# PHOSPHORYLATION WITHOUT PROTONMOTIVE FORCE

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## 1. Introduction

The total protonmotive force was estimated in metabolizing isolated mitochondria. Estimates of the electric potential across the mitochondrial membrane were obtained using an electrofluorimetric cyanine dye [diS-$C_3$-(5)]* and the pH gradient was estimated from the distribution of DMO under steady state conditions. The presumed protonmotive force was found to be a small fraction of that required for phosphorylation of ADP.

The chemiosmotic hypothesis proposes that phosphorylation is the result of the flux of protons in the direction of their electrochemical potential gradient, the protonmotive force, generated by the passage of electrons through the respiratory chain [1–5]. In mitochondria, the electric potential across the mitochondrial membrane is presumed to be a large component of the protonmotive force. Previous attempts [5–10] to estimate the membrane potential from the $K^+$ distribution in the presence of valinomycin have been challenged [11].

Recently, the magnitude of the mitochondrial membrane potential has been estimated by means of electrofluorimetric cyanine dyes [12,13]. However, the results are contradictory. One report suggests a metabolically induced change in the membrane potential of about −180 mV [13] and the other an insignificant change [12]. Both studies were carried out under a very narrow range of conditions. This question was re-examined to resolve this conflict. Furthermore, fluorescence responses could result from events unrelated to membrane potential changes, see [14,15] (e.g. the cationic dye could accumulate in exchange for $H^+$). The results of the present experiments demonstrate that phosphorylation can occur in the presence of a presumed protonmotive force which is a small fraction of that required for phosphorylation.

* *Abbreviations:* diS-$C_3$-(5): 3,3' dipropylthiocarbocyanine; DMO: 5,5 dimethyl-oxazolidine 2,4 dione; tris: tris(hydroxymethyl)amino methane.

## 2. Methods

Mitochondria were isolated by the method of Tedeschi [16] from male Holzman rats weighing 340–490 g. The mitochondria were washed once after isolation and then suspended in one of a variety of media (see legend of table 1).

The fluorescence of diS-$C_3$-(5)* was estimated with a Perkin Elmer-Hitachi spectrofluorimeter Model MPF 3 at a wavelength of 622 nm for the excitation and 670 nm for the emission [14]. In fig.1, the fluorescence is expressed in arbitrary units. Rotenone and antimycin A were used in a concentration of 0.33 μg/ml and diS-$C_3$-(5) in a concentration of 3.8 μM. Rotenone was present in all suspensions except in the medium used by Laris et al. [13] (medium C of the legend of table 1). For energization, succinate was added to a final concentration of 3.3 mM. When present, ADP was in a concentration of 1.4 mM. The changes in fluorescence corresponding to given membrane potentials are estimated after addition of 0.151 μM valinomycin to the mitochondria suspended in the incubation media (without KCl) and after the addition of the appropriate aliquots of stock KCl solutions.

To measure the [$^{14}$C]DMO distribution and the mitochondrial spaces, aliquots of the suspensions

Table 1
Presumed membrane potential of mitochondria in various media

| 1 Medium | | | | | 2 $-\Delta E_a \pm$ SD (mV) | | | 3 | 4 |
|---|---|---|---|---|---|---|---|---|---|
| Exp. | Solution | KCl (mM) | $P_i$ (mM) | $Mg^{++}$ (mM) | + Succinate | + Succinate + ADP | + Control | Phosphate* Potential required (mV) | $\sim$P (nmoles $min^{-1}$ mg $Pr^{-1}$ |
| 1 | C | 20 | 10 | 5 | 175 ± 18 | – | – | – | – |
| 2a | C | 20 | 10 | 5 | 209 ± 4 | – | – | – | – |
| b | C | 20 | 0 | 5 | 112 ± 12 | – | – | – | – |
| 3a | C | 20 | 0.5 | 5 | 185 ± 8 | – | – | – | 27 |
| b | A | 0 | 0 | 0 | 16 ± 4 | – | 15 ± 6 | – | – |
| 4a | C | 20 | 10 | 5 | 312 ± 15 | – | 14 ± 4 | – | 84 |
| b | A | 0 | 0 | 0 | 17 ± 4 | – | 7 ± 2 | – | – |
| 5 | A | 5 | 0.5 | 0 | 54 ± 10 | 50 ± 6 | 7.9 | (230) 460 | 57 ± 6 |
| 6 | A | 2.5 | 0.5 | 0 | 24 ± 4 | 48 ± 2 | 6.3 | (220) 440 | 29 |
| 7a | C | 20 | 10 | 5 | 218 ± 10 | – | – | – | 47 |
| b | B | 0 | 0 | 0 | 22 ± 6 | – | – | – | 26 |
| 8a | B | 5 | 0.025 | 0 | – | 45 ± 6** | 6 | (270) 540 | 20 |
| b | B | 5 | 0 | 0 | 22 ± 7 | – | 6.2 | – | 20 |
| c | C | 20 | 0.5 | 5 | – | 170 ± 18** | 25 | (210) 420 | 66 |
| d | C | 20 | 0.5 | 5 | 185 ± 9 | – | 35 | – | – |
| 9a | B | 5 | 0.5 | 0 | 62 ± 4 | – | 7 | – | – |
| b | B | 5 | 0.5 | 0 | 69 ± 2 | 72 ± 2 | 7 | (230) 460 | 48 |
| 10 | B | 5 | 0.5 | 0 | 29 ± 2 | 51 ± 7 | 14 | (210) 420 | 20 |
| 11 | B | 5 | 0.5 | 0 | 26 ± 4 | 53 ± 7 | 12 | (220) 440 | 57 |

The following incubation media were used: A: 0.3 osmolal sucrose, 3 mM glycylglycine, pH 7.4; B: 0.3 osmolal sucrose, 10 mM tris, pH 7.4; C: 0.225 M sucrose, 2 mM triethanolamine, pH 7.4. KCl, $P_i$ and $MgCl_2$ were added to these as shown below. The stock suspensions were generally suspended in the appropriate incubation media without the ionic additions. The mitochondrial protein concentration ranged between 0.090 and 0.181 mg/ml. Phosphate potentials were estimated from the ATP, ADP, and $P_i$ concentrations at four minutes. The experimental design follows the details given in fig.1. *The potentials shown in parentheses correspond to those necessary for the synthesis of 1 ATP per 2 $H^+$ transferred. **ADP added prior to succinate.

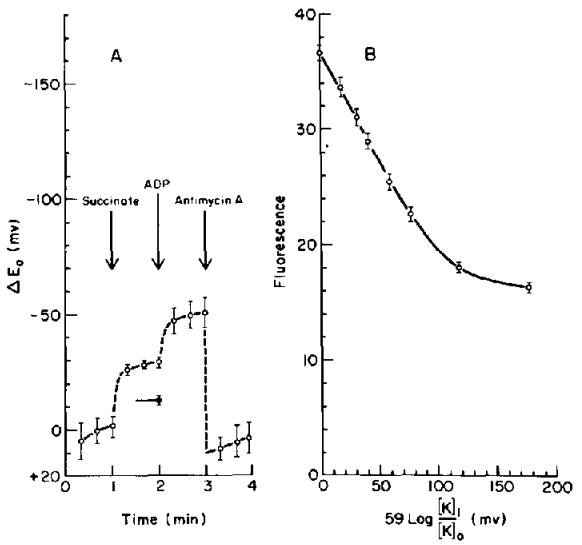


Fig.1. (A) Changes in fluorescence following the additions indicated by the arrows (see Methods). The $\Delta E_a$ were estimated from part (B). (B) Dependence of the fluorescence on the concentrations of $K^+$ in the presence of valinomycin. The mitochondria, 0.118 mg protein/ml were suspended in 0.30 osmolal sucrose, 3 mM glycylglycine, and 0.5 mM $P_i$, pH 7.4.

were sedimented through a silicon layer (General Electric SP 1154, sp. gr. 1.05 at 25°C) into 15% $HClO_4$ by centrifuging for 2.5 min in a Beckman-Spinco Microfuge, model 152, see [17,18]. The internal mitochondrial volume was estimated from [$^3$H] $H_2O$ (16.7–30 $\mu$Ci/ml, 1 mCi/g), the [$^3$H] methoxydextran (3.3 $\mu$Ci/ml, 70.4 mCi/g) and the [$^{14}$C]sucrose (0.8–1.6 $\mu$Ci/ml, 13 mCi/g) spaces. All radioactive compounds were from New England Nuclear (Boston, Mass.). The mitochondrial suspensions were more concentrated than those used for the fluorimetric assays. The oxygen level was monitored with a Clark electrode to be certain that the oxygen was not depleted during the incubation. The external pH was estimated at steady state with a glass electrode (Beckman # 39505) and a Brinkman Model 102 pH meter. The internal pH was calculated by the method published previously by others [19,20].

Parallel determinations of phosphorylative rate were carried out by monitoring the disappearance of $P_i$ using the method of Hurst [22] and a Technicon Autoanalyzer. The $P_i$ concentration was estimated by continuous sampling. The reaction was stopped by the monitoring system with 3 N $H_2SO_4$. The disappearance of $P_i$ was found to be completely oligomycin sensitive and hence represents the phosphorylation of ADP. The P : O ratios estimated by this method (monitoring the oxygen concentration with a Clark electrode) correspond closely to those calculated from the ADP : O ratios employing the method of Estabrook [23]. The concentrations of ADP and ATP were calculated from the ADP and $P_i$ present at zero time and the $P_i$ removed from the medium. The $\Delta G$s for the synthesis of ATP were calculated from these concentrations using the appropriate $\Delta G^0$ of Rosing and Slater [24].

All incubations were done at 25°C in 3 ml. Standard deviations are stated. Protein was estimated by the Biuret reaction [25].

## 3. Results and discussion

The effect of $K^+$ concentration on the fluorescence intensity in the presence of valinomycin can be expressed as a function of $[59 \log_{10} (K^+)_i/(K^+)_o]$, the presumed diffusion potential ($\Delta E_a$ in mV), where the subscripts i and o correspond to the internal and external $K^+$ concentration respectively. The values of $(K^+)_i$ used in these estimates follow the assumptions of Laris et al. [13] that $[K^+]$ is 100 mM and the mitochondrial volume remains approximately constant. Deviations from these assumptions would lead to significant but small deviations in $\Delta E_a$. The dependence of the fluorescence on the presumed diffusion potential $\Delta E_a$ is shown in fig.1B. The fluorescence response takes place in the presence of rotenone or antimycin and rotenone at concentrations which block metabolism entirely. The same relationship between fluorescence and $\Delta E_a$ are obtained regardless of valinomycin concentration in the range of 0.0003 to 1.7 $\mu$g/ml (the mitochondrial protein concentration was 0.139 mg/ml). The mean of the slopes (fluorescence units/ $\Delta E_a$) at the various valinomycin concentrations was 14.9 ± 0.9 and the mean of the intercepts on the ordinate was 20.0 ± 1.4 fluorescence units. The relationship between fluorescence and $\Delta E_a$ suggests that under these conditions the fluorescence reflects a diffusion potential. Hence the effect of varying the $K^+$ concentration on fluorescence

in the presence of valinomycin can be used also to relate fluorescence to membrane potential. However, it should be noted that the calculations of $\Delta E_a$ probably reflect maximal estimates since other ions may be involved (e.g. anions) and the accumulation of dye need not be in response to an electric potential alone.

Experiments using the restricted conditions of Laris et al. [13] show apparent membrane potential changes ranging between −170 and −310 mV (table 1, exp. 1,2a,3a,4a,7a,8c,8d). The calibration curves (e.g. fig.1B) were linear at least over the range of 0 to 80 mV. The apparent potentials using the conditions of Laris et al. are beyond the limits of the calibration curves and were calculated by linear extrapolation. Therefore, these values might be considered underestimated (e.g. see fig.1B). However, it is likely from our evidence that changes in fluorescence under the conditions of Laris et al. do not reflect a true membrane potential. The results are expressed in this manner for convenience. Moreover, it should be noted that biological membranes generally cannot support potentials in excess of 150 to 200 mV, see [26–30].

The results obtained in other media are quite different (fig.1A, table 1, exp. 2b,3b,4b,5,6,7b,8a,8b, 9,10). Fig.1A summarizes an experiment in which the mitochondria were suspended in 0.3 osmolal sucrose, 10 mM tris chloride, 0.5 mM $P_i$ and 5 mM KCl, pH

Table 2
Phosphorylation without protonmotive force

| Additions | 1 $pH_o$ | 2 $pH_i$ | 3 $\Delta pH$ | 4 $59\Delta pH$ (mV) | 5 $\Delta E_a$ | 6 Proton motive force (mV) | 7 Phosphate* Potential (mV) | 8 $\sim$ P (nmoles $min^{-1}$ $mg\ Pr^{-1}$ |
|---|---|---|---|---|---|---|---|---|
| Succinate | 6.92 ± .01 | 7.22 ± .04 | 0.30 | 18 | 0 | 18 | – | – |
| | 7.20 ± .01 | 7.44 ± .02 | 0.24 | 14 | 28 | 42 | – | – |
| | 7.30 ± .01 | 7.58 ± .06 | 0.29 | 17 | 26 | 43 | – | – |
| Succinate + ADP | 6.98 ± .01 | 7.30 ± .04 | 0.32 | 19 | 8 | 27 | (210) 420 | 27 |
| | 7.21 ± .01 | 7.67 ± .04 | 0.46 | 27 | 49 | 77 | (210) 420 | 20 |
| | 7.31 ± .01 | 7.59 ± .02 | 0.28 | 17 | 53 | 70 | (220) 440 | 57 |
| Succinate + ADP + Antimycin A | 6.92 ± .01 | 6.89 ± .12 | 0.03 | 2 | | | | |
| | 7.21 ± .01 | 7.29 ± .02 | 0.08 | 5 | | | | |
| | 7.27 ± .01 | 7.31 ± .04 | 0.04 | 2 | | | | |

Mitochondria (0.105–0.181 mg protein/ml) were suspended in 0.30 osmolal sucrose, 0.5 mM sodium phosphate, 5 mM KCl, 10 mM tris, pH 7.4 for the fluorescence assays. ΔpH was estimated in parallel experiments using 2.72–3.16 mg protein/ml. Succinate, ADP and antimycin were added successively; antimycin A and rotenone were in a concentration of 3.3 μg/ml. The protonmotive force was calculated following Mitchell and Moyle [5]. See table 1 for estimates of the phosphate potentials.

* The potentials shown in parenthesis correspond to those necessary for the synthesis of 1 ATP per 2 $H^+$ transferred.

7.4. The upper curve of part A indicates the experimental determination, the lower curve a control to which succinate was not added (closed circle). The activation with succinate produced a $\Delta E_a$ slightly higher than the control. ADP (contrary to theoretical predictions of the chemiosmotic hypothesis) enhanced the $\Delta E_a$. Part B shows the calibration curve, i.e. the fluorimetric response as a function of $K^+$ concentration (and hence $\Delta E_a$). The $\Delta E_a$ upon energization was within the linear portion of the calibration curve from the same preparation. The results shown in table 1 show that there is little or no correlation between $\Delta E_a$ induced by metabolism (column 2) and phosphorylative ability (column 4). Column 3 shows the electric potential necessary for phosphorylation, the phosphate potential [i.e. $\frac{\Delta G}{F}$ + 59 log (ATP)/(ADP) ($P_i$)] after 4 min of incubation. The values in parentheses are those obtained by assuming that 2 $H^+$ are taken up per ATP synthesized. During this time, the fluorescence of the energized suspension did not change. The $\Delta G^0$ values at the appropriate pH, ionic strength and $Mg^{2+}$ were derived from Rosing and Slater [24]. The phosphate potential per two electrons in the absence of added ADP has been estimated to be well in excess of 300 mV, see [31]. Determinations of ΔpH (table 2, column 3) and $\Delta E_a$ (column 5) under steady state conditions show that the total protonmotive force (column 6) as defined by Mitchell is insufficient to meet the requirements calculated from phosphate potential (column 7).

Alternative interpretations are possible. For example, the influx of $H^+$ could be greater than the usually assumed 2 $H^+$ per ATP synthesized. In the experiments in which the protonmotive force is approximately −20 and −40 mV (see table 2), approximately 30 and 15 $H^+$ would have to be transferred per ATP. Considering that the P : O ratio obtained under these conditions is 1.7 ± 0.3 ( 4 experiments), this is not likely. It is also conceivable that some mitochondria possessing a high internal $K^+$ (and hence a high fluorescence response in the presence of valinomycin) are unable to be activated (i.e. they would give a low fluorescence response when activated). Again this is not likely since in many instances high $\Delta E_a$ and low $\Delta E_a$ are obtained from the same preparation depending on the medium. Generally, the $\Delta E_a$ estimated from the fluorescence response upon activation seems unrelated to phosphorylative ability.

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