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A Quantitative Correlation between the Kinetics of Solutes and Water Translocation in Liver Mitochondria

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Summary. A procedure is described for the calculation of solute fluxes in mitochondria from absorbance measurements. The procedure assumes that mitochondria behave as osmometers and that they are always at osmotic equilibrium.

The rates and amounts of K^+ translocation have been calculated simultaneously, with the photometric procedure and electrometrically, during passive K^+ efflux coupled to Ca^{++} uptake and during active K^+ uptake and passive K^+ release coupled with anion translocation. Good agreement has been found between the two sets of measurements. The data are compatible with the concept that the energy-linked, ion translocation-coupled, mitochondrial swelling is osmotic in nature. It is concluded that the changes of absorbance are quantitatively related to changes in the inner volume and therefore the photometric procedure can be used to calculate ion fluxes of osmotically active species under various circumstances.

In a companion paper (Massari, Frigeri & Azzone, 1972) wide ranges of quantitative correlation between gravimetric and absorbance measurements have been shown during osmotically induced variations of the mitochondrial volume. Changes of the mitochondrial volume can, however, be produced by other mechanisms; namely: (a) uptake or release of solutes which are associated with the movement of osmotically active water (Chappell & Crofts, 1965; Azzone & Azzi, 1966; Azzi & Azzone, 1966, 1967*a*; Rottenberg & Solomon, 1969); (b) conformational changes of the membrane which appear to be independent of ion translocation (Hackenbrock, 1966). The latter process involves a molecular rearrangement of the membrane structure and will be considered for comparison in the Discussion. The former process, on the other hand, which includes the low amplitude active ion uptake and passive ion release or the large amplitude passive solute uptake, may be thought of as an osmotic process whereby the water follows

the solute movement (Azzone & Massari, 1972). This conclusion is in accord with several observations. For example, in the case of the passive, large amplitude swelling, good agreement has been found between the amount of water and of solutes translocated into the mitochondria (Azzi & Azzone, 1967*a*). No kinetic correlation between water and solute translocation has, however, been reported in the case of the passive swelling. In the case of the active, ion translocation-linked swelling, the kinetics of the swelling-shrinkage cycle correlate qualitatively with that of the K^+ translocation (Azzi & Azzone, 1966). The correlation between water and cation uptake has also been measured by using ^{14}C sucrose and flame photometry (Rottenberg & Solomon, 1969). The concentration of the K^+ entering the inner compartment is compatible with an osmotic swelling process. In the present study, we have attempted to correlate quantitatively the kinetics of the K^+ and of the water translocation by developing a procedure to calculate ion fluxes from photometric data and thereby measuring simultaneously the two processes. The results obtained indicate that: (a) the absorbance changes at 546 nm during active ion translocation are essentially caused by changes of the inner osmotic space of the mitochondria; (b) the change of mitochondrial volume during active ion uptake is essentially a process of osmotic nature; and (c) the photometric measurements can be used to follow quantitatively ion fluxes involving osmotically active species in mitochondria.

Materials and Methods

Mitochondria were prepared as described in the preceding paper (Massari *et al.*, 1972). The absorbance of the mitochondrial suspension was followed with an Eppendorf Photometer adapted to contain a special cuvette, 2 cm wide, 1 cm of light path, with H^+ and K^+ electrodes inserted from the side walls. The cuvette was stirred from above at the maximal rate compatible with the electrometric measurements. The distance of the cell from the photomultiplier was 5 cm. The H^+ and K^+ electrodes were connected with a Radiometer pH meter Model 26 and a Beckman pH meter Expandomatic, respectively. The output of the pH meters and of the Photometer were recorded, after suitable coupling, on a Texas Instruments multichannel recorder.

All measurements were made at 546 nm where only slight variations of absorption of the cytochromes occur (Chance & Williams, 1955). The conversion of the absorbance measurements into ion fluxes was critically dependent on the calibration curve relating absorbance to volume data (*cf.* Fig. 1 of the preceding paper by Massari *et al.*, 1972). Possible variations of the absorbance $\times$ mg protein^{-1} of various batches of mitochondria, therefore had to be taken into account. This was done by (a) constructing a standard calibration curve, μliters $H_2O \times$ mg protein^{-1} *vs.* osmolarity^{-1}, and (b) by plotting for each mitochondrial preparation, μliters $H_2O \times$ mg protein^{-1} *vs.* mg protein $\times$ absorbance^{-1}. The latter values were obtained by measuring the absorbance of the mitochondria at 20, 40, 60, 100 and, 200 mosm sucrose. By this procedure, the slope of the calibration

curve of Fig. 1 of the preceding paper, was repeated for each mitochondrial preparation assuming only a constant mitochondrial inner volume among the various preparations. Care was taken to limit the mitochondrial concentration below 2.6 mg protein/ml.

Theory

The following assumptions are made: (1) The system is always at osmotic equilibrium; i.e., the rate of solute translocation is smaller than the maximal rate of water translocation. (2) The membrane is symmetrical in respect to the process of influx and efflux of H_2O. (3) Volume changes are presumed due exclusively to H_2O translocation. (4) Solutions are dilute.

The osmolarity of the outer medium is not appreciably changed by the uptake of K^+, being maintained mostly by sucrose. From assumptions (1) and (4) we may write for v the rate of water translocation:

$$v = P \cdot S(\sum c_i - \sum c_o) \tag{1}$$

where v is expressed in moles sec^{-1}/g protein. P is the permeability coefficient in cm sec^{-1}, S the surface in cm^2/g protein, and $\sum c$ the sum of the concentrations of the osmotically active material in mole m^3. The subscripts i and o refer to inner and outer compartments, respectively. Eq. (1) is similar to that derived in the preceding paper and therefore the process of water translocation is assumed to be similar in the presence or in the absence of solute translocation. Also, the rate and amount of water translocation has been calculated as indicated in the preceding paper [*cf.* Eqs. (2) and (3)].

On the basis of assumption (2), P is considered to be the same during the processes of influx and efflux of water.

From assumption (3):

$$\frac{dV}{dt} = vw \tag{2}$$

where w is the volume (in cm^3) of 1 mole of water and V is the volume of the mitochondria.

The concentration of osmotically active material inside the mitochondria at the instant t is given by:

$$\sum c_i = \frac{m + \int_0^t \phi(t)\, dt}{V(t)} \tag{3}$$

where m refers to the moles of osmotically active material in the initial state and $\phi(t)$ is the rate of translocation of the osmotically active material at the instant t.

By replacing in Eq. (2) the expression for v given by Eq. (1), and expressing $\sum c_i$ as defined by Eq. (3), we obtain:

$$\frac{V}{P \cdot S \cdot w} \frac{dV}{dt} = m + \int_0^t \phi(t)\, dt - V \sum c_0. \tag{4}$$

By differentiating Eq. (4) and knowing that $S \propto V^{2/3}$, we obtain:

$$\phi = \sum c_0 \frac{dV}{dt} + \frac{1}{3P \cdot S \cdot w} \left(\frac{dV}{dt}\right)^2 + \frac{V}{P \cdot S \cdot w} \frac{d^2V}{dt^2}. \tag{5}$$

Since the rate of water translocation is experimentally found to be constant in the first instants we have from Eq. (2) $d^2V/dt^2 = 0$. Furthermore, it can be calculated that, by introducing the value of $P \cdot S$ found in the preceding paper into Eq. (5), the contribution of the second term amounts to about 5% for a rate of H_2O translocation of 10 µliters sec^{-1}/g protein at the osmolarity used. This rate has however seldom been attained in the present study. Therefore, provided the rate of solute translocation is not very high, the contribution of the second term of Eq. (5) also becomes negligible. Eq. (5) then becomes:

$$\phi = \sum c_0 v \tag{6}$$

where ϕ is expressed in moles sec^{-1}/g protein, $\sum c_0$ in moles/liter and v in liters sec^{-1}/g protein. Eq. (6) states that when the osmolarity of the external medium is maintained only by the solute being translocated, the concentration of the solute in the translocated fluid is equal to that of the outer medium. This is in agreement with the observation of Leaf (1956).

The total amount of solute translocated q_s is defined as number of moles translocated × sec^{-1}/g protein. The value for q_s is obtained by putting $m = \sum c_0 V_1$ where V_1 is the initial volume and by writing Eq. (4) as follows:

$$\frac{V}{P \cdot S \cdot w} \frac{dV}{dt} = \sum c_0 (V_1 - V) + \int_0^t \phi(t)\, dt \tag{7}$$

when we have reached the equilibrium state ($t \to \infty$), $dV/dt = 0$, and Eq. (7) reduces to:

$$\sum c_0 (V_1 - V_2) = -\int_0^\infty \phi(t)\, dt = q_s \tag{8}$$

where V_2 is the final volume.

Therefore, the total amount of water translocated q defined as the difference between initial and final volume of mitochondria is related to the total amount of solutes translocated q_s by Eq. (9):

$$q_s = \sum c_0 q. \tag{9}$$

Eq. (9) has a more general validity than that found by Tedeschi and Harris (1955):

$$q_s = c_s(V_1 - V_2)$$

where c_s is the initial concentration of solutes. In fact, the equation of Tedeschi and Harris is valid only if $\sum c_0 = c_s$.

Results

Anaerobic K^+ Efflux Coupled to Ca^{++} Uptake

K^+ efflux coupled to Ca^{++} uptake from valinomycin-treated mitochondria has already been reported (Azzi & Azzone, 1966; Rossi, Azzi & Azzone, 1967; Scarpa & Azzone, 1970). The K^+ translocation is accompanied by mitochondrial shrinkage since K^+ behaves as an osmotically active species whereas Ca^{++} does not. It is therefore possible to calculate the rate and amount of K^+ efflux from the increase of absorbance following the addition of valinomycin. The photometric calculation of the trans-

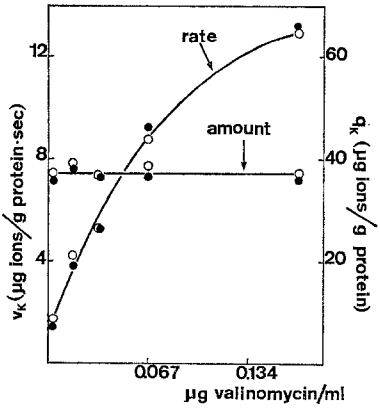

Fig. 1. Comparison between electrometric and photometric measurements for K^+ efflux coupled to Ca^{++} uptake. The incubation medium contained: 400 µM $CaCl_2$, 2 µM rotenone, 2.5 mM Tris-Cl, pH 7, 200 mM sucrose, 0.5 mM KCl. The reaction started by adding valinomycin, variable amount. The experimental points are averages of two different mitochondrial preparations. •—•, photometric measurements; ○—○, electrometric measurements

located K^+ is made on the basis of Eqs. (10) and (11):

$$v_k = \sum c_0 v \tag{10}$$

$$q_k = \sum c_0 q \tag{11}$$

where v_k and q_k are the rate and the amount of K^+ translocated. Fig. 1 shows experiments where rate and amount of K^+ translocation were measured simultaneously by photometric and electrometric procedures at various valinomycin concentrations. A good agreement was found between the two types of measurements.

Aerobic K^+ Uptake

The aerobic K^+ uptake by valinomycin-treated mitochondria is a well-known phenomenon (Moore & Pressman, 1964; Pressman, 1965; Azzi & Azzone, 1966). The overall process consists of two exchanges: H^+/K^+ and OH^-/A^-. The uptake of K^+ plus anion is accompanied by a large mitochondrial swelling which is reversed when the energy supply is inhibited by respiratory inhibitors or uncouplers. In this case, therefore, which differs from the previous one, the increase of osmotic pressure inside the mitochondria is caused by the translocation of two osmotically active species: K^+ and the anion. The rate of translocation of osmotically active material is:

$$\phi(t) = v_K + \frac{1}{z}(v_K - v_H) \tag{12}$$

where the first term is due to K^+ translocation, and the second to anion translocation. v_H is the rate of H^+ translocation. z may be empirically defined as the stoicheometry of the anion translocation, i.e., the OH^-/A^- ratio, since its value is determined by the net number of OH^- released from the mitochondria per number of anions penetrated. The value of z is essentially determined by the pK of the anion and the pH of the outer compartment. Under conditions where the pH of the medium is more than one unit higher than the pK of the transported anion, the value of z will be 1, 2, or 3 for univalent divalent and trivalent anions, respectively. When the pH is in the pK range of the acidic group, the value of z is a function of the percentage of ionization; i.e. 0.5 at 50% ionization. It is important here to note that the value of z is independent of whether the internal pH is more alkaline than the external pH.

Using Eq. (12), Eq. (6) becomes:

$$v_K = \frac{z}{z+1}\left(\sum c_0 v + \frac{v_H}{z}\right) \tag{13}$$

and the amount:

$$q_K = \frac{z}{z+1}\left(\sum c_0 q + \frac{q_H}{z}\right). \tag{14}$$

We have tested these equations using phosphate, lactate, acetate and succinate as anions.

Phosphate. Figs. 2–4 report three groups of experiments carried out with phosphate as anion, at various osmolarities, at various K^+ concentrations and at various phosphate concentrations. Since at pH 7 the first acidic group of phosphate is completely ionized whereas the second is 50% ionized, the value of z was taken as 1.5 (*cf. also* McGivan & Klingenberg, 1971). A good agreement was found between photometric and electrometric procedures in all three cases. It is to be noted that the experiments of Figs. 2–4 cover a wide range of experimental conditions from the point of view of the amount and rate of ion translocation, of the extent to which the K^+ uptake is limited by the alkalinization of the mitochondrial interior (variable phosphate concentrations), of the stretching of the membrane due to the variable degree of swelling (variable K^+ concentrations), and of the osmotic activity of the translocated species (variable osmolarities). It may be added that a good agreement was also obtained when the reverse process was analyzed; i.e., the K^+ release and the parallel increase of absorbance following the addition of uncoupling agents such as FCCP or DNP. Therefore, the experiments of Figs. 2–4 extend the range of applicability of the photometric procedure to conditions under which other light-scattering changes, for example those deriving from energy-linked conformational rearrangements, may interfere with the absorbance/volume relationship.

Acetate and Lactate. In the case of acetate and lactate, where the pK of the acidic group is much lower than the pH of the medium, z was assumed to be one. Fig. 5 shows that the correspondence between photometric and electrometric measurements was very good for the rate of K^+ uptake, while for the amount of K^+ uptake, especially at high lactate concentrations, the photometric measurements were nearly 10% lower than the electrometric measurements. The agreement between the two procedures, for the K^+ release after addition of 2 μM FCCP, was also good (not shown in the figure).

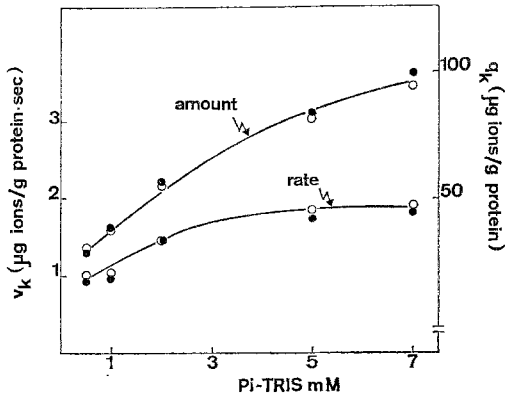

Fig. 2. Comparison between electrometric and photometric measurements for aerobic K^+ uptake at various phosphate concentrations. The incubation medium contained: 1 mM β-hydroxybutyrate, 2.5 mM Tris-Cl, pH 7.0, 200 mM sucrose, 0.5 mM KCl, 0.1 mM EGTA, 5 mM LiCl. The uptake started after addition of 0.5 µg of valinomycin. 2 µM FCCP was added after reaching equilibrium, causing the K^+ release. Final volume 2.5 ml. Amount of protein: 2.12 mg/ml. •—•, photometric measurements; ○—○, electrometric measurements

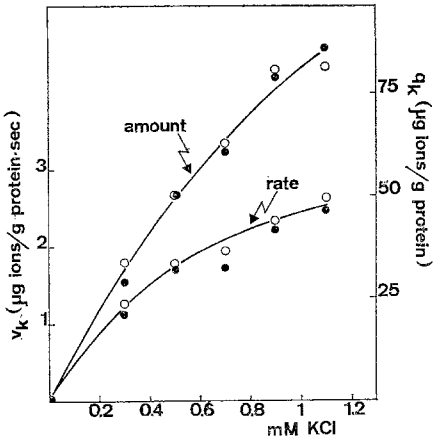

Fig. 3

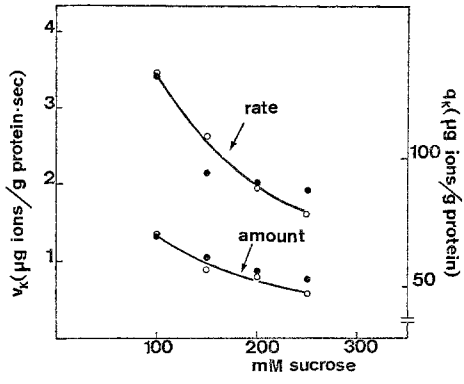

Fig. 4

Fig. 3. Comparison between electrometric and photometric measurements for aerobic K^+ uptake at various KCl concentrations. The incubation medium was the same as that used in Fig. 2 except that 1 mM Tris-phosphate was always present. •—•, photometric measurements; ○—○, electrometric measurements

Fig. 4. Comparison between electrometric and photometric measurements for aerobic K^+ uptake at various osmolarities. The incubation medium was the same as that used in Fig. 3. 0.5 mM KCl. •—•, photometric measurements; ○—○, electrometric measurements

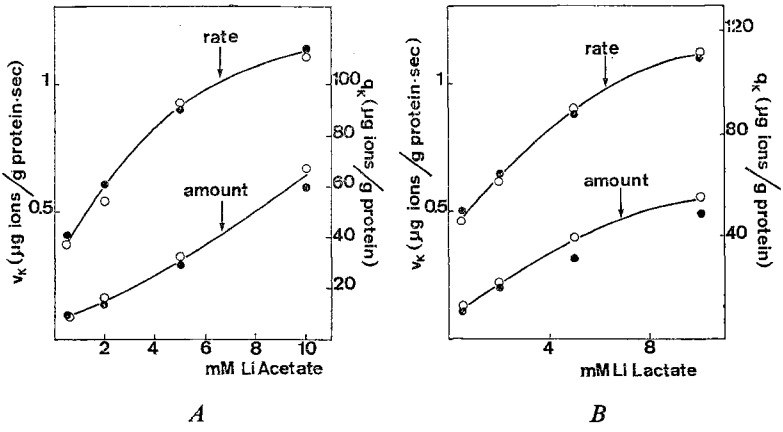

Fig. 5*A* and *B*. Comparison between electrometric and photometric measurements for aerobic K^+ uptake in the presence of acetate or lactate. The incubation medium was identical to that used in Fig. 2. The experimental points are averages of six different mitochondrial preparations. •—•, photometric measurements; o—o, electrometric measurements

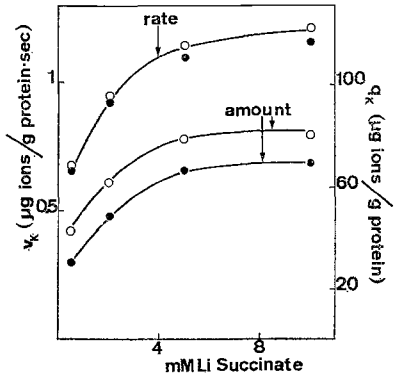

Fig. 6. Comparison between electrometric and photometric measurements for aerobic K^+ uptake in the presence of succinate. The incubation medium was identical to that used in Fig. 2. The experimental points are averages of eight different mitochondrial preparations. •—•, photometric measurements; o—o, electrometric measurements

Succinate. In the case of succinate, both acidic groups are practically completely ionized at pH 7.0. Therefore, the value of z was taken as two. Fig. 6 shows that the correspondence for the rate of K^+ uptake was very good. On the other hand, the amount of K^+ uptake calculated by the photometric procedure was somewhat lower with respect to the amount of uptake found by the electrometric measurements. The reason for this disagreement

is not clear. It is conceivable, however, that accumulation of a large amount of succinate in the matrix may result in damage to the membranes with alteration of light-scattering properties.

Discussion

Harris and van Dam (1967), have recently concluded that the light-scattering properties of the mitochondria are only qualitatively related to the mitochondrial volume for three reasons: (a) possible loss of protein during swelling with change of refractive index; (b) shape variations; (c) lack of distinction between variation of sucrose accessible and inaccessible spaces. We do not agree with the reservation of Harris and van Dam for the following reasons: (a) Tedeschi and Harris (1955) have shown that penetration of solutes into the mitochondria is accompanied by negligible changes of the refractive index; (b) as shown in the preceding paper (Massari *et al.*, 1972), during osmotic changes of mitochondria there is neither loss of material nor shape changes; (c) the variations of the sucrose accessible space are not osmotic in nature and do not appear to interfere either with gravimetric or photometric measurements. The kinetic correlation between photometric and electrometric measurements of the ion fluxes does not support the reservations on either the osmotic nature of the energy-dependent, ion translocation-linked mitochondrial swelling (Pressman, 1965) or the use of the photometric technique to follow the changes of mitochondrial volume quantitatively (Lehninger, 1962).

The absorbance changes analyzed in the present study, appear to represent a different process from that studied by Hackenbrock (1966), who has reported extensive ultrastructural transformations during variations of the metabolic state of the mitochondria. Hackenbrock (1968) has calculated that the inner volume of the mitochondria is doubled during the transformation to the orthodox configuration, which would mean an increase of the amount of water of about 1 μliter/mg protein. In the Hackenbrock experiments (1968) there is no translocation of osmotically active species. The interpretation of Hackenbrock (1968) is difficult to understand in view of the osmotic behavior of the mitochondria. In fact, a 100% increase of the amount of osmotic water in the absence of ion translocation would lead to a violation of the osmotic equilibrium between two spaces separated by a water permeable membrane.

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