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ENERGY-LINKED PROTONATION OF QUINACRINE IN BEEF HEART SUBMITOCHONDRIAL MEMBRANES

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SUMMARY

1. The absorption spectrum of quinacrine in aqueous solution, in the visible region, changes with the pH of the medium in the pH range from 6.0 to 9.0 with an isosbestic point at 353 nm. This indicates that the monoprotonated (quinacrine · H^+) and the diprotonated (quinacrine · $2H^+$) forms of quinacrine at equilibrium in this pH range have a 1 to 1 stoichiometry.

2. The monoprotonated and the diprotonated forms of quinacrine exhibit similar fluorescence emission spectra, but distinctive fluorescence excitation spectra.

3. The relative fluorescence quantum yields of quinacrine in aqueous media of various pH values are estimated. The relative fluorescence quantum yield of quinacrine at pH 9.0 is more than 3 fold of that at pH 6.0.

4. The fluorescence excitation and emission spectra, as well as the relative fluorescence quantum yield of quinacrine associated with non-energized submitochondrial membranes, are similar to those of quinacrine alone.

5. Analyses of the absorption spectra, the fluorescence excitation spectra and the relative fluorescence quantum yield indicate that the energy-linked fluorescence decrease of quinacrine associated with the energized submitochondrial membranes results from the protonation of quinacrine · H^+ to form quinacrine · $2H^+$.

6. Quantitative data are provided indicating that the maximal efficiency of protonation of quinacrine · H^+ to form quinacrine · $2H^+$ depends on the concentration of H^+ in the membranes generated through energy coupling, and the concentration of quinacrine · H^+ initially present in the reaction medium. Under optimal conditions virtually complete conversion of quinacrine · H^+ into quinacrine · $2H^+$ is observed.

7. The fluorescence intensity of quinacrine, either alone or associated with non-energized submitochondrial membranes, decreases with increasing temperature. When quinacrine is associated with the energized membranes, however, its fluorescence intensity increases slightly with increasing temperature. This unusual fluorescence behavior towards temperature, together with the fact that under optimal conditions virtually all the quinacrine molecules associated with the energized membranes are in the diprotonated form, further substantiate our earlier conclusion that the diprotonated quinacrine molecules are tightly bound to the energized membranes in a fashion which does not permit ready equilibration with the external medium.

INTRODUCTION

The application of aminoacridine dyes as fluorescent probes in the field of bioenergetics has drawn wide attention in recent years (cf. refs. 1–4). The use of quinacrine as a fluorescent probe for the energized state of biological membranes was introduced by Kraayenhof [5] in 1970 with a chloroplast membrane preparation. Subsequently, it has been used in various laboratories to study the energy-linked functions of bacterial membrane [6], chromatophores [7] and beef heart submitochondrial membranes [8, 9].

A number of hypotheses have been proposed for the mechanism by which energy-linked fluorescence decreases of quinacrine occur. They are: (A) a tighter binding of the dye molecules to the energized membrane [10–12]; (B) the accumulation of dye molecules in an osmotically active space of the membrane vesicles as a consequence of the established pH gradient across the membrane [7, 13–16]; (C) the dimerization or stacking of dye molecules to the energized membranes [17–19]; and (D) protonation of the dye molecules and their subsequent tighter binding to the energized membranes [1, 8, 20, 21].

In this paper a systematic spectrophotometric analysis of quinacrine either alone or associated with beef heart submitochondrial membranes at various metabolic states has been made. Quantitative evidence is provided which indicates that the energy-linked fluorescence decrease of quinacrine results primarily from the formation of the diprotonated species via protonation of the monoprotonated species of quinacrine, followed by a tigher binding to the energized membranes. Part of this work has been reported in a preliminary form [22].

MATERIALS AND METHODS

Submitochondrial particles derived from heavy beef heart mitochondria by sonic disruption in the presence of EDTA were prepared as described previously [23, 24]. The particles were then treated with oligomycin (1 μg/mg of protein) and the excess oligomycin was removed by centrifugation. This preparation is designated as OESP [8]. All experiments were performed with OESP unless otherwise indicated. Quinacrine hydrochloride and oligomycin were obtained from Sigma Chemical Co., all other chemicals were of the highest purity available commercially. Glass redistilled water was used throughout the present study.

The fluorescence emission and excitation spectra of quinacrine were measured with a Hitachi-Perkin Elmer (model MPF-2A) fluorescence spectrophotometer. An Aminco DW-2 UV/VIS spectrophotometer was used for absorption measurements. Both fluorescence and absorption spectra were recorded at 25 °C unless otherwise indicated. Protein was determined by the biuret method [25]. Further experimental details are specified in the figure and table legends.

RESULTS AND DISCUSSION

Effect of pH on the absorption spectra of quinacrine

Quinacrine is a strong diacidic base with pK_a values of 10.3 and 7.7, respectively [26]. At pH 9.0, quinacrine is present in aqueous solution mainly as the monoproton-

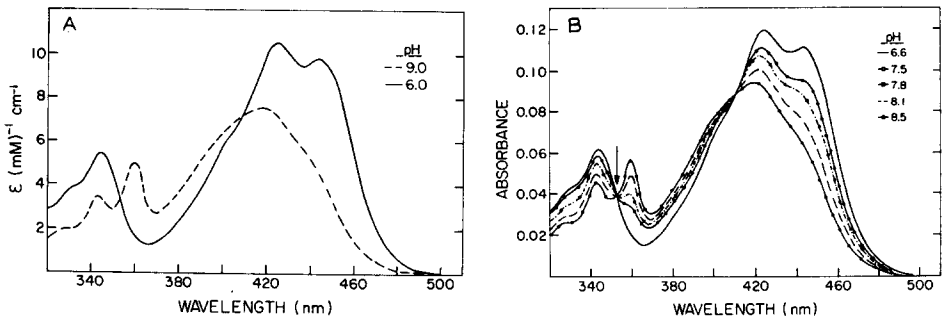

Fig. 1. Absorption spectra of quinacrine. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris/maleate buffer of various pH and 12 μM quinacrine. (A) pH 9.0 and 6.0; (B) pH 6.6, 7.5, 7.8, 8.1 and 8.5. The arrow in (B) indicates the isosbestic point at 353 nm.

ated form (quinacrine · H^+), whereas at pH 6.0, mainly as the diprotonated form (quinacrine · $2H^+$). Fig. 1A shows the absorption spectra of quinacrine · H^+ and quinacrine · $2H^+$, respectively. The absorption spectrum of quinacrine in the visible region changes with the pH of the medium in the range from pH 6.6 to 8.5, with an isosbestic point at 353 nm (Fig. 1B) which indicates that the two forms of quinacrine at equilibrium in this pH range have a 1 to 1 stoichiometry (Eqn. 1):

$$\text{quinacrine} \cdot H^+ + H^+ \rightleftharpoons \text{quinacrine} \cdot 2H^+ \quad (1)$$

The relative molar concentration of quinacrine · H^+ and quinacrine · $2H^+$ in the quinacrine solution of a given pH can be estimated through the following equation (Eqn. 2):

$$\frac{[\text{quinacrine} \cdot H^+]}{[\text{quinacrine} \cdot 2H^+]} = \frac{A_x - A_{6.0}}{A_{9.0} - A_x} \quad (2)$$

Where A_x, $A_{6.0}$ and $A_{9.0}$ are the absorbances at a given wavelength of quinacrine in aqueous medium of pH X, 6.0 and 9.0, respectively. The distribution of quinacrine · H^+ and quinacrine · $2H^+$ so estimated is given in Fig. 2. A pK_a value of 7.85 is derived which is remarkably close to the reported value of 7.7 estimated at 30 °C for quinacrine in aqueous solution [26]. The [quinacrine · H^+] % as a function of pH is also shown in Fig. 2.

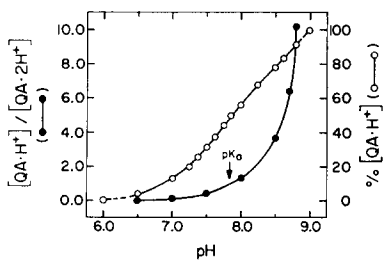

Fig. 2. Distribution of the monoprotonated (quinacrine · H^+) and the diprotonated (quinacrine · $2H^+$) forms of quinacrine as a function of pH. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris/maleate buffer of various pH and 3.3 μM quinacrine. The absorption spectra were recorded as shown in Fig. 1. The estimations were made according to Eqn. 2 shown in the text. QA, quinacrine.

Fluorescence emission and excitation spectra of quinacrine

The fluorescence emission spectra of quinacrine · H^+ and quinacrine · $2H^+$ exhibit similar characteristics with a maximum at 496 nm, but quinacrine · H^+ has greater emission intensity than quinacrine · $2H^+$. The excitation spectra are more complex, however. As shown in Fig. 3, the excitation spectrum of quinacrine · H^+ (spectrum I) differs markedly from that of quinacrine · $2H^+$ (spectrum III), both in intensity and in band structure. The excitation spectrum of the mixture of the two species at pH 7.5, the pH at which most experiments were carried out with submitochondrial membranes, is shown as spectrum II in Fig. 3. It is intermediate in band structure and intensity between the spectra of the individual components. This shows that neither quinacrine · H^+ nor quinacrine · $2H^+$ perturb the fluorescence characteristics of the other due to complex formation, a result in line with the distinct isosbestic point seen in the absorption spectra (Fig. 1).

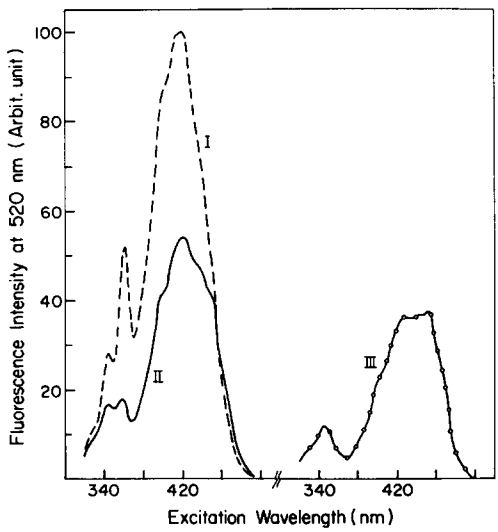

Fig. 3. Fluorescence excitation spectra of quinacrine. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris/sulfate buffer of various pH and 3.3 μM quinacrine. Spectrum I: pH 9.0; Spectrum II: pH 7.5; and Spectrum III: pH 6.5. The emission wavelength of 520 nm was used in all three cases.

Relative fluorescence quantum yield of quinacrine

The relative fluorescence quantum yield (Y) of quinacrine in solutions of various pH values can be estimated from the following equation (Eqn. 3):

$$Y_x = Y_{6.0} \cdot \frac{F_x}{F_{6.0}} \cdot \frac{A_{6.0}}{A_x} \tag{3}$$

Where F_x, $F_{6.0}$ and A_x, $A_{6.0}$ are the relative fluorescence and absorbance (at a given wavelength) of quinacrine at pH X and 6.0, respectively. Y_x and $Y_{6.0}$ are the fluorescence quantum yields of quinacrine at pH X and 6.0, respectively. The relative fluorescence quantum yields of quinacrine at various pH values, normalized to that at pH 6.0, are shown in Fig. 4.

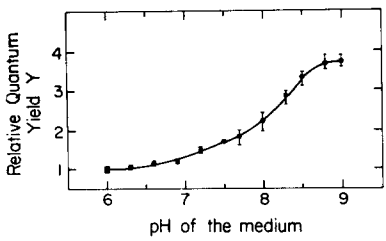

Fig. 4. The relative quantum yield (Y) of quinacrine as a function of the pH. The relative fluorescence quantum yields of quinacrine at various pH were computed according to Eqn. 3 shown in the text. Experimental conditions were as those indicated in Fig. 1.

Quinacrine associated with submitochondrial membranes

The presence of beef heart submitochondrial membranes in the non-energized state has virtually no effect on the fluorescence intensity of quinacrine. However, an appreciable reduction of the fluorescence intensity of quinacrine has been observed when the membranes become energized. A typical experiment is shown in Fig. 5, in which a low concentration of NaSCN was added in order to facilitate the energy-linked fluorescence response [8].

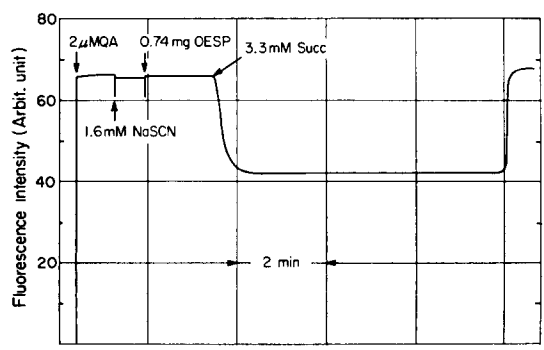

Fig. 5. Energy-linked fluorescence decrease of quinacrine associated with submitochondrial membranes. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris/sulfate, pH 7.5 and others as indicated. Total volume, 3.0 ml. The fluorescence intensity was measured at 500 nm with the excitation wavelength of 420 nm.

(*A*) *Relative fluorescence quantum yield and excitation spectra of quinacrine.* The fluorescence emission spectra of quinacrine associated with submitochondrial membranes, either in the non-energized or energized state, are very similar to those of quinacrine alone [8]. The relative fluorescence quantum yields of quinacrine associated with submitochondrial membranes in the non-energized and energized states are estimated to be 1.89 and 1.14, respectively (cf. Fig. 4). A similar fluorescence excitation spectrum of quinacrine associated with the non-energized submitochondrial membranes as compared with quinacrine alone, is also seen (Fig. 6, spectrum I). However, upon energization of the submitochondrial membranes, the excitation spectrum of quinacrine changes markedly with respect to its band structure

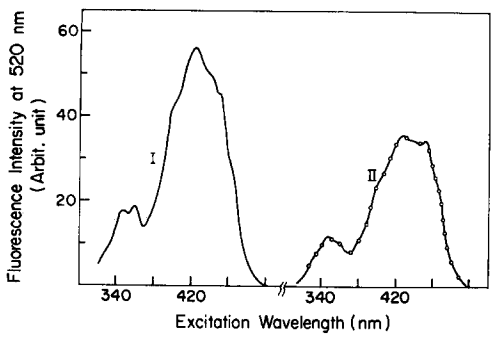

Fig. 6. Fluorescence excitation spectra of quinacrine associated with submitochondrial membranes. Conditions were as in Fig. 5. Spectrum I was recorded before the addition of succinate; and spectrum II was recorded during the aerobic steady state after the addition of succinate (cf. Fig. 5).

and intensity (Fig. 6, spectrum II) which bear a striking resemblance to that of quinacrine alone at a lower pH (cf. Fig. 3) in which virtually all quinacrine molecules are in the quinacrine · $2H^+$ form. These data suggest that the formation of quinacrine · $2H^+$ resulting from protonation of quinacrine · H^+ is responsible for the energy-linked fluorescence decrease of quinacrine. A similar conclusion was drawn from the change in the absorption spectrum of quinacrine under the same conditions [1].

(*B*) *Dependence on the concentration of submitochondrial membranes.* If protonation of quinacrine · H^+ is the origin of the energy-linked fluorescence decrease of quinacrine it would be expected that the extent of protonation of quinacrine · H^+ would be dependent on the amount of protons generated by the energized membranes and the concentration of quinacrine · H^+. Fig. 7 shows the relationship between the concentration of submitochondrial membranes (OESP) and the extent of the fluorescence decrease of quinacrine expressed as an increase in the ratio F°/F, where F° and F are the fluorescence intensities of quinacrine associated with submitochondrial membranes in the non-energized and energized states, respectively. F°/F increases with increasing concentrations of OESP until a plateau is reached. This would indicate

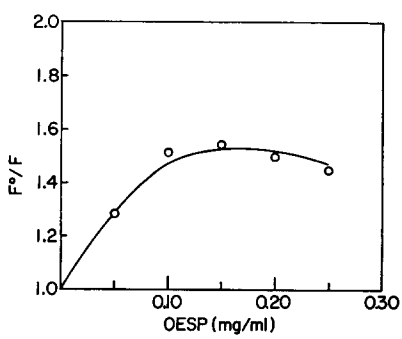

Fig. 7. Relationship between the energy-linked fluorescence decrease of quinacrine and the concentration of submitochondrial membranes. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris/sulfate, pH 7.5, 1.6 mM NaSCN and 3.3 μM quinacrine. 3.3 mM succinate was employed as the energy yielding substrate when indicated. F° and F are the fluorescence intensities of quinacrine associated with non-energized and energized submitochondrial membranes, respectively.

that the extent of fluorescence decrease of quinacrine is not only a function of the concentration of OESP, but also a function of the concentration of the active species of quinacrine molecules, presumably quinacrine · H^+.

(*C*) *Dependence on the concentration of quinacrine* · H^+. Under optimal conditions, when a saturating amount of H^+ is available in the energized membranes and the fluorescence intensity of quinacrine is proportional to its concentration, one would expect a quantitative relationship between the decrease in fluorescence intensity and the amount of quinacrine · H^+ being protonated. A quantitative analysis has been made accordingly as indicated below.

$$\text{quinacrine} \cdot H^+ + H^+ \rightarrow \text{quinacrine} \cdot 2H^+ \tag{4}$$

In the non-energized state:

$$[\text{quinacrine}] = M + D \tag{5}$$

Where [quinacrine] is the total concentration of quinacrine employed, M and D are the concentrations of the monoprotonated and diprotonated form of quinacrine, respectively. The fluorescence intensity (F°) of quinacrine in the non-energized state can be expressed as:

$$F^\circ = k''D + k'M \tag{6}$$

Where k' and k'' are the proportionality constants (fluorescence intensity/concentration) of quinacrine fluorescence for quinacrine · H^+ and quinacrine · $2H^+$, respectively.

Upon energization, a fraction (α) of quinacrine · H^+ is being protonated with the formation of quinacrine · $2H^+$. The fluorescence intensity (F) of quinacrine associated with the energized membranes can therefore be expressed as:

$$F = k''D + k'(1-\alpha)M + k''\alpha M \tag{7}$$

From Eqns. 6 and 7 it follows that

$$\Delta F = F^\circ - F = (k' - k'')\alpha M \tag{8}$$

hence

$$(\alpha M) = \Delta F/(k' - k'') = (1 - F/F^\circ)F^\circ/(k' - k'') \tag{9}$$

and

$$\%(\alpha M) = [(\alpha M)/(D+M)] \cdot 100 \tag{10}$$

where F/F°, F°, k', k'', M and D are all experimentally derived parameters. A number of $(\alpha M)_{max}$ and $\%(\alpha M)$ derived under various experimental conditions are given in Table I. The data show that the maximal amounts of quinacrine being protonated, in all cases studied, are approximately equal to the concentrations of quinacrine · H^+ initially present in the quinacrine solutions. The virtually complete conversion of quinacrine · H^+ into quinacrine · $2H^+$ implies that the quinacrine · $2H^+$ molecules formed are associated with the energized membranes in such a fashion that they are not readily equilibrated with the external medium and the reverse reaction of Eqn. 4 becomes negligible.

TABLE I

COMPARISON OF THE MAXIMAL AMOUNT OF QUINACRINE BEING PROTONATED WITH THE PERCENTAGE OF [QUINACRINE · H^+] PRESENT IN A QUINACRINE SOLUTION OF DEFINED pH

The reaction mixture consisted of 150 mM sucrose, 30 mM Tris/sulfate buffer of various pH and 2.0 μM quinacrine and 0.20–0.25 mg/ml OESP. 3.3 mM succinate was used as the energy yielding substrate unless otherwise indicated.

pH	$(\alpha M)_{max}$ (μM)	% $(\alpha M)_{max}$	% [quinacrine · H^+]**
8.5	1.44	72	78
	1.63	81	
8.1	1.36	68	62
	1.26	63	
	1.30	65	
	1.13	57	
	1.16	58	
7.8	0.91	45	46
	0.85	43	
7.5	0.70	35	31
	0.69	35	
	1.16*	35*	

* 3.3 μM instead of 2.0 μM quinacrine was employed; and NADH instead of succinate was employed as the energy yielding substrate.

** Data of Fig. 2.

(*D*) *Effect of temperature on the fluorescence intensity of quinacrine.* Fig. 8A shows the effect of temperature on the fluorescence intensity of quinacrine at pH 9.0, 8.1 and 6.6. The effect of temperature on the fluorescence intensity of quinacrine associated with non-energized (●---●) and energized (▲---▲) membranes is shown in Fig. 8B. In line with our earlier observations [1], the fluorescence intensity of quinacrine decreased with increasing temperature when quinacrine molecules were either alone in solution or associated with the non-energized membranes. However,

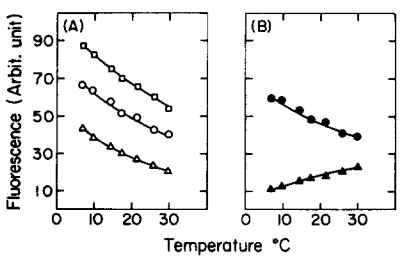

Fig. 8. Effect of temperature on the fluorescence intensity of quinacrine. (A) The reaction mixture consisted of 150 mM sucrose, 2 μM quinacrine and 30 mM Tris/sulfate of pH 9.0 (□---□), pH 8.1 (○---○) and pH 6.3 (△---△). (B) The reaction mixture consisted of 150 mM sucrose, 30 mM Tris/sulfate, pH 8.1, 2 μM quinacrine and 0.42 mg OESP. 3.3 mM succinate was employed as the energy yielding substrate. (●---●) and (▲---▲) are the quinacrine fluorescence responses associated with the non-energized and energized submitochondrial membranes, respectively.

when quinacrine molecules are associated with the energized membranes, the responses towards temperature are greatly altered, a slight increase in fluorescence intensity with increasing in temperature is observed. This unusual behavior towards temperature, together with the demonstration in the preceeding section that, under optimal conditions, virtually all the quinacrine molecules associated with the energized membranes are in the diprotonated form, further substantiate our earlier contention [1] that the diprotonated quinacrine molecules are tightly bound to the energized membranes in such a way that can not readily equilibrate with the external medium. This change in the environment of the quinacrine molecule may account for the unusual behavior of its fluorescence, and implies that the protonation reaction being sampled by the quinacrine probe occurs in the membrane.

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