total chlorophyll concentration or by changing the osmolarity of the incubation medium

Fig 2A shows the results of an experiment in which the total osmotic volume has been changed by increasing the amount of chlorophyll per incubation This experiment was carried out at four different 9-aminoacridine concentrations At the higher probe concentrations the predicted linear relationship is found, although the slope is dependent on the probe concentration At lower probe concentrations the linear part of the curves gradually disappears, while at the same time the slope increases somewhat

In Fig 2B the results of a binding experiment are described, carried out under the same conditions The remaining fluorescence in the supernatant was measured after centrifuging the chloroplasts in the dark or in the light From these data the percentage of 9-aminoacridine bound in the light was calculated after correction for binding that occurred in the dark Obviously, the results obtained with the two different methods are quite similar (compare Fig 2A)

Energized chloroplasts maintaining a constant ΔpH should maintain also a constant ratio of internal to external probe concentration Increasing the total osmotic volume by an increase in the chlorophyll concentration at constant probe concentration leads to a lower absolute internal probe concentration, whereas the ratio of inside to outside concentration remains constant To exclude the possibility

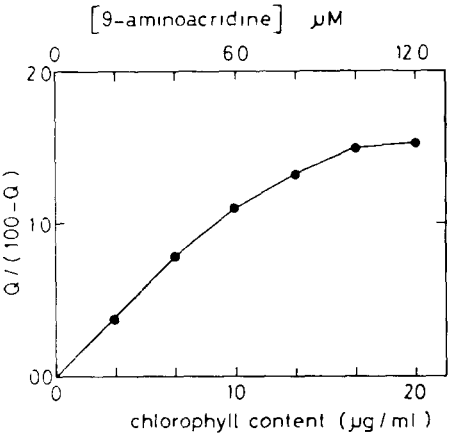

Fig 3 Determination of the light-induced fluorescence quenching of 9-aminoacridine in dependence of the chlorophyll concentration at a constant ratio of chlorophyll to 9-aminoacridine as indicated in the figure Conditions were as described under Fig 2A

that this fact could be responsible for the observed non-linearity, the experiment of Fig 2A was repeated at constant chlorophyll to probe concentration ratio Under these conditions also a non-linearity is found, as shown in Fig 3

Sorbitol has been shown to serve as a measure of the total non-osmotic space [10], which means that it is impermeant to the osmotically active membrane in chloroplasts Fig 4 shows that the percentage of fluorescence quenching of 9-aminoacridine is hardly sensitive to changes in the osmolarity of the incubation medium up to 500 mM sorbitol If it is assumed that the ΔpH is not affected under these conditions, the insensitivity of the fluorescence response towards changes in the medium osmolarity is contradictory to the model

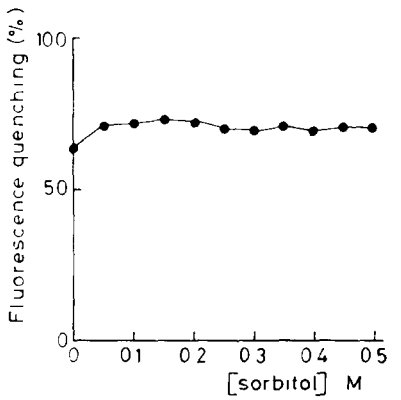

Fig 4 Fluorescence quenching of 9-aminoacridine in dependence of the osmolarity The incubation medium contained 2 5 mM P_i, 5 mM $MgCl_2$, 10 μM pyocyanine, 5 μM 9-aminoacridine, 2 mM tricine (pH 8 0) and in addition sorbitol as indicated in the figure The chlorophyll content was 30 μg per ml The final volume was 2 5 ml

Comparison of 9-amino-substituted acridines

A third prediction of the model is a quantitative relationship between the fluorescence quenching of different fluorescent amines upon energization of chloroplasts, provided that the degree of energization is the same and that the fluorescent amines fulfill the above mentioned pK_a requirements From equation 1 it follows that at equal ΔpH the fluorescence quenching of two different monoamines is related according to equation 3

$$\log\left(\frac{Q}{100-Q}\right)_{\text{monoamine 1}} = \log\left(\frac{Q}{100-Q}\right)_{\text{monoamine 2}} \tag{3}$$

Comparison of a monoamine with a diamine yields the following relationship

$$\log\left(\frac{Q}{100-Q}\right)_{\text{diamine}} = 2\log\left(\frac{Q}{100-Q}\right)_{\text{monoamine}} + \log\frac{V}{v} \tag{4}$$

The relationships (3) and (4) have been tested for 9-aminoacridine versus ACMA and atebrin, respectively The degree of energization was varied by the addition of different amounts of the uncoupler S_{13} The concentrations of the probes were chosen so low that no additional uncoupling occurred by the probes themselves This was checked by measuring the phosphorylation simultaneously at every S_{13} concentration plus and minus added probe

In the experiment of Fig 5, equation 3 was put to a test for 9-aminoacridine and ACMA, two monoamines with high pK_a values The predicted linearity is found and the slope of the straight line is 1 as expected from equation 3 However, the absolute values of the percentage of fluorescence quenching of the two probes should also be equal at every point in the figure, this is clearly not the case The

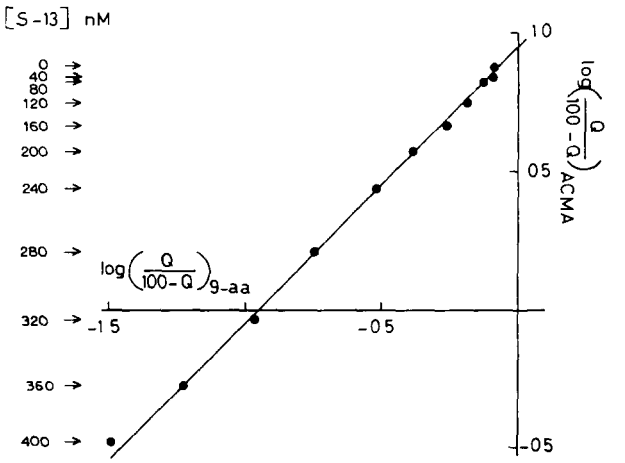

Fig 5 Comparison of the light-induced fluorescence quenching of 9-aminoacridine (9-aa) and ACMA under identical conditions The incubation medium contained 50 mM NaCl, 50 mM KCl 5 mM $MgCl_2$, 2 5 mM P_i, 1 mM ADP, 2 mM tricine (pH 7 9), 10 mM pyocyanin and 30 μg chlorophyll per ml The final volume was 2 5 ml 5 μM 9-aminoacridine or 2 5 μM ACMA were present The amount of fluorescence quenching was varied by addition of S_{13} at concentrations as indicated in the figure

fluorescence of ACMA is quenched more strongly at every level of energization

Equation 4 cannot be applied if atebrin is used as the diamine, without the introduction of a correction term, because the second pK_a of atebrin is 7 92, which

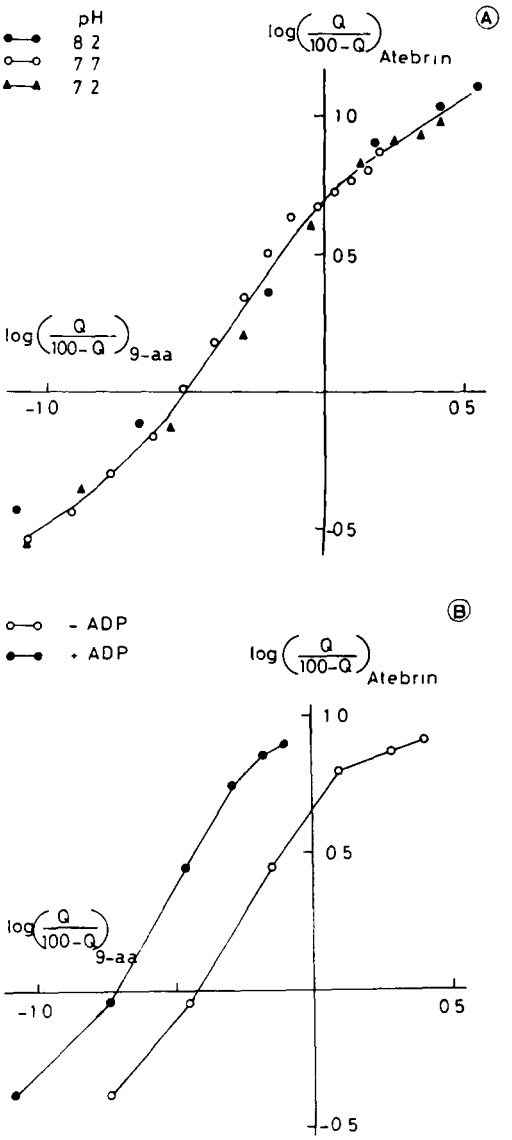

Fig 6 Comparison of the light-induced fluorescence quenching of 9-aminoacridine (9-aa) and atebrin under identical conditions The incubation medium and chlorophyll content was as described under Fig 5 A 5 μM 9-aminoacridine or 5 μM atebrin was present The fluorescence quenching was varied by addition of S_{13} in a concentration range of 0–0 5 μM The pH of the medium was as indicated in the figure For every point in the figure the rate of phosphorylation was the same in the presence or absence of 9-aminoacridine or atebrin B Conditions were the same as in Fig 6A with the exception that in the incubations indicated by the closed circles (●—●) ADP was omitted

is close to the pH of the incubation medium Introducing for this dissociation constant

$K = \alpha[H^+_{out}]$

equations (2) and (4) can be rewritten as follows

$$\Delta pH = \tfrac{1}{2} \log \frac{Q}{100-Q} + \tfrac{1}{2} \log \frac{V}{v} + \tfrac{1}{2} \log (1+\alpha) \quad (5)$$

$$\log \left(\frac{Q}{100-Q}\right)_{atebrin} = 2 \log \left(\frac{Q}{100-Q}\right)_{9\text{-aminoacridine}} + \log \frac{V}{v} - \log (1+\alpha) \quad (6)$$

The correction term log $(1+\alpha)$ varies from 0 08 to 0 48 when the outside pH is changed from 7 2 to 8 2

Fig 6A shows the relationship between the fluorescence changes of atebrin and 9-aminoacridine in chloroplasts at different levels of energization and of outside pH The energization has been varied by addition of the uncoupler S_{13} as in Fig 5 The results differ from the predictions (equation 6) in three aspects Firstly, no linearity is observed, secondly, the slope of the steepest part of the curves is about 1 5 instead of 2 and, thirdly, there is no dependence on the outside pH, i e no correction term in equation 6 seems necessary Moreover, calculation of the total osmotic volume in the sample is possible from the intercept of the curve with the ordinate with formula 6 (assuming $\alpha = 1$) Calculation yields an osmotic volume of about 250 μl per 66 μg of chlorophyll, which is 10 times the total volume of chloroplast suspension added to the cuvette (25 μl) Calculation from the intercept with the abscissa gives a value of 125 μl for v

Recently it was reported that going from non-phosphorylating to phosphorylating conditions by addition of ADP, there is a drop in the ΔpH as read out by the fluorescence quenching of 9-aminoacridine [20]

In Fig 6B the experiment of Fig 6A was repeated under phosphorylating and non-phosphorylating conditions The addition of ADP causes a decrease in fluorescence quenching of 9-aminoacridine whereas the fluorescence quenching of atebrin is not affected One would expect that at the higher S_{13} concentrations, which completely uncouple the system with respect to ATP synthesis, there would be no effect on the fluorescence of either of the probes upon introducing phosphorylating conditions This is not found experimentally

9-Aminoacridine in a liposome system

The quantitative model of pH-induced fluorescence quenching of acridine derivatives received considerable support from experiments in artificial membrane systems as mentioned above [11] In the light of the apparent discrepancies between the model and the experimental findings in energized chloroplasts, it was decided to re-examine the fluorescence behaviour of 9-aminoacridine in liposomes

In Fig 7A the results are shown of an experiment in which the amount of liposomes was varied at constant 9-aminoacridine concentration The predicted linearity is found at every outside pH, except at pH 8 0 and 9 0, where a deviation is found at the highest phospholipid concentrations This deviation is not due to acidification of the medium by the addition the liposome suspension The slope of the linear part of the curves is about 1 as it should be according to the model (cf equation 1)

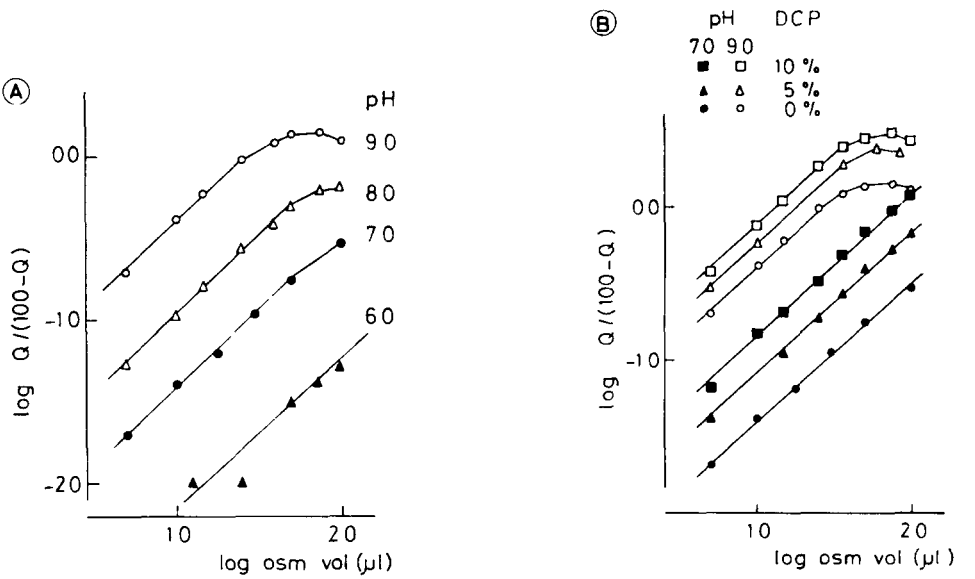

Fig 7 The fluorescence quenching of 9-aminoacridine (9-aa) added to liposomes, as a function of lipid concentration, with varying pH of the incubation medium (A) and varying lipid composition (B) Liposomes containing phosphate buffer (pH 4 0) were added to 2 5 ml 100 mM phosphate buffer ranging from pH 6 0 to 9 0 Titration with liposomes was carried out with a solution which contained 4 mg lipid per ml On the abscissa is plotted the logarithm of the amount of liposome suspension added (μl) This unit differs from the real osmotic volume by a constant only 10 μM 9-aminoacridine was present The lipid composition of the liposomes was varied with amounts of dicetylphosphate (DCP) as indicated in the figure

Fig 7B shows that the linearity as well as the slope do not depend on the composition of the liposomes However, as can be seen, increasing the net negative charge by increasing the percentage of dicetylphosphate causes more quenching of the fluorescence of 9-aminoacridine This increase in the fluorescence quenching is dependent on the pH of the incubation medium If negatively charged liposomes are compared with liposomes positively charged with HDTAB the same phenomena are observed (not shown) more fluorescence quenching is observed in negatively charged liposomes and this effect was more pronounced at the lower pH values

Fig 8 shows a plot of the external pH (and the ΔpH) against the fluorescence-quenching function The model (cf equation 1) predicts straight lines with a slope of 1 Linearity is found, but the slope depends on the charge density of the liposomes and in none of the cases a slope of 1 is reached (contrast ref 11) By extrapolation of the lines in Fig 8 to ΔpH = 0 calculation of the osmotic volume is possible The calculated values turn out to be 50 μl at 10 % dicetylphoshate, 16 μl at 5 % dicetylphosphate and about 3 μl when no dicetylphosphate was added All those values are for 180 μg of phospholipid in about 20 μl of solution

From Fig 9 it becomes clear that, as in the case of chloroplasts, the percentage of fluorescence quenched is very much dependent on the probe concentration used Also in this case linearity between quenching and osmotic volume (or phospholipid

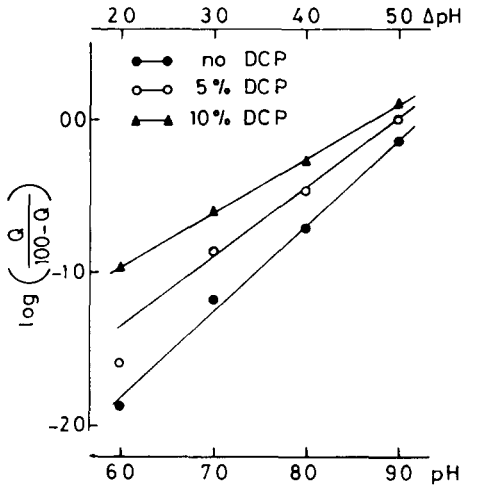

Fig 8 Effect of varying pH gradients on the fluorescence quenching of 9-aminoacridine in liposomes containing varying amounts of dicetylphosphate (DCP) Conditions as described under Fig 7

concentration) exists only at the higher probe concentrations

It should be noted that the fluorescence quenching in liposomes is always sensitive to nigericin

As in the case of chloroplasts, the fluorescence of ACMA added to liposomes was quenched more completely than that of 9-aminoacridine under identical conditions (not shown)

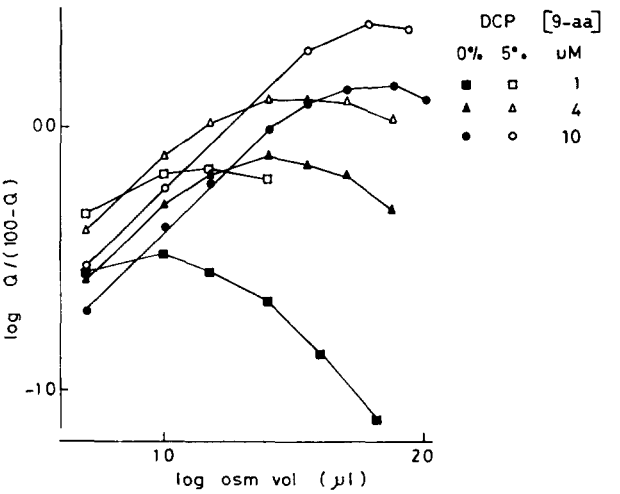

Fig 9 The fluorescence quenching of 9-aminoacridine (9-aa) added to liposomes as a function of the lipid concentration, dependence on the 9-aminoacridine concentration The incubations were carried out at pH 9 0 Further conditions as described under Fig 7 DCP, dicetylphosphate

DISCUSSION

Calculation of the ΔpH across the thylakoid membrane from the fluorescence quenching of 9-aminoacridine according to the model of Rottenberg and coworkers yields values of 3–4 pH units at an outside pH of about 8 0 at saturating light intensities Estimations of the light-induced ΔpH that have been made previously, by measurements of the buffer capacity of the thylakoids and the proton uptake [21] and the ammonium uptake method [22] on the one hand, and spectroscopic measurements on the other hand [23] also yield values in this range This agreement between the values found is in fact the main argument for the validity of the model It should be noted, however, that this value is mainly determined by the volume term in equation 1 and that the fluorescence term has only a minor influence

Other support for the model came from the experiments of Deamer et al [11], who studied the fluorescence behaviour of 9-aminoacridine and atebrin in a liposome system It was found that the 9-aminoacridine behaved ideally with respect to equation 1 as a monoamine whereas atebrin did not behave as would be expected for a diamine

Although we consider the existence of a proton gradient in energized chloroplasts very likely and although thermodynamics require a distribution of the 9-aminoacridine cation in equilibrium with the pH differential across the membrane, the experiments presented in this paper show that a significant fraction of 9-aminoacridine is not behaving as predicted and must therefore be bound to the membrane For this reason we feel that the validity of the calculation of the ΔpH by means of the fluorescence quenching of acridines should be reconsidered

As noted, there is a considerable dependence of the percentage of fluorescence quenching on both the probe concentration and on the concentration of chloroplasts or liposomes (cf Figs 1, 2 and 9) These observations are not necessarily contradictory to the model It could be argued that the fluorescence of 9-aminoacridine is not completely quenched at the lower probe concentrations This would not be unreasonable, since under these conditions the intra-thylakoid concentration will be relatively low In aqueous media the fluorescence of 9-aminoacridine is very sensitive to concentration quenching [17] If this quenching mechanism is partly responsible for the observed light-induced fluorescence quenching the quenching should be more extensive at the higher inside concentrations The fluorescence term in equation 1 should then be rewritten $\log Q/(100-\beta)-Q$ in which β is the fraction of remaining fluorescence Indeed, β itself could be a function of the probe concentration The non-linearity in Fig 3 could be explained in this way at least qualitatively Also the results in Fig 5, the comparison of 9-aminoacridine and ACMA and also the more complete fluorescence quenching of ACMA in liposomes could be explained in this way assuming a smaller value for β (i e more complete quenching) for ACMA

On the other hand, the fact that direct-binding experiments give the same results as fluorescence-quenching measurements suggests that the fluorescence quenching is complete

The insensitivity of the fluorescence quenching to changes in the osmolarity of the medium (cf Fig 4) is in contradiction to the model from a more fundamental point of view This observation raises the question whether the osmotically active volume is involved in the quenching process at all As shown in Fig 6A for chloro-

plasts and Fig 8 for liposomes, calculation of the osmotic volume using the derived equations yields values which are physically impossible Also the pH independence of the curve shown in Fig 6A does not fit into the model

If the fluorescence quenching, then, is not related to the osmotically active space we must conclude that the observed effects are due to interactions on or inside the membrane itself On the one hand, there is a direct effect of the charge of liposomes on the percentage of fluorescence quenching (cf Fig 7B) On the other hand, the ionic strength of the medium has considerable effects on the interaction between 9-aminoacridine and illuminated chloroplasts (cf Fig 1) Both phenomena are compatible with the above conclusion It could be argued that the presence of charged components in the liposomal membrane tends to increase the total internal volume giving rise to more fluorescence quenching at the same ΔpH However, this effect should have been observed in negatively as well as positively charged liposomes, which is not found experimentally Still, this possibility cannot be ruled out completely

The results in Fig 5 could be explained on the basis of different association constants of 9-aminoacridine and ACMA respectively for activated membrane sites The existence of a pH-gradient across the membrane apparently influences the binding (cf Fig 7), which may be explained on the basis of an increased proton activity inside or in the neighbourhood of the membrane, which in turn will influence the binding sites

In terms of a binding concept the function $Q/100-Q$ represents the concentration ratio of bound over free 9-aminoacridine with respect to the total incubation volume provided that 100 % quenching occurs on binding The fact that this function also fits the Rottenberg relation under some conditions (e g high probe concentrations and low particle concentrations, Figs 2 and 9) is purely accidental in our opinion

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