# Energy Dependent Hydrogen Ion Accumulation in Submitochondrial Particles†

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ABSTRACT: The fluorescence quenching of 9-aminoacridine (9AA) in suspension of beef heart EDTA submitochondrial particles was studied and was used to calculate the pH gradient between these particles and the medium. This pH gradient, which is energy dependent, is also dependent strongly on the presence of anion species in the medium. It is 2.2 pH units in acetate medium and can be as high as 3.6 units in the presence of other highly lyophilic anions. The anions tested were found to be effective in the following order: $SCN^- > I^- > NO_3^- > Br^- > Cl^-$. The validity of the $\Delta pH$ calculations was confirmed by comparison with $\Delta pH$ values calculated from $NH_4^+$ uptake. In contrast, calculations based on quinacrine (QA) fluorescence quenching under the same assumption used for 9AA did not agree with $NH_4^+$ measurements and show quantitative and in some cases even qualitative differences. Both carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and $NH_4^+$ decreased $\Delta pH$ significantly. When the rate of electron transport is slow, i.e., with succinate as substrate or with NADH and low concentration of rotenone, very low concentration of nigericin (<20 ng/ml) decreased $\Delta pH$. Under these conditions, valinomycin antagonized the nigericin effect and restored $\Delta pH$ to its original value. Upon increasing nigericin concentration (>100 ng/ml) the valinomycin effect is gradually replaced by a slower response of further reduction of $\Delta pH$.

Beef heart submitochondrial particles retain most of the energy linked processes which are observed in mitochondria (cf. Ernster and Lee, 1964). However, because of the apparent reversed polarity of these vesicles with respect to intact mitochondria (Lee and Ernster, 1966; Mitchell, 1966; Racker, 1969), vectorial processes, in particular ion transport and related reactions, are different from the parallel reactions in intact mitochondria (Montal et al., 1969, 1970; Cockrell and Racker, 1969; Chance and Montal, 1971). It was shown that in contrast to intact mitochondria, energy-dependent proton uptake is directed inward (Mitchell and Moyle, 1965) and as a result there is internal acidification of the particles and an alkalinization of external surrounding medium (Chance and Mela, 1967; Montal et al., 1970). Although proton uptake reactions have been studied in great detail (Papa, 1969; Papa et al., 1972, 1973), a quantitative estimation of internal pH is still lacking since Bromothymol Blue absorption and distribution changes are complex in nature and difficult to interpret (Mitchell et al., 1968). The unknown internal buffering capacity of the particles does not allow an accurate calculation of the internal pH from the extent of proton uptake. Recently, several methods for estimation of internal pH in chloroplasts have been developed (Rottenberg et al., 1972; Schuldiner et al., 1972; Rottenberg and Grunwald, 1972). These methods are based on measuring the distribution of amines which are greatly concentrated in acidic vesicles. The technique utilized a labeled amine such as [$^{14}$C]methylamine, an amine sensitive electrode, such as the $NH_4^+$ electrode, or the use of fluorescent amines, such as 9-aminoacridine (9AA).[1] The fluorescence intensity of 9AA is quenched when taken into the particles. These methods have been successfully applied to several other systems, such as subchloroplast particles (Rottenberg and Grunwald, 1972), chromatophores (Melandri et al., 1972; Casadio et al., 1974; Schuldiner et al., 1975), bacterial cells (Kashket and Wilson, 1973), liposomes (Deamer et al., 1972), and lysosomes (Goldman and Rottenberg, 1973). It has been shown that $NH_4^+$ uncouples submitochondrial particles (Papa, 1969; Cockrell and Racker, 1969) and that energization induces $NH_4^+$ uptake (Montal et al., 1970). Quinacrine (Atebrin) has been employed as a fluorescent probe for the energized state of submitochondrial particles (Lee, 1971, 1972, 1973, 1974; Azzi et al., 1971; Azzone et al., 1972, 1973; Dell'Antone et al., 1971a,b; Massari et al., 1974). The energy-dependent fluorescence quenching of quinacrine was interpreted by Lee (1973) as an indication of the acidification of the submitochondrial membranes. In chloroplasts, chromatophores, and liposomes quinacrine fluorescence quenching is qualitatively similar to that exhibited by 9-aminoacridine, although quantitatively it is less satisfactory as an internal pH probe (Deamer et al., 1973; Schuldiner et al., 1972; Casadio et al., 1974). It has been shown that in submitochondrial particles the quinacrine fluorescence response is rather complex as both enhancement and quenching effects could be achieved dependent upon the experimental conditions (Lee, 1973; Massari et al., 1974).

In this study we have compared the fluorescence responses of quinacrine and 9-aminoacridine with $NH_4^+$ uptake in beef heart submitochondrial particles under various conditions to test their applicability as internal pH indicator. The results indicate that both the $NH_4^+$ uptake and the 9-aminoacridine fluorescence quenching reflect mainly the internal pH of the particle while the quinacrine response is more complex and cannot be used simply as an indicator of internal pH.

## Materials and Methods

Submitochondrial particles derived from heavy beef heart mitochondria by sonic disruption in the presence of

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[1] Abbreviations used are: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; 9AA, 9-aminoacridine; QA, quinacrine.

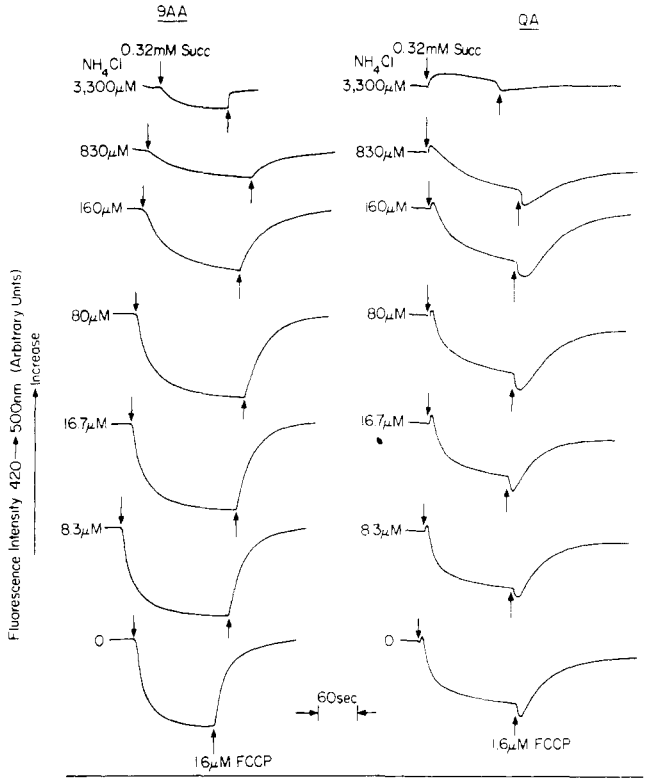


FIGURE 1: The dependence of 9-aminoacridine (9AA) and quinacrine (QA) fluorescence quenching on $NH_4Cl$. The medium contained 100 m*M* choline-Cl, 1.5 m*M* Tris-Cl (pH 7.5), 0.51 mg of protein/ml of OESP, and either 3.3 μ*M* 9AA or 3.3 μ*M* QA. Other additions were as indicated.

EDTA were prepared as described previously (Lee and Ernster, 1967). The particles were then treated with oligomycin (1 μg/mg of protein) and the excess of oligomycin was removed by centrifugation. This preparation is designated as OESP (Lee, 1971). All experiments were performed with OESP unless otherwise indicated. Fluorescence measurements were made at 25° with a Hitachi MPF-2A spectrofluorometer using 420 nm for excitation and 500 nm for emission. Changes in the concentration of $NH_4^+$ were monitored with a cationic Beckman electrode 39137 and a standard Calomel electrode with a choline-Cl salt bridge. Quinacrine hydrochloride was from Sigma Company and 9-aminoacridine hydrochloride was from the Aldrich Chemical Company. Nigericin was kindly supplied by Drs. David Wong and J. M. McQuire of the Lilly Research Laboratories. All other chemicals used were of the highest purity available commercially. Glass-redistilled water was used throughout the experiments.

## Results

Figure 1 shows the effect of $NH_4Cl$ on the fluorescence quenching of 9-aminoacridine (9AA) and quinacrine (QA) in choline-Cl medium with succinate as the energy yielding substrate. In the absence of added $NH_4Cl$ (bottom trace of Figure 1), 9AA fluorescence was quenched upon the addition of succinate and the fluorescence intensity was recovered upon the addition of FCCP. On the other hand, in the case of QA, an initial rapid fluorescence enhancement, though small in extent, was followed by a slow but more extensive quenching. Subsequent addition of FCCP initiated an additional fluorescence quenching which was followed by a slow but more extensive fluorescence enhancement.

Table I: Comparison of Quinacrine and 9-Aminoacridine Fluorescence Quenching and Calculated ΔpH.[a]

| Medium | Quinacrine | | 9-Aminoacridine | |
|---|---|---|---|---|
| | *Q* | ΔpH | *Q* | ΔpH |
| Tris-sulfate | 0.60 | (2.3) | 0.40 | 3.3 |
| +1.6 m*M* NaSCN | 0.45 | (2.1) | 0.50 | 3.5 |
| Tris-acetate | 0.07 | (1.6) | 0.04 | 2.2 |
| +1.6 m*M* NaSCN | 0.50 | (2.2) | 0.21 | 2.9 |

[a] EDTA submitochondrial particles pretreated with oligomycin (0.2 mg of protein /ml) were added to a medium containing 180 m*M* sucrose and 30 m*M* of the indicated Tris buffer (pH 7.5). 9AA or QA were added to a final concentration of 3.3 μ*M*. The extent of quenching (*Q*) induced by the addition of 190 μ*M* NADH and the ΔpH values calculated for these values are shown.

Thus it appears that energization of submitochondrial particles produces two effects on quinacrine fluorescence which are unequal in magnitude and rate. The difference between these two effects becomes more apparent with higher $NH_4Cl$ concentration. The extent of the energy-dependent 9AA fluorescence quenching is inversely proportional to the concentration of $NH_4Cl$. In the case of QA, the fluorescence quenching is reduced at high concentrations of $NH_4Cl$, while the fluorescence enhancement effect remains. As shown in Figure 1, 9AA lacks the enhancement effect. The fluorescence enhancement observed here may be related to the enhancement observed with relatively low concentrations of QA in earlier work (Lee, 1973), which showed that the distinctive separation of the fluorescence enhancement and quenching of QA depends on the composition of the reaction medium. The effect of changing the composition of the reaction medium on the responses of QA and 9AA is shown in Table I. In Tris-sulfate medium with NADH as substrate, the extent of fluorescence quenching of QA is retarded slightly by the addition of NaSCN (from 0.60 to 0.45). This is in contrast to the response observed in Tris-acetate medium in which the extent of fluorescence quenching is greatly increased by NaSCN (from 0.07 to 0.50). The extent of fluorescence quenching of 9AA increased in both buffers by the addition of NaSCN from 0.40 to 0.50 in Tris-sulfate, and from 0.04 to 0.21 in Tris-acetate medium. It can therefore be concluded that 9AA and QA exhibit not only quantitatively but also qualitatively different responses to the energized submitochondrial membrane. The responses of 9AA appear to be solely those of an indicator of internal pH, while at least two different reactions are involved in the case of QA.

Assuming the fluorescence quenching of these amines is a measure of its extent of uptake, we can calculate ΔpH, defined as the change of internal pH during the transition from the deenergized to the energized state, from the extent of fluorescence quenching and the internal particles volume (Schuldiner et al., 1972). For these calculations we have used the volume of 1.5 μl/mg of protein which was determined by Papa et al. (1973). Although the particle volume may change with conditions, these are relatively small and would not significantly affect the calculated ΔpH. For ΔpH calculation from 9AA fluorescence quenching we used the equation of Schuldiner et al. (1972). Since the particles contain 1.5 μl/mg of water, $V = 1000C/1.5$.

Thus

$$\Delta pH = \log\left(\frac{Q}{V(1-Q)}\right) = \log\left(\frac{Q}{(1-Q)}\frac{666}{C}\right)$$

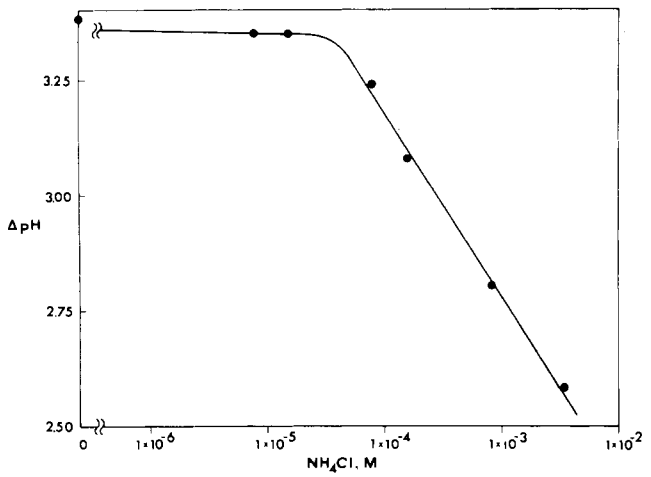


FIGURE 2: The dependence of ΔpH on $NH_4Cl$. The results are calculated from the data shown in Figure 1. For details of calculation, see the text.

where $Q$ is the fraction of the fluorescence that was quenched, $V$ is the ratio of the particle internal volume to the external solution volume, and $C$ is the concentration of particles in the solution in mg of protein/ml. We have also calculated ΔpH from the QA fluorescence quenching, taking $pK_{a_2} = 7.5$, $pK_{a_1} = 10.1$ (Schuldiner et al., 1972), and using the relation

$$\frac{Q}{(1-Q)V} = \frac{C_{in}}{C_{out}} = \frac{K_2(H)_{in} + (H)^2_{in}}{K_2(H)_{out} + (H)^2_{out}}$$

The calculated values are given in Table I. The difference between the two calculations is apparent both in magnitude and direction of change. As in other systems, QA gives values which are lower than 9AA (Schuldiner et al., 1972; Deamer et al., 1973). Because of the complex nature of the QA fluorescence responses (cf. Figure 1), we have employed 9AA as the probe for the estimation of ΔpH in further experiments.

The effect of $NH_4Cl$ on ΔpH, as computed from the fluorescence quenching of 9AA (cf. Figure 1) is shown in Figure 2. Up to $1 \times 10^{-4}$ $M$ $NH_4^+$, the effect of $NH_4^+$ on ΔpH is very small and above this concentration ΔpH drops sharply. ΔpH can be calculated also from the extent of $NH_4^+$ uptake (Rottenberg and Grunwald, 1972). A check on the validity of both estimations would be a comparison of ΔpH calculated from 9AA fluorescence quenching and ΔpH calculated from $NH_4^+$ uptake under identical conditions. Since $NH_4^+$ itself, at concentrations higher than $1 \times 10^{-4}$ $M$ reduces ΔpH significantly, lower concentrations of $NH_4^+$ for these determinations were employed. Figure 3a shows the effect of increasing FCCP concentrations on 9AA fluorescence quenching (in the presence of 8 $\mu M$ $NH_4^+$). A parallel experiment performed under identical conditions demonstrating $NH_4^+$ uptake as a function of increasing FCCP concentrations is shown in Figure 3b. The similarity between the two measurements is striking when the two sets of curves are compared. The values of ΔpH calculated from these two types of experiments are shown in Figure 4. The good agreement between these two methods indicates that both amines respond to the same parameter, presumably the internal pH, according to the mechanism suggested previously (Rottenberg et al., 1972). As can be inferred from Figure 1 and Table I, the values of ΔpH calculated from QA fluorescence quenching, under the same

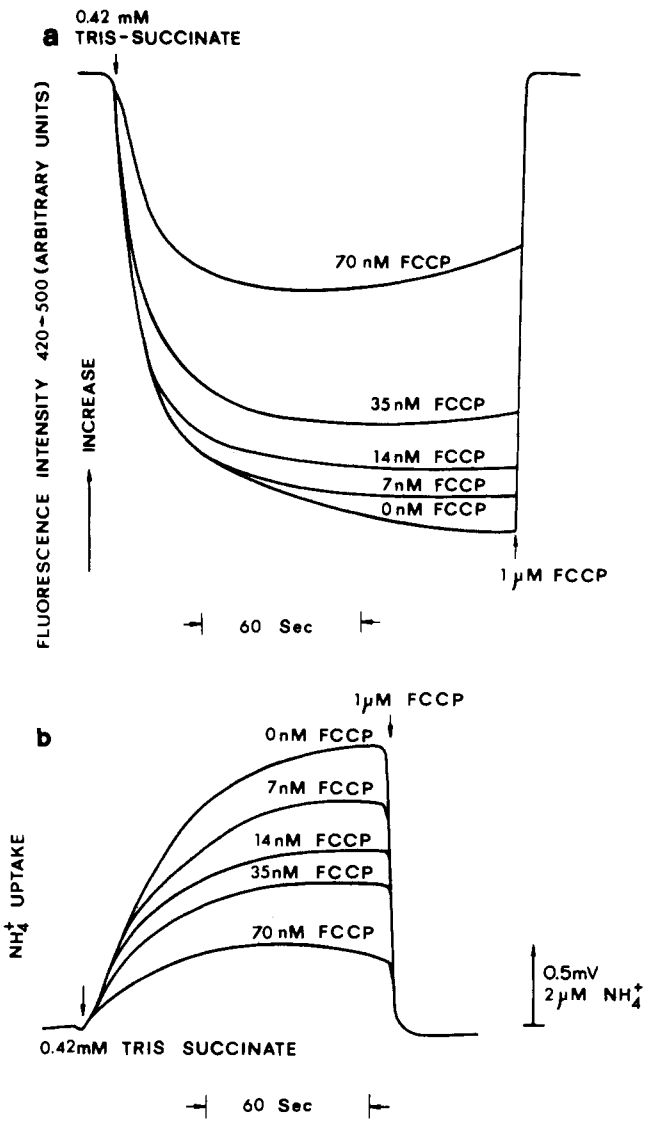


FIGURE 3: Comparison of the effect of FCCP on 9AA fluorescence quenching and $NH_4^+$ uptake. The medium employed for both experiments were identical: 100 m$M$ choline-Cl, 1.5 m$M$ Tris-Cl (pH 7.5), 8 $\mu M$ $NH_4Cl$, 3.3 $\mu M$ 9AA, and 0.36 mg of protein/ml of OESP. Figure 3a shows the fluorescence quenching of 9AA and Figure 3b shows the $NH_4^+$ uptake.

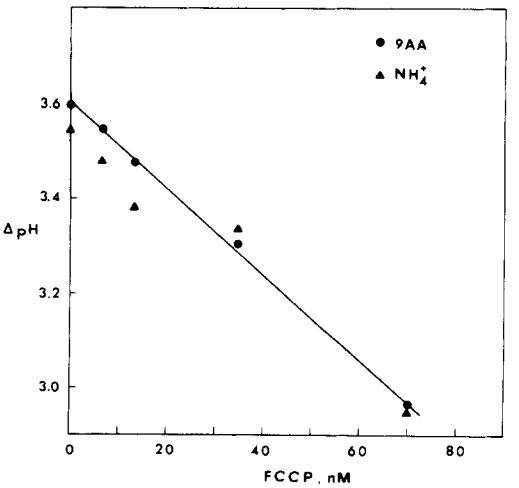


FIGURE 4: The dependence of ΔpH on FCCP. Data for calculations are taken from Figures 3a and b. (○) From the fluorescence quenching, (△) from the $NH_4^+$ uptake.

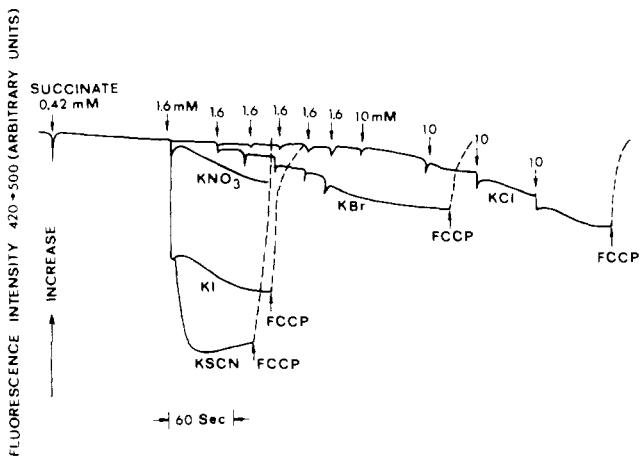


FIGURE 5: The effect of anions on the fluorescence quenching of 9AA. The medium contained 180 m$M$ sucrose, 30 m$M$ Tris-acetate (pH 7.5), 3.3 $\mu M$ 9AA, and 0.47 mg of protein/ml of OESP. Other additions are as indicated. The numbers indicated above the arrows are the final concentrations (m$M$) of the added salt.

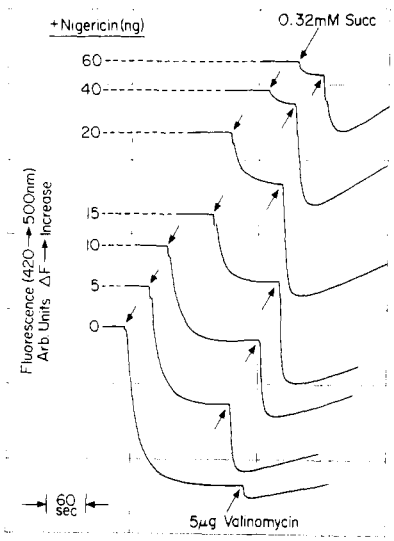


FIGURE 6: The effect of nigericin and valinomycin on the fluorescence quenching of 9AA. The medium contained 100 m$M$ choline-Cl, 1.5 m$M$ Tris-Cl (pH 7.5), 10 m$M$ KCl, 3.3 $\mu M$ 9AA, and 0.44 mg of protein/ml of OESP. Others are as indicated.

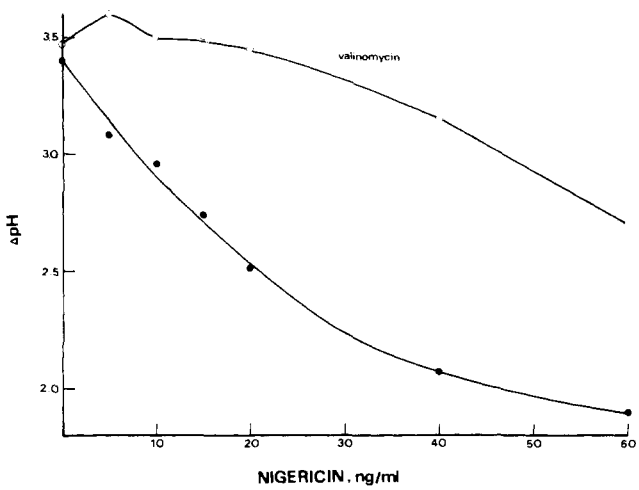


FIGURE 7: The effect of nigericin and valinomycin on ΔpH. The results are calculated from the data of Figure 6.

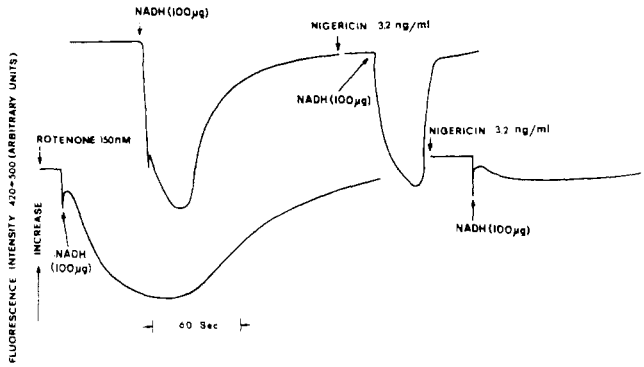


FIGURE 8: The dependence of the nigericin effect on 9AA fluorescence quenching on the rate of electron transport. The medium contained 100 m$M$ choline-Cl, 1.5 m$M$ Tris-Cl (pH 7.5), 10 m$M$ KCl, 3.3 $\mu M$ 9AA, and 0.4 mg of protein/ml of OESP.

assumptions used for 9AA, do not agree with those determined by the $NH_4^+$ electrode.

As in the case of QA, the fluorescence quenching of 9AA can be induced either by the aerobic oxidation with NADH (Table I), succinate (Figures 1 and 3), or ascorbate + PMS (not shown) as substrate. The effect of anions on the fluorescence responses of 9AA are similar to those observed with QA (Lee, 1972). Figure 5 shows that in Tris-acetate medium succinate produced a slight fluorescence quenching which is equivalent to a ΔpH of over 2.0 units, as shown in Table I. Addition of 1.6 m$M$ NaSCN increased the extent of quenching, corresponding to an increase in ΔpH to approximately 3.0 units. Addition of 1.6 m$M$ KI or $KNO_3$ gave somewhat lesser effect, while $Br^-$ and $Cl^-$ hardly show any effect. At higher concentrations $Br^-$ (10 m$M$) and $Cl^-$ (40 m$M$) can also induce fluorescence quenching (Figure 5). The spectrum of effectiveness of these anions in increasing ΔpH is in accordance with the following order: $SCN^- > I^- > NO_3^- > Br^- > Cl^-$. Sulfate is also effective, as shown in Table I.

Figure 6 shows that when succinate is the energy yielding substrate, very low concentrations (<20 ng/ml) of nigericin are sufficient to decrease the extent of the energy dependent 9AA fluorescence quenching. However, addition of valinomycin to the nigericin supplemented system restorates fully the fluorescence quenching. When the nigericin concentration is increased (>20 ng/ml), inhibition of fluorescence quenching is increased and valinomycin becomes less effective in restoring it. As nigericin concentrations are further increased, the fluorescence quenching of 9AA induced by valinomycin is gradually reversed with time in a process which becomes faster at increasing nigericin concentrations. This reversal of quenching proceeded above the level that was established prior to the addition of valinomycin and fully restored the initial fluorescence. At the high nigericin concentrations used, no energy-dependent fluorescence response is detected and valinomycin is thus without detectable effect. The reduction of ΔpH by nigericin (with succinate as substrate) and its restoration by valinomycin is shown in Figure 7. These effects are only observed with low concentrations of nigericin under conditions of slow electron transport and hence slow proton transport. When NADH serves as substrate (Figure 8, top trace), the same concentration of nigericin which is effective in reducing ΔpH when succinate is the substrate has very little effect on ΔpH. However, if NADH oxidase is slowed down by rotenone (Figure 8, bottom trace), this same low concentration of

nigericin reduced the extent of fluorescence quenching considerably. An interesting feature shown in Figure 8 is that in the absence of rotenone, while nigericin has only a slight effect on the extent of fluorescence quenching at steady state but greatly accelerates its decay, rotenone reduced only slightly the steady-state level of fluorescence intensity, but slowed very much both the *on* and *off* rates of the fluorescence response. The increased sensitivity to nigericin which is caused by slowing NADH oxidase activity with rotenone is shown in Figure 9, in which the ΔpH induced by NADH oxidation is plotted as a function of nigericin concentration with and without partially inhibitory concentrations of rotenone.

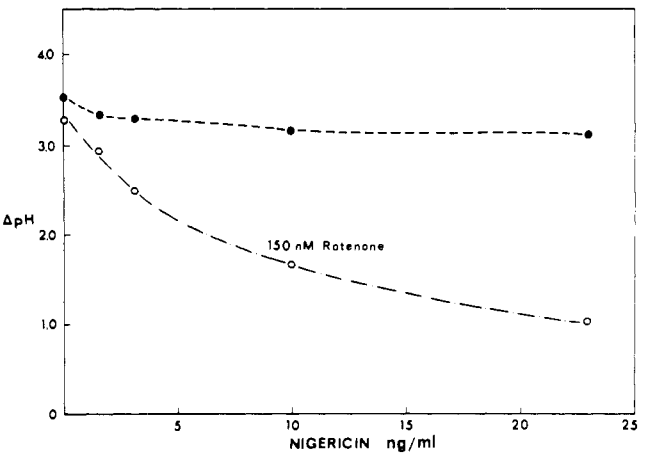


FIGURE 9: The dependence of the nigericin effect on ΔpH on the rate of electron transport. The results are calculated from experiments done under the same conditions as those described in Figure 8, except for the nigericin concentration.

## Discussion

The interpretation of the responses of various fluorescent probes to energization of submitochondrial membranes has been a matter of controversy (Kraayenhof et al., 1975) and should be reexamined with caution. In this study we have tested the interpretation that certain fluorescent amines, particularly 9-aminoacridine (9AA), exhibit a fluorescence quenching as a result of their extensive uptake by the particles which, in turn, is a result of the existence of an increased proton concentration of these particles. The fact that $NH_4^+$ uptake and 9AA fluorescence quenching (interpreted as uptake) follows the same pattern toward FCCP (Figure 3) and results in the same calculated ΔpH (Figure 4) suggests that this identical response depends on their identical property of being a strong monoamine. Because of their high $pK_a$ values, at pH 7.5 virtually all the ammonium and 9AA cations should retain their positive charge when taken up by the particle and thus most probably be located in the hydrated rather than in the lipid region of the particles. Although the fluorescence quenching of QA follows qualitatively the same pattern toward $NH_4^+$ as that shown with 9AA (Figure 1), it does not allow a quantitative estimation of ΔpH (Table I). It is possible that the fluorescence enhancement of QA is partially related to hydrophobic interactions. Since no fluorescence enhancement effect was observed with 9AA and there is a good agreement between ΔpH values calculated from this probe and from $NH_4^+$ uptake, it appears that 9AA is suitable for quantitative estimation of internal pH of submitochondrial particles. The comparison of 9AA and QA fluorescence responses in liposomes (Deamer et al., 1973) and chromatophores (Melandri et al., 1972; Casadio et al., 1974) has led to similar conclusions.

The fluorescence responses of 9AA toward various anions is qualitatively similar to those observed with QA (Lee, 1971, 1972, 1973). $SCN^-$ is found to be most effective in increasing ΔpH in acetate medium in which ΔpH is relatively low (Table I) and is less effective in $SO_4^{2-}$ medium in which ΔpH is considerably high (Table I). However, if the anion concentration is high enough (>10 m*M*) all the anions of strong acids that were tested, including $Cl^-$, could increase ΔpH. In high $Cl^-$ medium (>30 m*M*) as in most of the experiments reported here (Figures 1–4, 6, and 7), ΔpH was as high as in the presence of $SCN^-$ (Table I).

The present interpretation of the energy-dependent formation of ΔpH is based on the assumption of the existence of an electrogenic proton pump in the particles. The maximal value of ΔpH which was estimated in the present study, 3.6 units, is similar in magnitude to those observed in other energy conserving membrane preparations (Rottenberg, 1975; Rottenberg and Grunwald, 1972). Under these conditions, the membrane potential (Δψ) in these particles is presumably small; this value (ΔpH = 3.6) may be considered as the maximal energetic potential of the proton electrochemical gradient. It should be mentioned that attempts in estimating the membrane potential have not been made in this study. The fact that addition of NaSCN considerably increased the ΔpH in submitochondrial particles when assayed in acetate medium indicating that in acetate medium a large portion of the energy pool is conserved in the form other than ΔpH, presumably in the form of a membrane potential (Chance and Montal, 1971). The magnitude of ΔpH was found to be very sensitive to uncoupler (FCCP), to amines, and to $K^+$ + very low concentrations of nigericin. However, owing to the limitation of assay techniques we were unable to follow the complete reduction of ΔpH by these agents. The experiments with nigericin and valinomycin could also be interpreted as a redistribution of ΔpH and Δψ due to the action of the ionophores. In choline-Cl medium at aerobic steady state with succinate as the energy yielding substrate, ΔpH approaches a maximal level and presumably Δψ is very small. The addition of low concentrations of nigericin would result in a neutral $K^+/H^+$ exchange. Although the rate of the exchange is slow, it would increase significantly the proton leak and as a result ΔpH would decrease. In terms of the proton pump mechanism, the proton pump is electrogenic and a large part of the nigericin induced leak is electroneutral, so that the increased rate of proton pumping could result in an increase in Δψ. Subsequent addition of valinomycin would induce an electrogenic $K^+$ efflux resulting in a reduction of Δψ and allowing the pump to rebuild ΔpH (Figures 6 and 7). This process is possible only with low concentrations (<20 ng/ml) of nigericin, and can affect ΔpH only when electron transport is slow as when succinate is the substrate or when NADH oxidase is partially inhibited by rotenone. When high concentrations of nigericin are used, the rate of the $K^+/H^+$ exchange is limited by the $K^+$ gradient (Montal et al., 1970). On subsequent addition of valinomycin, in addition to collapsing the membrane potential, the $K^+$ gradient is decreased. This, in turn, will increase the rate of the nigericin-induced $K^+/H^+$ exchange, resulting in further reduction of ΔpH when the rate of exchange exceeds the maximal possible rate of proton pumping. The two effects follow separate kinetics since the collapsing of Δψ is instantaneous whereas the reduction of the $K^+$ gradient is relatively slow,

thus at moderate nigericin concentration (Figure 7) valinomycin first increases ΔpH and then slowly decreases it. At relatively high concentrations of nigericin only the latter effect can be detected (Montal et al., 1970; Lee, 1972) and the effect of nigericin and valinomycin on ΔpH, rates of respiration, and phosphorylation are synergistic. In contrast, the effects in these particles are antagonistic at low concentrations of nigericin, similar to the antagonistic effects of valinomycin and low concentrations of nigericin on ΔpH and $\Delta\psi$ in intact mitochondria as reflected in $Ca^{2+}$ uptake (Rottenberg and Scarpa, 1974).

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