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# Protonation Equilibrium of Ellipticine Bound to the Energy-Transducing Membrane of Mitochondria

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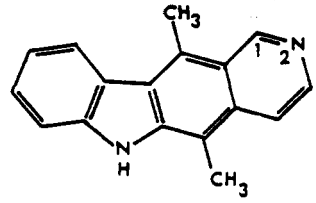
The relationship between the protonation state of ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole), an antitumoral alkaloid, and the transmembrane proton electrochemical gradient associated with mitochondrial membranes has been investigated. The binding parameters of ellipticine to mitochondria are independent of either the transmembrane ΔpH or membrane potential, which are both components of proton gradient (association constant = $1.5 \times 10^6$ $M^{-1}$; maximal binding ratio = one bound ellipticine *per* 25 phospholipids). Since the apparent p*K* of bound ellipticine is 7.1, its two protonation states can be detected from their specific fluorescence emission spectra near physiological pH. It is shown that a shift in the drug protonation equilibrium toward the neutral form occurs in mitochondrial membrane during the generation of the proton electrochemical gradient. This shift is sensitive to transmembrane ΔpH but insensitive to membrane potential, indicting that the protonation equilibrium of membrane bound ellipticine detects the $H^+$ movement in mitochondria.

## Introduction

As many other antitumor alkaloids, ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole)displays a high affinity for artificial[1-3]




ellipticine

and natural membranes,[4] including mitochondrial membranes.[5,6] Ellipticine binding to the inner mitochondrial membrane is of particular interest since mitochondria are involved in the mechanism of energy transduction via a process involving the coupling of electron transport and ATP-driven proton pumps by an electrochemical gradient of $H^+$ ions across the inner mitochondrial membrane.[7] The difference in the proton electrochemical potential ($\Delta\mu H^+$) is composed of electrical and concentration terms according to the relation $\Delta\mu H^+ = -2.3RT\Delta pH + F\Delta\Psi$, where $\Delta\Psi$ and ΔpH are the potential and the pH differences across the membrane, respectively.

We have shown previously that at high concentrations ellipticine leads "*in vitro*" to uncoupling of oxidative phosphorylation and inhibition of electron transfer at the level of cytochrome *c* oxidase activity.[5,6] These effects suggested that mitochondria could be one of the potential targets involved in the drug cytotoxicity mechanism. This hypothesis was recently strengthened by the observation that ellipticine also interacts with mitochondria at low concentrations in single living cells.[8]

In the present study, we have investigated the protonation equilibrium of ellipticine bound to isolated mitochondria and its possible displacements according to variations in mitochondrial transmembrane ΔpH and $\Delta\Psi$. We have observed that the protonation equilibrium of the bound drug was only sensitive to the generation of ΔpH at the inner interface of the inner mitochondrial membrane. The purpose of this report is to present and interpret these shifts in protonation equilibrium and postulate a mechanism.

## Experimental Section

Mitochondria from Potato tuber were isolated and subsequently purified on a self-generated Percoll gradient according to previously described procedures.[9] Mitochondrial protein was determined by the biuret reaction using bovine serum albumin as a standard. In binding experiments, the mitochondria concentration was expressed in phospholipids.

Ellipticine was purified by liquid chromatography and checked for purity as previously described.[10]

Fluorescence experiments were performed using a LS50 Perkin-Elmer spectrofluorometer in quartz fluorescence cells (1-cm path length) containing 3 mL of buffered solution: 100 mM Tris-HCl (pH 7.40), 0.4 M sucrose, and, when present, 50 mM KCl and 5 mM $MgCl_2$. For p*K* determinations, the same medium was used containing buffers obtained from Merck instead of Tris-HCl: pH 5 (0.025 M acetate), pH 7 (0.025 M phosphate), pH 8 (0.025 M borate), pH 9 (0.025 M borate), pH 11 (0.025 M borate); mixtures of pH 9 and pH 11 were used to obtained various pHs within this range.

In the binding studies, the concentration of bound ellipticine was calculated from its fluorescence increase upon binding at $\lambda_{ex}$ = 330 nm and $\lambda_{em}$ = 475 nm (isosbestic point between the neutral and protonated forms of ellipticine) as previously described.[10]

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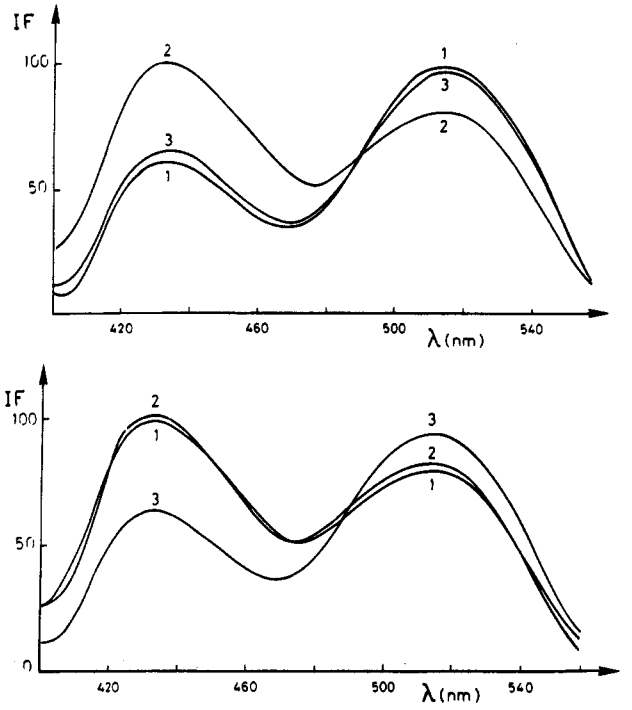


**Figure 1.** Fluorescence emission spectra ($\lambda_{exc}$ = 300 nm) of ellipticine bound to isolated mitochondria. (a, top) Effect of generation and dissipation of $\Delta\mu H^+$: nonrespiring mitochondria (1); addition of 5 mM succinate (2); addition of 1 $\mu$M FCCP (3). Reaction medium contained 0.4 M sucrose, 100 mM Tris-HCl (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$ 0.5 $\mu$M ellipticine, and 1.5 mg/mL of mitochondrial protein at 25 °C. (b, bottom) Effect of dissipation of $\Delta\Psi$ by addition of 2 $\mu$M valinomycin (spectrum 2) or $\Delta$pH by addition of 0.5 $\mu$g/mL nigericin (spectrum 3) on respiring mitochondria (spectrum 1).

Binding parameters were determined from Scatchard isotherms

$$r/c = K(n - r)$$

where $r$ is the ratio of bound ellipticine to total phospholipid concentration, $c$ the free ellipticine concentration, $n$ the maximal number of bound ellipticine per phospholipid, and $K$ the binding constant.

For studies on anaerobic to aerobic transitions, the medium was maintained at 25 °C in a stoppered cuvette under bubbling nitrogen until completely anaerobic. Aerobic transitions were initiated by the injection of $H_2O_2$ into the stoppered cuvette containing 3 $\mu$g of catalase.

## Results and Discussion

Ellipticine is a weak base with a protonation p$K$ of 7.4 on its N-2 nitrogen atom[11] and therefore exists under two protonation states in biological media:[8] the neutral (unprotonated) and cationic (protonated) form which exhibit specific fluorescence emission bands respectively centered at 430 and 529 nm.[8]

When purified mitochondria (0.5 mg of protein/mL) were added to an aerobic buffer (pH 7.0) containing 0.5 $\mu$M ellipticine (a mitochondrial phospholipid to ellipticine ratio of *ca.* 200), a 15-fold increase of ellipticine fluorescence was observed whereas the relative intensities of the two bands remain unchanged with respect to the spectrum of free ellipticine (Figure 1a, spectrum 1). The marked increase of ellipticine fluorescence upon binding to mitochondria was used for the determination of the dye binding parameters to mitochondria. A Scatchard plot of the dye binding to respiring and nonrespiring mitochondria leads to similar binding parameters with an association constant of *ca.* $1.5 \times 10^6$ $M^{-1}$ and a maximal ratio of ellipticine bound to phospholipids of *ca.* 0.04, corresponding to a maximum of one bound ellipticine *per* 25 phospholipids (Figure 2). The binding parameters determined

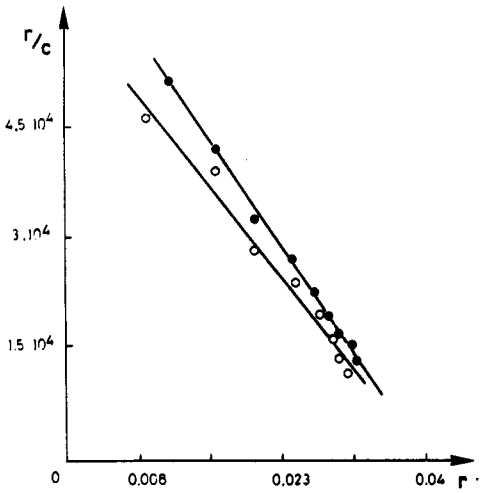


**Figure 2.** Scatchard isotherms for the binding of ellipticine to mitochondria: (○) nonrespiring mitochondria; (●) respiring mitochondria; $\lambda_{exc}$ = 330 nm, $\lambda_{em}$ = 475 nm. Same medium as in Figure 1. The binding constants ($K$) and maximum number of bound ellipticine per phospholipid ($n$) were respectively obtained from the slope and the intercept on $r$ abscissa (see Experimental Section).

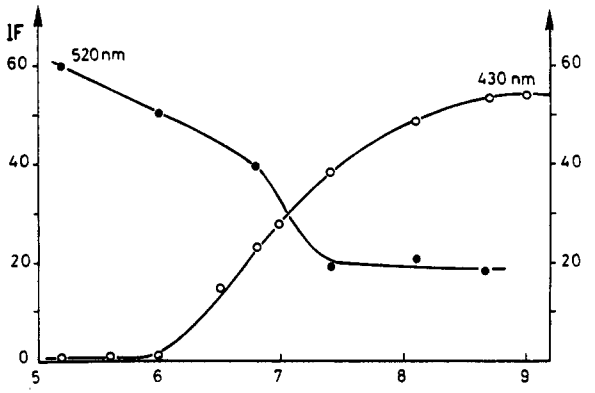


**Figure 3.** Variation of fluorescence intensities at 520 and 430 nm of ellipticine bound to mitochondria *vs* pH; $\lambda_{exc}$ = 300 nm, phospholipids/ellipticine ratio = 200. Same medium as in Figure 1 (see Experimental Section).

with mitochondria are very close to those previously inferred for ellipticine binding to negatively charged liposomes,[3] which suggests a similar binding mechanism. Moreover, the binding parameters of ellipticine to mitochondrial phospholipids do not vary with the mitochondrial energetic state, which suggests that the molecule was not redistributed or reoriented in response to the electrogenic $H^+$ movement.

On the other hand, the apparent p$K$ of ellipticine bound to mitochondria, estimated from variation in the fluorescnece intensities at 430 and 520 nm *vs* pH (Figure 3), was *ca.* 7.1, a value close to its intrinsic p$K$ (7.4).

Energization of mitochondria by addition of 5 mM succinate as a respiratory substrate led to an increase of the blue emission band (characteristic of the neutral form) whereas the green band decreases (Figure 1a, spectrum 2). As compared to this energized state, addition of 1 $\mu$M FCCP, a protonophore which collapses the proton electrochemical gradient,[12] the protonation equilibrium of ellipticine was shifted back toward the cationic form with a $I_{520}/I_{430}$ ratio almost identical to its initial value observed with nonenergized mitochondria (Figure 1a, spectra 3 and 1). The data suggested that the protonation equilibrium of ellipticine was responding to $\Delta\mu H^+$ or one of its components, namely, to either $\Delta\Psi$ or $\Delta$pH, generated by the respiratory chain. Consequently, the effects of two specific ionophores, valinomycin[13] and nigericin,[13] were investigated. In the presence of 2 $\mu$M valinomycin (a $K^+$ ionophore which depolarizes the mitochondrial membrane potential[13]) the $I_{520}/I_{430}$ ratio was practically unaf-

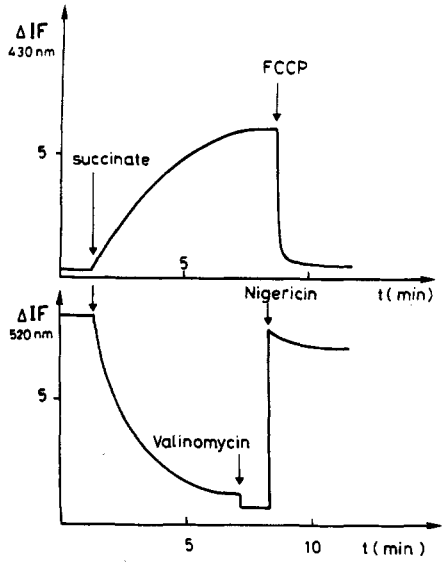


**Figure 4.** Kinetics of the ellipticine fluorescence response during generation and dissipation of $\Delta\mu H^+$ monitored at 430 nm (upper trace) and effect of dissipation of its two components, $\Delta\Psi$ and $\Delta pH$, monitored at 520 nm (lower trace). $\lambda_{exc}$ = 300 nm. Same medium as in Figure 1.

fected (Figure 1b, spectrum 2). In contrast, in the presence of 5 μg/mL nigericin (an ionophore which induces an electrically neutral exchange of protons for potassium ions and results in the dissipation of the pH gradient across the inner mitochondrial membrane[13]), the protonation equilibrium of ellipticine was shifted toward the protonated from (Figure 1b, spectrum 3). Taken together, the data indicated that the $\Delta pH$ was exclusively responsible for the shift of the protonation equilibrium of ellipticine toward the neutral form in respiring mitochondria.

The kinetics of the spectral changes induced by generation and dissipation of $\Delta\mu H^+$ were monitored using a wavelength of 430 nm, which corresponds to the neutral form, as well as 520 nm, which corresponds to the protonated form (Figure 4). The decrease in protonated form fluorescence mirrored the increase of the neutral form upon energization. Similar fluorescence variations were observed when valinomycin was added before energization by succinate (data not shown), which suggests that the $\Delta\Psi$ was not required for ellipticine $\Delta pH$ probing.

The effect of $\Delta\mu H^+$ generation on mitochondria-bound ellipticine may also be seen in the transition from anaerobic to aerobic conditions upon addition of oxygen to mitochondria incubated anaerobically with ellipticine. The transition, monitored at 430 nm, showed a rapid fluorescence increase which was complete

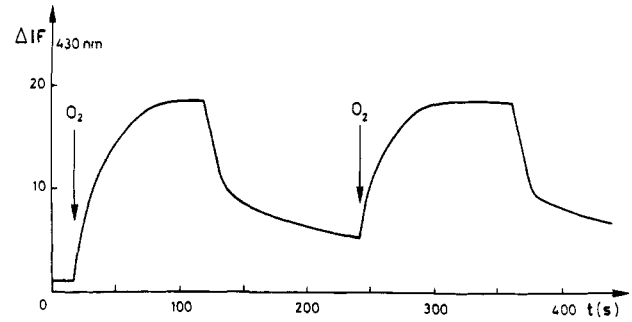


**Figure 5.** Ellipticine fluorescence response to an anaerobic–aerobic transition. The reaction medium contained 0.4 M sucrose, 100 mM Tris-HCl (pH 7.0), 3 μg of catalase, 0.5 μM ellipticine, and 0.5 mg of protein/mL mitochondria. The stoppered cuvette was maintained for 20 min under bubbling nitrogen until completely anaerobic. Two successive oxygen pulses (60 nmol of $O_2$ and 80 nmol of $O_2$) were initiated by addition of $H_2O_2$.

within 60 s and a plateau phase proportional to the amount of oxygen added which continued until anaerobiosis (Figure 5).

In conclusion, we have shown that ellipticine may be incorporated into the phospholipidic membrane of mitochondria. In addition, insensitivity of ellipticine binding parameters to $\Delta\mu H^+$ and of ellipticine optical response to $\Delta\Psi$ indicates that the topology of the ellipticine phospholipids complexes are not modified by variations in the transmembrane electrical fields. Thus, it appears that ellipticine remains localized within the inner mitochondrial membrane. The protonation equilibrium of bound ellipticine directly reflects the electrogenic $H^+$ ions movement which occurs during energy coupling.

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