

MEMBRANE POTENTIALS AND RESISTANCES OF GIANT MITOCHONDRIA

Metabolic Dependence and the Effects of Valinomycin

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ABSTRACT

The membrane potentials and resistances of giant mitochondria from mice fed cuprizonone have been studied. They were found to correspond approx. 10–20 mV, positive inside, and 2 M Ω , respectively. These properties were found to be independent of the metabolic state. The microelectrodes were in the inner mitochondrial space since (a) the potentials in the presence of valinomycin depended on the K⁺ concentration of the medium and the magnitude of the K⁺ diffusion potentials was consistent with the presence of a high internal concentration of K⁺, (b) almost identical results were obtained with mitochondria from which the external membrane had been removed and the cristae were evaginated, and (c) punch-through experiments, in which the microelectrodes were advanced until they emerged through the other side of the mitochondria, showed an identical membrane potential both in the presence and in the absence of valinomycin. The potentials were stable under a variety of conditions and showed no sign of decay or membrane leakiness.

Detailed evidence that the impaled mitochondria are metabolically viable will be presented in a separate publication.

KEY WORDS mitochondrial potentials ·
microelectrodes · oxidative phosphorylation

The Mitchell chemiosmotic hypothesis proposes that the electrochemical potential gradient of H⁺ across membranes (the so-called protonmotive force) plays the major role in mitochondrial phosphorylation and the transport of ions. This hypothesis has been described in detail in a series of publications (e.g., references 28–34). The electrochemical potential gradient of H⁺, which is the result of proton pumps (in the form of the so-

called loops of Mitchell), is constituted of the H⁺ concentration gradient and the membrane potential.

Tupper and Tedeschi (50, 52–54) were able to measure membrane potentials and membrane resistances directly in *Drosophila* giant mitochondria by means of microelectrodes driven by a piezoelectric device. The membrane potential was found to range from 10 to 20 mV, positive inside, and the resistance was 1–4 Ωcm^2 . Metabolism was not found to affect either the potentials or resistances significantly (53). The specific resistances were

probably underestimated by a factor of 2 to 4, since the assumed surface area did not take into account the extensive invaginations of the surface membrane (see electron micrographs of reference 52). The measured potentials are likely to approximate the potentials across the mitochondrial membrane, since the potentials (*a*) respond predictably to the osmotic pressure of the medium (52), (*b*) reflect quantitatively the distribution of organic anions between the inner and outer phases (54), and (*c*) decay when the mitochondria visibly deteriorate (50). These findings, however, have been questioned by several scientists. Liberman and Skulachev (24) suggested that the low resistances are indicative of a damaged mitochondrial membrane. The resistance they propose ($10^9 \Omega\text{cm}^2$) is that calculated by them and Mitchell (e.g., references 30 and 33) for the permeability of the membrane to H^+ . The applicability of this estimate is questionable since they only consider the conductivity of H^+ . The lack of permeability of other ions (e.g., K^+ and Cl^-) is assumed. Low resistances are not unusual for plasma membranes. The resistances were found to be approx. $3\text{--}10 \Omega\text{cm}^2$ for glial cells in tissue culture (13), 1 to 5 (36) and 4 to 13 (19) in the excitable face of electric eel plates and as low as 0.1 to 0.4 in the non-excitable face (19). In the erythrocyte cell, they have been reported to be approx. $10 \Omega\text{cm}^2$ in experiments using microelectrodes (23) or a sucrose gap technique (16).

Lassen et al., working with Ehrlich ascites tumor cells (21) or *Amphiuma* erythrocytes (22), found that following impalement there was a rapid decay of the membrane potential with a half time of about 1 ms. After the decay, the steady-state potential was no longer sensitive to the K^+ concentration of the medium. They calculate that, given the lesser surface areas of mitochondria, the decay after impalement should be much faster (21). The reasoning and the general applicability of their results are questionable. The time-course of the decay is about the same in Ehrlich cells and *Amphiuma* erythrocytes (cf. Figs. 4 and 5 of reference 21 with Fig. 2 of reference 22), despite a fourfold difference in the surface area (sevenfold difference in volume). This suggests that the decay is primarily a function of the microelectrode and the piezoelectric drive system used.

The finding of Sekiya (45) of much slower decays in the Ehrlich cells (with a half time of about 10 s) supports this view. Other experiments show a much slower decay (from seconds to 1

min, not milliseconds) in *Amphiuma* erythrocytes (48). In addition, a great number of experiments have been carried out with a variety of cells (some smaller than those used by Lassen et al. [see references 5, 6, 7, 11, 12, 17, 18, 37, 39, 40, 41]). Potentials which do not decay significantly with time have been recorded (5, 6, 7, 12, 17, 37–41). Some of these potentials were found to be sensitive to the K^+ concentration in the medium (5, 6, 12, 39, 40). In some of these cells, biologically important electrical responses can be detected (e.g., action potentials [11, 18] and electrogenic effects [7, 17, 37–40]). Clearly, the results are not in harmony with those of Lassen et al. The present experiments, using microelectrodes to impale the giant liver mitochondria of mice fed on cuprizone, substantiate the results obtained with *Drosophila*.

The giant mitochondria from cuprizone-fed mice were used in this study since they are large and reasonably well coupled (e.g., reference 49 and 55). The potentials and the membrane resistances measured in giant mice mitochondria were found to be of the same order of magnitude (+10–20 mV, $1\text{--}5 \Omega\text{cm}^2$) as those from *Drosophila*. The membrane potential was not found to be dependent on metabolism.

MATERIALS AND METHODS

Chemicals

Antimycin A and valinomycin were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was supplied through the courtesy of Dr. P. Heyter from the DuPont Research Department (DuPont Instruments, Wilmington, Del.). Rotenone was purchased from K and K Laboratories, Inc. (Plainview, N.Y.), 2(*N*-morpholino)ethane sulfonic acid (MES, buffer) from Calbiochem (San Diego, Cal.) and potassium methanesulfonate was prepared from methanesulfonic acid supplied by Eastman Kodak. Digitonin was recrystallized from the chemical supplied by Fisher Scientific Co. (Pittsburgh, Pa.).

When present, the reagents were used in the following final concentrations: sodium succinate, 3 mM; rotenone, 8×10^{-7} M; antimycin A, 1×10^{-6} M; FCCP, 5.9×10^{-8} M; and valinomycin, 1.4×10^{-7} M.

Preparation of the Mitochondria

In the present study, mice were fed cuprizone as described by Suchy and Cooper (49). Mitochondria were isolated from homogenates (0.25 M sucrose, pH 7–8) of the liver. The solutions and preparations were maintained between 0° and 5°C . Cells, nuclei (swollen), and

debris were discarded by centrifugation at 120 g for 80 s. After filtration through a quadruple layer of cheesecloth, the mitochondria were isolated by centrifugation at 3,000 g for 5 min. Generally, the isolated mitochondria were resuspended in 0.30 osmol/l sucrose, 10 mM KCl, and 1 mM MES, pH 7.4.

Preparation of Mitoplasts

Mitoplasts were prepared from mitochondria by the method of Schnaitman et al. (44) with 1 mg digitonin/10 mg of mitochondrial protein in one preparation (preparation 1) and 1.2 in another (preparation 2). Preparatory procedures for electron microscopy were carried out by the method detailed by Hackenbrock (10) except that the epoxy resin of Spurr was used (47).

Preparation of Microelectrodes

The glass microelectrodes were pulled on a Chowdhury micropipette puller (produced by ISEW, Calcutta, India; distributed by Scientific International, Midwest City, Okla.) (4) from Corning 9530 melting point glass tubing (Corning Glass Works, Science Products Div., Corning, N.Y.). The microelectrode tip diameters were estimated to be 0.1–0.2 μm by Peter M. O'Day (State University of New York at Albany) and William D. Radigan (Dudley Observatory, Albany, N.Y.) with a scanning electron microscope. The microelectrodes were filled with 2 M KCl by boiling under reduced pressure. The microelectrode resistances were between 10 and 80 M Ω , and the tip potentials were less than 14 mV. The tip potentials were unaffected by the addition of valinomycin. The microelectrodes were connected to an amplifier by a Ag-AgCl junction. The reference electrode was a Ag-AgCl wire.

More conventional microelectrodes, with a more

gradual taper, were used for the "punch-through" experiments (Fig. 2). In one set of experiments the microelectrodes were filled with 2 M NaCl. The results were essentially indistinguishable from the experiment in Fig. 6.

Manipulations and Impalements

The mitochondria (approx. 1 mg protein/ml) were sandwiched between a thin layer of agar and a glass cover slip (0.15 mm in thickness). 1% Agar, maintained fluid at 45°C, was layered by using a bacterial loop. The optical system consisted of a Zeiss Universal microscope equipped for differential interference microscopy after Nomarski (1) with a water immersion lens (40 \times , 0.75 NA). A 546-nm interference filter (Edmund Scientific Co., Barrington, N.J.) with a Zeiss (HBO 200) mercury burner and a heat filter (Calflex, Carl Zeiss, Inc.) were used. The temperature was between 19° and 25°C.

The mitochondria were impaled manually using a Leitz micromanipulator (E. Leitz, Inc., Rockleigh, N.J.). The potential measurements were carried out with a high input impedance amplifier (10^{11} Ω) with a digital voltmeter or an appropriate oscilloscope. Generally, a negative feedback amplifier built after a design of J. P. Hervey of The Rockefeller University was used. The time response of the system was about 200 μs . Resistances were measured by passing 10^{-11} A pulses.

RESULTS

Electrical Properties of the Mitochondrial Membrane

In most cases, giant mitochondria, 5–10 μm in diameter, were impaled (Fig. 1). The flattened

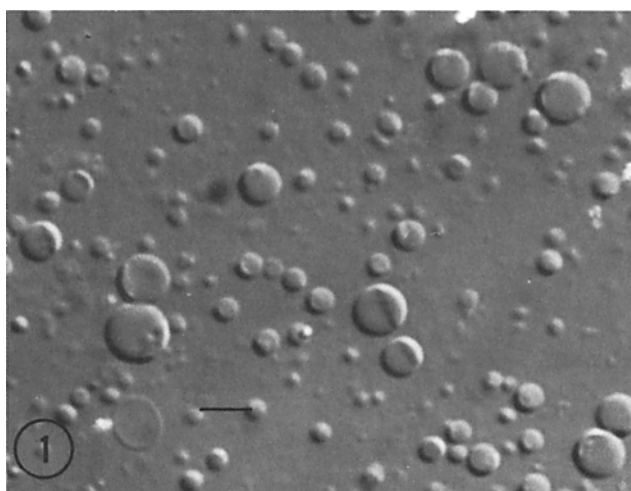


FIGURE 1 Cuprizone-fed mouse liver mitochondria seen with differential interference contrast optics after Nomarski (1). See Materials and Methods for details. Bar, 10 μm \times 750.

appearance is the result of the Nomarski differential optics which yield the image of an optical section of the object (1). Optical sectioning through the mitochondria reveals almost perfect spheres with a ratio of height to width of 1.03 ± 0.11 in 69 measurements. The tip potentials and the electrode resistances were measured before and after impalements. Generally, both measurements returned to approximately the same levels after either withdrawal of the electrode (e.g., Fig. 5), or in punch-through experiments in which the electrode was advanced until it traversed the mitochondrion and emerged from the other side (e.g., see Fig. 2*a* and 2*b*). The results of unusual cases in which the resistances or potentials did not return to approximately the original values were discarded.

Three basic sets of conditions were used: (a) without the addition of substrate and in the presence of metabolic blocking or uncoupling agents, (b) in the presence of succinate, and (c) in the presence of valinomycin, under conditions (a) and (b). The preparation has sufficient oxygen in solution to sustain respiration for a period two-five times longer than the experimental periods as shown in parallel experiments under identical conditions but in sealed cuvettes. Furthermore, as we shall present in a separate publication, each mitochondrion is capable of metabolically linked accumulation of calcium phosphate and the production of ATP.

No effects were noted from altering the metabolic state either in the presence or in the absence of valinomycin. The results are summarized in Table I, column 2 in the absence of valinomycin and in column 3 in the presence of valinomycin (see also Table II). In the experiments of Table I, the mitochondria were placed in the condition described before impalement. The results are identical if the reagents are added after impalement.

In the absence of valinomycin, the membrane potential ranges from 15 to 20 mV, positive inside (Fig. 2*a* and 3*a*), essentially the same results as those obtained previously for *Drosophila virilis*. Similarly, the resistances are approx. 2 M Ω (e.g., Table II). In all the experiments presented, the resistances were in the range of 1.8 to 2.5 M Ω . Since the mitochondria range in size between 4 and 10 μm , the specific resistances range from 1 to 6 Ωcm^2 , without considering the convolutions of the inner mitochondrial membrane. The actual specific resistance is probably much higher. The

resistance is independent of the K^+ concentration and the presence or absence of valinomycin.

In the presence of valinomycin, the polarity of the potential is reversed, as would be expected by valinomycin producing a K^+ diffusion potential (Figs. 2*b*, 3*b*, and 4*b*). This question was examined in more detail in other experiments presented below (Fig. 6).

Stability of the Measured Potentials

The measured potentials are maintained for relatively long periods without decay. This is shown in the fast sweep oscilloscope records (Fig. 3) in the absence (Fig. 3*a*) or the presence of valinomycin (Fig. 3*b*). The time response of the equipment corresponds to approx. 200 μs . Slower recordings also show no significant decay (Fig. 4*a* and 4*b*). In Fig. 4, four traces are superimposed so that they represent a period of approx. 180 s. Fig. 4*a* represents a record in the absence of valinomycin, and Fig. 4*b* represents a record in the presence of valinomycin. Some decay does take place when a second impalement is carried out either in the absence (Fig. 5*a*) or in the presence of valinomycin (Fig. 5*b*), but even in this case the decay is minimal. The lack of decay corresponds to the results previously reported for *Drosophila*.

Dependence of the Potential on the External Concentration of K^+

The dependence of the potentials on the K^+ concentration of the medium (K^+)_o is depicted in

TABLE I
Mitochondrial Membrane Potentials: Absence of a Metabolic Dependence

	E	
	- Valinomycin	+ Valinomycin
	mV	
Control, no ethanol	16.3 \pm 0.5	8.8 \pm 1.0
Control, ethanol	17.0 \pm 1.0	11.5 \pm 1.0
+ Rotenone ²	16.0 \pm 0.8	9.5 \pm 1.7
+ Rotenone	17.8 \pm 1.0	12.3 \pm 1.7
+ Na succinate		
+ Na succinate	16.3 \pm 0.5	9.5 \pm 0.6
+ Antimycin A		
+ Na succinate	15.8 \pm 1.0	8.5 \pm 1.3
+ FCCP		

The mitochondria were suspended in 55 mM potassium methanesulfonate, 0.20 osmol/l sucrose, 1 mM MES, pH 7.4. The reagents were present before impalement. The results represent means \pm SD.

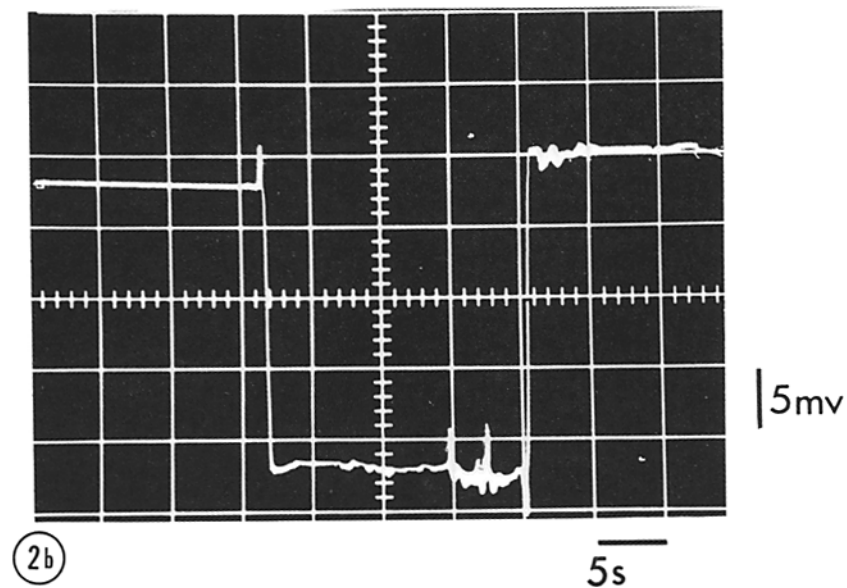
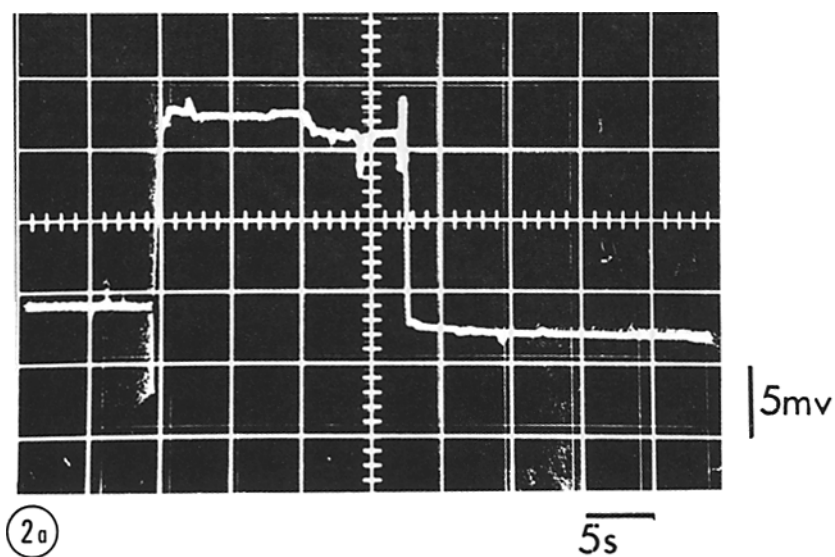


FIGURE 2 "Punch-through" experiments. The microelectrodes were advanced until they emerged from the mitochondrion after impalement. Fig. 2a and b correspond to oscilloscope tracings of potentials in the absence and in the presence of valinomycin, respectively. The mean \pm SD of four such impalements in Fig. 2a was 15.5 ± 1.6 mV. In Fig. 2b, the potential was -17.3 ± 2.2 mV.

Fig. 6. The upper line (closed circles) represents the results in the absence of valinomycin. The lower line (open circles) represents the results in the presence of valinomycin.

In the absence of valinomycin, the potential does not depend on the $(K^+)_o$. In some experiments, we have found a minor dependence on $(K^+)_o$ (involving 2–3 mV) at concentrations above

100 mM. The experiment with or without valinomycin shown in Fig. 6 was carried out with methanesulfonate as the anion. The results with or without valinomycin do not differ significantly when other anions such as Cl^- , acetate, or SO_4^{2-} substitute for methanesulfonate. The results with Cl^- are shown in Table II. In the presence of valinomycin, the potential shows a dependence on

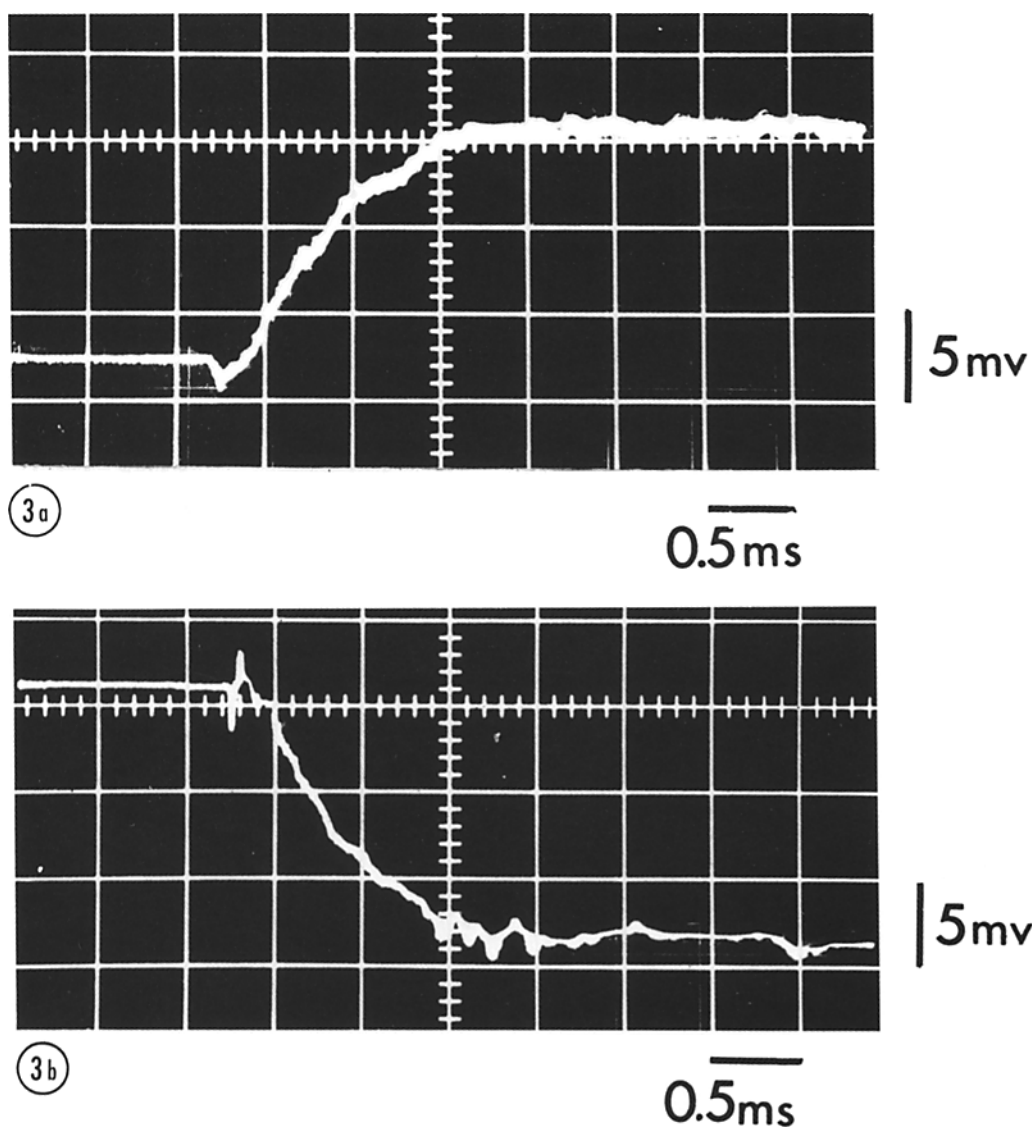


FIGURE 3 Fast sweep oscilloscope records of potentials from two impalements. The mitochondria were suspended in 10 mM KCl, 0.30 osmol/l sucrose, 1 mM MES, pH 7.4. The potentials recorded in Fig. 3a and b were in the absence and in the presence of valinomycin, respectively. In part a, the mean \pm SD of four impalements was 15.7 ± 1.0 mV. In part b, valinomycin was added before the impalement, the mean and SD were -17.8 ± 1.7 mV.

the K^+ concentration of the medium (open circles). Two features of this curve are noteworthy. The curve is linear over a limited range of $(K^+)_o$, then it levels off at low concentrations of K^+ . The leveling off at low K^+ concentrations probably results from the contribution of other ions to the potential at low K^+ and is characteristic of systems such as nerve (15) and muscle (14) which exhibit a K^+ diffusion potential. The linear part of the

curve has a slope which corresponds to 45 mV rather than the 59 predicted by the Nernst equation for a K^+ diffusion potential. This slope does not differ significantly from 59 when seven independent experiments are taken. The mean corresponds to 54 ± 8 mV.

The relationship of potential as a function of external K^+ concentration (such as in Fig. 6) is approximately the same regardless of ionic

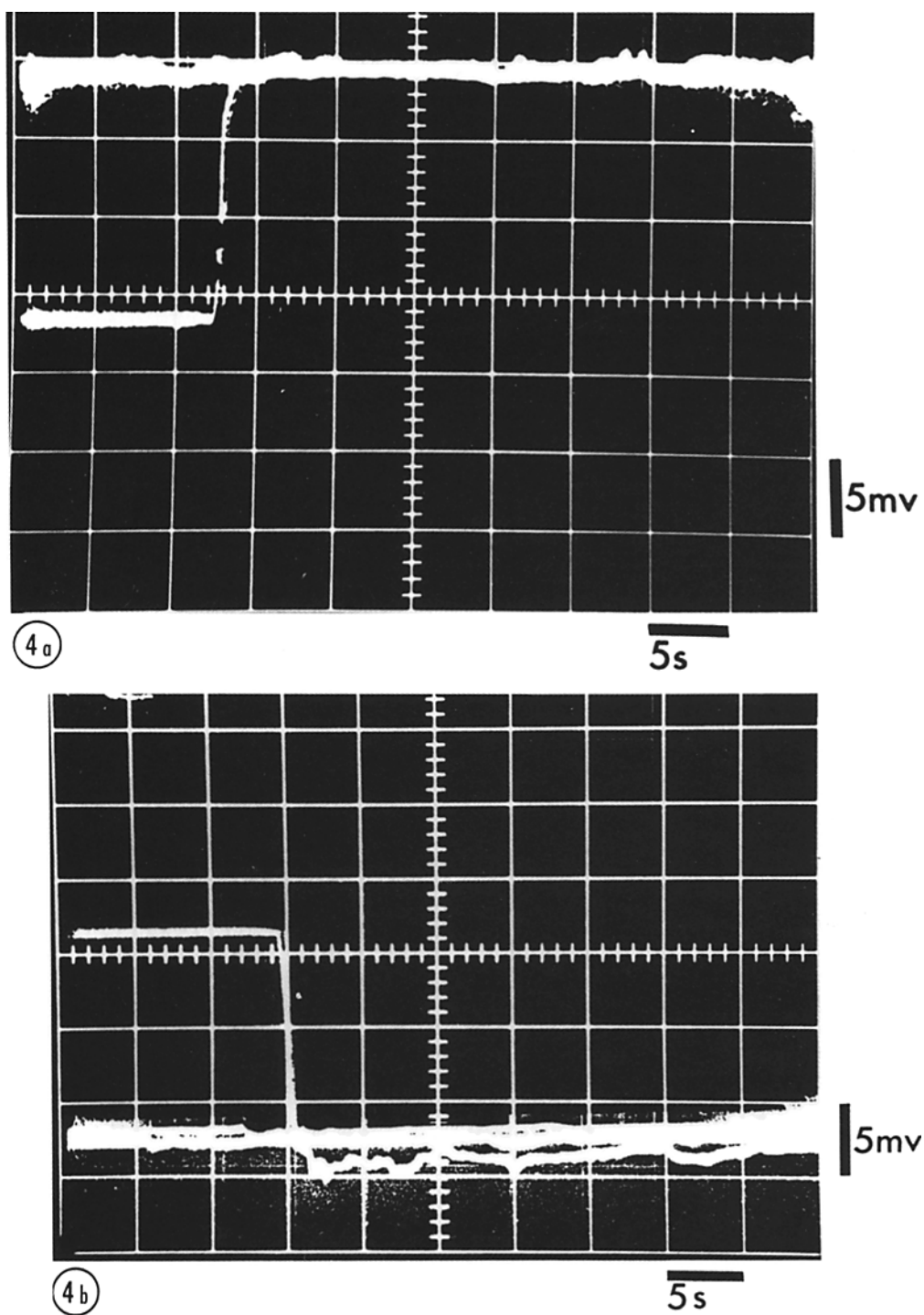


FIGURE 4 Slow sweep oscilloscope records of the potentials from two impalements. Each tracing represents a total of four sweeps, for a total period of about 3 min. The conditions correspond to those of Fig. 3. Part *a* has no valinomycin. Part *b* has valinomycin. The mean \pm SD for the membrane potential was 16.8 ± 1.2 mV and -16.2 ± 1.0 mV in the absence or presence of valinomycin, respectively.

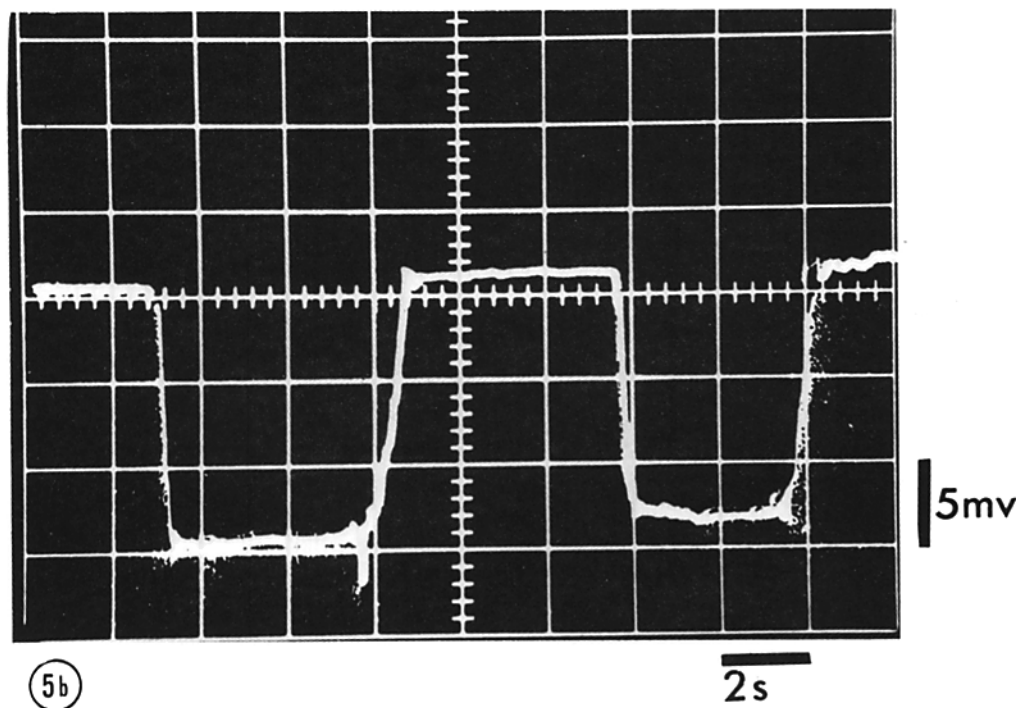
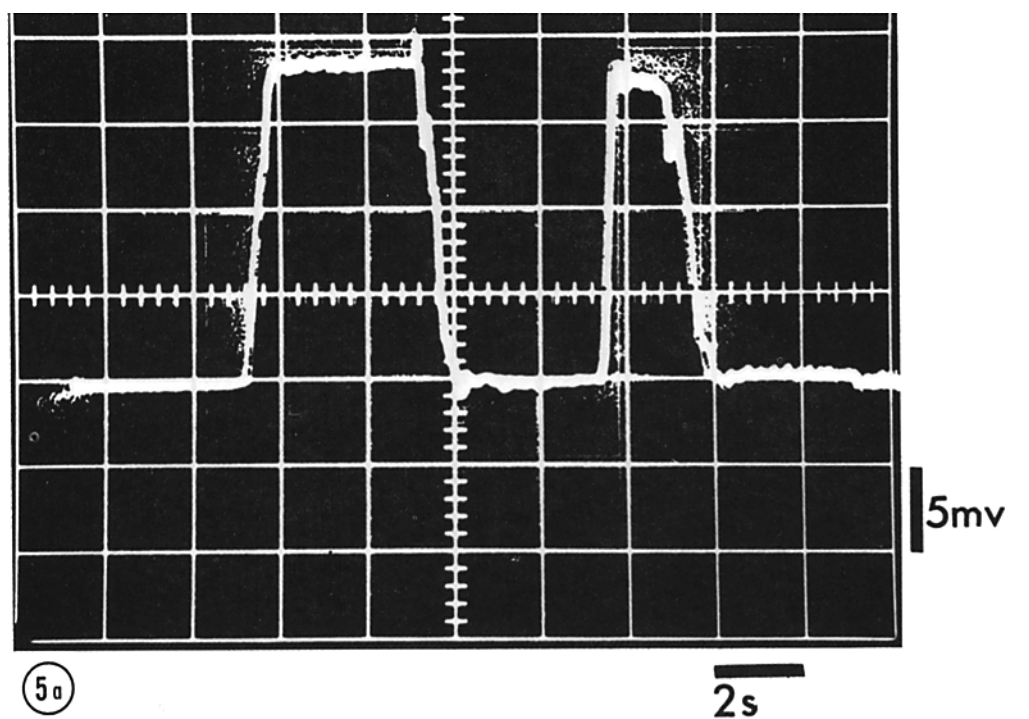


FIGURE 5 Double impalements. Each record represents an oscilloscope trace of the potentials in two sequential impalements of the same mitochondrion. The conditions correspond to those of Fig. 3. Part *a* is in the absence of valinomycin (mean \pm SD at potential peak of 19.7 ± 1.1 mV) and part *b* in the presence of valinomycin (mean \pm SD at peak of -15.5 ± 2.3 mV).

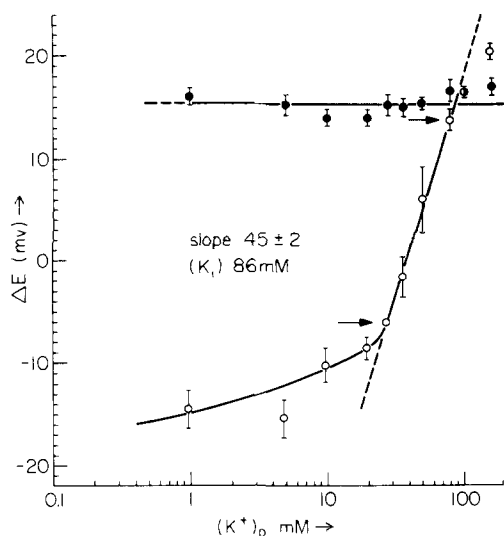


FIGURE 6 The dependence of the potentials on the external K^+ concentration. The mitochondria were impaled in solutions containing varying concentrations of potassium methanesulfonate, 1 mM MES, pH 7.4. The osmotic pressure was maintained at 0.30 osmol/l by adding the appropriate amounts of sucrose. Each point represents the mean of at least four determinations \pm SD. The closed circles represent the potentials in the absence of valinomycin, and the open circles, the potentials in the presence of valinomycin. The valinomycin was added after impalement so that each impalement represented by an open circle corresponds to a previously obtained record represented by a closed circle.

strength or nature of the ions. Similarly, the values in the nonlinear range of the curve in the presence of valinomycin are approximately the same. For example, in one experiment the membrane potential was -11.5 ± 2.0 mV ($n = 4$) in a medium containing 40 mM NaCl, 0.22 M sucrose, 1 mM Tris, and 1 mM KCl at pH 7.4 (cf. -14.0 ± 3.0 in Table II). In contrast, higher concentrations of Tris (e.g., 10 mM) produce higher potentials (i.e., more positive potentials) at the low K^+ concentrations.

At high $(K^+)_0$, the two curves intercept. At this point, the K^+ diffusion potential is zero. Hence the internal $(K^+)_i$ and the external $(K^+)_0$ concentrations are equal. The $(K^+)_i$ can be read directly from the abscissa, and in this experiment it corresponds to 86 mM. This concentration is generally somewhat lower as shown by the mean of seven experiments where it corresponds to 62 ± 13 mM.

Another similar experiment (using KCl) is detailed in Table II. In this experiment, membrane potentials and resistances were measured in the

presence and absence of valinomycin. The resistances are unchanged with varying experimental conditions.

Electrical Properties of Mitoplasts

The potentials and resistances obtained with mitochondria after the removal of the outer membrane are essentially the same as those of intact mitochondria as shown in Table III. A few measurements were atypical, having low potentials and high resistances (4 out of 30), and were not included. This experiment supports the notion that the microelectrodes are in the inside compartment. The electron micrographs showed complete removal of the outer membrane in the 365 randomly selected particles observed in preparation 1. In preparation 2, 276 mitoplasts were found devoid of outer membrane, 74 had a partially attached outer membrane, and 48 had the appearance of having both inner and outer membranes intact. The mitoplasts have a ruffled appearance with differential interference optics (Fig. 7a). This is expected from the evaginations of the inner space, typical of mitoplasts (42, 10). The presence of evaginations is confirmed by the electron micrographs, three samples of which are shown in Fig. 7b. However, a number of particles had a conventional arrangement of cristae (33%, $n = 638$).

Large mitoplasts (above 3 μ m) were a lesser proportion of the population than we expected from the differential interference microscopy. This might be the result of the low probability of sectioning a spherical particle through its major axis and, possibly, of the greater fragility of the larger vesicles. The 15 large mitoplasts which were looked at in detail showed the total absence of outer membrane.

DISCUSSION

The results are in general agreement with those observed with *Drosophila* mitochondria (50, 52-54). In particular, no evidence was found for a membrane potential directly dependent on metabolism, since the presence of succinate, antimycin A, and FCCP had no effect on the potentials either in the presence or in the absence of valinomycin. Further, the mitochondrial suspensions were in good physiological condition as the P:O ratio for succinate was 1.6 ± 0.2 ($n = 6$). The mitochondria were not leaky after impalement, as judged by the fact that the potential does not decay significantly with time. Therefore, there is no reason to believe they were not functioning

TABLE II
Resistances and Membrane Potentials

(K ⁺) _o mM	- Valinomycin		+ Valinomycin	
	R	E	R	E
	MΩ	mV	MΩ	mV
1	2.1 ± 0.1	16.8 ± 1.0	2.0 ± 0.1	-14.0 ± 3.0
5	2.0 ± 0.1	16.0 ± 1.0	2.0 ± 0.2	-15.0 ± 1.0
10	2.1 ± 0.0	17.0 ± 1.0	2.2 ± 0.1	-11.7 ± 1.0
20	2.3 ± 0.1	15.7 ± 0.5	2.2 ± 0.1	-5.2 ± 1.5
28	2.2 ± 0.5	16.2 ± 0.5	2.1 ± 0.0	-3.2 ± 0.5
36	2.1 ± 0.1	15.7 ± 1.0	2.0 ± 0.1	4.2 ± 2.8
50	2.1 ± 0.1	15.7 ± 0.5	2.0 ± 0.0	9.0 ± 0.8
80	2.1 ± 0.1	16.0 ± 0.8	2.0 ± 0.1	13.2 ± 1.0
100	2.0 ± 0.1	16.5 ± 0.6	2.0 ± 0.0	15.5 ± 0.8
160	2.0 ± 0.1	17.3 ± 0.5	2.0 ± 0.1	16.8 ± 0.5

The mitochondria were suspended in 1-160 mM KCl, as indicated, 1 mM MES, pH 7.4. The osmotic pressure was maintained at 0.30 osmol/l by the appropriate additions of sucrose. Four or more impalements were carried out in the absence of valinomycin. Valinomycin was added after the impalement. The values recorded correspond to potentials or resistances at the steady state (means ± S.D.).

TABLE III
Electrical Parameters of Digitonin-Treated Mitochondria

Exp	Condition	Potential	Resistance
		mV	MΩ
1	Control	15.2 ± 1.1	2.3 ± 0.4
	+ Succinate (3 mM)	15.6 ± 1.1	2.1 ± 0.1
	+ Rotenone (8 × 10 ⁻⁷ M)	15.4 ± 0.9	2.1 ± 0.1
	+ Succinate + Rotenone	14.6 ± 2.2	2.1 ± 0.1
	+ Valinomycin (1.4 × 10 ⁻⁷ M)	-14.0 ± 2.9	2.2 ± 0.2
2	Control (+ 8 × 10 ⁻⁷ M Rotenone)	16.8 ± 1.0	2.1 ± 0.1
	+ Succinate (3 mM)	15.6 ± 4.1	2.1 ± 0.1
	+ Antimycin A (1.0 × 10 ⁻⁶ M)	17.8 ± 1.0	2.2 ± 0.2
	+ Valinomycin (1.4 × 10 ⁻⁷ M)	-13.5 ± 2.2	2.1 ± 0.1

The mitoplasts were suspended in 0.30 osmol/l sucrose, 1 mM KCl, 1 mM MES, pH 7.4. The values represent means ± SD (n = 4). In exp 1, the mitoplasts were impaled after suspending them in the medium listed. In exp 2, the additions were made after the impalement of the control. The two experiments correspond in number to the preparatory procedures shown in Materials and Methods.

normally in terms of their transducing function (either because the intactness of the membrane is not necessary for function, or because the mitochondrial membrane can readily reseal). Evidence will be presented in a later publication supporting the notion that the individual impaled mitochondria are coupled.

An interesting feature of the data is the absence of an effect of valinomycin and the K⁺ concentration on the resistance of the mitochondria. This finding may indicate that the ions carrying the current used to measure the resistance are not K⁺. Previous studies with *Drosophila* (54) presented evidence for a role of anions in determining the

potential, positive inside, in the absence of valinomycin. Possibly, the electrical current used to measure resistance is in excess of the capacity of the valinomycin pathway to carry K⁺ across the mitochondrial inner membrane.

Three independent lines of evidence support the notion that the microelectrode is in the inner compartment of the mitochondria. The results in the presence of valinomycin indicate a K⁺ diffusion potential requiring a substantial internal concentration of K⁺ (between 60-80 mM). Other systems known to exhibit a diffusion potential dominated by K⁺, for example, nerve or muscle, exhibit a very similar dependence on the external

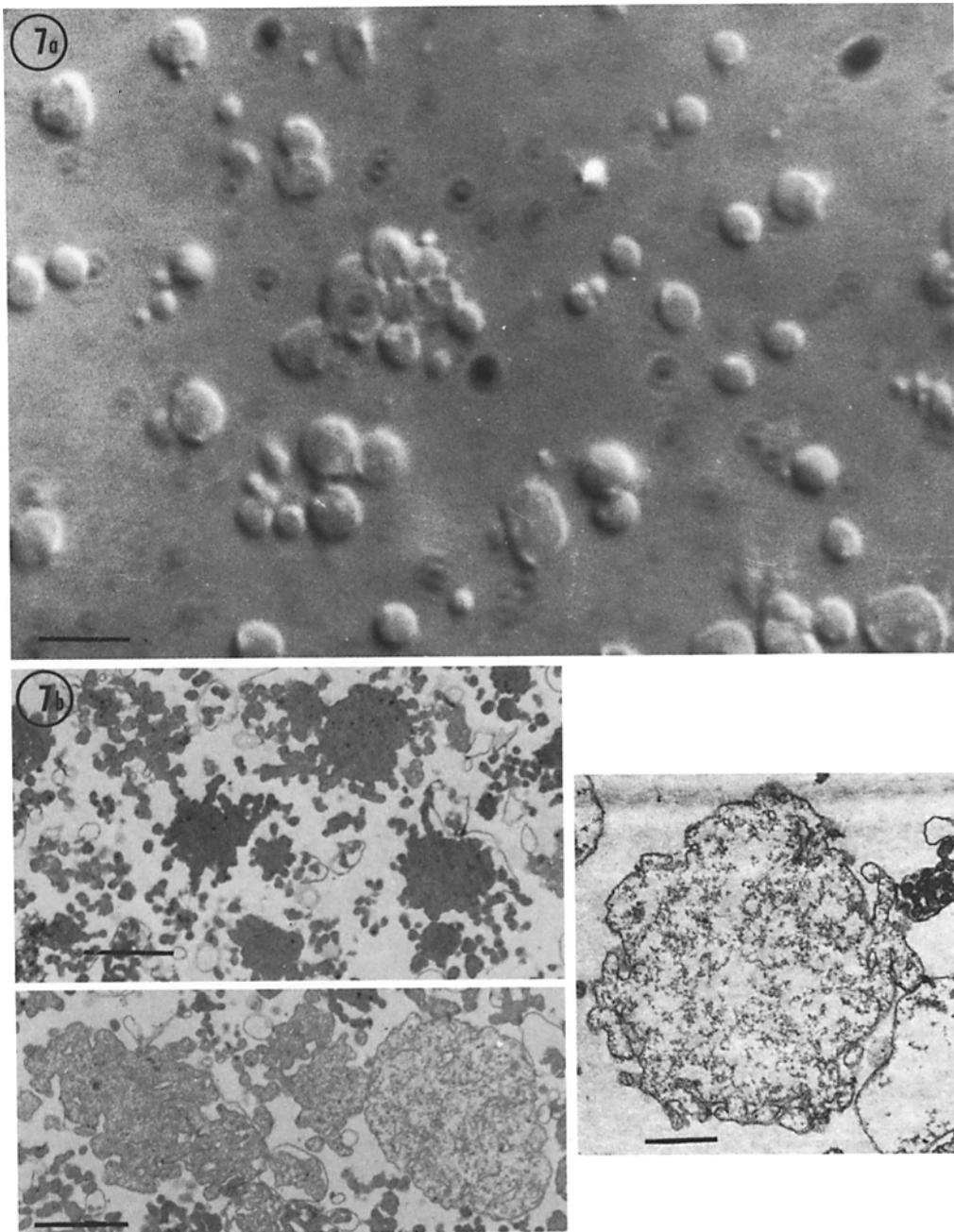


FIGURE 7 Morphology of the mitochondria after digitonin treatment. (a) Differential interference microscopy of the preparation showing the ruffled appearance of the mitochondria. (b) Electron micrographs of three fields, showing both evaginated and conventional arrangements of the inner membrane folds. The bars correspond to $10\ \mu\text{m}$ in Fig. 7a ($\times 1,250$) and $1\ \mu\text{m}$ in 7b ($\times 13,000$ for the upper two, and $\times 10,000$ for the lower one).

K^+ concentration. Furthermore, the “punch-through” experiments, in which the microelectrodes were advanced until they emerged from the opposite surface, having traversed the mitochondrion, showed a single potential. Similarly, the potentials and resistances of mitochondria from

which the outer membrane had been removed were essentially the same as those of intact mitochondria.

The potentials, whether in the presence or the absence of valinomycin, are steady. This stability indicates that the measured potentials closely correspond to the potentials across the mitochondrial membrane.

In summary, no evidence was found for a membrane potential induced by metabolism in mitochondria.

A number of studies have attempted to calculate the membrane potential induced by metabolism by using the Nernst equation and the K^+ distribution in the presence of valinomycin (e.g., references 35 and 43). This treatment assumes that the K^+ distribution can be imposed solely by a membrane potential. However, this would not be the case even in the presence of a significant membrane potential. The model requires the simultaneous presence of a membrane potential and a continuous proton pump to compensate for the K^+ inward leaking in response to the electrical gradient in the presence of valinomycin. Jointly, these two assumptions would result in a steady state in which the concentration of K^+ will depend on the rate of the H^+ pump, the backflow of H^+ as well as the net flux of K^+ . This can be readily appreciated by noting that the entry of $1 \mu\text{mol } K^+/\text{g}$ protein would depolarize a -250 mV potential (as calculated by Mitchell [33, 34]), where as many as $30\text{--}40 \mu\text{mol/g}$ protein of K^+ are accumulated in the experiments of Mitchell and Moyle (35). The K^+ concentrations can be interpreted readily with several possible models which do not invoke an electrogenic pump, but results in a 1:1, $K^+:\text{H}^+$ exchange (51) (see below).

The internal K^+ concentration at the steady state clearly is not the result of an equilibration of K^+ in relation to a membrane potential. For example, the internal K^+ concentration at steady state is a function of the valinomycin concentration (27). In addition, metabolizing mitochondria equilibrate very rapidly in relation to $^{42}\text{K}^+$ but continue taking up significant amounts until the content of K^+ inside the mitochondria almost doubles (Fig. 7 of reference 42). These results are incompatible with the postulated equilibration of K^+ in response to a membrane potential, which should occur within the time required for the $^{42}\text{K}^+$ equilibration.

The conclusion of the author that the uptake is a consequence of a passive, nonsaturable process

based on a presumed linear relationship between the external K^+ concentration and the rate of uptake is likewise a misinterpretation. Plotting the data on a linear scale (rather than the logarithmic scale used in Fig. 5 of reference 42) shows that the uptake of K^+ as a function of external K^+ concentration follows saturation kinetics.

The absence of a membrane potential which is dependent upon metabolism is shown by a variety of experiments. For example, Mitchell and Moyle (35) used an O_2 pulse to activate metabolism in an anaerobic suspension of mitochondria. The proton efflux corresponded quantitatively to the K^+ influx in the presence of valinomycin. In the absence of a metabolically dependent membrane potential (51), this situation leads to a Gibbs-Donnan distribution of K^+ . This distribution could be explained by a variety of models not requiring a membrane potential. Any of the following could account for the observed ion distributions: (a) an energy-dependent nonelectrogenic $\text{H}^+:\text{K}^+$ exchange mediated by an antiport carrier arrangement, (b) alternatively, an H^+ pump with a rapid passive K^+ exchange to maintain electric neutrality, (c) a carrier-mediated K^+ transport accompanied by OH^- , or (d) a carrier-mediated influx of K^+ accompanied by the passive efflux of H^+ to maintain electric neutrality. Indeed, such models have been proposed and are consistent with at least some of the data (see e.g., references 2, 8, 25, and 26).

Paradoxically, the presence of valinomycin could produce a K^+ diffusion potential as is obtained in K^+ -loaded liposomes or in erythrocytes (e.g., references 3 and 46). Such a potential would follow the Nernst equation over at least part of the K^+ concentration range. However, the potential would not be directly dependent on metabolism as in the observed cases of liposomes and erythrocytes (see Results).

The distribution of any permeable ion could also be imposed by a H^+ -pump without a membrane potential in the absence of valinomycin (comparable to mechanism *b*, discussed above). This could explain the distribution of lipophilic ions and electrofluorimetric dyes observed in several studies. The H^+ net flux would have to be matched quantitatively by the ion in question to maintain electric neutrality (see reference 20).

ADDENDUM

A recent report has been published on the membrane potential of the nucleus, the Golgi region

and mitochondria of impaled HeLa cells (9). The mitochondrial potential was found to be negative in relation to the cytoplasm (by about 10 mV). These results are in general agreement with our finding of a low potential in metabolizing mitochondria. However, the significance of the difference in the polarity of the potential between these results and those obtained by us either with *Drosophila* or giant mouse mitochondria is difficult to evaluate. The HeLa mitochondria are much smaller than ours and perhaps could be damaged more readily by microelectrodes. On the other hand, our medium is far removed in composition (e.g., the ions present) from the cytoplasmic environment provided by the HeLa cells.

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Note added in proof: The internal K^+ concentration may be much lower than the 50–60 mM calculated in this paper. If we were to assume that the diffusion of ions is responsible for the membrane potential both in the presence of valinomycin (i.e., a K^+ potential) or in its absence (i.e., involving some other ion, see reference 54), the $(K^+)_i$ would be much lower. It would correspond to the $(K^+)_o$ at which the membrane potential is 0, or approx. 38 ± 2 mM. Preliminary determination using atomic absorption with whole suspensions agree with the lower estimates (we found 25–39 mM K^+ in two experiments).

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