A Fluorescent Probe of the Hydrogen Ion Concentration in Ethylenediaminetetraacetic Acid Particles of Beef Heart Mitochondria*

Chuan-pu Lee

ABSTRACT: With low concentrations of quinacrine which do not give uncoupling of the oligomycin-coupled system, energization of the submitochondrial membrane supported by aerobic oxidation of NADH, succinate, or ascorbate plus phenazine methosulfate produces virtually no change in the intensity of the quinacrine fluorescence. Up to 50% decrease in intensity can be induced upon subsequent addition of salts with lyophilic monovalent anions. The order of potency ($SCN^- > ClO_4^- > I^- > NO_3^- > Br^-, Cl^-$) corresponds to the order of the lyotropic numbers of the anions. The decreased fluorescence can be restored upon subsequent addition of uncoupler. Nigericin, but not valinomycin, in combination with either K^+ or Na^+ can substitute for uncoupler in restoring the intensity of quinacrine fluorescence. Under similar conditions, valinomycin in combination with K^+, but not with Na^+, can induce a further decrease in quinacrine fluorescence in the presence of limiting concentration of lyophilic anions. These data indicate that under appropriate conditions quinacrine can act as a probe for the measurement of the H^+ concentration in EDTA particles of beef heart mitochondria.

Quinacrine (Figure 1) [9-(4-diethylamino-1-methylbutylamino)-3-chloro-7-methoxyacridine], an antimalarial agent and a flavin antagonist (Albert, 1966), has long been known to inhibit NADH–cytochrome *c* reductase and D-amino-oxidase reactions (Wright and Sabine, 1944; Haas, 1944; Hellerman *et al.*, 1946), to uncouple oxidative phosphorylation (Hunter, 1955), and to inhibit or stimulate the mitochondrial ATPase (Löw, 1959).

Recently quinacrine has been employed as a fluorescence probe to study the energized state in chloroplasts (Kraayenhof, 1970), bacterial membrane fragments (Eilermann, 1970, 1971), and chromatophores (Gromet-Elhanan, 1971). Kraayenhof (1970) has shown that the fluorescence of quinacrine, added to a suspension of chloroplasts, is completely suppressed when the chloroplasts are energized by light-induced electron transport, by ATP hydrolysis, or by a pH gradient. The fluorescene is restored when the system is uncoupled. It was therefore concluded that there is a stoichiometric relationship between the generation of energy and the quenching of quinacrine fluorescence in chloroplasts. This conclusion has been supported by Eilermann (1970, 1971) from studies with phosphorylating membrane fragments of *A. vinelandii* but challenged by Gromet-Elhanan (1971) from studies with *R. rubrum*. Gromet-Elhanan suggested that the decrease in fluorescence may reflect changes in binding of the quinacrine molecules by the chromatophores, resulting from the changes in pH between the inside and outside of the membrane, rather than being directly related to the generation of energy.

In the present paper, experimental evidence is reported which indicates that low concentrations of quinacrine which do not give uncoupling action can be used as a probe of the H^+ concentration in submitochondrial membranes from beef heart mitochondria. Correlation between the response of cytochromes, the energy-linked ANS[1] fluorescence enhancement, and the intensity of quinacrine fluorescence, and the possible role of lyophilic anions in submitochondrial membranes will be discussed. Part of these data have been communicated briefly (Lee, 1971).

Methods and Materials

Ethylenediaminetetraacetic acid particles derived from heavy beef heart mitochondria by sonic disruption were prepared as described previously (Lee and Ernster, 1967). When indicated, the particles were treated with oligomycin (1 μg/mg of protein) and the excess of oligomycin was removed by centrifugation. This preparation is designated as OESP. Oxygen consumption was measured polarographically with a Clark oxygen electrode. Cytochrome *b* reduction was measured with a dual-wavelength spectrophotometer at 561–575 nm. ANS and quinacrine fluorescence were measured routinely with a Hitachi MPF-2A spectrofluorometer. The following wavelength pair, 380 nm for excitation with 480 nm for emission, and 420 nm for excitation with 500 nm for emission, were employed for ANS and quinacrine, respectively.

ANS obtained commercially was purified as described previously (Lee and Radda, 1971). The crystalline form of quinacrine hydrochloride was obtained from Sigma Chemical Co. Nigericin was kindly supplied by Drs. David Wong and J. M. McGuire of the Lilly Research Laboratories. All other chemicals used were of the highest purity available commercially.

Results

Fluorescence Emission and Excitation Spectra of Quinacrine. The fluorescence emission spectra of quinacrine in

* From the Johnson Research Foundation, Department of Biophysics and Physical Biochemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received July 12, 1971.* This work has been supported by grants from the National Science Foundation (GB 23253) and the National Institutes of Health (GM 12202 and 1-K4 GM 38822). The author is a Career Development awardee of N.I.H.

[1] Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BTB, bromothymol blue; ANS, 8-aminonaphthalene-1-sulfonate.

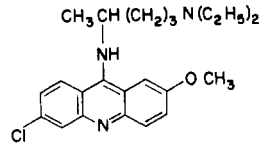

FIGURE 1: Structure formula of quinacrine. Other names of quinacrine are: acriquine, atabrin, atebrin, chinacrin, mepacrin, metoqine, italchin, acrichin, and erion SN 390 or 866 R.P.

the absence (curve 1) and presence (curve 2) of EDTA particles in 30 mM Tris-acetate buffer (pH 7.5) are shown in Figure 2A. Under these conditions, neither the peak position nor the fluorescence intensity of quinacrine was significantly

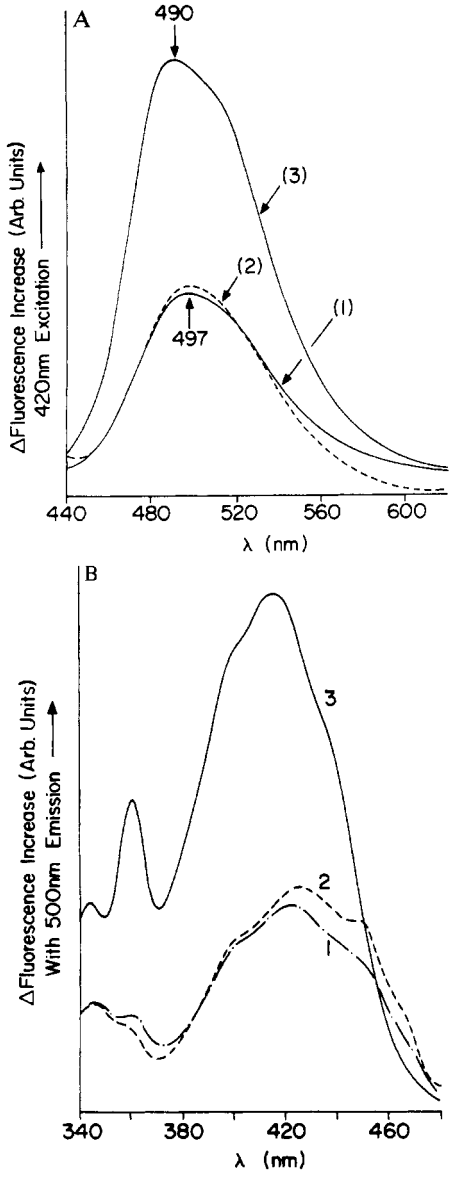

FIGURE 2: Emission (A) and excitation (B) spectra of quinacrine. (A) For spectra 1 and 2: the reaction mixture consisted of 200 mM sucrose, 30 mM Tris-acetate buffer (pH 7.5), and 3.3 μM quinacrine hydrochloride. When indicated, 0.9 mg of protein of oligomycin-pretreated EDTA particles (OESP) were added. For spectrum 3, 10 nmoles of quinacrine in 5 μl of water was added into 3.0 ml of absolute ethanol. The excitation wavelength of 420 nm was used in all these cases. (B) Conditions were as in A. The emission wavelength of 500 nm was used in all three cases.

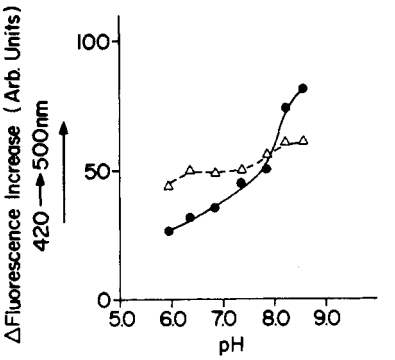

FIGURE 3: Effect of pH on the intensity of quinacrine fluorescence. The reaction mixture consisted of 200 mM sucrose, 30 mM MOPS–Tris buffer at the pH indicated, and 3.3 μM quinacrine hydrochloride. When indicated (dotted line) 0.9 mg of protein of OESP was also present.

affected upon the addition of submitochondrial membranes. For comparison, the fluorescence emission spectrum of quinacrine in ethanol is also presented (curve 3). An approximately 7-nm shift toward shorter wavelength and a twofold increase in intensity were observed from aqueous medium to ethanol. Similar results were also obtained with its excitation spectra (Figure 2B).

Effect of pH. As shown in Figure 3, in the absence of submitochondrial membranes a more than twofold increase in fluorescence intensity of quinacrine was observed from pH 6.5 to 8.5. This observation is in full agreement with that recently reported by Gromet-Elhanan (1971). In contrast with that observed with chromatophores (Gromet-Elhanan, 1971), the pH dependence of fluorescence intensity of quinacrine was diminished greatly upon the addition of EDTA particles. This would suggest some shielding of quinacrine molecules by the EDTA particles.

Effect of Monovalent Anions on the Fluorescence Intensity of ANS and Quinacrine. Low concentrations of perchlorate and thiocyanate which do not abolish the oligomycin-induced respiratory control of EDTA particles inhibit the energy-linked ANS fluorescence enhancement and the energy-linked binding of ANS to the membrane (Ernster *et al.*, 1971). Figure 4 summarizes the effect of various monovalent anions

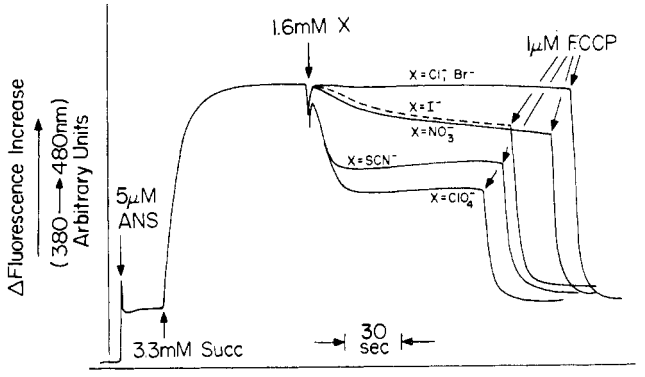

FIGURE 4: Effect of monovalent anions on the fluorescence intensity of ANS associated with the energized submitochondrial membrane. The reaction mixture consisted of 200 mM sucrose, 30 mM Tris-acetate buffer (pH 7.5), and 0.9 mg of protein of OESP. Potassium salts were used in all cases except ClO_4^- where sodium salt was used. Others are as indicated.

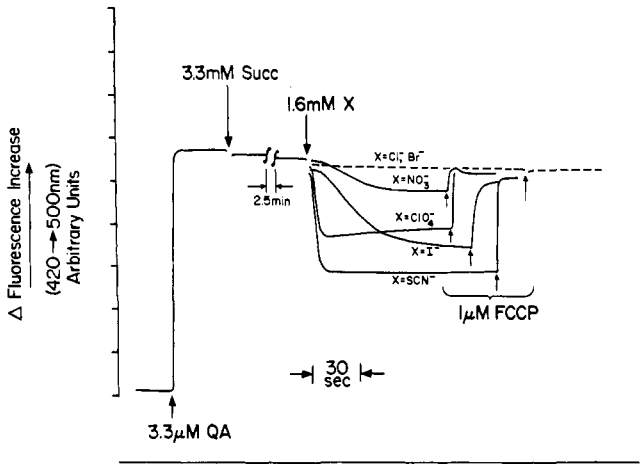

FIGURE 5: Effect of monovalent anions on the fluorescence intensity of quinacrine associated with the energized submitochondrial membrane. Conditions were those used in Figure 4 except 3.3 μM quinacrine (QA) replaced the ANS.

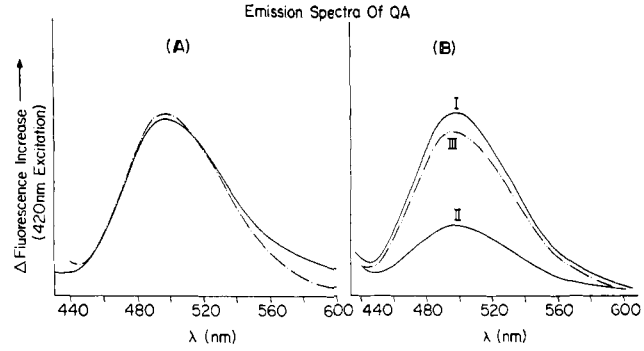

FIGURE 6: Effect of KSCN on the emission spectra of quinacrine at various metabolic states. (A) Solid line, 3.3 μM quinacrine in a medium consisting of 200 mM sucrose, 30 mM Tris-Ac (pH 7.5), and 1.6 mM KSCN. Dotted line, 0.9 mg of protein of OESP was added. (B) Spectrum I: 3.3 μM quinacrine in a medium consisting of 200 mM sucrose, 30 mM Tris-Ac (pH 7.5), 1.6 mM KSCN, and 0.9 mg of protein of OESP. Spectrum II: 3.3 mM Tris-succinate was added to the reagents used for spectrum I. Spectrum III: 1 μM FCCP was added to that for II.

on the energy-linked ANS fluorescence enhancement. Varying extent and rate of decrease in fluorescence can be induced upon the addition of KI, KNO_3, KSCN, and $NaClO_4$. Complete abolition of the energy-linked ANS fluorescence enhancement is achieved upon subsequent addition of FCCP. On the other hand, when ANS is replaced by quinacrine (Figure 5) the addition of succinate does not significantly change the intensity of quinacrine fluorescence. Up to 50% decrease in intensity can be induced upon subsequent addition of KSCN, as is observed with the energy-linked ANS fluorescence enhancement. Both the rate and extent of the decrease in fluorescence intensity are dependent on the anion species present, and the order of potency corresponds to the order of the lyotropic series (Hatefi and Hamstein, 1969; Wieth, 1970). Subsequent addition of FCCP reversed the fluorescence decrease induced by lyophilic anions, in contrast to the experiment with ANS (*cf.* Figure 4) in which a further decrease was observed. The distinct response of the energy-linked quinacrine fluorescence toward uncouplers eliminates the possible participation of the nonspecific light-scattering effect induced by these monovalent anions (Chance and Lee, 1969). These data indicated that the decrease of quinacrine fluorescence induced by lyophilic anions in the presence of respiring substrate is an energy-linked process. The decrease in fluorescence intensity induced by these anions occurs with no alteration of the quinacrine emission spectrum (Figure 6).

Figure 7 shows that in the presence of KSCN the energy required for the decrease of quinacrine fluorescence can be derived from aerobic oxidation of NADH, succinate, or ascorbate plus phenazine methosulfate (PMS). The decrease in fluorescence is reversed by electron-transfer inhibitors or by uncoupler (FCCP). Externally added ATP can also serve as the energy-yielding substrate (not shown). In this case, the decrease in fluorescence is reversed by oligomycin and FCCP. These results indicate that under appropriate conditions quinacrine can serve as a sensitive probe of the energy pool which is common to the three coupling sites of the respiratory chain.

Effect of Thiocyanate Concentration on the Intensity of Quinacrine Fluorescence. Figure 8 shows that both the rate (Figure 8A) and the extent (Figure 8B) of the decrease of quinacrine fluorescence supported by the aerobic oxidation of succinate in the oligomycin coupled state are dependent on the concentration of NaSCN. At these concentration levels, thiocyanate does not affect the oligomycin-induced respiratory control (Figure 8C), nor does it decrease the steady-state level of cytochrome *b* reduced by succinate in the oligomycin-coupled state (Figure 8D). A 50% decrease in the extent of quinacrine fluorescence intensity was observed with 2 mM NaSCN.

Effect of Valinomycin, Nigericin, and Monovalent Cations. In the presence of 33 mM KCl or NaCl, energization of submitochondrial membranes upon addition of succinate gave rise to a decrease of approximately 30% in quinacrine fluorescence with an initial delay of about 10 sec (Figure 9). Subsequent addition of valinomycin induced an additional 15% decrease in the case of KCl, whereas virtually no change was observed in the case of NaCl even with a second addition of valinomycin. On the other hand, when valinomycin was replaced by 1.3 mM NaSCN, an additional 15% decrease in fluorescence was observed with both KCl and NaCl. No fur-

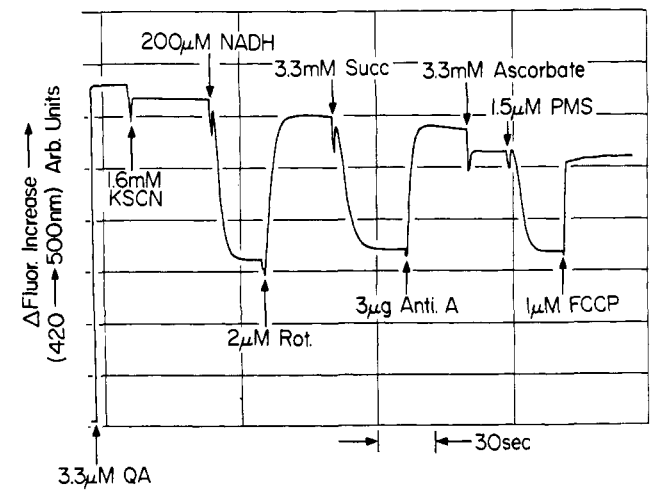

FIGURE 7: Decrease of fluorescence intensity of quinacrine induced with various energy-yielding substrates in the presence of KSCN. The reaction mixture consisted of 200 mM sucrose, 30 mM Tris-acetate buffer (pH 7.5), and 0.9 mg of protein of OESP. Others are as indicated.

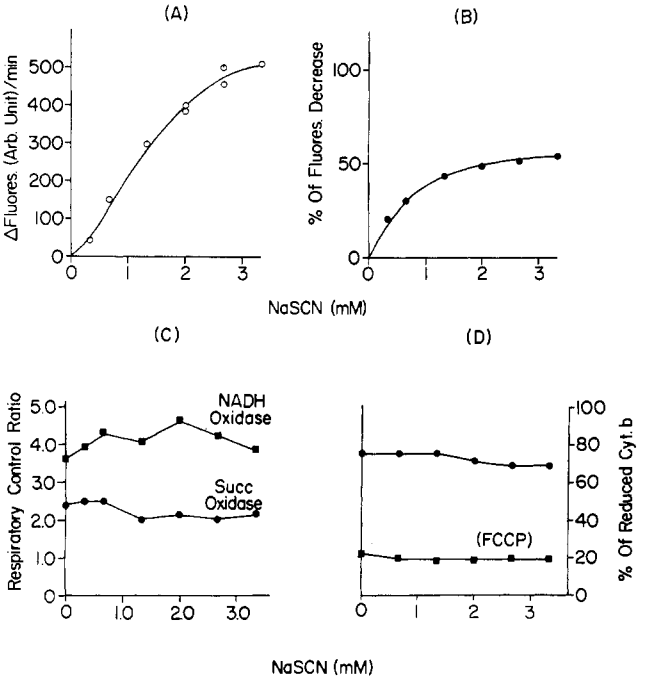

FIGURE 8: Effect of varying concentrations of NaSCN on the rate (A) and extent (B) of the decrease of fluorescence of quinacrine with succinate as the energy-yielding substrate, the oligomycin-induced respiratory control of NADH and succinate oxidase (C), and the steady-state level of reduced cytochrome *b* (D) supported by succinate oxidation. Conditions were the same as those indicated in Figure 7. Others are as indicated.

ther change was observed upon the addition of valinomycin to the NaSCN-pretreated system. Nigericin, in contrast to valinomycin, reversed the decrease of quinacrine fluorescence in all cases regardless of whether it was induced in the energized submitochondrial membranes by high concentrations of KCl or NaCl alone or in combination with valinomycin or NaSCN.

It should be noted that the response induced by nigericin is a relatively fast one. Unfortunately, the $t_{1/2}$ could not be estimated accurately with the instrument being used.

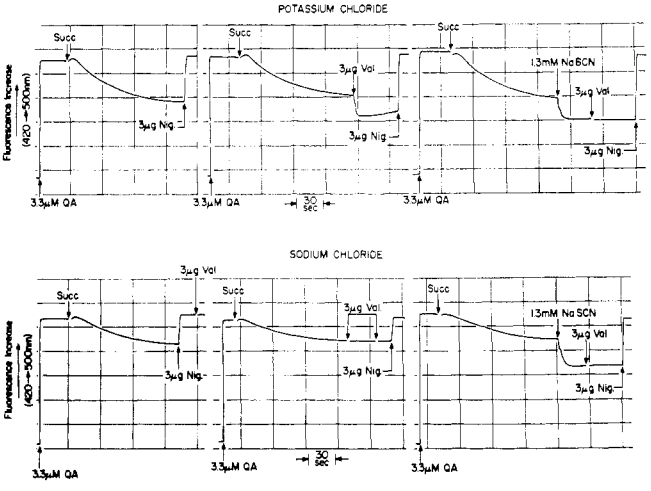

FIGURE 9: Effect of valinomycin and nigericin in combination with KCl or NaCl on the fluorescence intensity of quinacrine associated with the energized membrane. The reaction mixture consisted of 200 mM sucrose, 30 mM Tris-acetate buffer (pH 7.5), and 0.9 mg of protein of OESP. Others are 33 mM KCl, 33 mM NaCl, and 3.3 mM Tris-succinate.

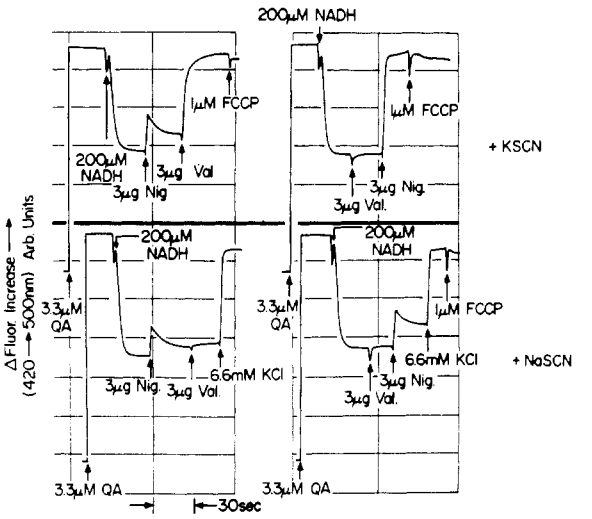

FIGURE 10: Cooperative action of valinomycin and nigericin in combination with K^+ in reversing the energy-dependent fluorescence decrease of quinacrine. The reaction mixture consisted of 200 mM sucrose, 30 mM Tris-acetate buffer (pH 7.5), and 0.9 mg of protein of OESP. Others are: 1.6 mM KSCN or 1.6 mM NaSCN.

Figure 10 shows that in the presence of low concentrations of K^+ (1.6 mM KSCN) both valinomycin and nigericin are required in order to achieve complete reversal of the energy-linked decrease of quinacrine fluorescence. These data are in accord with the observations previously established in this laboratory (Montal *et al.*, 1969a,b, 1970; Montal, 1971; Lee and Radda, 1971) and others (Cockrell and Racker, 1969; Papa *et al.*, 1969, 1970b,c) that K^+, in combination with valinomycin and nigericin, exhibits an uncoupling action. The K^+-dependent cooperative action of valinomycin and nigericin in uncoupling is further substantiated by the data shown in the lower portion of Figure 10.

Figure 11 illustrates the effect of varying concentrations of KCl on the extent of the energy-linked and the nigericin-induced responses of quinacrine fluorescence. It is clearly shown that KCl, at concentrations from 0.5 to 20 mM, did not alter the steady-state level of the energy-linked quinacrine fluorescence with succinate as the energy-yielding substrate

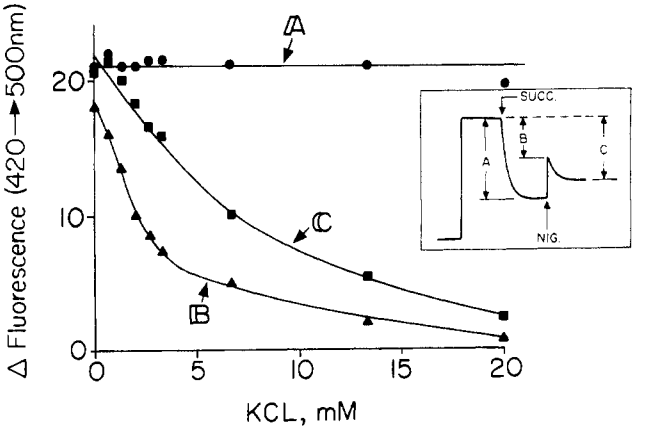

FIGURE 11: Effect of varying concentrations of KCl on the fluorescence intensity of quinacrine in the presence and absence of nigericin. The reaction mixture consisted of 200 mM sucrose, 30 mM Tris-acetate buffer (pH 7.5), 0.9 mg of protein of OESP, 1.6 mM KSCN, and 3.3 μM Tris-succinate. When indicated, 3 μg of nigericin was added. Others are as indicated.

TABLE I: Binding Parameters of Quinacrine to Submitochondrial Membrane at Various Metabolic States.[a]

		K_d (app) (μM)
1. Nonenergized	+1.6 mM KSCN	20
	+3.3 mM KSCN	20
2. Energized	+1.6 mM KSCN	2.2
	+3.3 mM KSCN	1.8

[a] Conditions were as in Figure 12B. The concentration range of quinacrine used is from 0.83 to 6.6 μM. The apparent K_d is the concentration of quinacrine which gives half-maximal change in fluorescence at 0.3 mg of protein/ml of the oligomycin-pretreated EDTA particles and is derived from the double-reciprocal plot of the fluorescence intensity and the concentration of quinacine.

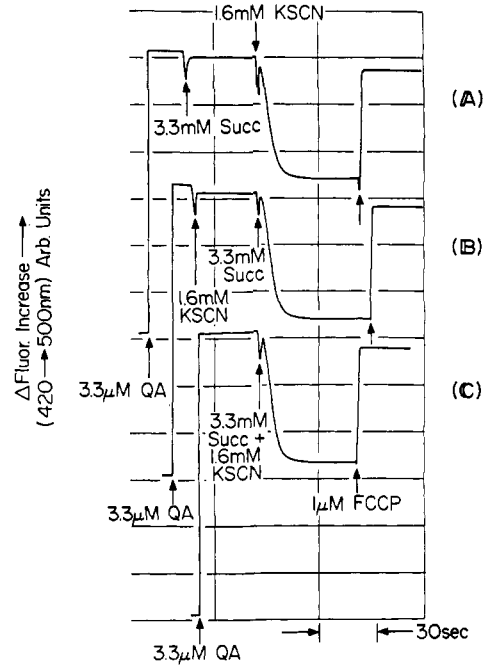

FIGURE 12: Effect of preincubation on the response of quinacrine fluorescence. The reaction mixture consisted of 200 mM sucrose, 30 mM Tris-acetate buffer (pH 7.5), and 0.9 mg of protein of OESP. Others are as indicated.

and 1.6 mM KSCN as the source of lyophilic anion. Nigericin induced a transient response; a fast increase was followed by a relatively slow diminution of fluorescence until a new steady state was reached. The dependence on KCl concentration of the nigericin-induced response either at the initial phase (curve B) or at the new steady state (curve C) is clearly illustrated. K^+ at concentrations of 3.5 and 7.5 mM is required to give 50% recovery of quinacrine fluorescence induced by nigericin, as estimated from its initial and steady-state level, respectively.

The distinct effects on quinacrine fluorescence exhibited by valinomycin and nigericin in combination with K^+ or Na^+ suggest that the energy-linked decrease of quinacrine fluorescence is a measure of the H^+ concentration in the membrane.

It is apparent that quinacrine can act as a probe of the H^+ concentration in EDTA particles, provided that a sufficient amount of lyophilic anion is also present. The question arises as to what role the lyophilic anion plays: does it promote the movement of quinacrine, the movement of H^+, or both into the mitochondrial membrane?

The dependence of lyophilic anion (A) and energy (B) on the decrease of quinacrine fluorescence is further illustrated in Figure 12. Virtually identical results with respect to both the extent and rate of decrease of fluorescence were obtained regardless of whether the mitochondrial membrane was preincubated with quinacrine alone (C) or in combination with succinate (A) or KSCN (B). These data indicate that lyophilic anions promote the movement of quinacrine molecules into the interior of the membrane where the H^+ generated from energization is accumulated. Data shown in Figure 12B suggest, although inconclusively, that lyophilic anions may also promote the movement of H^+ into the interior of the membrane. This conclusion is in accord with the recent observations reported by Papa *et al.* (1970b) that lyophilic anions promote H^+ uptake by submitochondrial membranes with aerobic oxidation of succinate as the energy-yielding substrate.

Binding of Quinacrine to EDTA Particles. Table I summarizes the binding parameters of quinacrine to EDTA particles at various metabolic states in the presence of 1.6 and 3.3 mM KSCN. A tenfold increase in binding affinity of quinacrine to EDTA particles from nonenergized to energized state was observed. An increase in thiocyanate concentration does not appear to affect the binding affinity of quinacrine to EDTA particles in the nonenergized state, but slightly enhanced it in the energized state.

Discussions and Conclusions

Quinacrine is a strong diacidic base with pK_a values of 10.3 and 7.7. The strongly basic diethylamino group (*cf.* Figure 1) is completely protonated at pH 7.5, but the second amino group, which is in resonance with the ring nitrogen of the aminoacrine moiety in the un-ionized form, is about 60% protonated at this pH. The third pK_a lies as low as -6.5 (Albert, 1966).

Nigericin is a low molecular weight, lipid-soluble, monobasic acid antibiotic which has been shown to induce monovalent cation–monovalent cation or monovalent cation–H^+ exchange in natural and artifical membrane in a nonenergy-dependent fashion. Valinomycin is a low molecular weight, lipid-soluble, cyclic dodecade-psipeptide with no ionizable group which confers selective ionic permeability of monovalent cations (except H^+) to a variety of natural and artificial membrane systems. Furthermore, it has been demonstrated that valinomycin mediates an energy-dependent *influx* of K^+, Cs^+, and Rb^+ (but not Na^+ or Li^+) into the membrane in the case of intact mitochondria (*cf.* Pressman, 1970) and an energy-dependent *efflux* of K^+, Cs^+, and Rb^+ (but not Na^+ or Li^+) from the membrane in the case of submitochondrial membrane derived from beef heart mitochondria by sonication (*cf.* Montal *et al.*, 1970).

The striking effect induced by valinomycin and nigericin in combination with K^+ or Na^+ on the energy-linked response of quinacrine fluorescence (*cf.* Figures 9 and 10) led to the conclusion that it is the H^+ content in the submitochondrial membrane which determines the fluorescence diminution of quinacrine. It is tempting to suggest that the decrease in quinacrine fluorescence upon energization in the presence of lyo-

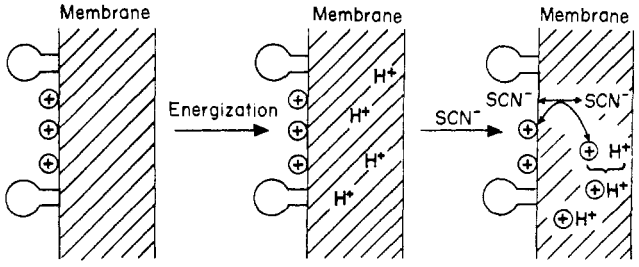

FIGURE 13: Hypothetical scheme of the relationship between the binding of quinacrine and its interaction with the H^+ associated with the energized membrane. ⊕ represents the quinacrine molecules with net positive charges.

philic anions may reflect a further protonation of the second basic group of the quinacrine molecule (*cf.* Figure 1) by the H^+ generated upon energization of submitochondrial membrane.

Figure 13 illustrates schematically our current interpretation of the observations presented in this paper. It is visualized that the quinacrine molecules bind first on the surface of the submitochondrial membrane, most probably through electrostatic forces. A net inward movement of H^+ into the membrane takes place upon energization (Lee and Ernster, 1965; Mitchell and Moyle, 1965; Chance and Mela, 1967; Papa and Lorusso, 1970; Papa *et al.*, 1970a). Under these conditions, quinacrine molecules are not accessible to the H^+ in the membrane. Addition of lyophilic anions results in a migration of quinacrine molecules into the membrane where it can react with the H^+ generated upon energization. The fluorescence decrease reflects the protonation of the second amino group of quinacrine. The migration of quinacrine molecules accompanied by SCN^- or by the lyophilic anions in general from the surface into the internal region of the membrane can be visualized to follow a "symport" mechanism where quinacrine molecules are the cations. The effectiveness of the monovalent anions in promoting the energy-linked response of quinacrine fluorescence parallels that obtained (Wieth, 1970) in promoting membrane permeability to cations ($SCN^- > I^- > NO_3^- > Br^- = Cl^-$). The increase in binding affinity of quinacrine to the membrane at the energized state may result from the increase of the net positive charge of the quinacrine molecules. However, the possible role of an environmental parameter cannot be ruled out at the present time. Further experiments are required to clarify this point.

It is interesting to note that under optimal conditions the rate of decrease of quinacrine fluorescence ($t_{1/2}$ = 3 sec) is of the same order of magnitude of proton uptake as estimated by Papa *et al.* (1970b) under similar conditions in the absence of quinacrine, but at least one order of magnitude slower than the rate of the associated turnover of the electron-transport carriers (Chance *et al.*, 1969). This would suggest that the energy-linked decrease of quinacrine fluorescence in the presence of SCN^- reports the *true* rate of proton uptake in the membrane. We may therefore conclude that accumulation of H^+ in the submitochondrial membrane represents one of the energy-linked functions, in the category of energy-linked pyridine nucleotide transhydrogenation (Lee and Ernster, 1965), energy-linked and valinomycin-mediated K^+ efflux from the membrane (*cf.* Montal *et al.*, 1970), energy-linked ANS fluorescence enhancement (Azzi *et al.*, 1969; Azzi, 1969; Chance *et al.*, 1970; Brocklehurst *et al.*, 1970; Datta and Penefsky, 1970; Chance, 1970; Nordenbrand and Ernster, 1971; Lee and Radda, 1971), and energy-linked BTB absorbance changes (Chance and Mela, 1967).

Acknowledgments

The technical assistance of Miss Barbara Cierkosz is greatly appreciated.

References

Albert, A. (1966), The Acridines, London, Edward Arnold Ltd.

Azzi, A. (1969), *Biochem. Biophys. Res. Commun. 37*, 254.

Azzi, A., Chance, B., Radda, G. K., and Lee, C. P. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 612.

Brocklehurst, J. F., Freedman, F. B., Hancock, D. J., and Radda, G. K. (1970), *Biochem. J. 116*, 721.

Chance, B. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 560.

Chance, B., Azzi, A., Lee, I.-Y., Lee, C. P., and Mela, L. (1969), *Fed. Eur. Biochem. Soc. Symp. 17, 1968*, 233.

Chance, B., and Lee, C. P. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 181.

Chance, B., and Mela, L. (1967), *J. Biol. Chem. 242*, 830.

Chance, B., Radda, G. K., and Lee, C. P. (1970), *in* Electron Transport and Energy Coupling, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Bari, Adriatica Editrice, p 551.

Cockrell, R. S., and Racker, E. (1969), *Biochem. Biophys. Res. Commun. 35*, 414.

Datta, A., and Penefsky, H. S. (1970), *J. Biol. Chem. 245*, 1537.

Eilermann, L. J. M. (1970), *Biochim. Biophys. Acta 211*, 231.

Eilermann, L. J. M. (1971), *in* Energy Transduction in Respiration and Photosynthesis, Quagliariello, E., Papa, S., and Rossi, C. S., Ed., Bari, Adriatica Editrice (in press).

Ernster, L., Nordenbrand, K., Lee, C. P., Avi-Dor, Y., and Hundal, T. (1971), *in* Energy Transduction in Respiration and Photosynthesis, Quagliariello, E., Papa, S., and Rossi, C. S., Ed., Bari, Adriatica Editrice (in press).

Gromet-Elhanan, Z. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 124.

Haas, E. (1944), *J. Biol. Chem. 155*, 321.

Hatefi, Y., and Hamstein, W. T. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1129.

Hellerman, L., Lindsay, A., and Bovarnick, M. R. (1946), *J. Biol. Chem. 163*, 553.

Hunter, F. E., Jr. (1955), *Methods Enzymol. 2*, 610.

Kraayenhof, R. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 6*, 161.

Lee, C. P. (1971), *in* International Symposium on the Biochemistry and Biophysics of Mitochondrial Membranes, Azzone, G. F., and Siliprandi, N., Ed., New York, N. Y., Academic Press (in press).

Lee, C. P., and Ernster, L. (1965), *Biochem. Biophys. Acta Libr. 7*, 218.

Lee, C. P., and Ernster, L. (1967), *Methods Enzymol. 10*, 543.

Lee, C. P. and Radda, G. K. (1971), *in* Energy Transduction in Respiration and Photosynthesis, Quagliariello, E., Papa, S., and Rossi, C. S., Ed., Bari, Adriatica Editrice (in press).

Löw, H. (1959), *Biochim. Biophys. Acta 32*, 1.

Mitchell, P., and Moyle, J. (1965), *Nature (London) 208*, 1205.

Montal, M. (1971), *in* Probes of Structure and Function of Macromolecules and Membranes, Vol. I, Chance, B., Lee, C. P., and Blasie, J. K., Ed., New York, N. Y., Academic Press, p 265.

Montal, M., Chance, B., and Lee, C. P. (1969a), *Biochem. Biophys. Res. Commun. 36*, 428.
Montal, M., Chance, B., and Lee, C. P. (1970), *J. Membrane Biol. 2*, 201.
Montal, M., Chance, B., Lee, C. P., and Azzi, A. (1969b), *Biochem. Biophys. Res. Commun. 34*, 104.
Nordenbrand, K., and Ernster, L. (1971), *Eur. J. Biochem. 18*, 258.
Papa, S., Guerrieri, F., Lorusso, M., and Quagliariello, E. (1970b), FEBS (*Fed. Eur. Biochem. Soc.*) *Lett. 10*, 295.
Papa, S., Guerrieri, F., Rossi-Bernardi, L., and Tager, J. M. (1970a), *Biochim. Biophys. Acta 197*, 100.
Papa, S., Guerrieri, F., Simone, S., Tager, J. M., and Quagliariello, E. (1970c), *in* Electron Transfer and Energy Conservation, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Bari, Adriatica Editrice, p 473.
Papa, S., and Lorusso, M. (1970), *in* Electron Transfer and Energy Conservation, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Bari, Adriatica Editrice, p 509.
Papa, S., Tager, J. M., Guerrieri, F., and Quagliariello, E. (1969), *Biochim. Biophys. Acta 172*, 184.
Pressman, B. C. (1970), *in* Membranes of Mitochondria and Chloroplasts, Racker, E., Ed., ACS Monograph 165, New York, N. Y., Van Nostrand-Reinhold, p 213.
Wieth, J. O. (1970), *J. Physiol. (London) 207*, 581.
Wright, C. I., and Sabine, J. C. (1944), *J. Biol. Chem. 155*, 315

Biochemical Analysis of the Naturally Repaired Sections of Bacteriophage T5 Deoxyribonucleic Acid. II. Conditions for Nucleotide Incorporation under Nonpermissive Conditions*

Robert K. Fujimura

ABSTRACT: The nucleotide incorporation into parental bacteriophage T5 DNA under nonpermissive conditions for DNA replication was characterized by means of CsCl equilibrium density gradient centrifugation and DNA–DNA hybridization. Bacteria were infected with a mutant of T5 which has temperature-sensitive T5 DNA polymerase. Such infected bacteria, when incubated at nonpermissive temperature in dBrUrd medium, synthesized small but definite amounts of DNA that band with parental T5 DNA in neutral and alkaline CsCl equilibrium density gradient centrifugation. Thus it is different from normal replication and very likely due to breakage and repair. DNA synthesis at the nonpermissive temperature occurred even in the mutants of *Escherichia coli* with negligible amounts of DNA polymerase I in their cell extracts as assayed by conventional methods. When chloramphenicol was added immediately after infection, the incorporation was completely inhibited. Thus this DNA synthesis depends on a phage-induced protein(s).

Bacteriophage T5ts53 is a mutant which has a temperature-sensitive T5 DNA polymerase (DeWaard *et al.*, 1965). When bacteria are infected with this mutant at the nonpermissive temperature, there is still some DNA synthesis. When such a synthesis occurs in the presence of dBrUrd,[1] there is no shift in the buoyant density of the newly synthesized DNA, indicating it is a repair-type synthesis (Fujimura and Volkin, 1967).

In the present paper it is confirmed that such a synthesis in the presence of dBrUrd occurs without any detectable density shift. Furthermore, it is shown that the synthesized DNA hybridize specifically to T5 DNA, that the synthesis is not affected by use of *Escherichia coli* mutants that have no detectable DNA polymerase I in their cell extracts as assayed by conventional methods (DeLucia and Cairns, 1969; Kato and Kondo, 1970), and that the synthesis is dependent on a T5-induced protein(s). The accompanying paper (Fujimura, 1971) is on the nucleotide composition of DNA synthesized under nonpermissive conditions.

Materials and Methods

For materials and methods not described here, see Fujimura and Volkin (1968). *E. coli* R15 was obtained from Dr. S. Kondo; and *E. coli* pol A$^-$, originally isolated in Dr. J. Cairns' laboratory, was obtained through Dr. J. Boyle. Bacteriophage T5ts53 labeled with ^{32}P was prepared from a medium containing 0.2–0.6 mCi of ^{32}P (H_3PO_4, New England Nuclear or Schwarz) per mg of phosphorous. Bacteriophage T7 DNA was a gift from Mrs. Ann C. Olson. Chloramphenicol was obtained from Parke Davis and Co. The dBrUrd-containing medium is MGM-CA medium containing 5 μg/ml of dFUrd, 200 μg/ml of dBrUrd, and 25 μg/ml of Urd as described by Fujimura and Volkin (1968).

Preparation of DNA from Infected Bacteria. Bacteria were grown at 37° in dBrUrd-containing medium from 1.5×10^8 to 3×10^8 per ml. They were centrifuged and resuspended in one-tenth the volume of MGM (Lanni, 1961) containing 10^{-3} M $CaCl_2$. The phage was added at a multiplicity of infection of 5 and adsorbed for 5 min. For *E. coli* F and R15, about 99% of the plaque-forming units was adsorbed within 5 min. For pol A$^-$, 94% was adsorbed. Infected bacteria were

* From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received November 2, 1970.* This research was sponsored by the U. S. Atomic Energy Commission under contract with Union Carbide Corp.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: dBrUrd, bromodeoxyuridine; TES, Tris–EDTA–saline; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.