# INFLUENCE OF ELECTROSTATIC SURFACE POTENTIAL ON MITOCHONDRIAL ADP-PHOSPHORYLATION

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## 1. Introduction

Induction of respiration in mitochondria leads to formation of the so-called 'energized state' of the membrane [1] and liberation of $H^+$-ions [2]. Both phenomena can be monitored by utilizing various chromophoric or fluorophoric probes [3,4], or in the latter case also sensitive glass electrodes [5]. Little is known, however, about the molecular nature of the membrane energization behind the response of these probes, which in most cases reply to multifunctional changes of their environment. This applies especially to ANS which binds equally well to lipids and to proteins [6].

It has been shown that the optical response of anionic dyes interacting with membranes is subject to a membrane surface potential [7]. There is good evidence that ANS in particular reports changes of these surface potentials ($\psi_0$) between a membrane and the aqueous bulk phase [8]. Our previous observation that ANS fluorescence truly resembles the hydrophobic fixation of positive charges by adsorption of alkyl-guanidines to various membrane preparations supports this assumption [9]. The present paper provides evidence, that the rate of oxidative phosphorylation may be controlled by the surface potential of isolated mitochondria.

## 2. Methods

Mitochondria from male Wistar rats were prepared according to standard methods by differential centrifugation in isotonic sucrose/manitol medium. Respiration was measured polarographically using succinate as substrate in presence of rotenone. $H^+$-uptake as a measure of phosphorylation-rate was monitored as previously reported [9]. Submitochondrial particles (SMP) were prepared from beefheart mitochondria by sonication according to Beyer [10]. Liposomes were prepared by sonication of dipalmitoyl-lecithin in 50 mM Tris-buffer to yield a final lipid concentration of 1.3 mM. The fluorescence of ANS was measured in a modified Eppendorf filter-fluorimeter.

*Abbreviation:* ANS, 8-anilinonaphtalene-sulfonic acid.

## 3. Results

Due to its high $pK_a$-values protonated alkyl-biguanides represent hydrophobic fixed positive charges when bound to phospholipid containing membranes. We have shown in previous studies on electrical conductance of lipid-bilayers that adsorption of *n*-octyl- or of phenethyl-biguanide generates a positive surface potential which could be directly determined [11]. This alteration of the membrane surface charge is also reflected by reversible binding of the anionic fluorescence dye ANS. With pure lipids the relation of ANS-binding and its fluorescence enhancement due to surface potential changes can be calibrated. Fig.1 demonstrates the influence of phenethyl-biguanide on ANS binding to phospholipid liposomes. The decrease of the ordinate intercept reflects an increase of the relative number of binding sites for ANS according to an increase of positive surface charges in presence of biguanide. This result may be theoretically expected. More important information is derived from comparison of liposomes with mito-

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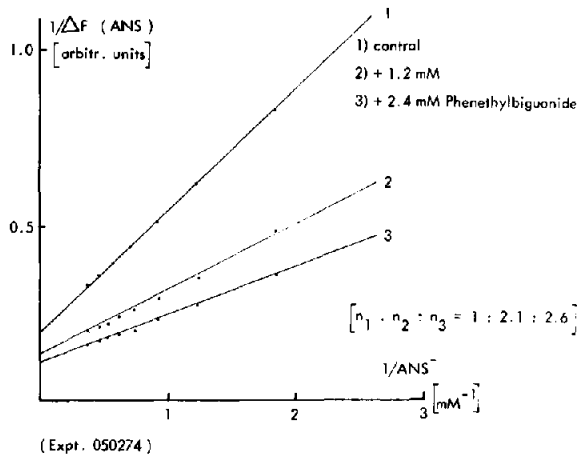



Fig.1. Reciprocal plot of fluorescence–titration (366→470 nm) of ANS-binding to phosphatidylcholine micells. Effect of increasing positive surface charge.

chondrial and submitochondrial membranes under identical conditions, as summarized in table 1. The excellent agreement of data clearly demonstrates that n-octyl- or phenethyl-biguanide in all membrane types causes the same response of ANS fluorescence. It is therefore concluded that also in mitochondria the surface potential is shifted more positive by these inhibitors of oxidative phosphorylation, and that ANS fluorescence can be taken as a relative measure of this shift, accordingly. The relative effectiveness of both inhibitors closely resembles their membrane-binding affinity as determined previously [9].

Fig.2 shows for both inhibitors the concentration dependence of mitochondrial state-3 respiration and

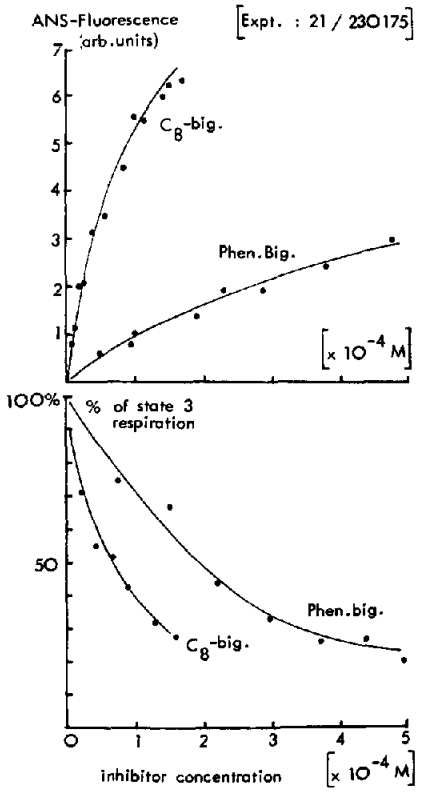



Fig.2. Dependence of mitochondrial state-3 respiration (ADP and $P_i$ present) and of ANS-fluorescence on concentration of *n*-octyl- or of phenethyl-biguanide.

the observed increase of ANS-fluorescence. The measured parameters exhibit an inverse response and further evaluation of data reveals a direct correlation between the degree of inhibition and the guanidine-

Table 1
The relative number of ANS-binding sites at various membranes as calculated from ANS-fluorescence-titrations. Maximum fluorescence without effector was referred to as 1.0.

| | Phenethyl-biguanide (× $10^{-3}$ M) | | | *n*-octylbiguanide (× $10^{-4}$ M) | | |
|---|---|---|---|---|---|---|
| Effector-concentration | 0 | 1.2 | 2.4 | 0 | 1.2 | 2.4 |
| | relative number of $ANS^-$ binding sites | | | | | |
| Liposomes | 1.0 | 2.1 | 2.5 | 1.0 | 1.9 | 2.6 |
| Liver mitochondria | 1.0 | 1.9 | 2.6 | 1.0 | 2.1 | 2.8 |
| SMP, beef-heart | 1.0 | 2.0 | 2.5 | 1.0 | 1.4 | 2.2 |

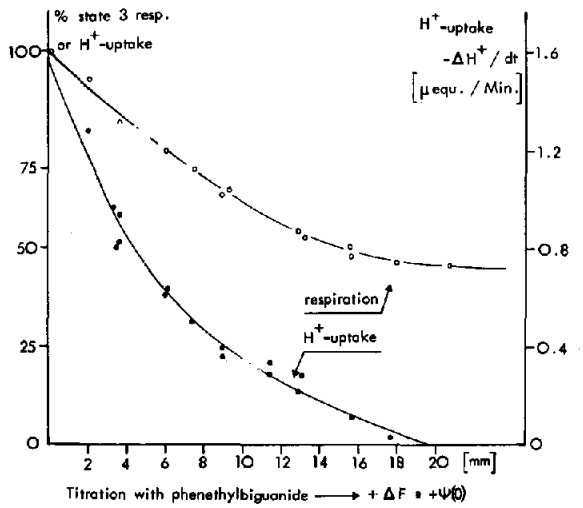
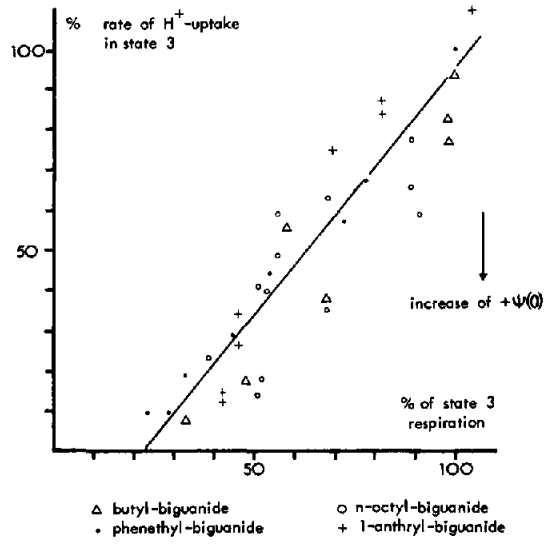



Fig.3. $H^+$-uptake and state-3 respiration of rat liver mitochondria; dependence on surface potential as indicated by ANS-fluorescence.




$y = 1.24x - 29.4$; $r = 0.94$; $n = 36$; $\overline{R.C.} = 4.22$

Fig.4. Correlation of state-3 respiration and $H^+$-uptake during ADP-phosphorylation under the influence of increasing positive surface potential.

induced ANS fluorescence, i.e. the change in surface potential. The inhibition levels off at the state-4 respiratory rate of the individual mitochondrial preparation. This can also be seen in fig.3, where the rate of ADP-phosphorylation is plotted versus the fluorescence of ANS as a relative measure of the surface potential. Phosphorylation has been measured by continuous registration of mitochondrial $H^+$-uptake on addition of ADP to the weakly buffered suspension. A 1:1 molar ratio of $H^+$-uptake to ADP-consumption was observed in all experiments, according to the chemical equation:

$$ADP^{3-} + HPO_4^{2-} + H^+ \rightarrow ATP^{4-} + H_2O \qquad (1)$$

With increasing surface potential the rate of $H^+$-uptake becomes zero, when respiration approaches the state-4 rate.

For conditions where different surface potentials become effective the correlation of the rate of $H^+$-uptake versus the rate of state-3 respiration yields a straight line (fig.4). These data were obtained from a large number of titrations as given in fig.3 using four different guanidine derivatives. The regression line intercepts the abscissa at the average state-4 respiratory rate, indicating an average respiratory control ratio of 4.3 under the employed conditions. The inhibitor concentration increases from the upper right to the lower left along the regression line. It becomes obvious that shifting the surface charge more positive (or less negative, respectively) affects the coupled phosphorylation, but does not directly affect respiratory electron transport. If the latter were the case the regression line should cross through the coordinate origin.

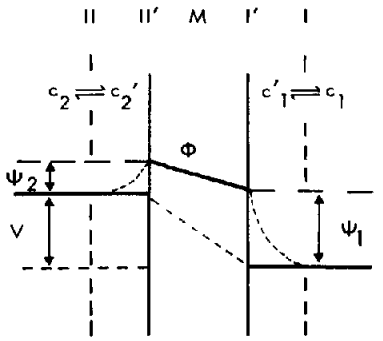



Fig.5. Schematic diagram of the potential-profile across a phase separating membrane.

## 4. Discussion

Fig.5 represents a model for the potential profile across a membrane with V denoting the membrane potential, with $\psi_1$ and $\psi_2$ denoting the surface potentials at sides I and II of the membrane, and with the unstirred layers I' and II' at interphase between a membrane and the bulk aqueous phase. $\psi_{1,2}$ is assumed positive for convenience. The presence of surface potentials not only determines the gradient of $\phi$ between the membrane surfaces but also the activity of charged species $[X^+]_m$ in the unstirred layers, as described by $[X^+]_m = [X^+]_b \cdot \exp(-\psi_{1,2}/kT)$, when $[X^+]_b$ is the activity of the positively charged species in the bulk solution.

Since considerable evidence has been presented that binding of biguanides to membrane-lipid causes a positive surface potential the above results may be interpreted by the assumption that the activity of $H^+$-ions at the membrane outside decreases gradually with an increase of bound inhibitor, until the availability of $H^+$ becomes rate-limiting for ADP-phosphorylation. Thus, the presence of these inhibitors is equivalent to a higher pH at the immediate surface of the mitochondrial membrane. Regarding the phosphorylation potential expressed by the ratio of [ATP]/[ADP] $[P_i]$ $[H^+]$ according to Eqn [1], it emerges that via the local concentration of $H^+$-ions the rate of ADP phosphorylation may be manipulated by the actual surface-potential. The reported release of biguanide inhibition by long-chain fatty acids [12] is explained by counteraction of adsorbed fatty acid anions rendering the surface potential more negative. On the basis of the above results it is obvious why a specific interaction of these inhibitors with catalytic units of oxidative phosphorylation was never detected, and why rather high concentrations are necessary to induce the inhibition and perhaps to overcome the local buffer capacity by means of surface-potential-induced $pK_a$ shifts [7]. Our earlier studies on ion movements across the mitochondrial membrane are in support of the above interpretation [13].

Accepting that large scale changes of ANS fluorescence at the mitochondrial membrane reflect changes of the surface potential ($\psi_0$) the lack of a fluorescence change due to state 4→3-transitions implies that $\psi_0$ remains constant at both kinetic states of the respiratory chain.

## References

[1] Azzi, A., Chance, B., Radda, G. K. and Lee, C. P. (1969) Proc. Nat. Acad. Sci. USA 62, 612.
[2] Mitchell, P., Moyle, J. (1967) in: Biochemistry of Mitochondria (Slater, Kaniuga and Wojtczak, eds) pp. 53–91, Acad. Press London.
[3] Radda, G. K. (1971) Curr. Topics Bioenerg. 4, 81.
[4] Azzi, A. (1975) in: Methods in Enzymology (Fleischer, S. and Packer, L. eds.) Vol. 32, p. 234, Acad. Press, N.Y.
[5] Papa, S., Guerrieri, F., Simone, S., Lorusso, M. and Larosa, D. (1973) Biochim. Biophys. Acta 292, 20.
[6] Radda, G. K. and Vanderkooi, J. (1972) Biochim. Biophys. Acta 265, 509.
[7] Montal, M. and Gitler, C. (1973) Bioenergetics 4, 363.
[8] Haynes, D. H. (1974) J. Membr. Biol. 17, 341.
[9] Schäfer, G. (1974) Europ. J. Biochem. 45, 57.
[10] Beyer, R. E. (1967) in: Methods in Enzymology (Estabrook, R. W. and Pullman, M. E. eds); Vol. X, p. 186, Acad. Press, N. Y.
[11] Schäfer, G., Rieger, E. and Speichert, M. (1974) Europ. J. Biochem. 46, 613.
[12] Davidoff, F. (1968) J. Clin. Invest. 47, 2331.
[13] Schäfer, G. (1974) Hoppe-Seyler's Z. Physiol. Chem. 335, 1247.