# Influence of Surface Potentials
# on the Mitochondrial H⁺ Pump
# and on Lipid–Phase Transitions

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## Abstract

It has been shown that the surface potential of lipid membranes, as well as of mitochondria, can be shifted more positive by absorption of alkylbiguanides. Both phospholipid vesicles and natural membranes respond in an analogous way to this shift. Ion activities at the immediate membrane surface are influenced by sign and magnitude of the surface charge. Corresponding effects on ion transport and on fluorescence-probe binding can be observed. The mitochondrial $H^+$ pump is inhibited when the surface charge is shifted more positive. In contrast, the absolute charge density determines the temperature of the ordered-fluid transition. The latter is increased by biguanides, suggesting that the membrane is rendered more rigid. The experiments make obvious that physical relations derived from model systems apply equally well to lipid-containing natural membranes.

## Introduction

Dynamic properties of membranes are not only determined by the chemical nature of their constituents but also by temperature [1] and by environmental factors such as pH, ionic strength [2], or adsorbed organic or inorganic ions [3, 4]. These latter parameters are capable of influencing the charge density $\sigma$ on the membrane surface. With phospholipid model membranes it has been shown that the area, $f$, per lipid molecule critically depends on the charge of the polar head groups

[5]. $\sigma$ is equivalent to $\sigma'/f$, where $\sigma'$ is the charge per molecule (specific charge).

Conditions influencing $\sigma$ modify the fluidity of liquid crystalline phospholipid structures, as well as the temperature, $T_t$, where an abrupt phase transition or phase separation occurs, with $T_t$ decreasing for increasing surface charge [2, 6]. Regarding biological membranes, which are much more complex systems, the question arises whether or not $\Delta\sigma$ induces an analogous shift of membrane fluidity and of the transition temperature of lipids. In mitochondria the latter is reflected by the temperature dependence of membrane-bound enzymes exhibiting pronounced breaks of their Arrhenius plots in the region of $21-23°C$ [7]. Fluorescence probes failed to indicate these phase changes with intact mitochondria; they could be verified with spin probes, however, at the respective temperatures [8].

In a previous paper we have shown that $H^+$ uptake during mitochondrial ADP phosphorylation is inhibited by making the surface potential more positive [9]. In the present investigation the influence of changes of membrane surface potential ($\psi_0$) on respiration-induced $H^+$ ejection of rat liver mitochondria and on passive $H^+$ uptake has been studied. The temperature dependence has been determined. Positive surface charges were generated by binding of alkyl- or aralkylbiguanides as described previously [9, 10].

## Experimental Procedures

Rat liver mitochondria were prepared according to standard procedures by differential centrifugation in 0.25 M sucrose. $H^+$ liberation and $H^+$ uptake was measured with a specially designed low-resistance glass electrode (Ingold) and a rapid recording device, the half-time of the setup being below 100 msec. The mitochondria were incubated in 0.25 M sucrose with 10 mM Na succinate as substrate and as a minimum buffer in a closed vessel of 0.9 ml vol. under vigorous magnetic stirring; 4.0 $\mu$g oligomycin and 0.5 $\mu$g rotenone were added. Prior to experiments the pH was adjusted to 7.2; the suspension was allowed to become anaerobic; $H^+$ ejection was induced by pulses of oxygen-saturated incubation buffer or by $H_2O_2$ in presence of excess catalase.

Phospholipid micelles were prepared by sonication of dipalmitoyl phosphatidic acid (DPA) in water, or in 50 mM Tris buffer in the case of egg lecithin; final concentration was 1.3 mM. The lipid-phase transition was monitored by fluorescence of $N$-phenylnaphtylamine (NPN) added as methanolic solution to yield $2.3 \times 10^{-6}$ M final NPN concentration [11]. The relative shift of surface charge was monitored by registration of ANS fluorescence. Phospholipids were purchased from Serdary; NPN from Riedel de Haen was recrystallized twice.

## Results

It has been shown that biguanides bind to lipids of mitochondrial and synthetic membranes and represent hydrophobically fixed charges. They provide a useful means to generate a positive surface potential [10, 12]. Successive increase of biguanide concentration gradually increases the positive surface charge or neutralizes negative charges. Since it is well established that ANS (8-anilinonaphtalene-1-sulfonic acid) fluorescence primarily reports the changes of membrane surface potential [10, 13–15], it is possible to demonstrate directly the relative changes of $\sigma$ induced by binding of biguanides. Figure 1 shows that phospholipid micelles and mitochondria respond in an analogous way to addition of biguanide. The increase of fluorescence is due to additional binding of ANS when the surface charge is shifted more positive [10, 12]. It is seen that binding of long-chain fatty acids reverses this effect and that additional biguanide again counteracts this negative shift of surface potential. This agrees well with the observation that biguanide inhibition of mitochondrial respiration is released by appropriate concentrations of long-chain fatty acids which do not uncouple oxidative phosphorylation [16].

The properties of the electrogenic mitochondrial proton pump in presence of biguanides are described in Fig. 2. The initial velocity $V_0$ of $H^+$ liberation [part (a)] decreases with increasing positive surface potential induced by $n$-octylbiguanide. Part (b) shows that the apparent first-order rate constant $k_1$ for the proton pulse is also gradually lowered. In contrast, the rate constant $k_2$ for anaerobic backflow of protons remains unchanged. In coupled mitochondria this anaerobic backflow is very slow and negligible for the forward reaction.

Figure 3 describes the temperature dependence of the kinetics of $H^+$ liberation and of $H^+$ uptake by rat liver mitochondria in presence or absence of phenethylbiguanide as a modifier of surface potential. The control experiment (Fig. 3a) reveals pronounced breaks of the Arrhenius plots at $20°$ C. This transition temperature agrees with that observed in other mitochondrial systems [7, 8]. Thus, the mitochondrial $H^+$ pump also reflects a temperature-induced membrane phase transition. At high inhibitor concentration (large positive shift of $\psi_0$) the transition appears to be completely suppressed (Fig. 3b). With lower inhibitor concentrations the transition temperature, $T_t$, could be gradually shifted from $20°$ to $30°$ C in a concentration-dependent manner, as shown in Fig. 3c. In these experiments the anaerobic backflow has been measured. $H^+$ uptake was accelerated by addition of uncoupler in concentrations yielding about an eightfold higher rate constant $k_2$, compared to coupled mitochondria.

In order to test the influence of a positive shift of surface charge on the fluidity state of pure phospholipids, a comparable model experiment
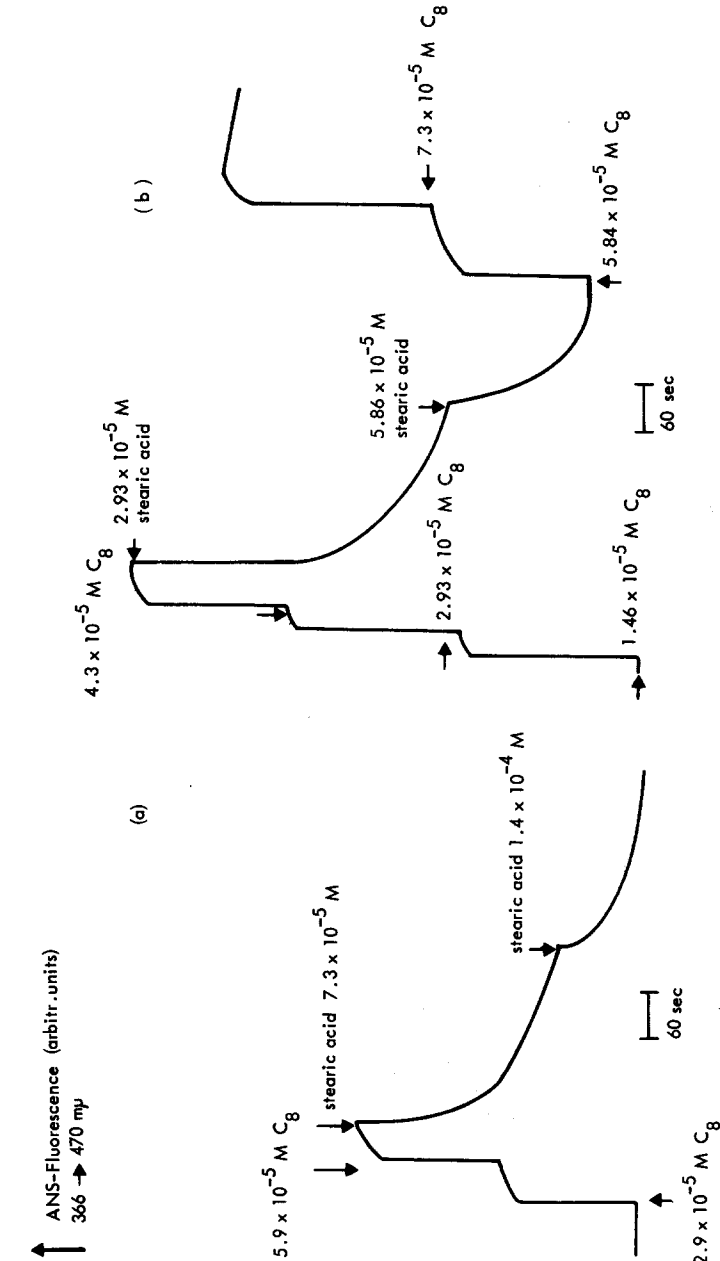
Figure 1. Relative changes of ANS fluorescence as a probe of the membrane surface potential induced by adsorption of *n*-octylbiguanide ($C_8$) or of stearic acid. (a) Electroneutral phospholipid micelles (lecithin) 1.3 mM in 50 mM Tris buffer, pH 7.2. (b) Rat liver mitochondria (1.3 mg protein) in 0.25 M sucrose (exp. 240675).
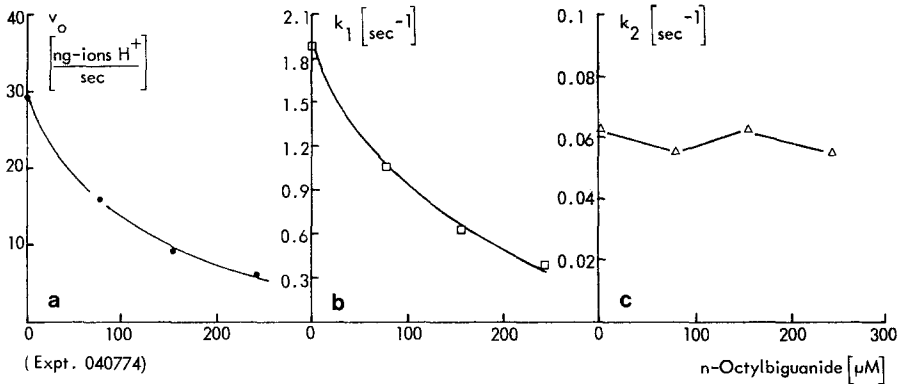
Figure 2. Kinetics of aerobic and anaerobic $H^+$ translocation across the membrane of coupled rat liver mitochondria. (a) Initial velocity $(V_0)$ of aerobic $H^+$ output; (b) first-order rate constant for active $H^+$ translocation; and (c) first-order rate constant of anaerobic $H^+$ backflow vs. concentration of $n$-octylbiguanide. Experimental conditions given in the text; mitochondrial protein 3.1 mg/ml (exp. 040774).

(Fig. 4) was performed with DPA vesicles bearing a large negative surface charge at pH 7 [6]. The phase transition is indicated by a large increase of NPN fluorescence [2, 11]. When the surface charge is made positive by adsorption of $n$-octylbiguanide, the transition is almost abolished and also shifted to higher temperatures. A slight plateau of the fluorescence trace appears about $10°C$ above normal $T_t$. A corresponding shift of $T_t$ can be achieved with these vesicles by decrease of pH by 2–3 units in absence of biguanide [6].

## Discussion

Regarding the surface charge, it emerges from the above observations as a main conclusion that physicochemical relations derived from model lipid membranes apply equally well to the lipid constituents of natural membranes; it is shown for the first time that mitochondrial membranes with respect to phase transitions exactly resemble the behavior of liquid crystalline phospholipid phases upon changes in the lipid environment. Such an analogy of phase transitions in intact membranes as compared to pure lipids has already been reported for *E. coli* membranes [11, 17] in great detail.

As revealed by studies with membrane-bound indicator dyes [18], application of a positive surface potential causes a decrease of $H^+$ activity at the immediate membrane surface and a lower $H^+$ affinity of
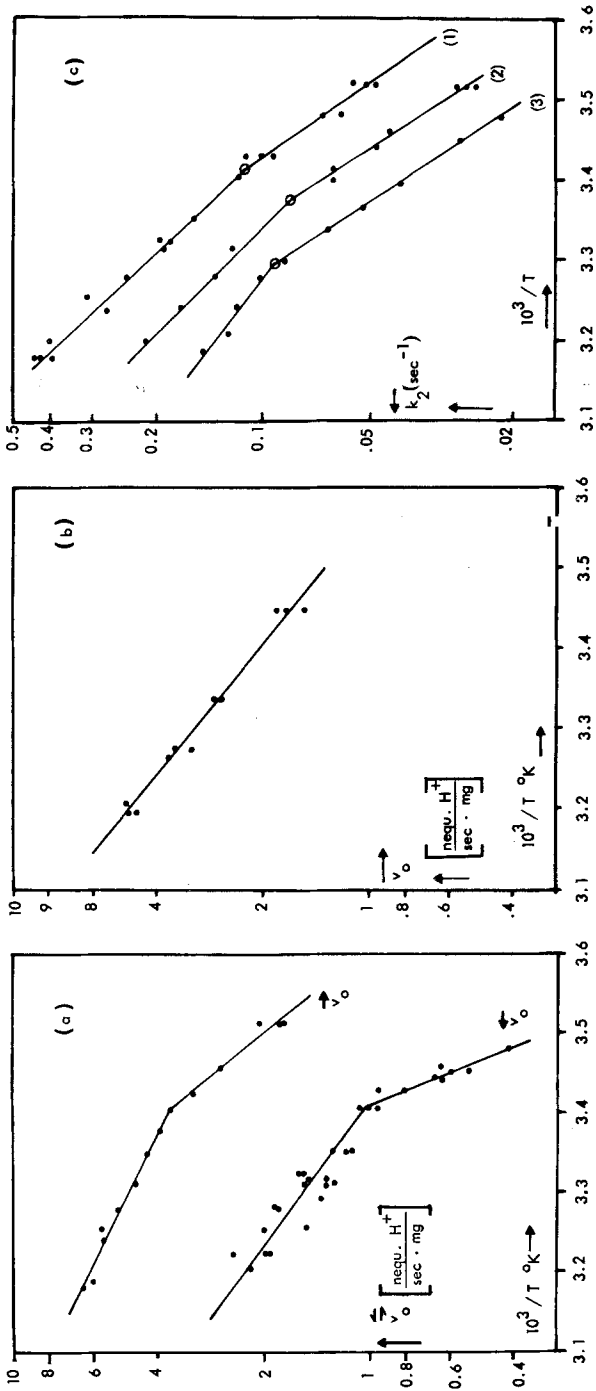
Figure 3. Arrhenius plots of respiration-induced H$^+$ ejection and uncoupler-mediated H$^+$ uptake. (a) Control initial velocity of H$^+$ ejection $\vec{V}_0$ and of H$^+$ uptake $\overleftarrow{V}_0$; (b) initial velocity $\overleftarrow{V}_0$ in presence of 3.0 mM phenethylbiguanide; (c) rate constants $k_2$ for H$^+$ uptake in presence of no (1), 0.8 mM (2), and 1.6 mM (3) phenethylbiguanide. To avoid superposition curves (2) and (3) were offset by a factor of −0.4 and −0.5, respectively; protein concentration 1.4–1.7 mg/ml (exp. 131174, 131274, 161274).
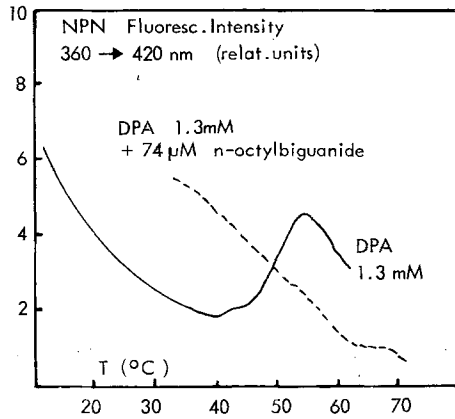
Figure 4. Temperature-induced phase transition of DPA micelles, monitored by fluorescence of NPN; pH 7.1; solid line = control; dashed line = 74 $\mu$M n-octylbiguanide present (exp. 030675).

dissociable acidic groups. The active translocation of protons presumably involves such nucleophilic sites at the membrane's surface becoming occupied or screened by adsorbed guanidinium ions. Part of the observed inhibition of the proton pump may therefore be explained by such a mechanism. At the applied concentrations of biguanides, mitochondrial respiratory rate and $H^+$ ejection were inhibited to the same degree without a change of $H^+/O$ ratio [12]. This supports mechanisms suggesting a primary coupling of active $H^+$ translocation and redox reactions on the level of the same components of the respiratory chain. The activation energies derived from Arrhenius plots are equivalent to breaking of 3–4 hydrogen bonds [19] and agree well with those observed for proton movements in submitochondrial particles [20] and for valinomycin-mediated $K^+$ uptake.

The anaerobic backflow of $H^+$ ions from the aqueous bulk phase into coupled mitochondria appears to proceed in an electroneutral form (undissociated acids, for instance), because no influence of the surface potential was seen on the apparent rate constant. This holds also for uncoupler-mediated $H^+$ flow. In a recent report we have shown [9] that $H^+$ uptake for mitochondrial ADP phosphorylation is also inhibited by biguanides and that the surface potential may thus regulate the turnover of oxidative phosphorylation. In addition it could be demonstrated that mitochondrial $Ca^{2+}$ uptake [21] or flux of potassium [12] responds to modification of the surface charge as theoretically expected from experiments with model membranes.

The analogous response of mitochondrial and model membranes with

regard to lipid phase transitions is explained by a decrease of absolute surface charge as a common basis. In fact, mitochondrial membranes, like DPA vesicles, exhibit a negative surface charge. This may be related to the high content of anionic phospholipids in mitochondria [17]. Binding of the positively charged biguanide molecules or lowering of pH, however, is equivalent to reduction of anionic net charge. This lowers the specific surface area, $f$, per lipid molecule because the latter is essentially determined by electrostatic interactions between the polar headgroups of the anionic phospholipids [5]. Therefore the whole assembly is rendered more rigid. In fact, the observed increase of $T_t$ is in favor of a more rigid membrane structure. The contrary would be expected if the charge density of a lipid-containing membrane is increased. As already mentioned, a positive shift of the intrinsic negative surface potential of mitochondrial membranes causes a reduction of the absolute net charge, however. Thus the results are in line with the theory.

These changes in the physical state of lipids may be transmitted to integral catalytic membrane proteins associated with the lipids; diffusion-controlled membrane transport will likewise be modified by the altered state of mobility of the membrane lipids.

It may be argued that a rather simplistic point of view has been taken by discussing only the interaction of biguanides with anionic lipids. However, lipids of the latter type are predominantly present in mitochondrial membranes. Regarding dipolar interactions (to be expected in lecithin-containing structures), a more sophisticated theory on the nature of structural perturbation and membrane fluidity would be required. Thus, the different lipid compositions of morphologically different membranes may at least partially explain the great variety of effects of blood-sugar-lowering biguanides described with various membrane-containing metabolic model systems. Nevertheless, creation of an excess of positive charges at the intimate membrane surface would persist and would exert the observed effect on activity and translocation of ionic species at a given temperature. It may be recalled in this context that with model membranes formed from pure dioleylphosphatidyl-choline, an increase of electrical capacitance has been found, which was tentatively explained in a thinning of the bimolecular lipid lamellae [10].

Besides inhibiting drugs, such as guanidines, physiological parameters influencing the surface charge, as for example divalent cations or the local pH, may also be considered to exert similar control on membrane structure and on the kinetics of membrane functions.

## Acknowledgment

## References

1. E. Oldfield and D. Chapman, *FEBS Lett.*, 23 (1972), 285.
2. H. Träuble and H. Eibl, *Proc. Natl Acad. Sci., U.S.A.*, 71 (1974), 214.
3. S.G.A. McLaughlin, *J. Membr. Biol.*, 9 (1972), 361.
4. B. Gomperts, F. Lantelme and R. Stock, *J. Membr. Biol.*, 3 (1970), 241.
5. D.E. Graham and E.J. Lea, *Biochim. Biophys. Acta*, 274 (1972), 286.
6. H.-J. Galla and E. Sackmann, *J. Am. Chem. Soc.*, 97 (1975), 4114.
7. J.K. Raison, *J. Bioenerg.*, 4 (1973), 285.
8. J.K. Raison, J.M. Lyons, R.J. Mehlhorn and A.D. Keith, *J. Biol. Chem.* 246 (1971), 4036.
9. G. Schäfer and G. Rowohl-Quisthoudt, *FEBS Lett.*, 59 (1975), 48.
10. G. Schäfer, E. Rieger and M. Speichert, *Eur. J. Biochem.*, 46 (1974), 613.
11. H. Träuble and P. Overath, *Biochim. Biophys. Acta*, 307 (1973), 491.
12. G. Schäfer, *Hoppe-Seyler's Z. Physiol. Chem.*, 335 (1974), 1247.
13. G.K. Radda, *Curr. Top. Bioenerg.*, 4 (1971), 81–126.
14. D.H. Haynes, *J. Membr. Biol.*, 17 (1974), 341–352.
15. M.B. Feinstein and H. Felsenfeld, *Biochemistry*, 14 (1975), 3041–3048.
16. F. Davidoff, *J. Clin. Invest.*, 47 (1968), 2344–2358.
17. P. Overath and H. Träuble, *Biochemistry*, 12 (1973), 2625–2634.
18. M. Montal and G. Gitler, *J. Bioenerg.*, 4 (1973), 363.
19. W.D. Stein, *The Movement of Molecules Across Cell Membranes*, Academic Press, New York and London, 1967.
20. S. Papa, F. Guerrieri, S. Simone, M. Lorusso and D. Larosa, *Biochim. Biophys. Acta*, 292 (1973), 20.
21. G. Schäfer and G. Rowohl-Quisthoudt, Abstr. Int. Symp. on *Electron Transfer Chains and Oxidative Phosphorylation*, Bari (1975).
22. M. Levy, R. Toury, M.T. Sauner and J. Andre, in: *Mitochondria, Structure and Function*, L. Ernster and Z. Drahota (eds.), Academic Press, New York, 1969, pp. 33–42.