Biochimica et Biophysica Acta, 459 (1976) 96–109

BBA 47214

PROTON ELECTROCHEMICAL POTENTIAL IN STEADY STATE RAT LIVER MITOCHONDRIA

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(Received July 12th, 1976)

SUMMARY

$\Delta\tilde{\mu}_H$ has been determined in steady state mitochondria, by measuring the magnitude of ΔpH on the distribution of acetate and of $\Delta\psi$ on the distribution of K^+, tetraphenylphosphonium, Ca^{2+}, Sr^{2+} and Mn^{2+}.

(1) The matrix concentration of divalent cations has been calculated from the total cation uptake, from the increase of matrix volume and from the ESR sextet signal of $Mn(H_2O)_6{}^{2+}$. The $[cat^{2+}]_i$ based on osmotic data is about five times higher than that based on ESR measurements. The $[cat^{2+}]_i$ based on total uptake is much higher than that based on osmotic data at low cation/protein ratios.

(2) In the presence of 10 mM acetate the maximal $\Delta\psi$ on Ca^{2+} is about 130 mV and on Sr^{2+} is 95 mV. $\Delta\psi$ on Mn^{2+} is 91 or 109 mV, according to whether $[cat^{2+}]_i$ is calculated from ESR or osmotic data. Under the same conditions, ΔpH is about 60 mV. Hence, $\Delta\tilde{\mu}_H$ on divalent cations is between 151 and 190 mV.

(3) $\Delta\psi$ on K^+, in valinomycin treated mitochondria with 10 mM acetate or 2 mM Pi, drops from 200 mV, at low $[K^+]_o$ to almost zero parallel to the increase of $[K^+]_o$. ΔpH is 30 mV at low $[K^+]$ and about 42 mV at 600 μM K^+. Hence $\Delta\tilde{\mu}_H$ drops from 227 mV to lower values with the increase of $[K^+]_o$.

(4) Maximal $\Delta\psi$ on triphenylmethylphosphonium is 143 mV.

(5) When $\Delta\tilde{\mu}_H$ is measured simultaneously on divalent cations and on K^+, the values on K^+ tend to approach those on Ca^{2+}, while those on Sr^{2+} are about 50 mV lower.

(6) It is concluded that the steady state mitochondrial energy potential is equivalent to a $\Delta\tilde{\mu}_H$ between 150 and approx. 190 mV.

INTRODUCTION

The determination of $\Delta\tilde{\mu}_H$ (the proton electrochemical potential) in intact mitochondria involves the measurement of the distribution of two permeant species, a weak acid and a strong base [1, 2]. The weak acid is thought of as reflecting ΔpH and the strong base, $\Delta\psi$ (where ΔpH indicates proton concentration gradient and $\Delta\psi$ the membrane potential). Application of the Nernst equation to the distribution of the strong bases provides $\Delta\psi$. $\Delta\tilde{\mu}_H$ is then given by:

$$\Delta\tilde{\mu}_H = -59\ \Delta pH + \Delta\psi$$

ΔpH has been determined through titration [1], and the distribution of 5,5-dimethyl-2,4 oxazolidinedione [2, 3] and of acetic acid [4]. The values of ΔpH in steady state mitochondria vary between 24 and 83 mV [1–6]. $\Delta\psi$ has been determined on the distribution of K^+ in valinomycin-treated mitochondria. The values of $\Delta\psi$ in steady state mitochondria vary between 136 and 198 mV [1–5]. Recently, Rottenberg [6] and Rottenberg and Scarpa [7] recorded a $\Delta\psi$ of 36 mV for Na^+ and K^+ in gramicidin-treated mitochondria and a $\Delta\psi$ between 33 and 100 mV for K^+ and Ca^{2+} in valinomycin-treated mitochondria. The discrepancy between higher and lower values of ΔpH and $\Delta\psi$ has not been explained.

The present work aims to extend previous studies on the determination of $\Delta\psi$ and ΔpH by comparing the values on the univalent and the divalent cations and by investigating the role of several factors, i.e. pH, osmolarity, cation/protein ratios etc. Two major discrepancies are observed: (a) in the case of divalent cation, $\Delta\psi$ decreases in the order $Ca^{2+} > Mn^{2+} > Sr^{2+}$; (b) in the case of K^+, $\Delta\psi$ is markedly dependent on the amount of K^+ added, and, at low $[K^+]_o$, is higher on the univalent than on divalent cations [8]. The latter discrepancy is reduced when $\Delta\psi$ is measured simultaneously on the univalent and divalent cations and increased with the increase of univalent and decrease of divalent cation permeability. Steady state $\Delta\tilde{\mu}_H$ varies between 150 and approx. 190 mV depending on the ion taken into consideration.

EXPERIMENTAL

Rat liver mitochondria were prepared as described previously [9] and washed free of EDTA. Incubation of mitochondria was carried out for 3 min in a standard medium of the following composition: 0.2 M sucrose, 10 mM acetate/Tris, 5 mM Hepes pH 7.0, 2 mM succinate/Tris, 1 mM β-hydroxybutyrate. Changes to this medium were as described in the legends to the figures. The medium was bubbled with oxygen and the reaction started with the addition of mitochondria. The reaction was terminated by centrifugation in the rotor S-12 of the Sorvall RC 2B centrifuge.

The matrix water was determined with a double labelling technique employing ^3H_2O and [C14] sucrose, where ^3H_2O measures the total pellet water and sucrose the extra matrix water [3, 7]. The mitochondrial pellet was dissolved in a Triton-containing liquid scintillation medium and counted in the TriCarb 2455 liquid scintillation spectrometer. In control experiments the extramatrix water was also measured with $^{36}Cl^-$ and [^{14}C]methylamine [4]. No difference was observed among the three measurements.

ΔpH was determined, essentially as indicated by Nicholls [4], by measuring the distribution of [^{3}H]acetate between supernatant and pellet. The number of counts of [^{3}H]acetate in the pellet may be overestimated for two reasons. One, the acetate present in the extramatrix water. Two, the acetate bound to the membrane. The first correction was made by measuring the extramatrix water with sucrose. The second correction was made by determining the number of counts in an uncoupler-supplemented sample. The concentration of acetate in the matrix was then calculated by dividing the corrected counts of acetate for the volume of matrix water as measured with ^3H_2O.

Ca^{2+} and Sr^{2+} were determined with $^{45}Ca^{2+}$ and $^{90}Sr^{2+}$. $^{45}Ca^{2+}$ was measured in the ^{14}C channel. $^{90}Sr^{2+}$ emits particles scattering in a wide energy range. The measurements were carried out on the particles with low energy by using the ^{14}C channel. In kinetic experiments, Ca^{2+} and Mn^{2+} were measured with a dual wavelength spectrophotometer in the presence of 100 μM murexide (wavelengths 540–500 nm). It is to be noted that the uptake of divalent cations in the presence of 10 mM acetate resulted in a large decrease of absorbance, due to swelling, which was not completely compensated by the dual wavelength spectrophotometer. In order to account for the swelling changes the absorbance change of the murexide supplemented sample was corrected for the absorbance change of the murexide unsupplemented sample.

$[K^+]_i/[K^+]_o$ was determined essentially as described by Nicholls [4]. The incubation medium was identical to that used for the divalent cations, except that valinomycin was always present. The procedure is based on the assumption that, in valinomycin-treated mitochondria, although a trace amount of $^{86}Rb^+$ is added, the distribution of $^{86}Rb^+$ is identical to that of K^+. This permits to calculate the $[K^+]_i/[K^+]_o$ ratio by determining in fact the $[Rb^+]_i/[Rb^+]_o$ ratio. K_i^+ has been calculated, when no other permeant cation is present, on the assumption that K^+ contributed to half the osmolarity of the matrix space [2]. A similar assumption has been used by Nicholls [4]. The values so obtained are very close to those calculated with other approaches [1]. In the absence of added K^+, $[K^+]_o$ was calculated, also on the basis of the $^{86}Rb^+$ distribution, by assuming an endogenous K^+ content of 80 nmol/mg protein [5]. In kinetic experiments K^+ was determined with the K^+ electrode [2].

Triphenylmethylphosphonium was determined by adding to the incubation medium, containing variable amounts of triphenylmethylphosphonium, a trace amount of [^{3}H]triphenylmethylphosphonium and then determining the distribution of the radioisotope between pellet and supernatant. [^{3}H]triphenylmethylphosphonium was kindly provided to us by Dr. R. Kabach.

The determination of Mn^{2+} was carried out by following the ESR spectra at X band (9100 M) with a Varian V-4502 spectrometer [10]. The microwave frequency was approximately 9.5 GHz. The modulation amplitude (M) was 16 G, the recorder time constant 0.3 s, and the scanning rate 250 G/min. All measurements were carried out with a quartz capillary permanently positioned into the resonance cavity. The capillary was connected with a continuous flow apparatus in order to avoid anaerobiosis [10]. Under the experimental conditions used, the ESR spectrum of Mn^{2+} bound to the membrane or chelated by EDTA was negligible. Then, the concentration of free Mn^{2+} in water was calculated from the signal height [11]. The height of the derivative curve from the maximum to the minimum was a linear function of the concentration of manganous ion in the range investigated 5–500 μM. To obtain the free Mn^{2+} in the matrix, excess EDTA was added; EDTA caused an instant quenching of the signal of Mn^{2+} free in the outer medium [12–14], while initiating a slow decay of the signal of Mn^{2+} free in the matrix. The decay is due to an EDTA-induced Mn^{2+} efflux from the matrix with quenching of the signal when the ion reaches the outer phase. Extrapolation of the Mn^{2+} signal at zero time after addition of EDTA indicates the amount of free Mn^{2+} in the matrix. The concentration of free Mn^{2+} in the matrix, is obtained by dividing the amount of internal Mn^{2+} for the matrix volume [10].

RESULTS

$\Delta\psi$ and $\Delta\tilde{\mu}_H$ with divalent cations

Fig. 1 shows the increase of matrix volume during uptake of Ca^{2+} and Sr^{2+} in the presence of 10 mM acetate. The cation uptake was accompanied by a marked increase of the matrix volume. The increase was slight up to 30 nmol per mg protein. Since the increase of matrix volume is due to osmotic equilibration, it may be expected that it should be dependent on the osmolarity of the medium. This is also shown in Fig. 1. Decrease of medium osmolarity induced a proportional increase of matrix volume at the various cation/protein ratios. A plot similar to that shown for Ca^{2+} and Sr^{2+} has been reported for Mn^{2+} [10].

Fig. 2 shows the calculation of $[cat^{2+}]_i$ in the case of Ca^{2+} and Sr^{2+} as based on two different assumptions. First, that the whole cation taken up is free in the matrix. Such an assumption has been used in the presence of either 1 mM [7] or 10 mM acetate [5]. In this calculation, the determination of the matrix volume is unable to correct for the extent of cation precipitated or bound. Second, that the increase of matrix water is due to the presence of osmotically-active calcium or strontium diacetate. In the latter case, within the assumption that the whole volume change is due to osmotic equilibration between outer and inner space during penetration of calcium diacetate, the uptake of 1 μl H_2O corresponds, in a 270 mosM medium, to the penetration of 90 nmol calcium diacetate. The concentration of free Ca^{2+} is then calculated by dividing the osmotically active Ca^{2+} by the total volume of the matrix. The above calculation also implies that the movement of H^+ ions is negligible. This is actually

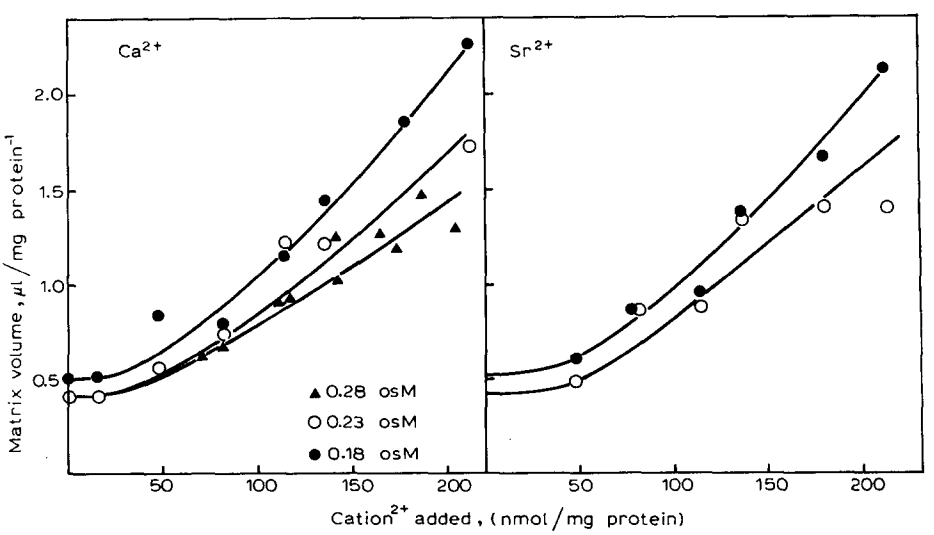

Fig. 1. Increase of matrix volume during uptake of Ca^{2+} and Sr^{2+} $(acetate)_2$. The medium contained in 2 ml, 5 mM Hepes, pH 7.0, 2 mM succinate/Tris, 1 mM β-hydroxybutyrate, 10 mM acetate/Tris, 6 mg mitochondrial protein and variable amounts of $CaCl_2$ or $SrCl_2$. The concentration of sucrose was 0.15, 0.2 or 0.25 M. Duration of incubation was 120 s in the presence of $CaCl_2$ and 240 s in the presence of $SrCl_2$. The matrix volume was determined with the double labelling technique as described in the Methods.

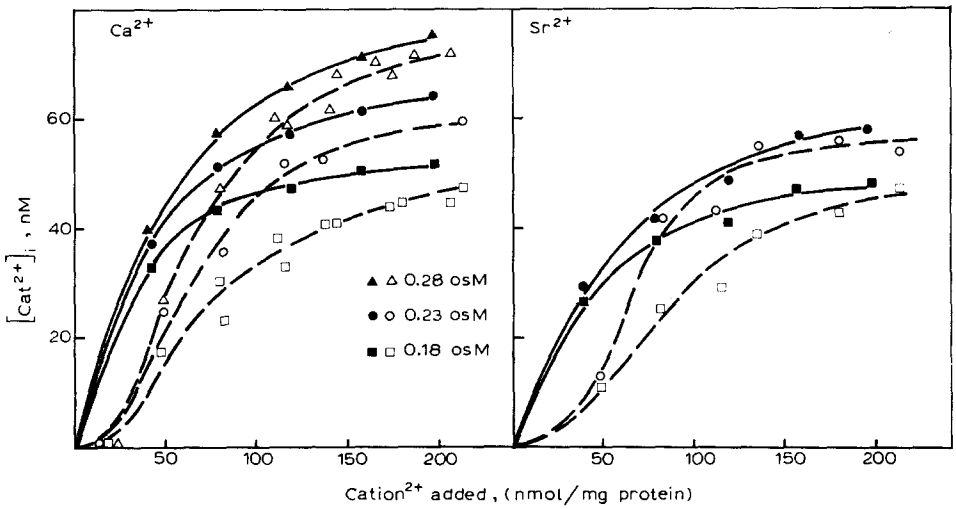

Fig. 2. Calculation of $[cat^{2+}]_i$. Experimental conditions as in Fig. 1. Continuous lines, $[cat^{2+}]_i$ calculated by assuming an activity coefficient of 1 for the total cation taken up. Dashed lines, $[cat^{2+}]_i$ calculated from the data of increase of matrix volume reported in Fig. 1. For further details, see the text.

the case with 10 mM acetate. It is seen that the use of the two assumptions leads to a large discrepancy in the calculation of $[cat^{2+}]_i$ at low cation/protein ratios. This is presumably due to the fact that the presence of acetate does not solubilize the osmotically inactive cations but rather brings into the matrix further cations in addition to those inactive [10, 12–14]. At high cation/protein ratios, on the other hand, where most of the cations are free in the matrix in the presence of acetate, the error due to the presence of an osmotically inactive fraction becomes smaller. Fig. 2 shows a close

TABLE I

EFFECT OF pH AND Mg^{2+} ON $\Delta\psi$ WITH Ca^{2+} AND Sr^{2+}

Experimental conditions as in Figs. 1 and 2. 0.2 M sucrose, 4.2 mg protein. $[cat^{+}]_i$ was calculated on the increase of matrix volume, see Fig. 2. Mg^{2+}, when added, was 10 mM. The two values of $\Delta\psi$ refer to two cation/protein ratios, i.e. 119 and 179 nmol cation/mg protein, respectively. The values of ΔpH recorded for Ca^{2+} were 61, 62, 62, 42 mV in the absence and 60, 62, 62, 46 mV in the presence of Mg^{2+} at pH 6.2, 6.7, 7.2 and 8.0, respectively. The values of ΔpH recorded for Sr^{2+} were 58, 60, 58, 30 mV in the absence, and 54, 57, 61, 30 mV in the presence of Mg^{2+} at pH 6.2, 6.7, 7.2 and 8.0 respectively.

pH of incubation medium	$\Delta\psi$ (mV)			
	Ca^{2+}		Sr^{2+}	
	without Mg^{2+}	with Mg^{2+}	without Mg^{2+}	with Mg^{2+}
6.2	114– 98	114–104	85–81	76–75
6.7	136–135	121–115	88–88	87–81
7.2	136–135	120–119	91–89	87–85
8.0	120– 76	118– 89	41–77	67–58

similarity for the calculation of $[cat^{2+}]_i$ between Ca^{2+} and Sr^{2+}, and also a similar correlation in respect to the osmolarity of the medium. Perhaps the extent of sigmoidicity was slightly enhanced in the case of Sr^{2+}.

Table I shows the effect of pH and Mg^{2+} on $\Delta\psi$ calculated on Ca^{2+} and Sr^{2+} distribution at two cation/protein ratios. $\Delta\psi$ was lower at more acidic and alkaline pH and highest near neutrality .10 mM Mg^{2+} caused a decrease of $\Delta\psi$ of 15 mV with Ca^{2+} but not with Sr^{2+}. Again, $\Delta\psi$ was considerably higher, i.e. up to 50 mV with Ca^{2+} as compared with Sr^{2+}. Since the values of ΔpH were about the same for the two cations, namely around 60 mV, $\Delta\tilde{\mu}_H$ was about 180–190 mV in the case of Ca^{2+} and 150 mV in the case of Sr^{2+}.

The increase of the amount of cation added was accompanied by a decrease of $\Delta\psi$ of about 10–15 mV between 40 and 200 nmol/mg protein. At 40 nmol/mg protein, the values of $\Delta\psi$ were about 10–15 mV higher when $[cat^{2+}]_i$ was calculated on the total cation uptake rather than on the osmotic changes. The difference was much higher below 40 nmol/mg protein and negligible above 150 nmol/mg protein. $\Delta\psi$ was almost independent of the osmolarity of the medium within the range 0.13–0.28 osM.

Fig. 3 shows the determination of $[Mn^{2+}]_i$, $[Mn^{2+}]_o$ and $\Delta\psi$ through the ESR technique. The curve relating $[Mn^{2+}]_i$ to the increase of cation/protein ratio was sigmoid similar to that based on the osmotic data (cf. Fig. 2). However $[Mn^{2+}]_i$ was about 4–5 times lower when based on direct measurement of the ESR sextet of $Mn^{2+} \cdot (H_2O)_6$ as compared to the osmotic values (cf. Fig. 2 and ref. 10). This is presumably due to a shift in membrane bound ionic species. The concentration of $[Mn^{2+}]_o$ in steady state was more similar to that of Ca^{2+} than to that of Sr^{2+}. $\Delta\psi$ decreased from 91 mV at 40 nmol/mg protein to 73 mV at 200 nmol/mg protein. When $[Mn^{2+}]_i$ was calculated on the osmotic data, $\Delta\psi$ was about 16 mV higher and thus approached the values for Ca^{2+} (not shown).

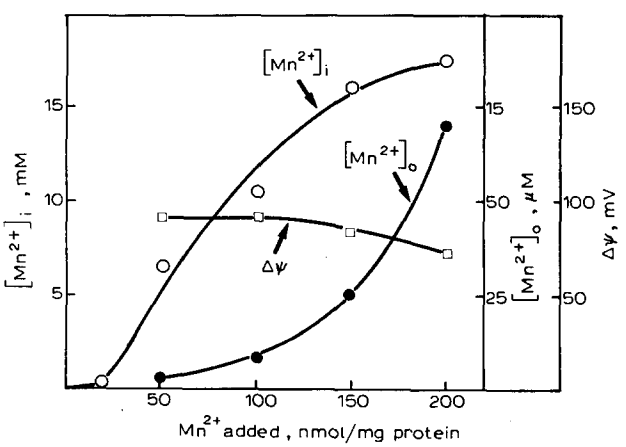

Fig. 3. ESR determination of accumulation ratio for Mn^{2+}. Experimental conditions as in Fig. 1. For details as to the determination of Mn^{2+} see the Methods and ref. 10. Sucrose concentration: 0.2 M.

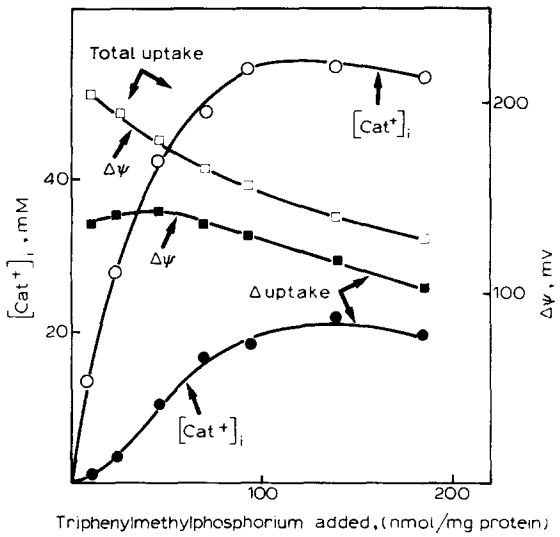

Fig. 4. $\Delta\psi$ for triphenylmethylphosphonium at various cation/protein ratios. Experimental conditions as in Fig. 1. Sucrose concentration, 0.2 M. Protein, 3.2 mg. Calculation of [triphenylmethylphosphonium$^+$]$_i$ was done either by assuming an activity coefficient of 1 for the total cation taken up or by assuming as free only the difference in cation uptake $\pm$10 mM acetate.

$\Delta\psi$ and $\Delta\tilde{\mu}_H$ with univalent cations

Fig. 4 shows the calculation of $[cat^+]_i$ and $\Delta\psi$ in the presence of triphenylmethylphosphonium. $[cat^+]_i$ was calculated either on the assumption that the whole cation taken up in the presence of 10 mM acetate was osmotically active, or that the fraction of osmotically active cation corresponded to the difference in cation uptake $\pm$10 mM acetate. The second assumption is more likely to be correct because of the negligible osmotic changes occurring in the absence of anions. It is seen that a sigmoid curve was obtained with the latter but not with the former assumption. The discrepancy between the two values of $[cat^+]_i$ was particularly large at low cation/protein ratios. $\Delta\psi$ reached values of 204 mV when calculated on the former and 144 mV when calculated on the latter assumption. The uptake of triphenylmethylphosphonium was not accompanied by a restoration of the state 4 respiratory rate. The respiratory rate increased proportionally to the increase of triphenylmethylphosphonium indicating a progressive membrane damage.

The K^+ distribution was remarkably constant and was not greatly affected by changing the pH from 6.2 to 8.0, by replacement of P_i with acetate, addition of Mg^{2+} or of EDTA (Fig. 5). 5 mM Mg^{2+} caused a decrease of $\Delta\psi$ of 5–8 mV only in the absence of added K^+. $\Delta\psi$ decreased, in the absence of acetate, from 188 mV in absence of added K^+ to 130 mV with 600 μM K^+, and, in the presence of 10 mM acetate, from 194 mV in absence of added K^+ to 148 mV in the presence of 600 μM K^+ [1–5].

Measurements in the presence of both univalent and divalent cations

The question arises as to whether the discrepancy between the results on

TABLE II

SIMULTANEOUS DETERMINATION OF $\Delta\psi$ WITH K^+ AND Ca^{2+}. EFFECT OF VALINOMYCIN

Ca^{2+} (nmol/mg protein)	Valinomycin (nmol/mg protein)	Matrix volume $\mu l \times$ mg protein	$[K^+]_i$ (mM)	$\Delta\psi$ (mV)	$[Ca^{2+}]_i$ (mM)	$\Delta\psi$ (mV)
107	0.08	1.2	29.3	136	57.0	135
107	0.2	1.2	34.5	144	53.4	132
107	0.6	1.3	34.5	144	53.4	130
107	0.9	1.3	28.8	137	57.4	127
167	0.08	1.4	22.1	127	62.0	122
167	0.2	1.6	24.2	131	60.5	123
167	0.6	1.5	33.7	146	54.0	124
167	0.9	1.5	31.5	143	55.7	124

Experimental conditions as in Figs. 1 and 5. 0.2 M sucrose, 10 mM acetate/Tris, 5 mM Hepes, pH 7.0, 2 mM succinate/Tris, 1 mM β-hydroxybutyrate, 100 μM KCl and 4.16 mg mitochondrial protein. Time of incubation 120 s. The matrix volume, the $^{86}Rb^+$ and $^{45}Ca^{2+}$ distribution were measured in parallel samples.

univalent and divalent cations occurs also when $\Delta\psi$ is measured in the presence of both cations. This is shown in Table II. The calculation of $[cat^+]_i$ in this experiment deserves a comment. In the case of $[K^+]_i$, the calculation is done by dividing the total K^+ content by the matrix volume. The calculation implies an activity coefficient of 1 for K^+. In the case of $[Ca^{2+}]_i$ the calculation is done by assuming osmotic equilibrium between matrix and outer space. The osmotic activity of calcium acetate cannot exceed the difference between total osmolarity and the contribution to the internal osmolarity of K acetate. This calculation permits to correct the internal Ca^{2+} concentration for the degree of precipitation and binding of divalent cations. The data for $[Ca^{2+}]_i$ so obtained were slightly higher in respect to those obtained in the experiment of Fig. 2 in the absence of valinomycin. Two points emerge from the data of Table II. First, $\Delta\psi$ on divalent cations was very close to that observed in the absence of valinomycin, while $\Delta\psi$ on univalent cations was diminished by 50–60 mV. Second, $[K^+]_i$ and hence $\Delta\psi$ on univalent cations first increased with increase of valinomycin, and then, at the highest amount of valinomycin, decreased. The increase of $\Delta\psi$ is in accord with a correlation of $\Delta\psi$ with the K^+ permeability while the inhibition is presumably due to an uncoupling effect of valinomycin (lack of discrimination between K^+ and H^+). $\Delta\psi$ was also measured simultaneously for K^+ and Sr^{2+}. The results were comparable with those obtained in the case of Ca^{2+}. The $\Delta\psi$ on K^+ decreased from 200 to about 147 mV, while that on Sr^{2+} remained at about 90 mV. The data of Table II are different from those of Rottenberg and Scarpa [6]. This is due to two facts. First, the requirement for osmotic equilibrium in the calculation of $[cat^+]_i$ was not taken into account by Rottenberg and Scarpa [6]. From their Table I, it is seen that in 0.22 osM, $[K^+]_i$ was calculated to be 34, 78, 100–230 and 180 mM at 1, 2, 5 and 10 mM $[K^+]_o$, respectively. Second, in the experiments of Rottenberg and Scarpa, mitochondria were presumably in state 3 (cf ref. 15). The release of respiratory control increases parallel to $[K^+]_o$ and the respiratory rate is already three times the resting rate above 1 mM $[K^+]_o$.

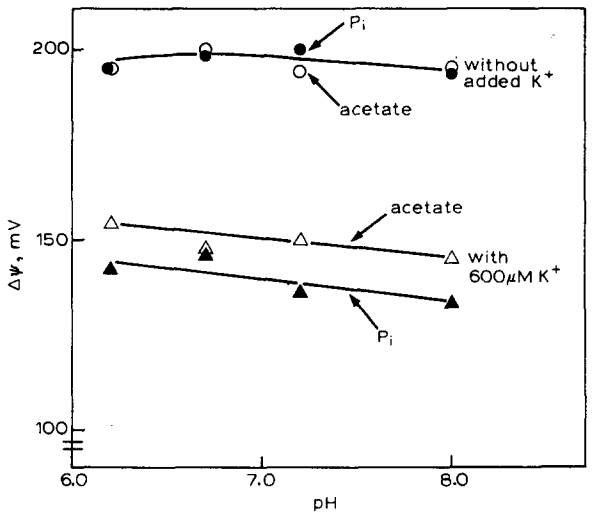

Fig. 5. Effect of $[K^+]_o$ pH and weak acids on $\Delta\psi$. Experimental conditions as in Fig. 4. When indicated, P_i was 2 mM and acetate 10 mM. Protein, 4.3 mg.

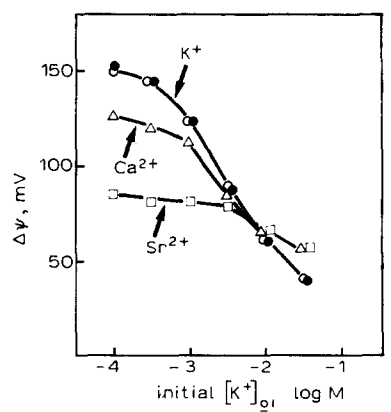

Fig. 6. Effect of K^+ on the simultaneous determination of $\Delta\psi$ with K^+, Ca^{2+} and Sr^{2+}. Experimental conditions as in Table II. 0.2 M sucrose 4.1 mg mitochondrial protein, 0.7 nmol valinomycin/mg protein. 170 nmol Cat^{2+}/mg protein. Determinations as indicated in Table III. $\Delta\psi$ on K^+ in presence of Ca^{2+} (○—○) and Sr^{2+} (●—●), respectively.

Fig. 6 shows the determination of $\Delta\psi$ on K^+, Ca^{2+} or Sr^{2+} at increasing $[K^+]_o$. The values at 0.1 mM $[K^+]_o$ were similar to those of Table. II. Above 0.5 mM $[K^+]_o$, there was a gradual decrease of $\Delta\psi$ on K^+ to values close to those reported by Rottenberg and Scarpa [6]. At low $[K^+]_o$, the $\Delta\psi$ calculated on Ca^{2+} and Sr^{2+} were 127 and 86 mV, respectively. At high $[K^+]_o$, the $\Delta\psi$ on divalent cations decreased almost parallel to the $\Delta\psi$ on K^+. The values of $\Delta\psi$ on K^+ and Ca^{2+} were closest between 1 and 10 mM $[K^+]_o$ [6]. Fig. 7 shows that addition of Mg^{2+} increased the discrepancy between the values of $\Delta\psi$ determined in the presence of K^+ and Ca^{2+} from about 15 to more than 30 mV. Table III summarizes the values of ΔpH, $\Delta\psi$ and $\Delta\tilde{\mu}_H$ measured

TABLE III

COMPARISON BETWEEN VALUES OF $\Delta\tilde{\mu}_H$ WITH VARIOUS CATIONS

The medium contained 0.2 M sucrose, 10 mM acetate/Tris, 2 mM succinate/Tris, 1 mM β-hydroxybutyrate, 0.5 μg valinomycin/ml, and 4 mg mitochondrial protein. When added, Ca^{2+} and Sr^{2+} were 200 μM. Incubation for 180 s. The distribution of K^+, Ca^{2+} and Sr^{2+}, and ΔpH were measured as indicated in the Methods.

Additions	Δ pH (mV)		$\Delta\psi$ (mV)				$\Delta\tilde{\mu}_H$ (mV)			
			on K^+		on Ca^{2+}	on Sr^{2+}	on K^+		on Ca^{2+}	on Sr^{2+}
	without Cat^{2+}	with Cat^{2+}	without Cat^{2+}	with Cat^{2+}			without Cat^{2+}	with Cat^{2+}		
without K^+	30	56	197	155	136	86	227	211	192	142
with 500 μM KCl	42	56	150	142	135	85	192	198	191	141

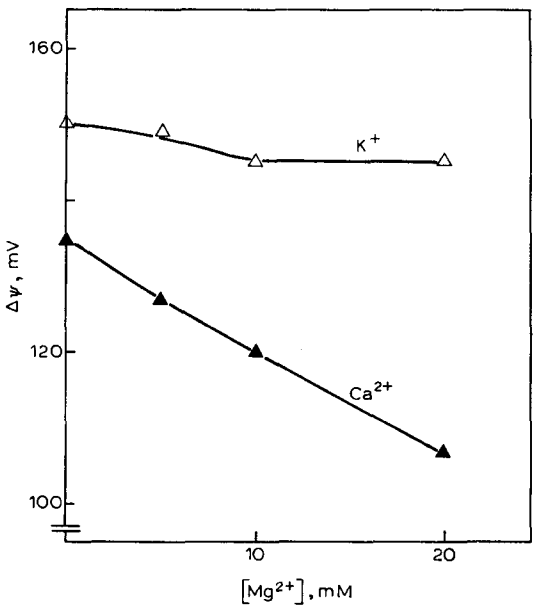

Fig. 7. Effect of Mg^{2+} on the simultaneous determination of $\Delta\psi$ with K^+ and Ca^{2+}. Experimental conditions as in Table II, 0.2 M sucrose, 50 μM KCl, 4.2 mg mitochondrial protein and 0.6 nmol valinomycin/mg protein. 180 s of incubation. Determination of $\Delta\psi$ as indicated in Table II. Amount of Ca^{2+} was 169 nmol/mg protein.

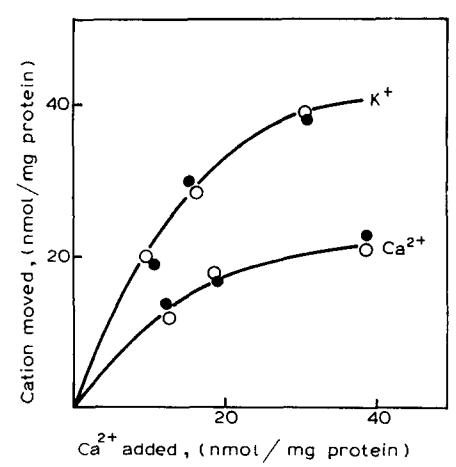

Fig. 8. Charge stoicheometry during K^+-driven Ca^{2+} uptake. The medium contained 0.2 M sucrose, 20 mM LiCl, 5 mM Tris · Cl, pH 7.0, 2 μM rotenone, 150 μM KCl, 3.2 mg protein/ml. The Ca^{2+} uptake was initiated by 0.2 μg valinomycin. The K^+ efflux was followed with the K^+ specific electrode. The Ca^{2+} uptake was determined with 40 μM murexide. Open and closed symbols indicate presence and absence of 2 mM Li acetate, respectively.

in the presence of univalent and divalent cations. It is seen that the presence of divalent cations resulted at low $[K^+]_0$ in a lowering of the values of $\Delta\tilde{\mu}_H$ calculated on K^+. Furthermore, the values of $\Delta\tilde{\mu}_H$ on divalent cations were always lower than those on K^+ and the difference was more marked when Sr^{2+} was taken into account.

Stoicheometry of passive divalent cation transport

It has been shown [16–18] that addition of valinomycin to intact mitochondria causes an efflux of K^+ down the concentration gradient, which may be coupled to Ca^{2+} uptake. Fig. 8 shows that the K^+/Ca^{2+} stoicheometry was 2 at all Ca^{2+} concentrations, whether acetate was present or not. Thus, under concentrations where the K^+/Ca^{2+} exchange occurred in the presence of either an excess of K^+ or an excess of Ca^{2+}, and whether or not acetate was providing protons for acidification of the matrix, hydrogen ions were not involved in the transport of Ca^{2+}. Incubation of mitochondria in $Ca(SCN)_2$ results in a large swelling interpreted as due to electrophoretic influx of Ca^{2+} driven by the SCN^- gradient [19, 20]. Incubation of mitochondria in media containing varying proportions of $Ca(SCN)_2$ and calcium diacetate resulted in a decrease of swelling proportional to the decrease of $Ca(SCN)_2$. This indicates that the restriction to Ca^{2+} penetration is due to lack of SCN^- charges and not to alkalinization of the matrix which could be compensated by acetate. The lack of swelling in calcium diacetate further indicates that the extent of operation of a H^+/Ca^{2+} antiporter in intact mitochondria is negligible.

DISCUSSION

$\Delta\psi$ for divalent cations have been reported previously [5, 7]. Ca^{2+} was assumed as completely free in the matrix, in the presence of either 1 mM acetate [7] or of 10 mM acetate [5]. Both assumptions are unlikely to be correct at low cation/protein ratios because of the sigmoidicity of the plot, increase of matrix volume vs. amount of cation added (Fig. 2). Direct measurements of free Mn^{2+} in the matrix by ESR technique [10] also indicate that the presence of acetate does not eliminate precipitation or binding which precede the accumulation of free calcium diacetate in the matrix [10]. Thus the assumption of an activity coefficient of 1 for divalent cations, whatever the concentration of acetate, causes an error which is greater at low cation concentrations and becomes negligible above 200 nmol cation/mg protein. Furthermore $[cat^{2+}]_i$ calculated on the osmotic data is 5 times higher than $[cat^{2+}]_i$ from direct ESR measurements. Presumably, a part of the Mn^{2+} penetrating in the matrix replaces K^+ bound to the negative charge of the phospholipids. Thus the volume increase reflects the increase of divalent cation concentration but not of divalent cation activity. Rottenberg and Scarpa [6] found in the presence of 160 nmol Ca^{2+}/mg protein a $\Delta\psi$ for Ca^{2+} of 99 mV which decreased to 75 mV in the presence of 10 mM KCl. Massari and Pozzan [5] found a $\Delta\psi$ of 126 mV at 44 nmol/mg protein where the binding error is higher, and a $\Delta\psi$ of 82 mV at 178 nmol Ca^{2+}/mg protein.

In the present work, maximal $\Delta\psi$ is about 130 mV for Ca^{2+} and 109 mV for Mn^{2+} when $[cat^{2+}]_i$ is calculated on the increase of matrix volume. On the other hand, the $\Delta\psi$ for Mn^{2+} is 95 mV when $[Mn^{2+}]_i$ is calculated on the ESR signal. The $\Delta\psi$ for Sr^{2+} is still lower than that for Ca^{2+} and Mn^{2+}, reaching values of 95 mV when $[cat^{2+}]_i$ is calculated on the osmotic data. Addition of Mg^{2+} or extreme pH

cause appreciable changes in the steady state $\Delta\psi$. The latter effect is presumably due to interference with energy coupling.

A large variation in $\Delta\psi$ has been recorded in the case of the univalent cations. Rottenberg and Scarpa [7] reported $\Delta\psi$ for K^+ between 67 and 100 mV. These values are somewhat lower than those reported by Padan and Rottenberg [3] i.e. 136 mV, and considerably lower than those observed by Rossi and Azzone [2], Mitchell and Moyle [1], Nicholls [4], Massari and Pozzan [5] and observed here. A major reason for the difference in K^+ accumulation ratios resides in the $[K^+]_o$ at which the experiments are carried out. The mitochondrial membrane being highly permeable to water, the internal osmotic pressure is identical to the external and the K^+ matrix concentration approaches half the osmolarity of the medium [2]. At high $[K^+]_o$, since $[K^+]_i$ cannot exceed half the osmolarity of the medium, the attainment of a large accumulation ratio requires a large reduction of $[K^+]_o$ and therefore a massive uptake. At 1–2 mM $[K^+]_o$, the K^+ uptake already leads to extensive swelling [15], which is reflected by a high respiratory rate (state 3). At still higher $[K^+]_o$, say above 5 mM, the extent of active K^+ uptake becomes negligible in respect to $[K^+]_o$ and the accumulation ratio becomes an expression of the contribution of the cation to the osmolarity of the supporting medium, i.e. the ratio is equal to 1 when K^+ provides half the osmolarity of the medium.

Maximal $\Delta\psi$ for triphenylmethyl phosphonium is 204 mV when calculated on the assumption of an activity coefficient of 1 for the total cation taken up and 143 mV when calculated on the assumption that only the increase of cation uptake due to the presence of anions corresponds to cations free in the matrix. The $[cat]_i$ calculated on the second assumption is more likely to be correct. Thus the $\Delta\psi$ in the presence of triphenylmethylphosphonium is not far from that observed in the presence of 500 μM K^+.

The discrepancy between the values of $\Delta\psi$ on Ca^{2+}, Mn^{2+} and Sr^{2+} on one side, and between univalent and divalent cations on the other gives rise to two questions. First, the reason(s) for the discrepancy; second, the validity of these measurements for the evaluation of the mitochondrial energy potential. The higher $\Delta\psi$ on K^+, in respect to the divalent cations, is unlikely to be due to the operation of a divalent H^+/Cat^{2+} antiporter leading to cation extrusion. This would cause energy dissipation and is therefore in contrast with the low rate of state 4 respiration. A net charge transfer of 1 during divalent cation transport is also unlikely. The analysis of the passive fluxes of divalent cation indicates that the K^+/Ca^{2+} ratio is always 2, whether Ca^{2+} is in excess or not, and is unmodified by acetate. A kinetic restriction is also unlikely since the accumulation ratio with all the cations used is time independent. Two arguments support the view that the accumulation ratio for K^+ at low $[K^+]_o$ is not a valid indication of the dimension of $\Delta\psi$ in steady state mitochondria. First, as will be discussed in a future paper (Azzone, G. F. and Pozzan, T., unpublished) a part of the K^+ distribution is uncoupler-insensitive; this suggests that K^+ distribution depends partly on osmotic equilibrium [2]. Second, that steady-state mitochondria possess a primary respiratory chain-generated $\Delta\psi$ of 200 mV is in contrast with a physiological consideration [8] concerning the regulation of the concentrations of free Ca^{2+} in the muscle cell. A $\Delta\psi$ of 200 mV corresponds to an accumulation ratio of 10^7. Since the matrix concentration of Ca^{2+} hardly exceeds 10^{-3} M under physiological conditions an accumulation ratio of 10^7 in steady state mitochondria would

cause a decrease of $[Ca^{2+}]_i$ down to 10^{-10} M. However, in the cytosol of the muscle cell, 10^{-10} M Ca^{2+} is too low a concentration to permit muscle contraction. On the other hand, an accumulation ratio of 10^4 for Ca^{2+} is compatible with a $[Ca^{2+}]_i$ in the matrix of 10^{-3} M and a $[Ca^{2+}]_o$ in the cytosol of 10^{-7} M, which is the limit for the physiological operation of the sarcotubular system.

The relative constancy of the values of $\Delta\tilde{\mu}_H$ suggest that this parameter may be a valid indicator of the mitochondrial energy potential, provided that the measurements are made on several permeant species. The mitochondrial energy potential is "equivalent" under steady state conditions to a $\Delta\tilde{\mu}_H$ between 150 and 190 mV [21–23]. In the present paper we have used the terminology $\Delta\tilde{\mu}_H$, which is more widely accepted, instead of osmotic potential used by us in preceding papers [21, 22]. Although the term osmotic potential implies broader assumptions than $\Delta\tilde{\mu}_H$, both osmotic potential and $\Delta\tilde{\mu}_H$ are calculated on the H^+ and cation distributions. The assumption of electrochemical equilibrium required for calculating $\Delta\psi$, by applying the Nernst equation to the cation distribution, is independent of whether $\Delta\psi$ is originated by a primary electrogenic proton pump or by a primary electroneutral proton pump followed by a diffusion potential. That $\Delta\psi$ may originate as a diffusion potential cannot be excluded; for example the magnitude of the discrepancy between the values of $\Delta\psi$ on K^+ and Ca^{2+} is dependent on the relative permeability of the membrane for the two cations. Also the magnitude of $\Delta\psi$ on the various divalent cations correlates with the membrane permeability for the cations.

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