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THE PERMEABILITY OF THE MITOCHONDRIAL INNER MEMBRANE TO SUCROSE

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SUMMARY

The permeability of the inner membrane to sucrose in fresh and aged mitochondria is described. Theoretical aspects of the use of the distribution of sucrose as a reference for movements of ions and water are discussed. Experimental findings support the following conclusions:

1. When mitochondria are exposed to labelled sucrose for less than 5 min, penetration across the inner membrane is not significant; and the [^{14}C]sucrose space is a reliable estimate of the volume external to this membrane. The size of the inner, ion-containing compartment can then be estimated by difference (total water *minus* sucrose-accessible water).

2. With longer incubations, sucrose gains entrance into the inner compartment. Under these conditions, the sucrose-free space may continue to have meaning as a virtual space in which the endogenous solutes are isotonic to the external medium. Changes in this virtual space remain useful for evaluating movements of ions and water across the inner membrane.

3. Entrance of sucrose, and also of Cl^-, into the inner compartment during aging was found to be closely linked to reciprocal losses of K^+ and water-soluble phosphate compounds.

INTRODUCTION

Future studies of ion and water exchanges in mitochondria will place increasing reliance on determinations of dilution volumes of normally impermeant solutes to measure changes in the size of the internal, ion-containing compartment. It is now generally accepted that the space within the inner membrane is osmotically active[1–11] and contains water-soluble components consisting chiefly of K^+ and various phosphate compounds[2,4,12]. Mitochondria contain a second compartment between the inner and outer membranes. However, the outer space is believed to be freely accessible to solutes of low molecular weight[3,6,9]. Electron micrographs provide strong evidence that it is the inner membrane that expands or shrinks with osmotic changes, while the outer membrane appears not to be involved[5,10,11].

The size of the inner compartment is estimated as the difference between total water and the volume of water that is accessible to such solutes as sucrose[2–4,7], mannitol[9] or Cl^- (ref. 7). The boundaries of the semipermeable membrane system are therefore defined in functional terms. Impermeability to sucrose has been a tacit assumption in experiments in which sucrose is a major constituent of the incubation medium. Since the initial studies by HOGEBOOM *et al.*[13], sucrose has been the solute of choice in isolation and preparation media. Penetration of sucrose during these procedures, with continued retention of endogenous solutes, would result in swelling. In addition, most observations on the osmometric properties of mitochondria, which depend on impermeability to medium solute, have been carried out in sucrose solutions. Finally, most studies on bioenergetic phenomena are carried out in sucrose-containing suspensions. It is evident that the characteristics of inner membrane permeability to sucrose are of major importance in mitochondrial research.

A particularly difficult problem is to define the permeability of the inner membrane under abnormal conditions. It has been noted by several investigators that sucrose becomes distributed throughout essentially all of mitochondrial water during the large amplitude swelling observed in hypotonic solutions or in the presence of inorganic phosphate[4,7,14,15]. This may be a result of extensive non-specific damage to the mitochondrial membrane which under these conditions can be likened to an expanding fish net. Described in the present report is evidence of penetration of sucrose under quite different circumstances. Extended incubations at 30° resulted in entrance of sucrose with displacement of equivalent amounts of endogenous solute and with little change in the volume of the inner compartment. In these experiments the membrane remained sufficiently intact to retain accumulated sucrose and residual amounts of K^+ and phosphate through a washing procedure and, also, to transport K^+ against a concentration gradient.

METHODS

Mitochondria were isolated from homogenates of rabbit liver by differential centrifugation according to conventional procedures[16]. 0.25 M sucrose buffered with 2 mM Tris–HCl (pH 7.5) was used to suspend the mitochondria during the preparative and post-incubation washing procedures. Centrifugations were carried out at 15000 × *g* for 5 min at 0°.

Water-soluble phosphate, organic and inorganic, was determined by the method of FISKE AND SUBBAROW[17] and Na^+ and K^+ by flame photometry. Protein was determined by a biuret method[18]. Accumulation of Cl^- was measured with radioactive $^{36}Cl^-$. The quantity retained was calculated by dividing the radioactivity of the washed mitochondria by the specific activity of the chloride of the medium. Total water and sucrose-free water were determined by the procedures described previously[7]. L-[1-^{14}C]Glutamic acid, L-[4-^{14}C]malic acid and uniformly labelled [^{14}C]-sucrose were obtained from Calbiochem. Radioactivity was measured with a Packard scintillation counter (Series 314E). Samples of mitochondria were prepared for counting by digesting them with 2 ml of Hyamine (Packard Instrument Co.), and by subsequently mixing them with 20 ml of toluene with primary and secondary scintillators.

RESULTS AND DISCUSSION

Effects of repeated washing and centrifugation in the cold

Centrifugation was employed in these studies to isolate mitochondria for gravimetric measurements; and, in some experiments, a second centrifugation was used as part of a washing procedure to remove materials of the incubation medium prior to analysis. As demonstrated in Fig. 1, repeated centrifugation and re-suspensions will produce significant losses of protein, but other parameters remain relatively constant when expressed in relation to the surviving solid material. There is no evidence of swelling. These data provide a baseline with respect to freshly isolated mitochondria maintained in the cold (0–4°). In additional studies in the report, centrifugation was used after experimental procedures which included incubations at 30°. Values for total solute and for sucrose-free water in these mitochondria were similar to those found in fresh preparations when the data were expressed in relation to protein content. It is recognized that centrifugation under certain conditions will produce losses of endogenous components. As reported by HARRIS *et al.*[19], large accumulations of K^+ obtained in the presence of valinomycin do not survive centrifugation. Presumably, the fragility of the membrane is increased with excessive swelling.

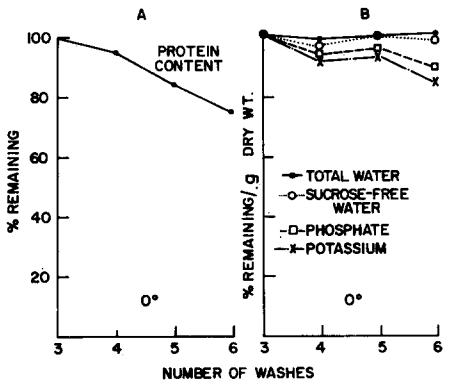

Fig. 1. Effects of repeated washing procedures in the cold (0–4°). Each wash consisted of resuspension of the mitochondria in fresh media followed by centrifugation to obtain a new pellet. The mitochondria were washed 3 times during the initial preparative procedure, and the data refer to changes observed after each of three additional washes. All procedures were carried out in the cold in media containing 0.25 M sucrose buffered with 2 mM Tris–HCl. Phosphate refers to total water-soluble phosphate, organic and inorganic.

The data in Figs. 1 and 2 are given as percent remaining. Representative values (per g protein) for pellets of thrice-washed, freshly prepared mitochondria isolated in 0.25 M isotonic sucrose are: wet wt., 4.1 g; total water, 2.9 g; sucrose-accessible water, 1.85 g; sucrose-inaccessible water, 1.05 g; K^+, 149 μmoles; total phosphate, 126 μmoles.

Thus, the sucrose-free space, the measure of the internal volume, is a relatively small fraction of the total pellet water. The larger sucrose-accessible compartment is believed to include the space between the inner and outer membranes in addition to the space between the mitochondria[3,6,9].

Effects of aging

Shown in Fig. 2 are effects of extended incubations (aging) at 0 and at 30°. The mitochondria were suspended in 0.25 M [^{14}C]sucrose; samples were removed at the times indicated, centrifuged and analyzed. At 0°, sucrose appeared to reach its maximum volume of distribution within the time required for mixing and centrifugation (5 min). Samples removed after 2, 5 and 10 min of incubation showed

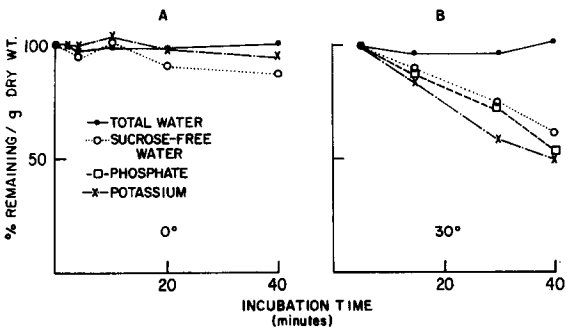

Fig. 2. Changes observed with aging at 0 and at 30°. Freshly prepared, thrice-washed mitochondria (10–12 mg protein per ml) were incubated in 0.25 M sucrose containing 0.1 mM EDTA (pH 7.5). Aliquots were removed at the times indicated, centrifuged in the cold (0–4°) and analyzed. The figures on the abscissa indicate the total incubation times, but they do not include the centrifugation time (5 min for each sample).

no further increase in sucrose-accessible water; or, as shown in the figure, decrease in sucrose-inaccessible water. This is evidence that sucrose equilibrates rapidly across the outer membrane. The losses of K^+ and phosphate were small. In contrast, at 30°, there was a progressive fall in these solutes, and this decrease was associated with a proportional reduction in sucrose-free water. These decreases will also occur in the cold if the incubation times are extended sufficiently[2].

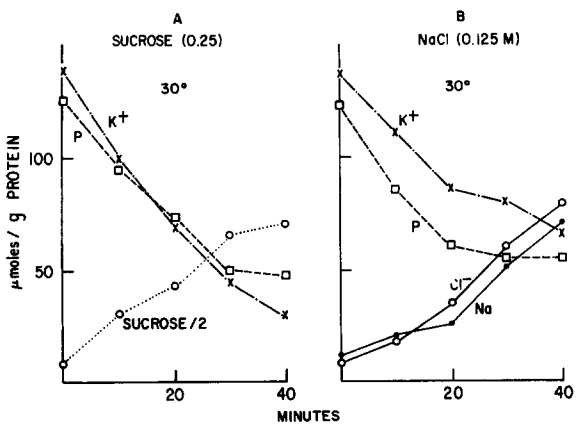

Fig. 3. Retention of endogenous and exogenous solute after washing. Mitochondria were incubated in 0.25 M sucrose or 0.125 M NaCl at 30°. At the times indicated the samples were removed and centrifuged. They were re-suspended in 20 ml of unlabelled cold sucrose and re-centrifuged prior to analysis. P refers to total phosphate, organic and inorganic.

TABLE I

DISTRIBUTION OF [^{14}C]SUCROSE IN RELATION TO TIME OF EXPOSURE IN AGED MITOCHONDRIA

Mitochondria (54 mg protein) were incubated for the times indicated in 5 ml 0.25 M sucrose containing 0.1 mM EDTA (pH 7.5) at 30°. Trace amounts of [^{14}C]sucrose were added at the start in the first two samples but only for the final 5 min in the third. Data are expressed as ml or μmoles per g protein.

Mitochondria	*Total incubation time (min)*	*Time exposed to [^{14}C]sucrose (min)*	*Distribution of water (ml)*			*K^+ (μmoles)*
			Total	*[^{14}C]Sucrose-accessible*	*[^{14}C]Sucrose-inaccessible*	
Fresh	5	0–5	2.92	1.94	0.98	141
Aged	35	0–35	2.94	2.45	0.49	48
		30–35	2.96	2.02	0.94	46

As noted previously[4,7], the quantities of K^+ and phosphate in fresh mitochondria are appropriate to provide isotonic concentrations if contained in the sucrose-free water of the inner compartment. Therefore, the proportional loss of electrolytes and of sucrose-free water seen at 30° (Fig. 2B) could be due to shrinkage of the inner compartment. However, a second possibility is suggested by the lack of change in total water. The decrease in the sucrose-free portion would also result if there were penetration of sucrose into an inner compartment of constant volume. Evidence that the above explanation is correct is presented in Fig. 3A. In this experiment, the initial procedure was identical to that of Fig. 2B. Following the incubation at 30°, the mitochondria were removed and centrifuged as before, but they were then resuspended in unlabelled sucrose at 0° and re-centrifuged prior to analysis. The resulting washed pellets (Fig. 3A) showed retentions of labelled sucrose that increased with incubation time. The increased sucrose retentions are reciprocal to the decreases in sucrose-free water, as measured before washing (Fig. 2B). We interpret these results as demonstrating that the labelled sucrose entered the inner compartment during the incubation at 30°, and was retained during the brief washing procedure at 0°.

Additional evaluation of these findings is provided by the data summarized in Table I. The numbers in the first two lines illustrate changes similar to those shown in Fig. 2B. After incubation for 35 min at 30°, there was a decrease in sucrose-free water and in K^+ content. The data in the third line were obtained under identical conditions except that exposure to the labelled sucrose was limited to the final 5 min of a 35-min incubation. It can be seen that the measured distribution of the label obtained during the final 5 min was nearly identical to that obtained during the first 5 min. This similarity is particularly striking in view of the fact that, by the end of the incubation period, there were large losses of endogenous solutes and, presumably, large gains of unlabelled sucrose. The results suggest that the volume of the inner compartment did not change, but the evidence is not conclusive since the alterations in permeability in these aged mitochondria could affect the distribution of the sucrose. There are two additional reasons for believing that the volume relationships were not greatly altered. There was little change in total water content (Fig. 2B and Table I), and total solute content, at least as measured after washing, remained

relatively constant. The loss of K^+ and phosphate in the experiments of Fig. 3A was balanced by the accumulation of sucrose.

The stoichiometric exchange of endogenous for exogenous solute with little change in volume is not a unique effect of sucrose. This is shown by the comparable changes obtained when incubation is carried out in NaCl media (Fig. 3B). Measurements made after washing again demonstrated a progressive increase in the retention of the medium solute (NaCl), and this again was balanced by the losses of K^+ and phosphate.

EDTA was included in the medium in accordance with the procedure described by CHRISTIE *et al.*[20]. In its absence, losses of K^+ and phosphate occurred more rapidly, and accumulations of sucrose and NaCl could not be consistently demonstrated after washing. It is believed that EDTA serves to sustain the integrity of the membrane during the aging incubations.

Solute and water movements and the "sucrose-free" space

In addition to the fundamental postulate that the inner membrane is the site of discrimination between small molecules, we believe that mitochondria are in osmotic equilibrium with their surroundings. We assume further that endogenous K^+ and phosphate are the predominant osmotically active solutes. Under these assumptions, when sucrose does not cross the inner membrane, changes in the sucrose-free space will be equal to changes in inner compartment volume. On the other hand, if sucrose enters the inner compartment, it will carry with it an obligatory volume of water to maintain osmotic equilibrium. Swelling will result unless there is an equivalent loss of endogenous solute. Let us consider the special case where medium sucrose contributes all, or nearly all, of the osmotic strength of the medium. Whether sucrose penetrates or not, the derived value for the sucrose-inaccessible water will continue to be a measure of the volume necessary to contain, isosmotically, the other internal solutes. This interpretation is examined experimentally in the following section.

Application of the parameter "sucrose-free water" after penetration of sucrose into the inner compartment

Considered next is an example of respiration-induced K^+ uptake in the presence of sucrose penetration. As the first step, mitochondria were aged in labelled sucrose at 30° for 40 min. Samples removed at this time were found to have a reduced content of K^+ and a decreased volume of sucrose-free water, in accordance with the results given in Fig. 2B and Table I. The medium was then supplemented with KCl and oxidizable substrates, and the incubation continued for an additional 5 min. Samples taken before and after this brief incubation were isolated by centrifugation. The resulting pellets were resuspended in aliquots of the original labelled sucrose medium (K^+-free) and re-centrifuged. The results of determinations of sucrose-free water and K^+ on these washed pellets are shown in Table II. The K^+ uptake is similar to that described by CHRISTIE *et al.*[20]. Since labelled sucrose was present during the entire 40–45-min incubation, it may be presumed to have entered the inner compartment (*cf.* Figs. 2B and 3A).

As shown in Table II, initiation of respiration induced an average increase (per g protein) of 80 μmoles of K^+ and 0.27 g of sucrose-free water. If this gain in

TABLE II

INCREASE IN THE CONTENT OF K^+ AND WATER AFTER INITIATION OF RESPIRATION IN AGED MITOCHONDRIA

Mitochondria (10 mg protein per ml) were incubated at 30° in 0.25 M [^{14}C]sucrose with 0.1 mM EDTA pH 7.5. First samples were removed at 40 min. The media were then supplemented with 0.02 M KCl and with malate and glutamate (0.002 M each), and the incubation was continued for an additional 5 min before removing the second sample. First and second samples were washed once prior to determining total water, sucrose-free water, and K^+ content. The wash consisted of re-suspension in a chilled aliquot of the original K^+-free, [^{14}C]sucrose medium with immediate re-centrifugation to form the final pellet on which the measurements were made. Quantities per g protein.

Expt. No.	*Time sample removed* (*min*)	K^+ (*μmoles*)	*Total water* (*g*)	*Sucrose-free water* (*g*)	*Increase* K^+ (*μmoles*)	*Increase* *Sucrose-free water* (*g*)
1	40	62	2.84	0.66		
	45	147	2.91	0.91	85	0.25
2	40	56	2.79	0.57		
	45	141	2.85	0.86	85	0.29
3	40	50	2.71	0.60		
	45	130	2.77	0.83	80	0.23
4	40	71	2.81	0.68		
	45	146	2.88	0.98	75	0.30

TABLE III

ANIONS ACCUMULATED WITH K^+

Mitochondria (3 mg protein per ml) were incubated at 30° in 0.25 M sucrose with 0.1 mM EDTA (pH 7.5) for 30 and for 40 min. At these times 5-ml aliquots were removed and incubated for 5 min in three separate incubation mixtures containing 0.02 M KCl and malate and glutamate (both 2 mM). The separate mixtures were labelled with $^{36}Cl^-$, [^{14}C]malate and [^{14}C]glutamate, respectively. The mitochondria were washed once prior to analysis, and the accumulation of the labelled materials was calculated by dividing the activity retained by the specific activity (counts/min per μmole) of the medium. The aliquots removed at 30 min contained 1.13 μmoles of K^+ and 0.93 μmole of total phosphate. After 40 min of aging these values were 1.04 and 0.92.

Accumulated ion	*Increase* (*μmole*)	
Aging time:	*30 min*	*40 min*
K^+ (average)	0.82	0.80
Phosphate (average)	0.04	−0.02
Cl^-	0.01	0.02
Malate	0.15	0.08
Glutamate	0.07	0.03

K^+ were contained in this amount of water, the concentration would be 295 mosmoles/kg water, and hence uptake of the cation by itself is sufficient for isosmolality of the accumulated water. The experiments were repeated to measure accumulations of anions under these conditions, and as shown in Table III these were relatively small. The ability to accumulate malate, which is readily demonstrable in fresh preparations[21], was much reduced in these aged mitochondria. It may be concluded that K^+ was accumulated in exchange for H^+ [refs. 20 and 22]. Internal buffering would be contributed chiefly by remaining phosphate compounds and proteins of

the matrix. With conversion of protonated components or weak acids to salts there would be a gain of 1 osmoequiv per equiv of entering K^+.

The merits of analysis after washing are evident. One can obtain, simultaneously, a measure of each of the retained solutes and of the water available for their solution. On the other hand, the interposition of the additional step may lead to losses or exchanges of water and ions prior to final analysis. ROTTENBERG AND SOLOMON[15] reported uptake of succinate with K^+ in the presence of valinomycin. In our experiments, one cannot exclude the possibility of accumulation of potassium malate during the incubation with loss of malic acid during the wash.

Mathematical description of interrelationships between sucrose-free water and inner compartment solute and water content

The theoretical basis for the observations made in the previous sections is the postulate that mitochondria are in osmotic equilibrium with their surroundings. That the sucrose-free water remains a useful and meaningful quantity in the presence of sucrose penetration is demonstrated by the following equations. Molal activity coefficients are assumed to be equal on both sides of the inner membrane, and the outer membrane is assumed to be permeable to medium solutes.

Definition of terms: W_p, total pellet water, determined gravimetrically (kg); W_i, water contained in the inner compartment (kg); W_o, water contained in the osmotically inactive spaces of the pellet (kg); W_{sf}, the sucrose-free water in the pellet (kg); $[s]_o$, medium sucrose concentration (molal); $[m]_o$, summed osmolality of all medium solutes other than sucrose; S_i, amount of sucrose present within the inner compartment (moles); N_i, total inner compartment solutes, other than sucrose (osmoles).

Sucrose-accessible water is determined experimentally by dividing the [^{14}C]-sucrose content of the pellet by the [^{14}C]sucrose concentration in the medium. Sucrose-free water is total pellet water *minus* sucrose-accessible water. The relationships between sucrose-free water, inner compartment water and sucrose penetration are shown in Eqns. 1–3:

$$W_p = W_i + W_o \tag{1}$$

by definition; therefore

$$W_{sf} = W_i + W_o - \frac{([s]_o W_o + S_i)}{[s]_o} \tag{2}$$

which reduces to

$$W_{sf} = W_i - \frac{S_i}{[s]_o} \tag{3}$$

Thus, the sucrose-free space is a measure of inner compartment water provided there is no penetration of sucrose. Since the inner compartment is assumed to be in osmotic equilibrium with the medium,

$$W_i = \frac{S_i + N_i}{[s]_o + [m]_o} \tag{4}$$

By substitution, Eqn. 3 may now be expressed in terms of solute content:

$$W_{sf} = \frac{N_i}{[s]_o + [m]_o} - \frac{[m]_o S_i}{[s]_o([s]_o + [m]_o)} \tag{5}$$

Eqn. 5 is of general applicability to studies of mitochondrial swelling and shrinking, and the relationships between the sucrose-free space and inner compartment solute content. We wish to make two observations relating to the experiments described in this paper:

First, in experiments carried out in media containing only sucrose ($[m]_o = 0$), Eqn. 5 reduces to

$$\frac{N_i}{W_{sf}} = [s]_o \tag{6}$$

That is, the derived sucrose-free space equals that required to hold endogenous solutes isosmotically with the medium. In the experiments described in Fig. 2B, $[s]_o$ was held constant at 0.25 M. The experimental results are consistent with Eqn. 6, which says that W_{sf} is dependent only on endogenous solute content under these conditions. In particular, W_{sf} is independent of sucrose penetration which, occurring by itself, would cause swelling (increase in W_i) with no change in W_{sf}.

The second observation relates to the experiments described in the preceding section, in which ion uptake is associated with uptake of water. If $[m]_o$ and $[s]_o$ are held constant in the control and experimental media, Eqn. 5 can be expressed as follows:

$$\frac{\Delta N_i}{\Delta W_{sf}} = [s]_o + [m]_o + \frac{[m]_o \Delta S_i}{[s]_o \Delta W_{sf}} \tag{7}$$

If sucrose uptake (ΔS_i) during the brief experimental interval is sufficiently small, the osmolality of ion uptake will equal that of the medium, as was demonstrated experimentally in Table II. This result is independent of the amount of sucrose that entered during the control period.

For the purposes of this discussion, it was assumed that all the water in the inner compartment was osmotically active. If there is a constant, non-solvent water fraction within W_i (ref. 8), this will introduce a constant into Eqns. 4–6. However, this constant will cancel when differences in W_{sf} are used.

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