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The Measurement of Transmembrane Electrochemical Proton Gradients

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Introduction

The recent interest in testing Mitchell's chemiosmotic hypothesis [1, 2] has stimulated the study of transmembrane electrochemical proton gradients in various biological systems. The main postulate of the chemiosmotic hypothesis is that in energy conserving membranes there are electrogenic proton pumps, driven by electron transport, which builds up a proton electrochemical potential difference, $\Delta\tilde{\mu}_H$. This potential is postulated to be the driving force for ATP synthesis by a reversal of an ATPase proton pump. The proton electrochemical potential difference, $\Delta\tilde{\mu}_H$ (or Δp as it is called by Mitchell [2]), is divided into electrical and concentration terms according to the relation:

$$\Delta\tilde{\mu}_H = \Delta\Psi - \frac{2.3RT}{F}\Delta pH \text{ (in mV)}$$

Where $\Delta\Psi$ is the potential difference across the membrane, and ΔpH is the pH difference (the expression 2.3RT/F at room temperature is equal to 60 mV). In macroscopic systems these can be determined simply by the use of electrodes (or microelectrodes in large cells), a technique which will not be discussed in this review.* We shall discuss the measurement of ΔpH and $\Delta\Psi$ in microscopic vesicular systems such as

* Microelectrodes have been used in an attempt to determine membrane potential in mitochondria [3]. We consider these measurements as highly questionable both because of the uncertainty regarding the exact location of the inserted electrode tip and because of the probable damage of the organelle.

mitochondria and their particles, chloroplasts and their particles and other closely related systems. These systems are often referred to as "energy conserving membranes" and various aspects of the coupling of electron transport, proton and other ions transport, and phosphorylation have been recently reviewed [4, 5, 6]. We shall limit our discussion to the methods of $\Delta\Psi$ and ΔpH determination and shall discuss briefly the major implications of these results. All the above mentioned systems, when studied *in vitro* are composed of a suspension of microscopic vesicles enclosed by (one or more) phospholipid-protein membranes in which the vesicles internal volume is only a small fraction of the total suspension volume. In many of the methods, which will be discussed below, the accurate determination of the particles internal water volume is of crucial importance for the calculation of ΔpH or $\Delta\Psi$. The vesicle volume determination (which we will not discuss in detail) usually involves sedimentation of the vesicles by centrifugation, determination of the pellet total water volume and the water volume which is external to the vesicles volume. In several systems (such as mitochondria and chloroplasts) when more than one membrane system exists it is necessary to find a suitable marker molecule that can penetrate the organelle but do not penetrate the inner membrane across which ΔpH and $\Delta\Psi$ are to be determined. [7, 8, 9, 10].

I. *The Determination of ΔpH*

An extensive review on intracellular pH, which discusses in great detail the principles and problems of some of the methods discussed here was published recently [11].

(a) *Titrametric methods.* A simple method which was employed in Lehninger's laboratory [12] (titrametric I) consists of separating mitochondria by centrifugation, dissolving the mitochondrial pellet in the non-ionic detergent Lubrol and measuring the pH of this solution. This is obviously a crude method which can only give a very rough estimation.

Mitchell and Moyle [13] have developed a titrametric method for mitochondrial suspensions (titrametric II) in which the buffering capacity of the matrix and of the solution external to the matrix are estimated from titrations in the presence of the detergent triton and in its absence, and ΔpH is estimated, for any metabolic state, from measurement of the extent of proton transport on the transition from a reference state (anaerobic) to the measured state. The main weakness of this method is the assumption that detergent treatment do not change the total buffering power of the suspension. Since the buffering power of phospholipid-protein complexes certainly depends on their interaction such an assumption is questionable. From comparison with other methods (see Table I) it appears that Mitchell's method tends to produce

values of ΔpH which are much higher than all other methods. An interesting work on the buffering capacity of chloroplast suspension in the dark and the light [14] demonstrates that if one knows the internal buffering capacity (which, however, was estimated from comparison of proton transport and ΔpH by an independent method [10]) it is possible to estimate the difference in internal pH between light and dark from the difference in the titration curves.

(b) *Internal pH indicators.* This method, when free from complications, should enable the continuous spectroscopic monitoring of internal pH with fast response time and great sensitivity. However, for a nonambiguous interpretation it requires a pH indicator which is exclusively located inside the particles. Unfortunately, none of the known indicators fulfil this requirement. If the indicator is distributed between various phases (i.e. external medium, membrane and internal volume) and, moreover, if this distribution is dependent on the metabolic state of the system, such measurement becomes extremely ambiguous. Chance [15, 16], has introduced the use of Bromthymol Blue (BTB) as an internal pH indicator. However, it is clear from Chance's group work as well as from the work of others [17, 18] that most of the BTB is bound to the membrane and its distribution between the membrane internal and external side is state dependent. Lynn [19, 20] has used both BTB and neutral red in chloroplasts suspension in which, upon illumination, BTB indicated pH increase and neutral red a decrease. It was suggested that neutral red indicates the pH in the internal thylakoid space. However, the low values obtained and their insensitivity to NH_4Cl and nigericin [20] which are known to abolish pH gradients in chloroplasts and subchloroplast particles shed doubt on the interpretation of these measurements.

(c) *Distribution of permeant acids.* An acid which permeates a membrane only in its neutral form will reach equilibrium when $AH_{in} = AH_{out}$. Since

$$K_a = \frac{H^+_{in} A^-_{in}}{AH_{in}} = \frac{H^+_{out} A^-_{out}}{AH_{out}}$$

it follows that $H^+_{in}/H^+_{in} = A^-_{out}/A^-_{in}$. Thus in equilibrium, the total species concentration ($A^T = A^- + AH$) on both sides of the membrane is given by

$$\frac{A^T_{in}}{A^T_{out}} = \frac{1/K_a + 1/H^+_{in}}{1/K_a + 1/H^+_{out}}$$

for acids with $K_a \gg H^+_{in}$ and H^+_{out} $A^T_{in}/A^T_{out} = H^+_{out}/H^+_{in}$. Wadell and Butler [21] have introduced the use of 5,5-Dimethyl-2,4-oxazolidedione (DMO) as a permeating acid in ΔpH studies of muscle cells. Its advantages over other permeating acids are: fast equilibration, no binding to membranes and proteins and no metabolic effects. Addanki [22, 23] have introduced the use of ^{14}C-DMO for measurement of ΔpH in

TABLE I. ΔpH determination in various energy conserving systems: ($\Delta pH = PH_{in} - PH_{out}$)

System	Metabolic state	pH	Method	Reference	Comments
Mitochondria					
Rat liver	non energized	+ 0.6 − 0.4	Titrametric II	47	$pH_0 = 6$ $pH_0 = 8$
	non energized	+ 0.7	DMO	48	$pH_0 = 7.2$
	non energized	+ 0.5	DMO	24	$pH_0 = 6.5$
	state 4 state 3	+ 0.8 + 0.7	Titrametric II	13	
	state 4 state 3	+ 0.4 + 0.3	DMO	49	corrected with ^{14}C-sucrose
Beef heart	state 4 state 3	+ 0.35 + 0.2	DMO	23	corrected with ^{14}C-dextran
	+ Ca^{+2} + Pi	+ 1.8 + 0.05	DMO	23, 22	
Rat liver	+ Ca^{+2}	∿ + 1.0	BTB	15, 16	
	+ Ca^{+2}	∿ + 1.0	Titrametric I	12	
	K^+ + valinomycin	+ 0.3 + 0.8	DMO	49	$pH_0 = 7.4$ { 0.5 mM K^+ 150 mM K^+
	K^+ + valinomycin	+ 1.5 + 1.0	DMO	50	$pH_0 = 6.2$ $pH_0 = 7.5$ } 1.0 mM K^+
	K + valinomycin	+ 3.0	Titrametric II	13	10 mM K^+

TABLE I *(continued)*

System	Metabolic state	pH	Method	Reference	Comments
Submitochondrial particles	energized	−2.2	9-amino acridine	32	EDTA-oligomycin particles
	energized + permanent anion	−3.6			
Chloroplasts	dark	+1.0	DMO	51	
	dark	−0.5	^{14}C-methylamine	10, 26	
	light	−2.6	^{14}C-methylamine	10, 26	
	light	−3.5	9AA quenching, NH_4^+ uptake	28, 29	
	light	−0.9	neutral red	19, 20	
	light	−3.0	rate of electron transport	46	
	ATP	−3.0	9AA quenching	52	
Whole chloroplast	light	−2.6	^{14}C–MA, ^{14}C–DMO	44	
Subchloroplast particles	light	−2.6	9AA quenching, NH_4^+ uptake	28	
Chromatophores	light	−1.8	^{14}C-methylamine	33	
	light	−2.0	9AA quenching	30	
Bacteria (strep. faecalis)	energized	+1.0	DMO	42	

mitochondria. The mitochondria are precipitated by fast centrifugation and the ^{14}C-DMO distribution ratio is determined and corrected for extramitochondrial water (^{14}C-Dextran Space). Other workers, who used the method, have corrected for the extramatrix water (sucrose nonpermeable space) assuming that the pH gradient is established across the inner membrane (see Table I). The value of ΔpH determined in mitochondria by this method vary from 0 to 1.8 (the inside being more alkaline) depending on the metabolic state, presence of ions, ionophores etc. The highest values are obtained in the presence of high Ca^{2+} concentration and no permeant acids and the lowest value obtained in uncoupled systems, in the presence of permeant acid (such as phosphate or acetate) or proton translocating ionophores (nigericin). The ΔpH determination by this technique is limited to a stable steady state, since equilibration of the DMO is required. There is also the possibility that the permeant acid itself, at least in high concentrations, might affect the ΔpH (similar to other permeating acids). The method is also limited to vesicles which are more alkaline internally than the medium, since in acidic vesicles the acid is extruded and the determination of its internal concentration becomes impossible. Other permeating acids such as acetate equilibrate in similar ways to DMO and can be used instead [24].

(d) *Distribution of amines.* In principle this method is very similar to the acid distribution method. Since amines permeate chloroplasts and other biological membranes in their neutral form [25] they are distributed in equilibrium according to the relation $R{-}NH_{3\,in}^{+}/R{-}NH_{3\,out}^{+} = H_{in}^{+}/H_{out}^{+}$. Note, however, that in contrast to acids, amines are concentrated in acidic vesicles. This fact was utilized to develop a method for ΔpH determination in chloroplast which is based on the distribution of ^{14}C-methylamine [26, 10]. Other small organic amines such as ethylamine are taken up by illuminated chloroplasts and probably can serve for ΔpH estimation as well [27]. Another method is the estimation of ΔpH from the extent of NH_4^+ uptake, as measured by a specific electrode [28]. One of the most useful methods is the estimation of ΔpH from the extent of the fluorescence quenching of fluorescent amines which are quenched when taken in by the vesicles [29]. The most suitable probe in the latter method was found to be 9-aminoacridine and its use has been applied in addition to chloroplasts, to subchloroplast particles [28], chromatophores [30], liposomes [31], and submitochondrial particles [32]. The labelled amine technique was also applied to various other systems such as chromatophores [33], Lysosomes [34, 35] and bacteria [36]. The quenching of Atebrin fluorescence (quinacrine) was also suggested to indicate the extent of ΔpH [37, 38]. Although this is probably correct it appears that atebrin does not behave as an ideal amine in this respect [29, 31]. Moreover, in submitochondrial particles an enhancement effect which is different in origin from the quenching renders quantitative interpretation very

difficult [32, 39, 40]. The use of amines of high pK at low pH values was criticized on the ground that the very low concentration of free amine might limit the equilibration of these species [41]. However, various amines, with different pK, give similar results which indicates that this is not a limiting factor [28]. Moreover, the kinetic of amine uptake even at low pH is very fast and seems to be limited by the rate of proton uptake and not by the penetration of the amine (H. Rottenberg, unpublished).

These studies have shown that in chloroplasts light dependent ΔpH can reach values of up to 4.0 and can be reduced by amines, uncouplers and nigericin [10, 28, 29]. ΔpH values in chromatophores, submitochondrial particles and subchloroplast particles are somewhat lower but can be increased by permeant anions [28, 30, 32, 33]. In bacteria during metabolism the internal pH is more basic [42], but it can become more acidic by setting up a proper diffusion potential [36].

An early study of amine uptake by non metabolizing bacteria which have been transferred from acidic medium to neutral pH, indicate that their membranes are quite impermeable to proton and can maintain a rather large pH gradient [43]. An interesting study in which both methylamine and DMO were used to estimate internal pH of different compartments of whole chloroplasts [44] indicates that during illumination while the thylakoid becomes more acidic the stroma becomes more basic. Rumberg and Siggel [45] have suggested to use the effect of internal pH on the rate of electron transport in chloroplasts as an estimation of the internal pH. However, it was shown that in addition to the strong effect of internal pH, external pH as well as the magnitude of ΔpH determines the rate of electron transport and therefore only rough estimates under controlled conditions can be obtained by this method [46].

II. *The Determination of Membrane Potential*

(a) *Membrane probes.* A molecule located in the membrane or at its surface, and with spectroscopic properties that are influenced by changes in the electrical field, could serve as an indicator for membrane potential. However, a quantitative estimate is possible only if these effects can be properly calibrated. Witt [53] and his group have studied absorption shifts which are observed in chloroplasts upon illumination and have concluded that a certain spectrum, normally followed at 515 nM (the 515 shift), represents an electrochromic effect which is induced by the electrical field on the carotenes and chlorophyls in the membrane. A quantitative estimate was made, based on a simplified model for the primary light reaction [54]. The "515 shift" was also observed in bacterial chromatophores where it was possible to estimate the potential by setting up diffusion potentials of different values [55]. However, it is

questionable whether membrane potential, generated by events within the membrane such as light induced charge separation or electron transport, can be estimated on the basis of this calibration since the probe is responding to perturbations on a local field within the membrane. The relationships between membrane potential, which is the potential difference between the two solutions on the two membrane sides, and the potential profile within the phospholipid-pigment-protein membrane could be very complex, particularly when the potential is generated by events within the membrane.

The fluorescence changes of anilinonaphthalene sulfonate (ANS) in suspensions of mitochondria, submitochondrial particles and bacterial chromatophores were suggested to reflect changes in membrane potential in these vesicles [56-58]. The mechanism of this effect was suggested to be either binding on the membrane surface [56] or equilibrium distribution of the membrane-permeant anion [57]. Since all of the ANS fluorescence, which is very sensitive to the environment, is due to the bound dye, a quantitative estimation of potentials is not possible without independent calibration. Recently it was shown that cyanines fluorescence changes in red blood cells, liposomes and axons can be correlated with independently measured changes in membrane potential [59-61]. The mechanism of these effects is not entirely clear although it appears that the fluorescence response of these dyes is a reflection of the partition of the molecules between the medium and the cells [61]. No doubt, these dyes might prove valuable in energy conserving systems. However, a proper calibration with an independent measurement which would be valid for membrane potential indication, regardless of the origin of this potential, could prove difficult if indeed the mechanism of the probe response is dependent on interaction between the membrane and the probe.

(b) *Distribution of permeating ions.* An ion which is in an electrochemical equilibrium across the membrane is distributed according to the Nernst equation. Since in equilibrium

$$\Delta\tilde{\mu}_i = RT \ln (C_{in}/C_{out}) + ZF\Delta\Psi = 0$$

then

$$\Delta\Psi = -\frac{RT}{ZT} \ln (C_{in}/C_{out}).$$

In applying this classical method for determination of membrane potential in energy conserving membranes, it is necessary to insure that the selected ion freely permeates the membrane in its charged form and that a true equilibrium distribution is established. A direct coupling of its transport to other ions which are not in equilibrium or its direct pumping would of course invalidate the calculation. It is also important to insure that no significant binding to proteins or accumulation within

the membrane occur and if it does, it must be properly corrected for. Since it is only possible to determine accurately the internal concentrations of those ions that are more concentrated in the vesicles, cations must be used in cases when the potential is negative inside and anions when the potential is positive inside.

We have introduced the use of potassium distribution in the presence of valinomycin for potential calculations in mitochondria [62-64]. The method consists of the sedimentation of the mitochondrial pellet, determination of the matrix water content and the potassium distribution ratio. It was justified by the demonstration that in the presence of ionophores like valinomycin or gramicidin, potassium in mitochondria approaches true equilibrium [24]. However, when the mitochondrial potassium content is low it is necessary to correct for binding [24]. The full experimental description is given by Padan and Rottenberg [49]. Mitchell and Moyle [13] have introduced a similar method in which the potassium ratio is estimated from the extent of potassium uptake as measured by potassium electrode. The values obtained by Mitchell and Moyle are somewhat higher than the values obtained by us (see Table II). This discrepancy is due firstly to the fact that a very low external potassium concentration is used by Mitchell and Moyle, while there is no correction for binding, which becomes very significant in these concentrations. Secondly, matrix water volume, was not determined and the value used is much lower than those actually found for similar experimental conditions [7]. The cation distribution technique was extended [65] to the use of ^{86}Rb (in the presence of valinomycin) which is more convenient for handling than ^{42}K. In chloroplasts both ^{86}Rb, ^{36}Cl, and ^{131}I were used, however, the value of membrane potential obtained was very low [6]. ^{131}I distribution was used in subchloroplast particles where higher potentials (positive inside) were observed (Rottenberg, unpublished). In chromatophores, ^{14}C–SCN^- was used to estimate membrane potential and values of up to 90 mV were observed [33].

Skulachev [6] has introduced the use of organic lipid soluble cations that permeates phospholipid membranes. It was demonstrated that mitochondria accumulates cations, whereas submitochondrial particles and chromatophores accumulate anions which indicated the reverse polarity of membrane potential in these particles. A quantitative estimate of membrane potential in bacteria and bacterial vesicles, which is based on the distribution of these cations was made [66-68].

The main findings of the various determinations of membrane potential are summarized in Table II. In mitochondria values of up to 140 mV are obtained by ^{42}K (or ^{86}Rb) distribution and up to 200 mV by Mitchell and Moyle [13]. Potassium (in the presence of valinomycin) as well as uncouplers reduced $\Delta\Psi$. In chloroplast the potential is insignificant according to the ion distribution method, but estimated as

TABLE II. Determinations of $\Delta\Psi$ in energy conserving systems

System	Metabolic state	$\Delta\Psi$ mV	Method	References	Comments
Mitochondria (rat liver)	State 4	−168	Potassium electrode	13	
	State 3	−162			
	State 4	−136	^{42}K distribution	49	
	State 3	−128			
	+ Ca^{+2}	−144	Potassium electrode	13	
	+ Ca^{+2}	−100	^{86}Rb distribution	65	
	10 mM K	−83	Potassium electrode	13	
	10 mM K	−70	K distribution	64, 49	
	150 mM K	−35	^{42}K distribution	49	
	Gramcidin (+ K, Na)	−18	K, Na distribution	24	
	K^+ depleted + EGTA	−199	Potassium electrode	13	
	FCCP	−67	Potassium electrode	13	
Chloroplasts	Dark	−10	^{86}Rb, ^{36}Cl, ^{131}I	10	
	Light	0	^{86}Rb, ^{36}Cl, ^{131}I	10	
	Light	+100	515 nM shift	53	
Chromatophores	Dark	+12	^{14}C-SCN^- distribution	33	In the
	Light	+88	^{14}C-SCN distribution	33	presence
	Light	+144	515 nM shift	30	of KCl
	+ Val + KNO_3	+6	515 nM shift	30	
	+ FCCP	+6	^{14}C-SCN^- distribution	33	
Bacteria					
Strep. faecalis	Energized	−170	DDA^+ uptake	68	
E. coli	Energized	−140	DDA^+ uptake	66	
E. coli vesicles	Energized	−100	^3H-DDA^+distribution	67	

100 mV from the "515 shift". It is higher, however, in subchloroplast particles and even higher in chromatophores as calculated by both methods. Significant membrane potential with values and sign which is similar to mitochondria were also observed in bacteria and bacterial vesicles.

III. *Protons Electrochemical Gradients and Energy Conversion*

A comprehensive discussion of the implications of these and related measurements will be given elsewhere (H. Rottenberg, "Chemiosmotic activities in biological membranes" in International Review of Cytology, to be published). However, several brief comments are in line. (a) In mitochondria, submitochondrial particles, chloroplasts, subchloroplast particles, bacterial chromatophores, various bacteria and their vesicles energization by substrate or light induced the formation of large $\Delta\tilde{\mu}_H$ in

a direction which is determined by the direction of the energy dependent proton transport and thus confirming the suggestion that in these systems there are electrogenic proton pumps.

(b) If one assumes [2] that the stoichiometry of the ATPase proton pump is $2H^+$/ATP the value of $\Delta\tilde{\mu}_H$ obtained by various methods is not sufficient to maintain the very high phosphate potential which was measured in mitochondria [69] and chloroplasts [70] and is probably reached in other systems as well. This is demonstrated in Table III which summarizes the measured $\Delta\tilde{\mu}_H$ and phosphate potential in various systems. Moreover, ATP synthesis can take place even when $\Delta\tilde{\mu}_H$ is considerably reduced [64]. In the presence of high phosphate concentration, 10 mM potassium and valinomycin phosphorylation occurs with normal efficiency (P/O is 1.8 with succinate). Under these conditions $\Delta\tilde{\mu}_H$ is less than 75 mV, phosphate potential is at least 250 mV (in the presence of hexokinase), and can reach much higher values [64].

(c) In mitochondria the transition from state 4 to state 3 involves only a very small change in $\Delta\tilde{\mu}_H$ [49, 13] (Table I and II). This change cannot account for the large stimulation of respiration since a much larger reduction of $\Delta\tilde{\mu}_H$ (by uncouplers or ionophores) is necessary in order to induce equivalent stimulation of respiration [49]. Similarly, this small change is not compatible with the chemiosmotic mechanism if one considers the high efficiency of oxidative phosphorylation (H.

TABLE III. Maximal $\Delta\tilde{\mu}_H$ and phosphorylation free energy

System	ΔpH	$\Delta\Psi$	$\Delta\tilde{\mu}_H$(mV)	ΔG_{ATP}(mV)
Mitochondria				
(State 4)	+ 0.8	− 168	− 216[13]	
(State 4)	+ 0.4	− 136	− 160[49]	640[70]‡
Chloroplasts	− 3.0	+ 100	+ 280[53]	
	− 3.6	0	+ 210[25]	640[71]‡
Submitochondrial particles	− 3.6	−(∿0)*	+ 210[32]	
Subchloroplast particles	− 2.6[28]	+ 60†	+ 210[32]	
Chromatophores	− 2.5	+ 188	+ 341[30]	
	− 1.8	+ 88	+ 206[33]	
Bacteria (strep. faecalis)	+ 1.0[42]	− 170[67]	− 230	

* Estimated.

† H. Rottenberg, unpublished.

‡ This value is obtained by dividing ΔG by F (Faraday constant). To compare with $\Delta\tilde{\mu}_H$, it should be divided by the assumed stoichiometry. Thus, if the stoichiometry is $2H^+$/ATP one obtains 320 mv, for $3H^+$/ATP the value is 213 mV, and for $4H^+$/ATP 160 mV.

Rottenberg, in preparation). These facts might indicate that in the mitochondria there exists direct coupling between electron transport and phosphorylation which is not mediated by proton transport across the membrane boundaries. These and other data were rationalized in a scheme of parallel coupling both direct and chemiosmotic [71] (H. Rottenberg, in preparation). In chloroplasts phosphorylation reduced ΔpH considerably [72] in parallel to the effect on the rate of electron transport [46] and there is a high threshold value of ΔpH below which no phosphorylation is observed [73]. Thus, so far, the studies in chloroplasts have not indicated the existence of coupling which is not mediated by proton transport.

(d) Uncouplers and ionophores affect $\Delta\tilde{\mu}_H$ in a way which is compatible with the chemiosmotic explanation of their action. Moreover, the comparison of their effect on the rate of electron transport and $\Delta\tilde{\mu}_H$ indicates that their uncoupling of oxidative phosphorylation is the result of their effect on $\Delta\tilde{\mu}_H$ [49].

(e) All of the observed energy dependent ion movements in energy conserving membranes can be explained as either linked to the formation of ΔpH or $\Delta\Psi$ [24, 4].

In summary, the actual determinations of $\Delta\tilde{\mu}_H$ allows a quantitative approach to the testing of the chemiosmotic theory.

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