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Permeability to Water, Dimension of Surface, and Structural Changes during Swelling in Rat Liver Mitochondria

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Summary. Rates and amounts of water translocation across the mitochondrial membrane have been studied with a photometric technique. The process of water translocation can be described in terms of the diffusion equations, and the mitochondria behave as spherical bodies between 15 and 110 mosm. A permeability coefficient to water of 5.3×10^{-3} cm sec^{-1} has been calculated. The mitochondrial surface is about 1 m^2/g protein during incubation in 0.10 M KCl, and increases to 30 m^2/g protein during incubation in 0.005 M KCl.

The osmotic shrinkage of hypotonically swollen mitochondria has also been studied. Complete reversibility of hypotonic swelling occurs only after incubation of mitochondria in media below 60 to 90 mosm. The appearance of the reversibility is phenomenologically correlated with the rupture of the outer mitochondrial membrane. Below 30 mosm there is a change of the absorbance properties of the membrane. The change correlates with the complete unfolding of the cristae and is attributed to ultrastructural reorganization of the membrane following mechanical stretching.

From experiments carried out under equilibrium conditions, the general conclusion has been reached that rat liver mitochondria behave as perfect reversible osmometers in saline media and in a certain range of osmolarities (Tedeschi & Harris, 1955, 1958; Bentzel & Solomon, 1967; Yoshida & Sato, 1968; Stoner & Sirak, 1969). Either gravimetric or photometric techniques have been used for this purpose.

An attempt to find a correlation between gravimetric and photometric measurements has been made by Tedeschi and Harris (1958). Such a correlation has been brought to a quantitative level in the present work. Changes of absorbance have been used to calculate the volume changes of mitochondria and thereby the amount of water translocated across the mitochondrial membrane. By using the diffusion equation we have also tried to analyze the kinetics of the water translocation. This approach has

permitted the determination of the permeability coefficient of the mitochondrial membrane to water and the surface area of the mitochondria. Some new aspects of the osmometric behavior of mitochondria after hypotonic treatment will also be reported.

Materials and Methods

Rat liver mitochondria were prepared in 0.25 sucrose, 1 mM EDTA, 5 mM Tris-Cl, pH 7.4. After two washings they were resuspended in the same medium devoid of EDTA.

Gravimetric measurements were carried out on mitochondrial pellets after centrifugation with a Sorvall RC2B centrifuge for 10 min at 20,000 $\times g$. The volume of the inner mitochondrial space was determined by correcting the weight of the mitochondrial pellet for the amount of mitochondrial protein and the volume of the Cl^- accessible space. The Cl^- accessible space which was determined with $^{36}Cl^-$ included both the space between outer and inner membrane and the intermitochondrial space. Photometric measurements were carried out with an Eppendorf Photometer at 546 nm. The light path between the cuvette and the photomultiplier was 5 cm. All experiments were carried out at 20 °C.

Correlation between Gravimetric and Photometric Measurements

Tedeschi and Harris (1958) have shown that rat liver mitochondria undergo osmotically induced swelling-shrinkage cycles in accordance with the Boyle-van't Hoff equation:

$$V=\frac{k}{\sum c}+V_o \tag{1}$$

where V is the total volume, V_o is the so-called osmotic dead space, $\sum c$ is the sum of all osmotically active species in the external space, and k is a constant. According to Eq. (1), plotting the volume obtained from gravimetric measurements against the reciprocal of the osmolarity, a straight line should be obtained with the intercept giving the nonosmotic volume (Tedeschi & Harris, 1955).

Fig. 1 shows that a straight line is obtained, within a certain range of osmolarities, by plotting the volume against the reciprocal of the absorbance. The slope of Fig. 1

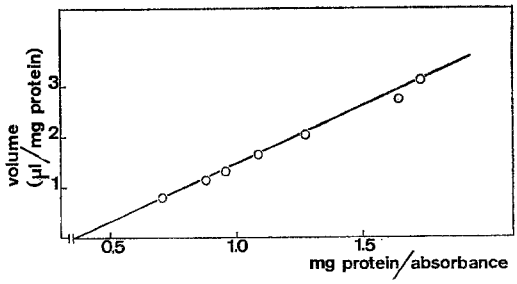

Fig. 1. Relationship between mitochondrial volume and absorbance. The incubation medium contained 2 µM rotenone, 2.5 mM Tris-Cl and variable concentrations of sucrose. The final pH was 7. Volume measurements were obtained from gravimetric measurement. The absorbance values were corrected for the changes of refractive index

was also constant in a range of protein concentration between 72 μg and 2.7 mg/ml. With the same mitochondrial preparation, the slope was not affected by replacing sucrose in the supporting medium with equivalent LiCl, NaCl, KCl or Tris-Cl. At 30 mosm the average volume per mg protein obtained from an average of 16 determinations on different mitochondrial preparations was 3.0 ± 0.5 μliters and the ratio mg protein/absorbance was 1.7 ± 0.2 (where 0.5 and 0.2 are mean root errors).

Special care was given to the problem of the variation of the refractive index *RI* of the medium. The absorbance of a mitochondrial suspension decreases 0.86% for an increase of ΔRI of 0.001 (Tedeschi & Harris, 1955, 1958), where ΔRI is the difference between the refractive index of the medium and that of water. The correction of the absorbance values for the changes of refractive index is therefore given by:

$$(\mathrm{Abs})_{\mathrm{corr}} = \frac{(\mathrm{Abs})_{\mathrm{obs}}}{1 - 8.6\Delta RI}$$

where $(\mathrm{Abs})_{\mathrm{corr}}$ is the corrected value of absorbance, and $(\mathrm{Abs})_{\mathrm{obs}}$ is the observed value.

In our study, there are two cases where corrections for the changes of refractive index may apply: (1) when a given amount of solute (a concentrated solution of KCl or sucrose) was added to the mitochondrial suspension to induce an osmotic shrinkage, and (2) when the mitochondria were suspended in media of variable osmolarity. In the former case, the maximal change of osmolarity was 80 mosm, this corresponding to a change of refractive index, and therefore of absorbance, which is negligible for KCl and almost negligible for sucrose. Furthermore, the change of absorbance, caused by the change of ΔRI, is much faster than that caused by the osmotic response of the particles. No correction was therefore introduced. In the latter case, suitable corrections were introduced when the change of absorbance caused by the change of ΔRI was appreciable.

Kinetic Measurements

The incubation was carried out in a cuvette equipped with a stirring device. The stirring rod was above the light beam and rotated at the maximal speed compatible with the lack of formation of air bubbles. Additions were made with a syringe. The absorbance changes were recorded on a Texas servoriter (pen speed, 0.4 sec, full scale; chart speed 20 cm per min). The mixing dead time for the instrument was determined with a dye. Full equilibration was obtained within 0.6 sec, which was satisfactory since only processes occurring at a linear rate for more than 0.6 sec were examined in the present study.

Calculation of the Rate and Amount of Water Translocation

The straight line of Fig. 1 satisfies the equation $y = ax + b$, where y = μliters/mg protein, x = mg protein/absorbance, and a is the slope. The rate v of water translocation in μliter sec^{-1}/mg protein is $\frac{dy}{dt} = a\frac{dx}{dt}$ where $dx = \frac{\text{mg protein}}{(\mathrm{Abs})^2}\, d(\mathrm{Abs})$.

The initial rate of water translocation is calculated from the slope of the tangent to the absorbance change trace. Therefore

$$v = \frac{\Delta y}{\Delta t} = a\frac{\Delta x}{\Delta t} = a\frac{\text{mg protein}}{(\mathrm{Abs})_1^2}\frac{\Delta(\mathrm{Abs})}{\Delta t} \tag{2}$$

where $(\mathrm{Abs})_1$ is the initial absorbance and $\frac{\Delta(\mathrm{Abs})}{\Delta t}$ is the absorbance change in the time interval Δt.

The amount q of water translocation in μliters/mg protein is given by Eq. (3):

$$q = a \frac{\Delta(\text{Abs})}{(\text{Abs})_1 \, (\text{Abs})_2} \times \text{mg protein.} \tag{3}$$

All the experiments reported in the present work were done by studying the shrinkage rather than the swelling phase because this required smaller volume changes of the medium.

Theory

We assume that the mitochondrial membrane behaves as semipermeable; i.e., it is permeable only to the molecules of the solvent (Tedeschi, 1961; Yoshida & Sato, 1968). This assumption is not affected by either (a) the presence of the anion translocation system in the membrane or (b) a slight leakiness of the membrane. In regard to (a), solutes not involving the operation of the ion translocation system were used. In regard to (b), the leakiness involves equilibration times several orders of magnitude greater than those investigated here. A relationship between fluxes and osmotic forces has been obtained by Cass and Finkelstein (1967) using the thermodynamics of irreversible processes. The classical diffusion equations also provide a means to derive the equation for the water fluxes:

$$v = P \cdot S(g_2 \sum c_2 - g_1 \sum c_1) = P \cdot S \, \Delta p \tag{4}$$

where v = rate of water efflux, in moles sec^{-1}/g protein; P = permeability coefficient, in cm sec^{-1}; S = surface, in cm^2/g protein; $\sum c$ = sum of the concentrations of external solutes, in moles cm^{-3}; and g = osmotic coefficients. The subscripts 1 and 2 indicate the initial and final state, respectively.

The osmotic change Δp is given by:

$$\Delta p = g_2 \sum c_2 - g_1 \sum c_1. \tag{5}$$

Strictly speaking, the term $\sum c$ should be expressed in molality. However, with low molecular weight compounds such as KCl or NaCl the concentration term can be expressed in molarity without introducing an appreciable error. With higher molecular weight compounds such as sucrose, replacement of molality with molarity involves a correction of 4.7% at 200 mM. However in the present study, concentrations higher than 100 mM have seldom been used. In this range the experimental errors are larger than those introduced through the molarity term.

The amount of water translocated is defined by:

$$q = V_1 - V_2 \tag{6}$$

where V_1 and V_2 are the initial and final mitochondrial osmotically active volumes.

The amount of osmotically active material inside the mitochondria m is constant during the osmotic change, and therefore we write:

$$\frac{m}{V_1} = g_1 \sum c_1$$

$$\frac{m}{V_2} = g_2 \sum c_2.$$

By expressing V_2 in function of V_1 we obtain:

$$V_2 = \frac{g_1 \sum c_1}{g_2 \sum c_2} V_1.$$

Then:

$$q = V_1 \left(\frac{g_2 \sum c_2 - g_1 \sum c_1}{g_2 \sum c_2} \right). \tag{7}$$

By combining Eqs. (5) and (7) we obtain:

$$q = V_1 \frac{\Delta p}{\Delta p + g_1 \sum c_1}. \tag{8}$$

Results and Discussion

The Permeability Coefficient to Water

According to Eq. (4), a plot of v *vs.* Δp, at constant initial osmolarity, should give a straight line. Fig. 2 shows that the rate of water translocation increased proportionally to the increase of the osmotic pressure of the external medium (up to a Δp of 150 mosm, not shown in the figure). The agreement with Eq. (4) at larger values of Δp could not be tested because of limitations of the measuring apparatus. Fig. 2 also shows that a plot of q *vs.* Δp gives a hyperbolic curve. This will be discussed in the next chapter.

In Eq. (4) there are two unknown terms: P and S. Determination of P, the permeability coefficient, is therefore dependent on the evaluation of the mitochondrial surface S. However, the dimension of the mitochondrial surface is not open to direct measurement. The assumption that the mitochondria are spherical can, on the other hand, be tested. Whittaker (1966) has reported electron micrographs where mitochondria change their shape from rods in 0.44 M sucrose to spheres in 0.25 M sucrose.

Fig. 3 shows the rate of water translocation after a constant Δp at variable osmolarity of the medium. The rate decreased, more markedly in

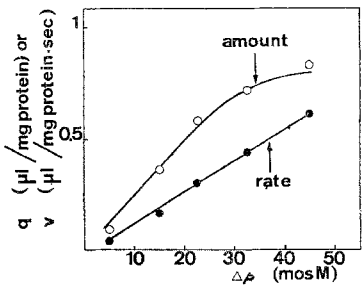

Fig. 2

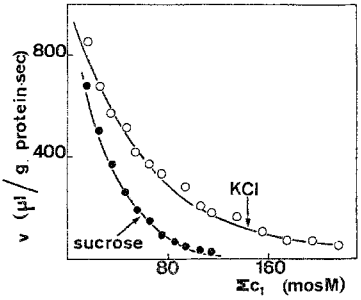

Fig. 3

Fig. 2. Rate and amount of water efflux after various changes of osmotic pressure. The incubation medium contained 2 μM rotenone, 0.1 mM EGTA, 2.5 mM Tris-Cl, pH 7 and 40 mM sucrose. Shrinkage of mitochondria was induced by addition of variable amounts of sucrose

Fig. 3. Rate of water efflux at variable osmolarity. The medium contained 2 μM rotenone, 2.5 mM Tris-Cl, pH 7 and variable concentrations of sucrose and KCl. The osmotic change Δp was caused by the addition of 50 mosm sucrose and 60 mosm KCl, respectively. The experimental points are means of four determinations of different preparations of mitochondria. ○—○, KCl; ●—●, sucrose

sucrose than in KCl, at the higher osmolarity. If the rate of water translocation is divided by the change of osmotic pressure Δp, the ratio $P \cdot S$ plotted *vs.* $V^{(2/3)}$, where V is the total volume of the mitochondria, should give a straight line if the mitochondria are spherical, provided that the permeability coefficient remains constant under the range of osmolarities studied.

Fig. 4 shows that the plot of $P \cdot S$ *vs.* $V^{(2/3)}$ gives a straight line in KCl as well as in sucrose, except at low osmolarities. The equation for this straight line may be written as:

$$P \cdot S = K_1 V^{2/3} - K_2 \tag{9}$$

where K_1 and K_2 are the experimental values of the slope and intercept. In Fig. 4 the lines do not pass through the origin indicating that the "osmotic" surface is zero when $V^{2/3}$ becomes equal to the nonosmotic volume.

The surface and the volume of 1 g protein of mitochondria are

$$S = N s_m \tag{10}$$

$$V = N v_m \tag{11}$$

where s_m and v_m are the surface and the volume of 1 mitochondrion. N is the number of mitochondria contained in 1 g protein, which is about

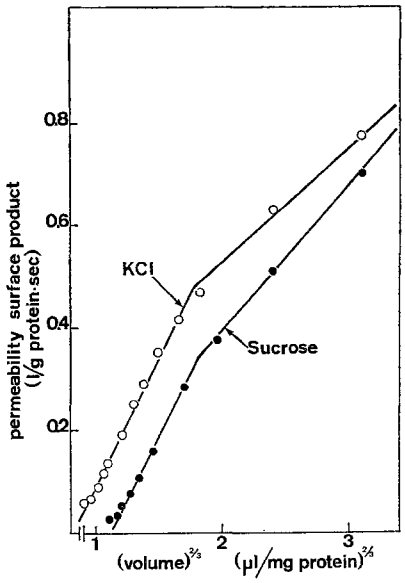

Fig. 4. Relationship between permeability surface product ($P \cdot S$) and mitochondrial volume. The points are obtained by dividing the rates of water efflux, (in moles of water $\times$ sec^{-1}/g protein $=$ 55.5 liters $\times$ sec^{-1}/g protein), from the data of Fig. 3, for the values of the osmotic change Δp (in osmoles/liter). The values of V are obtained from a titration curve of V *vs.* osmolarity

7.2×10^{12} (Estabrook & Holowinsky, 1961). The relation between volume and surface of a single spherical mitochondrion is:

$$v_m^{2/3} = 0.207 s_m. \tag{12}$$

By combining Eqs. (10)–(12) we have:

$$S = N \frac{v_m^{2/3}}{0.207} = \frac{N^{1/3}}{0.207} V^{2/3}. \tag{13}$$

Multiplying Eq. (13) by P, we have:

$$P \cdot S = P \frac{N^{1/3}}{0.207} V^{2/3} \tag{14}$$

which is similar to Eq. (9). We further write:

$$\frac{N^{1/3}}{0.207} P = K_1. \tag{15}$$

Therefore, by knowing the experimental value K_1 ($\sim$495 cm sec$^{-1} \times$ g protein$^{1/3}$) and $N^{1/3}$ (1.93×10 number of mitochondria $\times$ g protein$^{1/3}$)

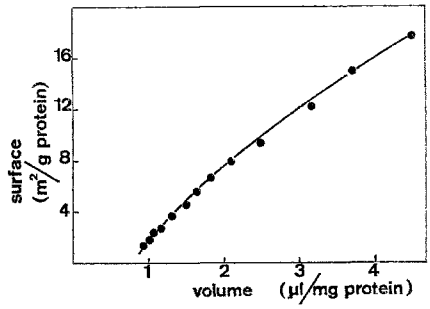

Fig. 5. Relationship between mitochondrial surface and volume. The points are obtained by dividing the values of the permeability surface product ($P \cdot S$), derived from the straight line of KCl in Fig. 4, for the permeability P

we obtain a value of P of nearly 5.3×10^{-3} cm sec^{-1}. Such a figure is within the range of values given by Müller and Rudin (1969) for the water permeability of the lipid bilayers (between 1.2×10^{-2} and 3.6×10^{-4} cm sec^{-1}). However, at variance from some artificial membranes, the permeability coefficient was not affected by replacing KCl with sucrose (Cass & Finkelstein, 1967). The permeability coefficient was also not affected by changes in the metabolic state of the mitochondria.

Fig. 5 shows the relationship between mitochondrial surface and volume. It is seen that the surface of mitochondria incubated in 0.10 M KCl is about 1 m^2/g protein. This figure may be compared with other theoretical estimates of the total mitochondrial surface which are 29 μ^2 per mitochondrion in the case of Lehninger (1965)[1], and 40 m^2/g protein in the case of Mitchell (1966). One possible reason for the discrepancy between the present measurements of the mitochondrial surface and the estimates of Mitchell (1966) may be due to the fact that the former is based on the amount of surface involved in the water flux whereas the latter is based on the assumption of a complete exposure of the cristae surface. It is possible that in 0.10 M KCl a large part of the cristae surface is folded and does not participate in the water translocation process. This suggestion is in agreement with our data for the mitochondrial surface at 10 mosm, where the cristae are probably completely unfolded (Stoner & Sirak, 1969), which is about 30 m^2/g protein. The increase of the total mitochondrial surface at decreasing osmolarity is in agreement with the hypothesis of an unfolding of the cristae parallel to the swelling of the mitochondria (Tedeschi, 1959; Stoner & Sirak, 1969).

1 By assuming 7.2×10^{12} mitochondria $\times$ g protein^{-1} (Estabrook & Holowinsky, 1961), the mitochondrial surface becomes 210 m^2/g protein in the case of Lehninger (1965).

Ultrastructural Rearrangement of the Membrane at Low Osmolarities

From Fig. 4 it appears that at low osmolarities there is no linear correlation between the term $P \cdot S$ and the mitochondrial volume function $V^{(2/3)}$. Fig. 6 shows that similar deviations from linearity were also apparent in the plot of the reciprocal of absorbance *vs.* reciprocal of osmolarity. The osmolarity range at which the deviation from linearity occurred, coincided with those of Fig. 4. These data are in agreement with those of Stoner and Sirak (1969) and therefore indicate that the deviation in Fig. 4 is due principally to changes in the absorbance values.

Tedeschi and Harris (1958) have suggested that deviations in linearity of light scattering at low osmolarity may be caused by membrane damage with leakage of internal solutes. Solute leakage during hypotonic treatment has also been suggested by Kaplan and Greenawalt (1966). Bangham, De Gier and Greville (1967) have observed a similar "break" in the absorbance of smectic mesophases at low osmolarities. However, plotting the volume of the mitochondria (determined *gravimetrically*) against $1/\sum c_1$, produces a straight line at low as well as at high osmolarities. Hence, the alterations observed must arise from the failure of light scattering to follow the expected function of volume at low osmolarities. This could result from stretching of the membrane causing rearrangement of membrane structure during the hypotonic treatment.

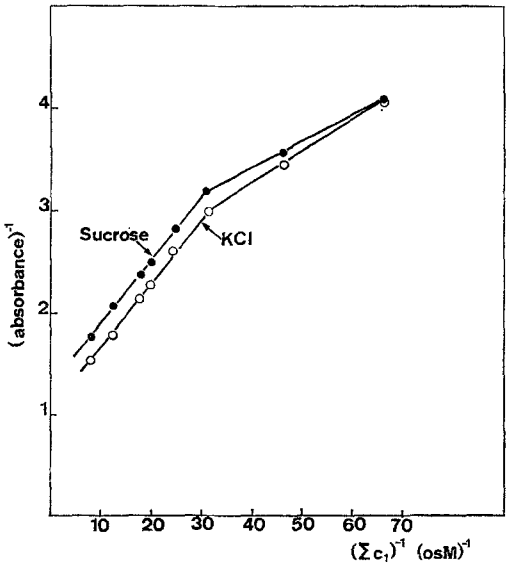

Fig. 6. Relationship between absorbance of mitochondria and osmolarity of the medium. The medium was identical to that of Fig. 3

Reversibility of the Osmotic Swelling

As shown in Fig. 2, the plot of q *vs.* Δp was hyperbolic. By inverting and then rearranging Eq. (8) we obtain:

$$\frac{V_1}{q} = g_1 \frac{\sum c_1}{\Delta p} + 1. \tag{16}$$

According to Eq. (16), a plot of $1/q$ *vs.* $1/\Delta p$, at constant osmolarity, should give a straight line. The intercept on the abscissa should give the reciprocal of the initial osmolarity; the intercept on the ordinate should give the reciprocal of the initial volume.

Fig. 7 shows a plot of $1/q$ *vs.* $1/\Delta p$. While at low KCl concentrations, i.e. 58 mosm, the osmometric behavior of mitochondria was in good agreement with Eq. (16); two types of deviations appeared in the data of Fig. 7: first, when the mitochondria were incubated at low osmolarity of sucrose, and second, when the mitochondria were incubated at high osmolarity either of sucrose or of KCl.

Consider first the deviation in sucrose. Mitochondria were incubated in 58 mM sucrose and the shrinkage was induced by the addition of variable amounts of sucrose. From Fig. 7 it appears that the shrinkage induced by sucrose was less than that predicted by Eq. (16), indicating an incomplete reversal of the hypotonic swelling after incubation in sucrose (Stoner &

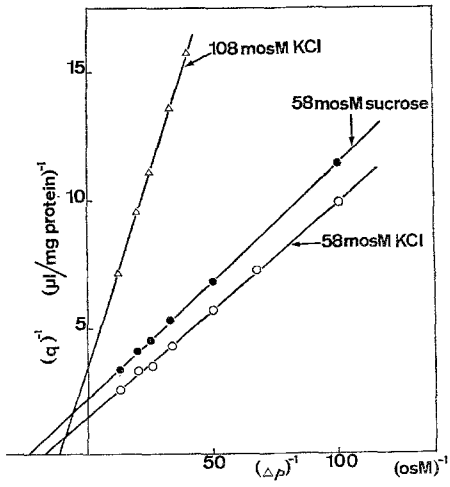

Fig. 7. Relationship between amount of water efflux and change of osmotic pressure. Mitochondria were incubated in 2 µM rotenone, 2.5 mM Tris-Cl, pH 7 and a constant concentration of sucrose or KCl. Shrinkage was induced through the addition of variable amounts of sucrose or KCl, respectively. The experimental points are means of four determinations of different preparations of mitochondria. o—o, 58 mosm KCl; ▵—▵, 108 mosm KCl; •—•, 58 mosm sucrose

Sirak, 1969). On the other hand, no deviation from Eq. (16) was observed when the mitochondria were incubated in KCl and the shrinkage induced by the addition of variable amounts of KCl. Parallel gravimetric measurements were in good agreement with the absorbance measurements either in sucrose or in KCl. Our interpretation of the incomplete reversibility is that of a partial modification of the osmotic properties of the membrane in the presence of sucrose. There is no support yet for this interpretation. It should be noted however that sucrose affects also the light-scattering properties of the membrane as indicated by the lower absorbance of the mitochondrial suspension in sucrose in respect to KCl, about 0.95% after correction for the change in ΔRI.

Consider now the deviation from Eq. (16) at high osmolarities. The data of Fig. 7 indicate that the shrinkage of mitochondria incubated in 108 mosm KCl and subjected to a KCl pulse was less than that predicted by Eq. (16).

To investigate the reversibility of the osmotic behavior of mitochondria in greater detail, the amount of water efflux was measured after a constant change of osmotic pressure at variable osmolarity. Eq. (16) was further rearranged by replacing $V_1 = k/\sum c_1$; Eq. (16) then becomes:

$$\frac{k}{q\sum c_1} = g_1 \frac{\sum c_1}{\Delta p} + 1. \tag{17}$$

According to Eq. (17) a plot of $1/(q\sum c_1)$ *vs.* $g_1 \sum c_1$, at constant Δp, should give a straight line. The intercept on the abscissa should give the osmotic change in the incubation medium, and that on the ordinate, the reciprocal of the slope of the van't Hoff line. Fig. 8 shows that the amount of water efflux following the pulse of KCl or of sucrose corresponded to the predictions of Eq. (17) only after incubation of the mitochondria in a rather narrow range of KCl concentrations. In KCl agreement with Eq. (17) is observed up to 90 mosm. At higher concentrations, linearity still obtains but a strong deviation from Eq. (17) appears. It is very interesting that the "break" in the plot corresponds to the disorganization of the outer mitochondrial membrane observed in the electron micrograph (Stoner & Sirak, 1969). The role of the phenomenon of the rupture of the outer membrane in affecting the osmotic reversibility of the mitochondria cannot yet be assessed. However, when the plot of Fig. 8 was carried out with mitochondria brought beyond the point of disorganization of the outer membrane by preincubation in 60 mosm sucrose, the experimental points were in good agreement with the predictions of Eq. (17) (*see* Fig. 9).

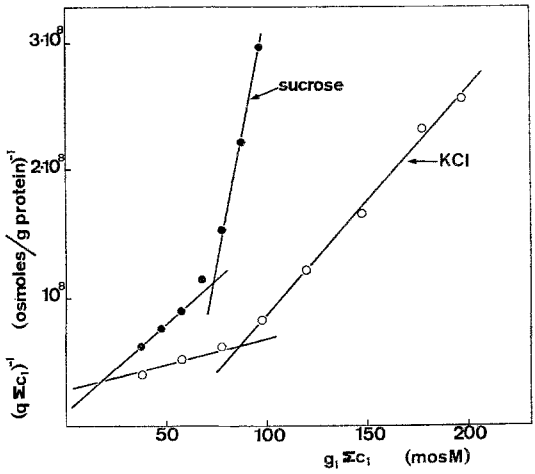

Fig. 8. Relationship between amount of water efflux and osmolarity of the medium. The experimental conditions were identical to those used in Fig. 3. The osmotic change Δp was 60 mosm KCl in the KCl and 50 mosm sucrose in the sucrose medium. ○—○, KCl; ●—●, sucrose

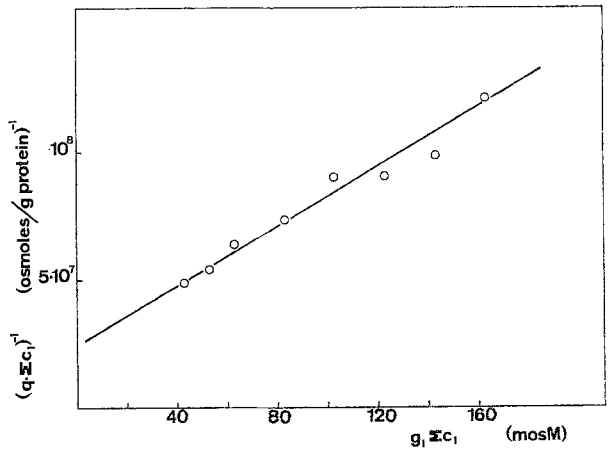

Fig. 9. Relationship between amount of water efflux and osmolarity of the medium in hypotonic treated mitochondria. The mitochondria were preincubated in 60 mosm sucrose and then transferred to a medium containing 2 μM rotenone, 2.5 mM Tris-Cl, pH 7 and variable KCl concentrations. The osmotic change Δp was 50 mosm KCl

Conclusion

The present data indicate that the mitochondrial membrane, as most other natural membranes, has a very high permeability to water. Therefore, the assumption that inner and outer mitochondrial spaces are always at osmotic equilibrium appears well founded (Rossi & Azzone, 1969).

The increase of mitochondrial volume, during hypotonic treatment, causes two effects. The first, after incubation at 80 to 100 mosm, involves the appearance of full osmotic reversibility which otherwise is not present in native mitochondria. Only after this hypotonic treatment the mitochondria behave as ideal osmometers. The second, after incubation at 20 to 30 mosm, involves large changes in the absorbance property of the membrane. The former is phenomenologically related with the rupture of the outer membrane, the latter with an almost complete unfolding of the cristae. The molecular mechanism underlying the changes of the inner membrane structure after mechanical stretching will be the subject of future investigations.

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