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# ION TRANSPORT BY HEART MITOCHONDRIA

## XIV. THE MANNITOL-IMPERMEABLE COMPARTMENT OF THE MITOCHONDRION AND ITS RELATION TO ION UPTAKE

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SUMMARY

1. The permeability of isolated heart mitochondria to mannitol and several other solutes has been examined using a dual isotope procedure. Mannitol enters a portion of the water volume of a centrifuged pellet of mitochondria strictly as a function of its concentration in the suspending medium. Sucrose, KCl, and a number of other solutes penetrate the mitochondrion to the same extent as does mannitol, but high molecular weight solutes such as dextran penetrate a considerably smaller volume. Certain permeant solutes such as glycerol and acetate penetrate to a greater extent than does mannitol.

2. The absolute and relative sizes of the mannitol-permeable and mannitol-impermeable volumes can be varied extensively as a function of the suspending medium. Hypotonic swelling and osmotic swelling which results from either energy-linked ion uptake or gramicidin-induced passive ion uptake cause large increases in the mannitol-impermeable space of the mitochondrion.

3. The size of the mannitol-permeable space does not increase as a function of time in either isotonic or hypotonic media. These results are compatible with earlier suggestions that mitochondria contain two aqueous compartments, one permeable to solutes such as mannitol and sucrose and one which excludes these solutes. In addition, the osmotic barrier to mannitol penetration appears to be the site of both active and passive salt uptake.

## INTRODUCTION

Studies of the penetration of solutes into isolated mitochondria indicate that a portion of the mitochondrial water is readily penetrated by solutes of low molecular weight, whereas another portion of the mitochondrial water volume appears not to be penetrated and to respond as an osmometer to such solutes[1–8]. It has been suggested that this apparent compartmentation of mitochondrial water results from permeabil-

Abbreviations: EGTA, ethyleneglycol-bis-($\beta$-aminoethylether)-*N*,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

ity differences between the outer and the inner membranes of the mitochondrion[1,3,6]. This interpretation that the inner membrane is the site of the osmotic response is strongly supported by the studies of PARSONS *et al.*[9]. These authors utilized osmotic swelling of the inner membrane to fracture the outer membrane, and after osmotic contraction of the inner portion, were able to separate the two membrane components. Since the available evidence favors the interpretation that mitochondrial cristae are infoldings of the inner membrane (see ref. 10, for example), the aqueous space between the inner and the outer membrane has been considered to provide a morphological explanation of the solute-permeable mitochondrial water compartment (*cf.* discussions in ref. 6). On the other hand, TEDESCHI[11] has presented evidence which can be explained on the basis of a slow penetration of solute into a single compartment of variable size, and other authors[12] consider that the mitochondrion is completely permeable to salts.

It is well known that the volume of the apparent compartment which is not penetrated by a solute such as sucrose can be varied extensively as a function of the osmolarity of the suspending medium[1–8]. It has also been established that the morphology of the mitochondrion can be varied by altering the tonicity of the suspending medium[1,13,14]. It therefore follows that if the explanation for the apparent compartmentation of mitochondrial water rests entirely in the differential permeability of the inner and the outer membrane, then simultaneous comparison of the solute-permeable water space and the morphology of the mitochondrion under various conditions should result in parallel changes in both parameters. The present study examines the penetration of mannitol into the isolated heart mitochondrion. This solute distributes in a way similar to sucrose and has some advantages when measuring permeability in mitochondria which are suspended in sucrose solutions. The accompanying study[15] examines the morphology of these particles as a function of their solute content. The results are quite consistent with the interpretation that the apparent compartmentation of mitochondrial water results from the permeability of the outer membrane to mannitol combined with the relative impermeability of the inner membrane to this solute. While these studies were in preparation the reports of PFAFF *et al.*[16] and BUTLER AND JUDAH[17] appeared. These authors have reached similar conclusions from simultaneous comparisons of solute permeability and the morphology of liver and kidney mitochondria.

## METHODS

Beef heart mitochondria were prepared by a modification of the Nagarse procedure of HATEFI *et al.*[18], which substitutes ethyleneglycol-bis-(β-aminoethyl-ether)-*N*,*N*′-tetraacetic acid (EGTA) for EDTA[19].

The permeability of isolated mitochondria to a solute such as $^{14}$C-labeled mannitol was estimated by the following modification of a procedure first described by MALAMED AND RECKNAGLE[2]. Mitochondria were incubated in the presence of the labeled solute under conditions which are specified for the individual experiments reported. The mitochondria were recovered by centrifugation in a Sorvall SE-12 rotor for 2–5 min at 20000 rev./min. Supernatants were decanted and the tubes carefully blotted dry. Residues (usually 10 or 15 mg of protein) were then extracted with 0.5 ml of 1.0 M $HClO_4$, diluted with an additional 0.5 ml of water, and the

denatured protein was removed by centrifugation. The radioactivity of duplicate 0.2-ml samples of this extract was then determined by liquid scintillation spectrometry in 10 ml of BRAY's medium[20] using a Packard instrument. In order to count the supernatant solutions under the same conditions as the residues, these solutions were diluted in 0.5 M $HClO_4$ to approximately the same activity as the mitochondrial extracts and 0.2-ml samples were counted. The amount of water in the pellet in equilibrium with the solute of the supernatant was calculated from the following expression:

$$\mu\text{l mitochondrial water in equilibrium with supernatant [}^{14}\text{C]mannitol} = \frac{\text{total disint./min [}^{14}\text{C]mannitol in pellet}}{\text{disint./min [}^{14}\text{C]mannitol in supernatant per } \mu\text{l of supernatant solution}}$$

The total water of the pellet was determined in our earliest studies by a gravimetric procedure using a second identical incubation. The procedure suffers a number of disadvantages including, (a) rather poor reproducibility, (b) the requirement for a second incubation of all experimental points, and (c) the fact that the [$^{14}$C]mannitol water and the total water are not measured in the same tube. In addition, it was found that polycarbonate tubes lose weight on drying and that this weight loss can significantly affect gravimetric water estimation unless an additional correction is made. Equilibration of the mitochondria with tritiated water was therefore investigated as an alternative method for estimating the total water content of the mitochondrial pellet. Fig. 1 compares the water content of mitochondrial pellets of increasing size as estimated by the gravimetric and by the tritiated water procedures. The total water was calculated by comparison of the radioactivity of the residue and supernatant as described above for mannitol. The two methods show quite acceptable agreement.

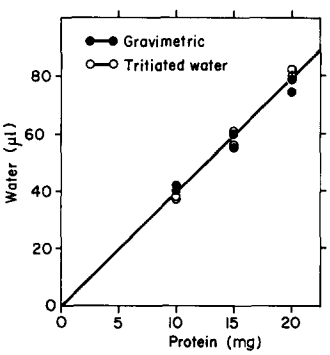


Fig. 1. Comparison of mitochondrial water volume as estimated by the gravimetric and the tritiated water procedures. The indicated amount of beef heart mitochondria was suspended for 5 min at 0–4° in 6 ml of a medium of sucrose (0.25 M) and Tris–HEPES (3 mM, pH 6.9). After centrifugation in a Sorvall SE-12 rotor (5 min at 20000 rev./min) the supernatants were decanted and the tubes carefully blotted dry. For the gravimetric determinations the tubes were weighed immediately and again after drying to constant weight at 100°. Tubes for the tritiated water assay contained $4 \cdot 10^6$ disint./min tritiated water. The residues from these tubes were extracted with 0.5 ml of 1 M $HClO_4$ followed by 0.5 ml of $H_2O$ and the radioactivity of 0.2-ml sample was determined by liquid scintillation spectrometry in 10 ml of BRAY's solution. The supernatants were diluted ten fold with 0.5 M $HClO_4$ and identical samples counted. The water content of the pellet was then calculated based on disint./min per $\mu$l of supernatant. The average water content was identical as estimated by the two procedures (3.9 $\mu$l/mg of protein).

Use of tritiated water to estimate the total water of the pellet permits simultaneous comparison of the total water and the water penetrated by a $^{14}C$-labeled solute by using both labels in the same incubation tube. When this modification was applied, $2 \cdot 10^6$ disint./min of tritiated water and $1.1 \cdot 10^5$ disint./min of a $^{14}C$-labeled solute were included in the incubation. The isotope ratio was found to give minimum error under our experimental conditions. The discriminator and gain settings were established for simultaneous estimation of these isotopes under our conditions of sample preparation and the disint./min of $^{14}C$ and $^{3}H$ were calculated by the simultaneous equation method of OKITA *et al.*[21]. The total water of the pellet and the water penetrated by solute were then calculated as described above. Total water and penetrated water established by this method agree well with the comparable values from parallel incubations with single isotopes (Table I). The penetration of $^{36}Cl$ into the mitochondrion was estimated in a similar manner. $K^+$ and $Na^+$ content of the isolated mitochondria was determined by atomic absorption spectroscopy of appropriate dilutions of the perchloric acid extracts.

All labeled reagents were purchased from New England Nuclear Corporation.

## RESULTS

### *Penetration of mannitol into the mitochondrion compared to other solutes*

The data presented in Table I establish that mannitol penetrates about 2.5 $\mu$l of the 3.7–3.9 $\mu$l of water per mg of protein found in a centrifuged pellet of beef heart mitochondria. Carboxydextran of 60000–90000 mol. wt. penetrates only about 1.0 $\mu$l of water under these conditions. If it is assumed that the dextran space of these pellets accurately estimates the extraparticulate suspending medium which is occlud-

TABLE I

COMPARISON OF SINGLE-LABEL, DOUBLE-LABEL AND GRAVIMETRIC PROCEDURES FOR ESTIMATION OF TOTAL WATER AND MANNITOL-PERMEABLE WATER OF ISOLATED MITOCHONDRIA

Mitochondria (10 mg of protein) were incubated for 3 min at 25° in a medium of sucrose (0.25 M), Tris–*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Tris–HEPES) (5 mM, pH 7.0), and mannitol (8 mM). Where indicated tritiated water and $^{14}C$-labeled mannitol were included. The mitochondria were centrifuged, extracted, and the radioactivity determined as described in the legend for Fig. 1. The total water and mannitol-permeable water were calculated as described in the text. The values ($\mu$l water/mg protein) tabulated are the means of duplicate counting vials of duplicate incubations with the range indicated. The percent of the intramitochondrial water (line D) which is permeable to mannitol is indicated in parentheses (line F) as is the percent which excludes this solute (line E).

| *Method:* | *Gravimetric* | *Single label* | *Dual label* |
|---|---|---|---|
| *Experimental* | | | |
| A. Total water | 3.88 ± 0.10 | 3.70 ± 0.04 | 3.75 ± 0.15 |
| B. Mannitol-permeable | — | 2.55 ± 0.10 | 2.53 ± 0.06 |
| C. Dextran-permeable | — | 1.10 ± 0.01 | 1.00 ± 0.10 |
| *Calculated* | | | |
| D. Intramitochondrial water (A−C) | | 2.60 | 2.75 |
| E. Mannitol-impermeable (A−B) | | 1.15 (44%) | 1.22 (44%) |
| F. Intramitochondrial mannitol-permeable (D−E or B−C) | | 1.45 (56%) | 1.53 (56%) |

ed in the pellets, then one can subtract the dextran-permeable water volume from the total water to obtain the intramitochondrial water volume[3] (Line D of Table I).

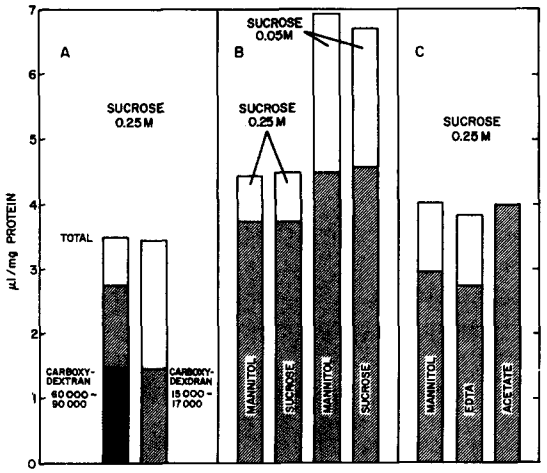


Fig. 2. Penetration of solutes into isolated beef heart mitochondria. The experiments were carried out by the dual-lable procedure as described in the text. A. The penetration of mannitol compared to that of carboxydextran of 60000–90000 mol. wt. and carboxydextran of 15000–17000 mol. wt. B. Comparison of sucrose and mannitol penetration into mitochondria suspended in isotonic (0.25 M sucrose) and hypotonic (0.05 M sucrose) media. C. Comparison of the penetration of mannitol, EDTA, and acetate into mitochondria suspended in 0.25 M sucrose containing 3 mM Tris–HEPES buffer, pH 7.1, 0.5 mM Tris EDTA, and potassium acetate (3 mM).

Under the conditions of the experiments described in Table I it can be seen that 1.5 µl (or 56 %) of the intramitochondrial water volume is permeable to mannitol whereas the remainder (1.2 µl or 44 %) excludes this solute. Carboxydextran of 15000–17000 mol. wt. distributes in the way as the higher molecular weight dextran in these experiments (Fig. 2A). The study shown in Fig. 2B is typical of a number of studies which establish that sucrose and mannitol penetrate the mitochondrion to the same extent in both isotonic and hypotonic media. It should be noted that the sucrose and mannitol-permeable volume of a mitochondrial pellet is subject to somewhat greater fluctuation from preparation to preparation than is the mannitol-impermeable space. The preparation shown in Table I, for example, has a mannitol-permeable space of 2.5 µl/mg, whereas that of Fig. 2B has a corresponding value of 3.7 µl/mg. The mannitol-impermeable spaces of these preparations were 1.2 and 0.8 µl/mg, respectively. A similar fluctuation of the sucrose-permeable volume of liver mitochondria has also been reported by PFAFF *et al.*[16].

A number of other low molecular weight molecules which are usually considered 'non-permeants' penetrate the mitochondrial water volume to nearly the same extent as sucrose and mannitol under similar conditions. These solutes include EDTA (Fig. 2C), AMP, and KCl (Fig. 3). The penetration of EDTA into the mitochondrial water volume is a complicated process and appears to be related to pH, the energy status of the mitochondrion, and the composition of the suspending medium[22]. The value shown in Fig. 2 is for penetration of EDTA in the absence of metabolism in a medium of cold, buffered (pH 7.0) 0.25 M sucrose.

TABLE II

CHANGES IN MANNITOL-IMPERMEABLE WATER WITH OSMOTIC SWELLING

Mitochondria (15 mg of protein) were suspended in 6 ml of either 0.12 M $NH_4Cl$ or 0.12 M ammonium acetate containing tritiated water and [$^{14}$C]mannitol (8 mM) for 5 min at 25°. A parallel incubation contained [$^{14}$C]dextran in place of [$^{14}$C]mannitol. The mitochondria were isolated by centrifugation, extracted, and the total water, mannitol-permeable and dextran-permeable water spaces determined as described in the text. Values are $\mu$l water/mg protein.

| | *Suspending medium* | |
|---|---|---|
| | *$NH_4Cl$* | *Ammonium acetate* |
| *Experimental* | | |
| A. Total water | 4.52 | 11.38 |
| B. Mannitol-permeable | 2.92 | 4.07 |
| C. Dextran-permeable | 1.40 | 2.62 |
| *Calculated* | | |
| D. Intramitochondrial water (A−C) | 3.12 | 8.76 |
| E. Mannitol-impermeable (A−B) | 1.60 (51%) | 7.31 (83%) |
| F. Intramitochondrial mannitol-permeable (B−C) | 1.52 (49%) | 1.45 (17%) |

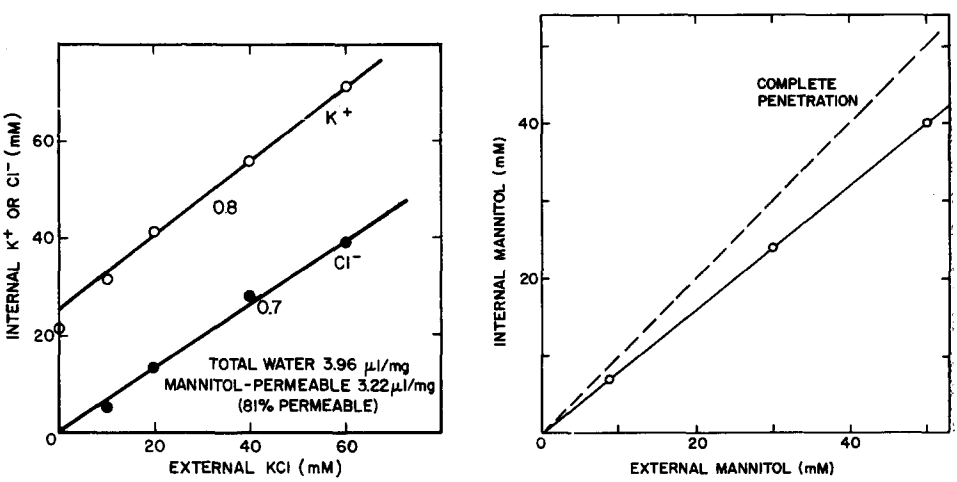


Fig. 3. Penetration of $K^+$ and $Cl^-$ into beef heart mitochondria. Mitochondria (10 mg of protein) were incubated for 5 min at 25° in a medium consisting of the indicated amount of KCl, Tris–HEPES (7 mM, pH 6.9) and sucrose to bring the final osmolarity to 0.25 osM. Tritiated water and [$^{14}$C]mannitol (8 mM) were included in one set of tubes and $K^{36}Cl$ was added to a parallel set of incubations. The mitochondria were isolated by centrifugation for 5 min at 20000 rev./min in a Sorvall SE-12 rotor, extracted with $HClO_4$, and the radioactivity and $K^+$ content determined. The average total water of all pellets was 3.96 $\mu$l/mg, and the average mannitol-permeable water volume was 3.22 $\mu$l/mg (81% permeable to mannitol). The $K^+$ permeability as evaluated from the slope of the plot shown was 80%; the $Cl^-$ permeability estimated in the same way was 70%.

Fig. 4. Mannitol content of centrifuged mitochondria as a function of the concentration of mannitol in the suspending medium. The experiment was carried out using the dual isotope procedure as described in the text. The mitochondria were suspended in a medium consisting of the indicated concentration of labeled mannitol, Tris–HEPES (7 mM, pH 7.0), and sucrose to give a final osmolarity of 0.25.

Evaluation of the extent of $K^+$ penetration is complicated by the presence of endogenous $K^+$ which is probably localized in the sucrose (or mannitol)-impermeable water volume[8] and by the tendency of the mitochondrial membrane to adsorb $K^+$ and other cations. In the experiment shown in Fig. 3 the KCl content of the suspending medium was increased, while the tonicity was maintained constant by varying the sucrose concentration. The $K^+$ content of the isolated pellets increases as a straight-line function of the $K^+$ concentration of the suspending medium. The slope of the line obtained should therefore give the degree of penetration of the pellet water volume by $K^+$ and the extrapolated intercept of such a plot should provide an estimate of the adsorbed and endogenous $K^+$. Since the endogenous $K^+$ is readily evaluated (83 nmoles/mg in this particular preparation) the difference between the extrapolated value and the endogenous $K^+$ (a difference of 16 nmoles/mg) gives an indication of the degree of absorption of this cation by the membrane under these conditions. In the studies shown in Fig. 3 the total water content of the pellets averaged 3.96 $\mu$l/mg and did not change as a function of the external KCl. Of this total, 3.22 $\mu$l/mg (81 %) was penetrated by mannitol. The slope of the $K^+$ penetration plot shows that 80 % of the mitochondrial water is in equilibrium with the suspending medium under these conditions. Evaluation of the permeability to $Cl^-$ by $^{36}Cl$ shows that about 70 % of the pellet water is also in equilibrium with this ion under these conditions. Mannitol, like KCl and the other solutes tested, enters the mitochondrion as a function of its concentration in the suspending medium (Fig. 4) and can be quantitatively removed by washing the particles in isotonic sucrose or KCl.

These data are consistent with the presence of 2 permeability barriers in a pellet of centrifuged mitochondria, one which excludes high molecular weight substances such as the dextrans and a second which excludes low molecular weight solutes. A third class of solutes is known to penetrate nearly the entire water volume of the pellet. Examples of this type of penetration include glycerol (which distributes into more than 90 % of the mitochondrial water in a time-dependent reaction) and acetate (Fig. 2C).

*Variation of mannitol-impermeable water volume with osmolarity*

When mitochondria are suspended in hypotonic sucrose or KCl a rapid osmotic swelling occurs[23,24] with expansion of the mannitol-impermeable water volume, but little change in the mannitol-permeable space (Fig. 5). This observation is in agreement with previous reports regarding expansion of the sucrose-impermeable water volume in hypotonic media[1–8]. As noted before (Fig. 2) the two solutes, sucrose and mannitol, penetrate mitochondria swollen in hypotonic media to the same extent. The penetration of mannitol into mitochondria occurs at a rate which is too rapid to be estimated by conventional centrifuge techniques. Studies of the penetration of this solute over rather extended periods of time at 0–4° are compatible with the suggestion that mannitol enters a mitochondrial compartment at a rapid rate and then is effectively excluded from the remaining water volume for periods of 30 min or more. Similar results are obtained when the rate of penetration of mannitol into hypotonically swollen mitochondria (in which the volume of water which excludes mannitol is considerably greater than in isotonic media) is estimated (Fig. 6).

Additional evidence in favor of the 2-compartment hypothesis comes from studies in which large osmotic volume changes are first induced in the absence of

labeled mannitol, and then a pulse of labeled solute is added just prior to centrifugation. The mannitol-permeable space of mitochondria suspended in isotonic and hypotonic media measured in this manner is indistinguishable from that measured in studies such as that shown in Fig. 6. Similar results are obtained with pulse-label studies and studies of the rate of entrance of sucrose, dextran, and EDTA. Glycerol, however, penetrates a larger and larger portion of the mitochondrial water as the incubation proceeds.

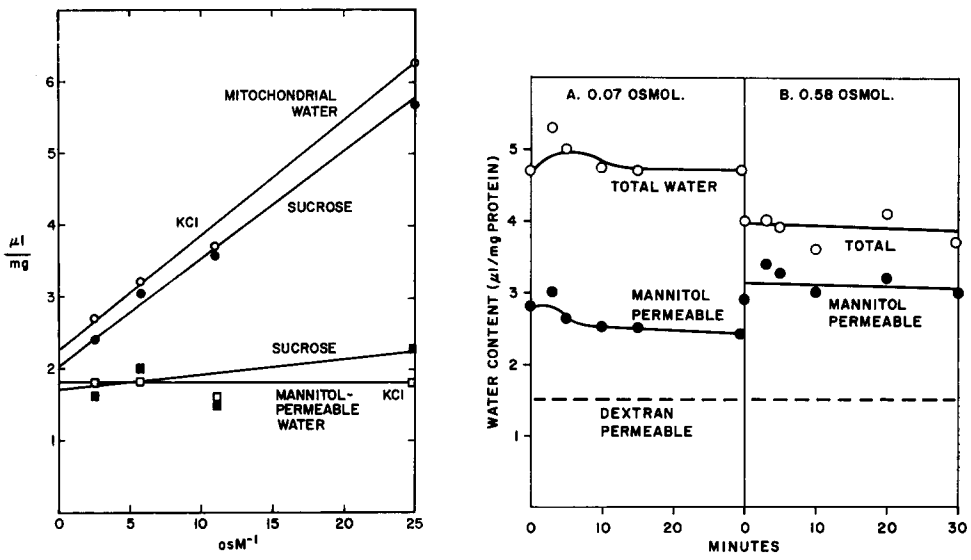


Fig. 5. Mannitol-permeable and total water volumes of isolated mitochondria as a function of decreasing osmolarity of the suspending medium. The open symbols show the total water and mannitol-permeable water volume of mitochondria isolated from the indicated concentration of KCl; the closed symbols the same data obtained in the presence of sucrose in place of KCl.

Fig. 6. Penetration of mannitol into heart mitochondria as a function of time of incubation and osmolarity. Heart mitochondria (15 mg of protein) were suspended in 3 ml of either 0.05 M sucrose (A) or 0.55 M sucrose (B). [$^{14}$C]Mannitol (25 mM) and tritiated water were added and the mitochondria isolated by rapid centrifugation at the indicated times. The dextran-permeable space was estimated from parallel incubations.

*Ion uptake and the mannitol-permeable space of the mitochondrion*

Mitochondria suspended in isotonic ammonium acetate swell rapidly and extensively in the absence of a source of energy due to the ready permeability of the membrane to $NH_4^+$ and to acetate$^-$ (probably in the un-ionized form)[23]. A corresponding swelling does not occur in isotonic $NH_4Cl$ due to the failure of $Cl^-$ to penetrate, and the resulting lack of a counter ion for charge neutralization[23]. The data summarized in Table II establish that the osmotic swelling which accompanies the uptake of $NH_4^+$ and acetate$^-$, like hypotonic swelling, results in a marked increase in the mannitol-impermeable space of the mitochondrion. The intramitochondrial mannitol-permeable space remains virtually the same as unswollen mitochondria suspended in $NH_4Cl$ (Table II). [$^{14}$C]Acetate equilibrates with the entire water volume of the pellet under these conditions (data not shown).

A response similar to the osmotic swelling in ammonium acetate is found in isotonic sodium acetate. It has been noted that $Na^+$ and acetate enter the mitochondrion and result in osmotic swelling in the absence of a source of energy in this medium[19]. The data of Fig. 7 show that total mitochondrial water and the $Na^+$ content of the centrifuged pellets increase in a parallel manner as a function of time when mitochondria are incubated in isotonic sodium acetate in the absence of an energy source. The mannitol-permeable space remains essentially constant during this incubation, however. If the water content of the pellets is predicted based on the observed $Na^+$ content of the pellets and assuming osmotic swelling (entire pellet water volume in equilibrium with 0.12 M sodium acetate) the curve obtained is virtually superimposable with the observed values for total water (Fig. 7).

Swelling and ion uptake do not occur in isotonic potassium acetate in the absence of a source of energy[19,23]. Mitochondria isolated by centrifugation from 0.12 M potassium acetate in the absence of energy contain about the same total water and mannitol-permeable water volume as preparations suspended in 0.25 M sucrose (Table III). The observed $K^+$ content under this condition is in close agreement with the value predicted from the sum of the endogenous $K^+$ (0.12 $\mu$moles/mg) and the penetration of 0.12 M potassium acetate into 3.1 $\mu$l/mg of mannitol-permeable water (0.37 $\mu$mole/mg). The sum of these values is 0.49 $\mu$mole/mg whereas the observed value is 0.53 $\mu$mole of $K^+$/mg. An energy-linked uptake of $K^+$ and acetate occurs in this medium[19], however, and the swelling which accompanies this energy-dependent ion uptake, like that seen in the presence of passive ammonium and sodium acetate uptake, results in an increase in the mannitol-impermeable space with little change in the mannitol-permeable volume (Table III). The $K^+$ content of the isolated pellets

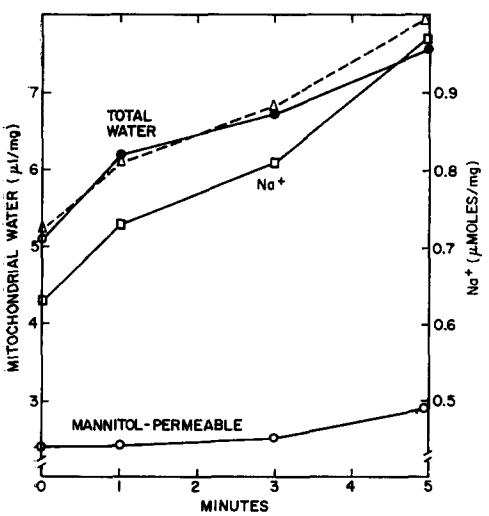


Fig. 7. Passive swelling and $Na^+$ accumulation as a function of time of incubation. Mitochondria (10 mg of protein) were treated with rotenone and oligomycin to suppress endogenous energy sources and incubated in 0.12 M sodium acetate containing Tris–acetate (7 mM, pH 7.0), 16 mM sucrose (added with the mitochondria), $^{14}C$-labeled mannitol (8 mM) and tritiated water. The mitochondria were isolated by centrifugation, extracted with $HClO_4$, and the radioactivity and $Na^+$ content estimated as described in the text. Also shown (dashed line) is the water content of the pellets as calculated from the $Na^+$ data with the assumption that the swelling is the result of the uptake of sodium acetate at 0.12 M (osmotic swelling).

also increases nearly 2-fold, and the observed value (1.0 $\mu$mole of $K^+$/mg) corresponds reasonably well to that predicted for complete equilibration of the entire mitochondrial pellet water volume with 0.12 M $K^+$ (7.6 $\mu$l/mg·0.12 M = 0.91 $\mu$mole $K^+$/mg). Extensive swelling of mitochondria suspended in isotonic potassium acetate can be induced in the absence of energy by the addition of reagents which increase the permeability of the membrane to $K^+$. We have previously noted that the combination of gramicidin and CCCP is particularly effective in this regard[19]. Mitochondria treated in this way swell extensively, take up $K^+$ and acetate, and show marked increase in the mannitol-impermeable space without a corresponding change in the mannitol-permeable volume (Table III).

TABLE III

INCREASE IN MANNITOL-IMPERMEABLE SPACE OF HEART MITOCHONDRIA WITH ACTIVE AND PASSIVE ACCUMULATION OF $K^+$

Beef heart mitochondria (10 mg of protein) were incubated for 3 min at 25° in 6 ml of a medium of either sucrose (0.25 M), sucrose (0.05 M), KCl (0.12 M), or potassium acetate (0.12 M). All incubations contained Tris buffer (3 mM, pH 6.9), and the $K^+$ media also contained sucrose (17 mM) added with the mitochondria. Each of the incubation tubes also contained tritiated water ($4 \cdot 10^6$ disint./min), [$^{14}$C]labeled mannitol (8 mM, $2 \cdot 10^5$ disint./min), and, where indicated, rotenone (0.7 $\mu$g/mg), CCCP (3 $\mu$M), and gramicidin (3 $\mu$M). The mitochondria were then isolated by centrifugation (Sorvall SE-12 rotor, 20000 rev./min for 5 min) and the supernatants decanted. The residues were blotted dry and extracted with 1.0 ml of 0.5 M $HClO_4$. Duplicate aliquots of this extract were counted in 10 ml of BRAY's medium[20] in a liquid scintillation counter. The $^3$H and $^{14}$C content were calculated from internal standards using a simultaneous equation procedure[21]. Other aliquots were diluted and the $K^+$ content estimated by atomic absorption spectrometry. The $K^+$ values reported are uncorrected for occluded and adsorbed $K^+$ (see the discussion in the text). The supernatants were diluted with 0.5 M $HClO_4$ to approximately the same counting rate as the residue and analyzed in the same way. The total water of the residue was calculated by comparing the specific activity of the supernatant ($^3$H disint./min per $\mu$l of water) with the total $^3$H radioactivity of the residue. The mannitol-permeable water was calculated by comparing the $^{14}$C radioactivity of the residue with the specific activity of the supernatant ($^{14}$C disint./min per $\mu$l). The mannitol-impermeable water was calculated by subtracting the mannitol-permeable water from the total water.

| *Expt. No.* | *Suspending medium* | *Total water* ($\mu$l/mg) | *Mannitol-permeable* ($\mu$l/mg) | *Mannitol-impermeable* ($\mu$l/mg) | *$K^+$ content* ($\mu$mole/mg) |
|---|---|---|---|---|---|
| 1 | Sucrose (0.25 M), rotenone | 4.08 | 3.29 | 0.79 | 0.12 |
| 2 | Sucrose (0.05 M), rotenone | 6.32 | 4.05 | 2.27 | — |
| 3 | Potassium acetate (0.12 M), rotenone | 4.34 | 3.10 | 1.24 | 0.53 |
| 4 | Potassium acetate, rotenone, gramicidin, CCCP | 7.84 | 3.32 | 4.52 | 0.99 |
| 5 | Potassium acetate, respiration | 7.59 | 3.94 | 3.65 | 1.00 |
| 6 | KCl (0.12 M), rotenone | 4.10 | 3.06 | 1.04 | 0.55 |
| 7 | KCl, rotenone, gramicidin, CCCP | 4.17 | 3.24 | 0.93 | 0.58 |
| 8 | KCl, respiration | 4.02 | 3.19 | 0.83 | 0.54 |

In the presence of isotonic KCl, however, both active and passive uptake of $K^+$ by the mitochondria is limited by the inability of $Cl^-$ to penetrate beyond the mannitol permeability barrier. In KCl media no swelling occurs in the presence of energy, and no passive swelling occurs in the presence of gramicidin and CCCP[19]. In neither case is there an increase in the $K^+$ content or in the mannitol-impermeable water volume (Table III).

## DISCUSSION

The present communication in conjunction with the report of HARRIS AND VAN DAM[25] establishes that a dual-isotope distribution procedure can be used to give a simultaneous estimate of total water and solute-permeable water in a centrifuged pellet of mitochondria. The method appears to be generally applicable to a number of isotopically labeled solutes and has been applied in this laboratory for studies of compartmentation in other organelles as well[26]. Previous estimates of permeability of isolated heart mitochondria to solutes have been calculated based on the amount of $^{14}C$-labeled solute which can be recovered in a mitochondrial pellet in excess of the dextran-permeable space[3]. The validity of estimates using dextran can be verified by comparing the mannitol-impermeable water space as estimated by the isotopic dilution procedure used to obtain the data of Table I (total water less the mannitol-permeable water) with the corresponding value calculated as described by O'BRIEN AND BRIERLEY[3]. The value for mannitol-impermeable water obtained as described in Table I (independent of dextran) is about 1.2 $\mu$l/mg and the corresponding value obtained using the dextran space as a correction for occluded suspending medium in the pellet is also 1.2 $\mu$l/mg.

If one subtracts the dextran-permeable water space from the total water of the mitochondrial pellet a calculated value of 2.65 $\mu$l of intramitochondrial water per mg of protein is obtained. Of this intramitochondrial water 1.53 $\mu$l/mg (56 %) appears to be permeable to mannitol. This value is considerably in excess of the 30–39 % penetration by sucrose observed by O'BRIEN AND BRIERLEY[3]. However, it must be noted that the earlier study used beef heart mitochondria prepared by the method of HATEFI and LESTER[27], a procedure which employs a blender treatment in the presence of potassium phosphate buffer and the absence of chelators, whereas the present study uses mitochondria prepared by Nagarse treatment in the presence of Tris buffer and EGTA[19]. As has been pointed out in this study, the ionic environment and tonicity of the suspending medium both have a marked effect on the ratio of the mannitol(or sucrose)-permeable space to the mannitol-impermeable space.

The present study also supplements the impressive body of evidence already in the literature which suggests that isolated mitochondria consist of at least two aqueous compartments, one which readily admits low molecular weight solutes such as sucrose, mannitol, and KCl, and one which excludes these solutes[1–8,16]. Evidence in favor of this concept includes the following points:

(a) A number of widely different solutes enter the mitochondrial water volume to the same extent. In the present work KCl, EDTA, mannitol, and sucrose have been shown to enter the identical water volume when measured either simultaneously or in parallel experiments. In addition PFAFF *et al.*[6,16] have reported that aspartate, sucrose, and adenine and pyridine nucleotides penetrate mitochondria to the same extent in simultaneous measurements. The failure of high molecular weight solutes such as the dextrans to penetrate the mitochondrial water volume to this extent would be explained by the presence of the outer envelope of the mitochondrion, a membrane which appears to exclude only rather large molecules. PFAFF *et al.*[16] have indicated that molecules with a maximum mol. wt. of 5000–12000 are unable to cross the outer membrane. This value is in line with the results of the present study. Solutes with appreciable lipid solubility (such as acetate, glycerol and others) appear to cross the

inner membrane, since they cause osmotic swelling[24] and penetrate the mitochondrial water volume to a substantially greater extent than mannitol.

(b) Penetration of a solute such as mannitol is essentially independent of the time of incubation. Similar degrees of penetration are recorded in both pulse-labeling experiments on preparations in which the size of the compartment has been altered prior to the addition of the label and experiments in which the penetrating solute is present during the alteration of the compartment.

The absolute and relative sizes of these two compartments, however, can be varied almost at will as a function of the metabolic state of the preparation and the composition of the suspending medium. In agreement with numerous previous reports[1–8,16] we find that hypotonic swelling appears to result from the intake of water into the sucrose(or mannitol)-impermeable space. The present study establishes that the osmotic swelling which results from the energy-linked accumulation of $K^+$ and a permeant anion also results in a marked increase in the mannitol-impermeable water volume. This result and the fact that the increased water volume can be accounted for quantitatively by an increase in $K^+$ and acetate content suggests that the ion uptake and osmotic swelling take place at the level of the barrier to mannitol penetration. This result is in substantial agreement with the recent findings of HARRIS AND VAN DAM[25]. The morphology of preparations swollen as a result of $K^+$ and acetate uptake is reported in the accompanying communication[15] and shows that the additional volume can be accounted for by an expansion of the matrix space. This observation is compatible with and supports the suggestion of a number of investigators that the morphological correlate of the mannitol-impermeable space is the matrix and that the inner membrane of the mitochondrion is therefore the barrier to sucrose and mannitol penetration[1,6,16,17].

A similar osmotic swelling and ion uptake can be observed in the direction of the concentration gradient in the absence of a source of energy if the membrane is made permeable to $K^+$ by addition of gramicidin, or if a permeant cation such as $NH_4^+$ or $Na^+$ is employed with a permeant anion. The passive swelling, like the active, results in an increase in the mannitol-impermeable volume and may be presumed to occur across the inner membrane in a similar manner.

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REFERENCES

1 M. KLINGENBERG AND E. PFAFF, in J.M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, Elsevier, Amsterdam, 1966, p. 180.
2 S. MALAMED AND R. O. RECKNAGEL, *J. Biol. Chem.*, 234 (1959) 3027.
3 R. L. O'BRIEN AND G. P. BRIERLEY, *J. Biol. Chem.*, 240 (1965) 4527.
4 W. C. WERKHEISER AND W. BARTLEY, *Biochem. J.*, 66 (1957) 79.
5 J. E. AMOORE AND W. BARTLEY, *Biochem. J.*, 69 (1958) 223.

6 E. PFAFF, in E. QUAGLIARIELLO, S. PAPA, E. C. SLATER AND J. M. TAGER, *Mitochondrial Structure and Compartmentation*, Adriatica Editrice, Bari, Italy, 1967, p. 165.
7 C. J. BENTZEL AND A. K. SOLOMON, *J. Gen. Physiol.*, 50 (1967) 1547.
8 J. S. TARR AND J. L. GAMBLE, *Am. J. Physiol.*, 211 (1966) 1187.
9 D. F. PARSONS, G. R. WILLIAMS AND B. CHANCE, *Ann. N.Y. Acad. Sci.*, 137 (1966) 643.
10 V. P. WHITTAKER, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, Elsevier, Amsterdam, 1966, p. 1.
11 H. TEDESCHI, *J. Cell Biol.*, 25 (1965) 229.
12 G. A. BLONDIN AND D. E. GREEN, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 612.
13 E. A. MUNN AND P. V. BLAIR, *Z. Zellforsch.*, 80 (1967) 205.
14 S. MALAMED, *Z. Zellforsch.*, 65 (1965) 10.
15 G. R. HUNTER, Y. KAMISHIMA AND G. P. BRIERLEY, *Biochim. Biophys. Acta*, 180 (1969) 81.
16 E. PFAFF, M. KLINGENBERG, E. RITT AND W. VOGELL, *Europ. J. Biochem.*, 5 (1968) 222.
17 W. H. BUTLER AND J. D. JUDAH, *Biochem. J.*, 106 (1968) 17P.
18 Y. HATEFI, P. JURTSHUK AND A. HAAVIK, *Arch. Biochem. Biophys.*, 94 (1961) 148.
19 G. P. BRIERLEY, C. T. SETTLEMIRE AND V. A. KNIGHT, *Arch. Biochem. Biophys.*, 126 (1968) 276.
20 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
21 G. T. OKITA, J. J. KABARA, G. V. LEROY AND F. RICHARDSON, *Nucleonics*, 15 (1957) 111.
22 C. T. SETTLEMIRE, G. R. HUNTER AND G. P. BRIERLEY, *Biochim. Biophys. Acta*, 162 (1968) 407.
23 J. B. CHAPPELL AND A. R. CROFTS, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, Elsevier, Amsterdam, 1966, p. 293.
24 H. TEDESCHI AND D. L. HARRIS, *Arch. Biochem. Biophys.*, 58 (1955) 52.
25 E. J. HARRIS AND K. VAN DAM, *Biochem. J.*, 106 (1968) 759.
26 G. P. BRIERLEY, D. FLEISCHMAN, S. D. HUGHES, G. R. HUNTER AND D. G. MCCONNELL, *Biochim. Biophys. Acta*, 163 (1968) 117.
27 Y. HATEFI AND R. L. LESTER, *Biochim. Biophys. Acta*, 27 (1958) 83.
28 G. R. HUNTER, Y. KAMISHIMA AND G. P. BRIERLEY, *J. Cell Biol.*, 39 (1968) 64A.