9-Aminoacridine as a fluorescent probe of the electrical diffuse layer associated with the membranes of plant mitochondria

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(Received 21 April 1980/Accepted 7 August 1980)

1. Mitochondria from Jerusalem artichoke (*Helianthus tuberosus*) tubers and *Arum maculatum* spadices caused a quenching of the fluorescence of 9-aminoacridine when mixed in a low-cation medium ($\sim 1 \text{ mM-K}^+$) and addition of chelators further decreased the fluorescence. Salts released the quenching of the 9-aminoacridine fluorescence and the efficiency of the release appeared to be mainly dependent on the valency of the cation ($\text{C}^{3+} > \text{C}^{2+} > \text{C}^+$). 2. The results are consistent with the theory of charge screening and demonstrate that 9-aminoacridine is a convenient probe of the behaviour of cations on the membranes of mitochondria and in the diffuse layer associated with these membranes. 3. The concentration of salt required to achieve half-maximal release of quenching of 9-aminoacridine fluorescence was proportional to the concentration of mitochondria in the solution and theoretical considerations show this effect to be inherent in the Gouy–Chapman theory. 4. 9-Aminoacridine was removed from the bulk of the solution by the mitochondria to a far greater extent than was Na^+ or K^+ , which is suggested to be due to the formation of bi- and poly-valent cations by aggregation of 9-aminoacridine molecules in the diffuse layer. This would have implications for the use of 9-aminoacridine to determine ΔpH across membranes. 5. Jerusalem-artichoke mitochondria removed more 9-aminoacridine and Ca^{2+} from the bulk of the solution and required more ions to screen the membranes than did an equal concentration (mg of protein/ml) of *Arum* mitochondria, indicating that Jerusalem-artichoke mitochondria contain more negative charges per mg of protein.

Wojtczak & Nalecz (1979) and Johnston *et al.* (1979) have demonstrated the usefulness of applying the Gouy–Chapman theory of the diffuse layer (see McLaughlin, 1977) to the prediction of the influence of cations on the behaviour of membrane-bound enzymes. The results were consistent with the view that the concentration of a charged substrate near a biological membrane is influenced by the number of fixed negative charges on the membrane (Wojtczak & Nalecz, 1979) and by the degree of screening of these fixed charges by cations (Johnston *et al.*, 1979).

The electrostatic properties of plant mitochondria are largely unknown. Screening of membrane charges has an effect on the oxidation of exogenous NADH by plant mitochondria (Johnston *et al.*, 1979) and since most other mito-

chondrial substrates are also negatively charged it appears desirable to have a probe of the behaviour of ions in the diffuse layer associated with the membranes.

The fluorescent amine 9-aminoacridine has been used extensively to measure ΔpH across biological membranes such as submitochondrial particles (Bashford & Thayer, 1977). The fluorescence quenching of 9-aminoacridine is here used to measure the accumulation in the compartment of low pH and the pH difference is then calculated assuming that (1) 9-aminoacridine crosses the membrane in the uncharged form only and (2) 9-aminoacridine is a univalent cation (Schuldiner *et al.*, 1972).

However, 9-aminoacridine has also been used to monitor the extent of electrostatic screening of fixed charges on the surface of thylakoid membranes under conditions where no pH gradient would be formed (Searle *et al.*, 1977; Chow & Barber, 1980). Under such conditions and in the presence of low concentrations of cations the fluorescence of 9-aminoacridine is quenched due to a concentration of

Abbreviations used: Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonate; σ , density of charges on the membrane ($\text{C}\cdot\text{m}^{-2}$); c_1 value, the concentration of a salt needed to give a release of quenching of 9-aminoacridine fluorescence equal to half that given by 20 mM-MgCl_2 .

the probe in the diffuse layer near the negatively charged thylakoid membranes. The addition of cations will now cause an increase in fluorescence as a result of increased degree of screening. It is noteworthy that when 9-aminoacridine is used to measure ΔpH , high concentrations of cations are usually present (Schuldiner *et al.*, 1972; Bashford & Thayer, 1977) and very little 9-aminoacridine will go into the diffuse layer.

In the present paper 9-aminoacridine is used with whole mitochondria under non-metabolizing conditions in a manner similar to that with thylakoid membranes. It is shown that the fluorescence of 9-aminoacridine provides useful information about bivalent cations bound to the mitochondrial membranes as well as about cations in the diffuse layer associated with these membranes. The behaviour of 9-aminoacridine in the diffuse layers associated with the membrane surfaces not facing the matrix can easily be distinguished from 9-aminoacridine accumulated in the matrix since the fluorescence quenching is partly brought about by chelators and rapidly released by cations, neither of which can cross the mitochondrial inner membrane. The present paper also provides evidence on the behaviour of 9-aminoacridine in the diffuse layer, the influence of protein concentration on the degree of screening by cations and electrostatic differences between Jerusalem-artichoke and *Arum* mitochondria.

Materials and methods

Preparation of mitochondria

Mitochondria were prepared from Jerusalem-artichoke (*Helianthus tuberosus*) tubers bought in the market and stored at 4°C until used. The procedure for isolation of mitochondria was that of Palmer & Kirk (1974) with the following modifications. The first centrifugation was at 48000g for 2 min. The pellets were resuspended in 0.4 M-sucrose/5 mM-Tes (pH 7.2)/0.1% (w/v) bovine serum albumin and, after a short centrifugation (accelerating to 12000g followed by immediate deceleration in a Sorvall RC-2B centrifuge with an SS-34 rotor) where the pellets were discarded, the mitochondria were recovered from the supernatant by centrifugation at 48000g for 2 min. These mitochondria were washed a second time in 0.4 M-sucrose/2 mM-potassium Tes (pH 7.2)/0.1% bovine serum albumin to minimize the number of cations present in the final preparation.

Arum maculatum spadices were collected in the wild and used the following day. The mitochondria were isolated by the method described by Cammack & Palmer (1977) except that the wash medium contained 0.3 M-sucrose, 5 mM-potassium Tes (pH 7.2) and 0.1% bovine serum albumin (no EDTA). The mitochondria thus obtained were

washed a second time in a medium containing 0.3 M-sucrose, 2 mM-potassium Tes (pH 7.2) and 0.1% bovine serum albumin.

The above procedures ensured that the mitochondria from both plant tissues were exposed to very low concentrations of cations (~1 mM-K⁺) during the last stage of the isolation.

The respiratory properties of both types of mitochondria have been well characterized with a variety of substrates in previous publications from this laboratory (Coleman & Palmer, 1971, 1972; Palmer & Passam, 1971; Palmer, 1972).

Measurement of fluorescence of 9-aminoacridine

The fluorescence of 9-aminoacridine was measured at room temperature with a Perkin-Elmer MPF-3 fluorescence spectrophotometer as described by Searle *et al.* (1977). The medium (3.0 ml) contained 0.3 M-sucrose, 2 mM-potassium Tes (pH 7.2) and 20 µM-9-aminoacridine. No respiratory substrate was present.

Determination of the concentrations of K⁺ and Ca²⁺ in the bulk of a suspension of mitochondria

Different concentrations of mitochondrial protein (Jerusalem artichoke, 0.02–0.2 mg/ml; *Arum*, 0.05–0.5 mg/ml) were incubated at room temperature in 0.3 M-sucrose/2 mM-potassium Tes (pH 7.2)/15 µM-EDTA/20 µM-9-aminoacridine. After incubation for at least 5 min, KCl or CaCl₂ was added to give concentrations of 0–20 mM and 0–200 µM respectively. The mitochondria were pelleted at 8000g for 10 min and discarded and the concentrations of 9-aminoacridine, K⁺ and Ca²⁺ were determined in the supernatant. The concentration of 9-aminoacridine was measured by determination of its fluorescence. K⁺ was determined by the use of flame photometry (Eel Flame Photometer, Evans Electro-selenium Ltd.) and Ca²⁺ by atomic-absorption spectrophotometry (Instrumentation Laboratory, Warrington, Cheshire, U.K.; model AA IL 151; Ca/Mg lamp, wavelength 422.7 nm, air/acetylene flame).

Determination of the concentration of 9-aminoacridine in the bulk of a suspension of mitochondria

Jerusalem-artichoke (0.02–0.15 mg/ml) and *Arum* mitochondria (0.04–0.58 mg/ml) were incubated at room temperature in 0.3 M-sucrose/2 mM-potassium Tes (pH 7.2)/100 µM-EDTA/20 µM-9-aminoacridine. Mitochondria were pelleted and the concentration of 9-aminoacridine was determined in the supernatant.

Determination of protein concentration

Protein concentration was determined by the method of Lowry *et al.* (1951) after solubilizing with

0.5% (w/v) deoxycholate. Bovine serum albumin (Sigma no. A-4503) was used as the standard.

Chemicals

9-Aminoacridine was from Koch-Light Laboratories (Colnbrook, Bucks., U.K.) and tris(ethylenediamine) cobalt (III) chloride from Alfa Products (Danvers, MA 01923, U.S.A.). All other chemicals were of analytical grade.

Results

The effect of chelators on 9-aminoacridine fluorescence in a mitochondrial suspension

The addition of both Jerusalem-artichoke and *Arum* mitochondria to a solution of 9-aminoacridine caused a decrease in fluorescence, which is shown in Fig. 1 for *Arum* mitochondria. Addition of EDTA caused a further decrease (Fig. 1), probably due to the removal of bivalent cations associated

with the membranes, thereby allowing more 9-aminoacridine to enter the diffuse layer and become quenched. In Fig. 1 about $35\mu\text{M}$ -EDTA gave maximal quenching of fluorescence, but the amount depended on the protein concentration. At the same protein concentration Jerusalem-artichoke mitochondria required more EDTA than did *Arum* mitochondria. In all cases the addition of 0.5 mM-EDTA gave rise to a small increase in fluorescence, which may be attributed to the screening effect of the K^+ in the EDTA solution.

EGTA was $34 \pm 5\%$ (mean $\pm$ s.d., $n = 5$ preparations of both kinds of mitochondria) less effective than EDTA in causing a decrease in fluorescence (Fig. 1). The addition of EDTA after EGTA brought the fluorescence down to the value reached with EDTA alone (Fig. 1), whereas EGTA added after EDTA had no effect.

Citrate, which inhibits NADH oxidation possibly by removing Ca^{2+} (Cowley & Palmer, 1978), also caused a decrease in the fluorescence of 9-aminoacridine, but even at $400\mu\text{M}$ it had less effect than EGTA (not shown).

The release of quenching of 9-aminoacridine fluorescence in a mitochondrial suspension by salts

The addition of salts to mitochondria suspended in a medium containing 9-aminoacridine and EDTA caused an increase in fluorescence due to a displacement of 9-aminoacridine from the diffuse layer associated with the membranes. In Fig. 2 this is shown for KCl but all other salts gave similar effects over different concentration ranges depending on the valency of the cation (see below). The release of quenching of 9-aminoacridine fluorescence after each addition of salt was a rapid process and was complete within 10s, which was the earliest time fluorescence could be measured by the technique adopted. At the end of each experiment 20 mM- MgCl_2 was added to give the maximal release of fluorescence quenching (Fig. 2; Searle *et al.*, 1977). This maximum fluorescence was always lower than the initial fluorescence of $20\mu\text{M}$ -9-aminoacridine (Fig. 2) and the difference was directly proportional to the protein concentration. The c_1 value for the effect of a given salt was taken to be the concentration of the salt needed to release the quenched fluorescence of 9-aminoacridine to half that achieved by 20 mM- MgCl_2 (Searle *et al.*, 1977), e.g. in Fig. 2 the c_1 value for KCl was found to be 12 mM.

Table 1 presents c_1 values determined on Jerusalem-artichoke and *Arum* mitochondria for a variety of salts containing uni-, bi- and ter-valent cations. The nature of the anion is unimportant since salts containing the same anion, e.g. NaCl , MgCl_2 and tris(ethylenediamine) cobalt (III) chloride, were efficient at bringing about release of fluorescence

Fig. 1. *The effect of EDTA and EGTA on 9-aminoacridine fluorescence in the presence of plant mitochondria*

The medium contained 0.3 M-sucrose, 2 mM-potassium Tes (pH 7.2), $20\mu\text{M}$ -9-aminoacridine and 0.36 mg of mitochondrial protein/ml. EDTA (×) and EGTA (●) were added stepwise and the fluorescence was determined after each addition (see the Materials and methods section). The data represent two experiments on one preparation of *Arum* mitochondria, but similar results were obtained with other preparations and with Jerusalem-artichoke mitochondria.

Table 1. c_1 values for a range of salts with Jerusalem-artichoke (0.08 mg of protein/ml) and Arum (0.29 mg of protein/ml) mitochondria

Mitochondria were suspended in a medium containing 0.3 M-sucrose, 2 mM-potassium Tes (pH 7.2) and 20 μ M-9-aminoacridine. In most cases 20 μ M-EDTA was added, but with LaCl_3 this was omitted as explained in the text. All measurements were performed on one preparation of each type of mitochondria.

Valency of cation	Salt	c_1 value (mM)	
		Jerusalem-artichoke mitochondria	Arum maculatum mitochondria
One	KCl	6.2	5.4
	NaCl	6.3	5.3
	KNO_3	6.0	5.3
	RbCl	5.4	4.8
	Choline chloride	10.0	8.3
Two	CaCl_2	0.026	0.022
	MgCl_2	0.025	0.027
	MgSO_4	0.022	0.024
	SrCl_2	0.025	0.032
	MnCl_2	0.021	0.021
	Decamethylenebistrimethylammonium bromide	0.16	0.16
	Lysyl-lysine	0.22	0.185
Three	Tris(ethylenediamine) cobalt (III) chloride	0.0045	0.0050
	LaCl_3	0.0083	0.0058

Fig. 2. The release of quenching of 9-aminoacridine fluorescence in a mitochondrial suspension by KCl

The medium was as described in the Materials and methods section plus 35 μ M-EDTA and Jerusalem-artichoke mitochondria at a concentration of 0.20 mg of protein/ml. KCl was added to the final concentrations indicated and the fluorescence of 9-aminoacridine was measured (see the Materials and methods section). The broken line indicates the determination of the c_1 value to be 12 mM.

quenching at widely different concentrations, whereas salts containing the same cation but different anions, like MgCl_2 and MgSO_4 , were equally efficient. It is also apparent that the valency of the

cation was more important than its chemical specificity; trivalent cations displaced 9-aminoacridine from the diffuse layer at concentrations 1000 times lower than for univalent cations, whereas bivalent cations showed an intermediary efficiency. The c_1 values obtained with the same cation in the two types of mitochondria were very similar (Table 1).

The bulky cations were less efficient than other cations of the same valency at releasing the quenching of 9-aminoacridine fluorescence. Choline chloride gave a c_1 value that was $56 \pm 13\%$ (five preparations of both types of mitochondria) higher than that of KCl determined on the same mitochondrial preparations. Decamethylenebistrimethylammonium bromide and lysyl-lysine were eight to ten times less efficient at displacing 9-aminoacridine from the diffuse layer than were the bivalent metal cations. In addition, with these bulky cations the maximum values of fluorescence release were markedly lower than that reached with MgCl_2 . An explanation for this observation was not pursued further.

Only 15–20 μ M-EDTA was used in the experiments presented in Table 1, since higher concentrations of EDTA were found to interfere with the measurements of 9-aminoacridine fluorescence when metal bivalent cations were used, probably due to chelation.

Because of the very high stability constant of La^{3+} with EDTA (10^6 times higher than for Ca^{2+} ; Sillén & Martell, 1971), the c_1 value for La^{3+} was measured in the absence of EDTA and a direct

comparison between the c_1 values for La^{3+} and for $[\text{tris}(\text{ethylenediamine})\text{cobalt}]^{3+}$, which is presumably non-complexing (Barber & Searle, 1978), should not be attempted since the experimental conditions differed. The two trivalent cations were, however, efficient in the same order of magnitude in releasing 9-aminoacridine fluorescence.

The effect of protein concentration on the release of quenching of 9-aminoacridine fluorescence by cations

The concentration of mitochondrial protein in the assay had a marked effect on the c_1 values obtained with all cations as shown in Fig. 3 for KCl, CaCl_2 and $[\text{tris}(\text{ethylenediamine})\text{cobalt (III) chloride}]$.

With both types of mitochondria there is an increase in the c_1 value with increasing protein concentration and the size of the increase is dependent on the valency of the cation as well as on the type of mitochondrion. Over the protein concentration range used (0.02–0.44 mg/ml) the c_1 value measured on Jerusalem-artichoke mitochondria increased 5-fold, 9-fold and 30-fold for K^+ , Ca^{2+} and $[\text{tris}(\text{ethylenediamine})\text{cobalt}]^{3+}$ respectively. The c_1 values measured on Jerusalem-artichoke mitochondria are higher than those measured on *Arum* mitochondria except at very low protein concentrations. Here minimal and constant c_1 values appear to be reached, which are the same for the two kinds of mitochondria: KCl, 6 mM; CaCl_2 , 20 μ M; $[\text{tris}(\text{ethylenediamine})\text{cobalt (III) chloride}]$, 2 μ M.

It is now apparent that the results presented in Table 1, which show that the c_1 values were similar for both types of mitochondria for any given ion, were obtained because relatively low concentrations of Jerusalem-artichoke mitochondria were employed.

Concentrations of 9-aminoacridine, K^+ and Ca^{2+} in the bulk of the solution

According to the Gouy–Chapman theory it is the concentration of a cation in the bulk of the solution

Fig. 3. The dependence of the c_1 values on the concentration of mitochondrial protein

(a), (b) and (c) show c_1 values for KCl, CaCl_2 and $[\text{tris}(\text{ethylenediamine})\text{cobalt (III) chloride}]$ respectively, determined as described in the text (see also Fig. 2). In all three Figures the symbols have the same meaning: ○ and ● are results from seven preparations of Jerusalem-artichoke mitochondria; □ and ■ are results from eight preparations of *Arum* mitochondria; ● and ■ denote results from single preparations. In (a) only the results from one preparation of *Arum* mitochondria are presented. Other preparations gave similar linear plots.

(at infinite distance from the membrane) that determines the degree of screening and, consequently, the degree to which the quenching of 9-aminoacridine fluorescence is released. The strong effect of protein concentration noted above seemed to indicate that perhaps a significant proportion of the cations was taken up into the diffuse layer at high protein concentrations, thereby decreasing the bulk concentration of the cation.

We set out to test this hypothesis by determining the concentrations of 9-aminoacridine, K^+ and Ca^{2+} in the bulk of the solution as described in the Materials and methods section.

When K^+ was added to a suspension of mitochondria all the K^+ remained in the supernatant after the mitochondria had been removed by centrifugation. On the other hand the concentration of 9-aminoacridine (also a univalent cation) in the bulk of the solution was markedly decreased in the presence of mitochondria and significantly more efficiently by Jerusalem-artichoke than by *Arum* mitochondria (Fig. 4). This difference in behaviour between 9-aminoacridine and K^+ was not due to K^+ present in the diffuse layers of the mitochondria, since an experiment showed that no significant exchange took place between Na^+ in the bulk of the solution and K^+ in the mitochondrial suspension (results not shown).

Both types of mitochondria removed a significant proportion of added Ca^{2+} from the bulk of the solution and Jerusalem-artichoke mitochondria were twice as efficient as *Arum* mitochondria in doing this at the same protein concentration (Table 2). The

Fig. 4. The concentration of 9-aminoacridine in the bulk of the solution at different concentrations of Jerusalem-artichoke (●) and *Arum* mitochondria (x).

The mitochondria were incubated in 0.3 M-sucrose/2 mM-potassium Tes (pH 7.2)/20 μM-9-aminoacridine plus 100 μM-EDTA and pelleted. The concentration of 9-aminoacridine was determined in the supernatant and in the medium to which no mitochondria had been added. Each point is the average of two determinations and the bars indicate standard errors. Where no bar is shown the error was smaller than 0.3 μM.

Table 2. The concentration of Ca^{2+} in the bulk of the solution in the presence of mitochondria measured at different concentrations of added Ca^{2+}

Mitochondria (0.2 mg of protein/ml for both types) were incubated in 0.3 M-sucrose/2 mM-potassium Tes (pH 7.2)/20 μM-9-aminoacridine/15 μM-EDTA and after 5 min Ca^{2+} was added at the concentrations given in the Table. The concentration of Ca^{2+} was determined in the mitochondrial supernatant by the use of atomic-absorption spectrometry. The medium used contained about 10 μM- Ca^{2+} .

Concentration of added Ca^{2+} (μM)	[Ca^{2+}] in the bulk (μM)					
	<i>Arum</i> mitochondria			Jerusalem-artichoke mitochondria		
	(a) Found*	(b) Calculated†	Difference (a-b)	(a) Found*	(b) Calculated†	Difference (a-b)
0	22	—	—	23	—	—
10	29	32	-3	25	33	-8
20	36	42	-6	31	43	-12
30	45	52	-7	38	53	-15
50	60	72	-12	49	73	-24
100	—	122	—	88	123	-35
150	147	172	-25	—	173	—
200	—	222	—	166	223	-59

* Experimentally determined.

† Calculated as the concentration of Ca^{2+} found in sample to which no Ca^{2+} had been added plus the concentration of added Ca^{2+} .

dependence of the amount of Ca^{2+} removed from the bulk solution on protein concentration was also much greater in Jerusalem-artichoke mitochondria (results not shown).

Discussion

9-Aminoacridine as a probe of the diffuse layers in mitochondria

The data in the Results section show that 9-aminoacridine is a useful probe of the behaviour of cations in the diffuse layer associated with mitochondrial membranes. The fluorescence of 9-aminoacridine in a low-cation medium is quenched when mitochondria are added (Fig. 1) and there is a concomitant decrease in the concentration of 9-aminoacridine in the bulk of the solution (Fig. 4). This indicates that 9-aminoacridine is concentrated into the diffuse layers associated with the mitochondrial membranes. These must be negatively charged and this was confirmed by a particle electrophoretic study in which both intact mitochondria and submitochondrial particles were found to have a net negative charge (I. M. Møller & H. Y. Nakatani, unpublished work). Addition of cations caused an increase in 9-aminoacridine fluorescence (Fig. 2). The relative efficiency of cations of different valencies and the independence of the chemical properties of the cation (Table 1) are consistent with the Gouy–Chapman theory (McLaughlin, 1977). With increasing concentration of cations the electrical potential will decrease due to screening of the fixed surface charges, 9-aminoacridine will be released into solution and fluorescence will increase. 9-Aminoacridine can thus be used to monitor the degree of screening in mitochondria as it has previously been used with thylakoid membranes (Searle *et al.*, 1977; Chow & Barber, 1980).

Since the outer membrane of mitochondria is permeable to all molecules of low molecular weights (Pfaff *et al.*, 1968), possibly with an upper limit corresponding to a diameter of about 1.5 nm (Parsons *et al.*, 1965; Colombini, 1979), all molecules used in the present study should have access to the intermembrane space and thus to three membrane surfaces. Neutral 9-aminoacridine can penetrate the mitochondrial inner membrane (Bashford & Thayer, 1977), so a significant proportion of the 9-aminoacridine taken up by the mitochondria (Fig. 4) could, in theory, be present in the matrix as a result of a Donnan potential (any pH gradient formed would make the matrix relatively basic and thus not favour accumulation of 9-aminoacridine). Two observations allow us to rule out this possibility. (1) A major portion of the quenching of 9-aminoacridine fluorescence was brought about by EDTA (Fig. 1), which does not cross the inner membrane (Reed & Bygrave, 1974; Wehrle *et al.*, 1976; Imedidze *et al.*,

1978). (2) Univalent cations take minutes to come into equilibrium across the inner membrane of plant mitochondria and the uptake of metal bivalent cations is energy-linked (Wilson & Graesser, 1976). Yet the effect of these cations on 9-aminoacridine fluorescence was fast and fluorescence was brought back close to the initial value (Fig. 2). It is possible that the small difference between the initial value and the recovered fluorescence was caused by 9-aminoacridine inside the matrix and not reached by the cations. However, the major part of the fluorescence quenching observed in the present study can be ascribed to entry of 9-aminoacridine into the diffuse layers associated with the membrane surfaces not facing the matrix.

The effect of chelators

The chelator-dependent reduction of 9-aminoacridine fluorescence shown in Fig. 1 is interpreted as being due to the removal of bivalent cations from the membranes. A similar observation has also been made with thylakoid membranes and EDTA (Chow & Barber, 1980). Because EDTA caused a greater quenching of fluorescence than EGTA (Fig. 1) it is assumed that a mixture of two-thirds Ca^{2+} and one-third Mg^{2+} was bound to the membranes. EGTA would remove only Ca^{2+} , since it complexes this cation 10^5 times better than Mg^{2+} (Sillén & Martell, 1964, 1971), whereas EDTA would remove both Ca^{2+} and Mg^{2+} .

The c_i value and the effect of protein concentration

An increase in c_i value for all cations irrespective of valency was observed with increasing concentration of mitochondrial protein (Figs. 3a–3c). It was suggested that this could be due to a significant decrease in the concentration of cations in the bulk of the solution as a consequence of the increase in the volume of the diffuse layer with increasing protein concentrations. Such a decrease in the bulk concentration would lead to overestimates of the c_i value since the added concentration of cations is used in the calculations, whereas it is the actual concentration of the cation in the bulk of the solution that is responsible for the degree of screening.

With Ca^{2+} the decrease in bulk concentration could partly explain the dependence of the c_i value on protein concentration (Fig. 3b, Table 2). However, neither *Arum* nor Jerusalem-artichoke mitochondria significantly decreased the concentration of K^+ in the bulk phase even at a protein concentration of Jerusalem-artichoke mitochondria of 0.2 mg/ml, which would give a 3-fold increase in c_i for K^+ (Fig. 3a). A mathematical analysis was then attempted to see if the relationship between the c_i value and the concentration of mitochondrial

protein (Fig. 3) is, in fact, inherent in the Gouy–Chapman theory.

The c_4 values for K^+ calculated as explained in the Appendix are shown in Fig. 5, together with experimentally obtained values taken from Fig. 3(a). There is a good resemblance between the shape of the experimental and calculated curves for both types of mitochondria. The difference between the absolute values could derive from the approximations and assumptions made in the analysis (see below). It is clear, however, that the Gouy–Chapman theory does predict an increase in the c_4 value with increasing area of negatively charged membranes per unit volume of suspension.

The behaviour of 9-aminoacridine in the diffuse layer

The concentration of 9-aminoacridine in the bulk solution was decreased markedly in the presence of mitochondria (Fig. 4). However, as discussed above, the concentration of K^+ was unchanged. The distribution of two ideal univalent cations between the diffuse layer associated with a negatively charged surface and the bulk solution would be expected to be the same and this was not the case for 9-aminoacridine and K^+ . The difference must lie with the special properties of 9-aminoacridine, since K^+ would be expected to behave very much like an ideal univalent cation.

9-Aminoacridine is known to aggregate even at $120\mu\text{M}$ in an aqueous solution (Albert, 1966). This

aggregation may be the cause of the concentration quenching observed with 9-aminoacridine (Searle *et al.*, 1977), but it would certainly have the effect of forming bi-, ter- or multi-valent cations (Albert, 1966), whose behaviour in the diffuse layer would be quite different from that expected for a univalent cation. This could explain the fact that 9-aminoacridine associates more readily with the diffuse layer than does K^+ . It could also explain the difference between the experimentally obtained and the calculated curves shown in Fig. 5. One of the assumptions made in the mathematical analysis (Appendix) was that 9-aminoacridine behaves like a univalent cation. The proposed aggregation would make 9-aminoacridine harder to displace by K^+ and lead to higher experimental values. This is precisely what was found (Fig. 5).

As mentioned in the introduction 9-aminoacridine has been used to measure pH gradients across biological membranes and the high-cation conditions used in these measurements would, as discussed above, prevent 9-aminoacridine accumulation into the diffuse layers. The phenomena described in the present paper would not be observed. However, one of the assumptions made in the calculations of ΔpH is that 9-aminoacridine behaves like a univalent cation in both compartments (Schuldiner *et al.*, 1972). The aggregation of 9-aminoacridine molecules in the acid compartment would violate this assumption and one would therefore expect values obtained with 9-aminoacridine to represent overestimates of ΔpH .

A comparison between Arum and Jerusalem-artichoke mitochondria

Jerusalem-artichoke and *Arum* mitochondria showed marked differences throughout the present investigation. At a given protein concentration Jerusalem-artichoke mitochondria required a higher cation concentration to release the quenching of 9-aminoacridine fluorescence (Figs. 3 and 5) and removed more Ca^{2+} and 9-aminoacridine from the bulk phase than did an equal amount of *Arum* mitochondria (Fig. 4, Table 2).

One explanation for these differences could be that Jerusalem-artichoke mitochondria contain more negative charges per mg of protein than do *Arum* mitochondria. This could be due to either a greater density of charges per unit area of membranes or a greater area of membrane per mg of protein.

Charges on the membranes have been shown to have a significant effect on NADH oxidation in Jerusalem-artichoke mitochondria (Johnston *et al.*, 1979). Thus the differences in electrostatic properties between the two kinds of plant mitochondria may have a special significance, since they also differ greatly in their capacity to oxidize exogenous NADH; *Arum* mitochondria oxidize added NADH

Fig. 5. Comparison between experimental and calculated c_4 values for K^+ at different concentrations of mitochondria

The experimental c_4 values are taken from Fig. 3(a) and have the same symbols as in that Figure (●, Jerusalem artichoke; ■, *Arum*; both sets of results from one preparation). The calculated values (○, □) are based on the 9-aminoacridine bulk concentrations presented in Fig. 4 and were derived as described in the Appendix using a background concentration of K^+ of 1.2 mM and $\sigma = -0.005\text{ C}\cdot\text{m}^{-2}$.

up to 10 times faster than do Jerusalem-artichoke mitochondria (Johnston *et al.*, 1979; Palmer, 1979).

We thank Mrs. Jill Farmer for expert technical assistance. I. M. M. is the recipient of a NATO Science Fellowship and grants no. 511-15019 and 511-20033 from the Danish Natural Science Research Council. This study was supported by grants from the Science Research Council, The Royal Society and the Central Research Fund, University of London.

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APPENDIX

The estimation of the $c^{\frac{1}{2}}$ value of K^+

Let [9-aminoacridine] be a_b in the bulk and a in the diffuse layer. Let A be the effective area of mitochondrial membranes in a suspension of volume V and let $c = [K^+]$ in the bulk. Assuming that 9-aminoacridine behaves purely as a univalent cation, the excess amount of 9-aminoacridine above the bulk values per unit area residing in the diffuse layer is related to the difference between the total concentration of 9-aminoacridine added (a_t) and the bulk concentration (a_b) by:

$$\begin{aligned} a_t - a_b &= \frac{A}{V} \int_0^{\infty} (a - a_b) dx \\ &= a_b \frac{A}{V} \int_0^{\infty} (e^{-F\psi/(RT)} - 1) \frac{dx}{d\psi} d\psi \end{aligned} \quad (1)$$

since $a = a_b e^{-F\psi/(RT)}$ according to the Boltzman

distribution, where ψ is the electrical potential at a distance x from the membrane surface, F the Faraday constant, R the gas constant and T the absolute temperature. For a 1:1 electrolyte solution ($c + a_b \approx c$) the Gouy–Chapman theory predicts that:

$$\frac{d\psi}{dx} \approx - \left(\frac{8cRT}{\epsilon_0 \epsilon_r} \right)^{\frac{1}{2}} \sinh \frac{F\psi}{2RT} \quad (2)$$

where ϵ_0 is the permittivity of vacuum and ϵ_r is the dielectric constant of water.

Substituting eqn. (2) into eqn. (1) and rearranging:

$$\frac{a_t - a_b}{a_b} \frac{V}{A} \left(\frac{8F^2}{RT\epsilon_0\epsilon_r} \right)^{\frac{1}{2}} = \frac{-1}{c^{\frac{1}{2}}} \int_0^{\infty} \frac{(e^{-y} - 1)}{\sinh(y/2)} dy \quad (3)$$

where $y = F\psi/RT$

$$y_0 = 2 \sinh^{-1} \left(\frac{\sigma}{(8RT\epsilon_0\epsilon_r c)^{\frac{1}{2}}} \right)$$

and σ is the surface charge density of the membrane in $C \cdot m^{-2}$. Assuming a certain charge density (σ) it is now possible to estimate V/A at a given protein concentration using an experimentally determined $a_{b \min}$ the minimum bulk concentration of 9-amino-acridine corresponding to maximum fluorescence quenching (this value is plotted against the protein concentration in Fig. 4 of the main paper).

We now wish to find the 'true c_i value', that is, the K^+ concentration at which a_b will have increased to:

$$a_{b, \frac{1}{2}} = \frac{0.02 + a_{b \min.}}{2}$$

($a_i = 0.02$ mM was the concentration of 9-amino-acridine used). This can be done by first numerically evaluating the right-hand side of eqn. (3) between the limits y_0 and y_d , where $d = 15$ nm, for a range of K^+ concentrations and then using a graphical method to find the K^+ corresponding to the required $(a_i - a_{b, \frac{1}{2}})/a_{b, \frac{1}{2}}$ as given in eqn. (3). The c_i (apparent) is now found by subtracting the background $[K^+]$ from the 'true c_i value'. The above procedure is repeated for other protein concentrations, with corresponding values of V/A .