Measurements of mitochondrial volumes are affected by the amount of mitochondria used in the determinations

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Mitochondrial water spaces were determined by centrifugal filtration, by using $^3\text{H}_2\text{O}$ and ^{14}C -sucrose, -mannitol, -inulin and -dextran. The volume (in $\mu\text{l}/\text{mg}$ of mitochondrial protein) of each of the spaces was inversely proportional to the amount of mitochondria (mg of protein) centrifuged. The dextran space (representing extramitochondrial water carried down with the mitochondria) decreased the most, and accounted for most of the changes observed in the other spaces. However, the calculated matrix and intermembrane spaces also decreased when increasing amounts of mitochondria were centrifuged. For each space, the same value was obtained when centrifugal filtration was done at 8000 and at 15600 g , and when the mitochondria were incubated with the markers for 15 s to 5 min, indicating that sucrose, mannitol and inulin do not penetrate the matrix, nor does dextran penetrate the intermembrane space, under the incubation and centrifugation conditions generally used to measure mitochondrial spaces.

INTRODUCTION

The measurement of mitochondrial water spaces is a matter of interest for practical and theoretical reasons. It is needed to calculate the concentrations of compounds in submitochondrial compartments, which, in turn, are required for interpreting metabolic and transport studies unequivocally; it is essential in order to understand how enzymes function in the mitochondrial matrix, in which water appears to be extremely restricted (Srere, 1981). There is some uncertainty about the validity of measurements made in isolated mitochondria by the methods now available. For example, although labelled sucrose (Harris & van Dam, 1968) has been most often used to calculate the matrix space, Halestrap & Quinlan (1983) propose that mannitol is a better marker for this purpose; also, the existence of the sucrose space has been questioned (Sambasivarao & Sitaramam, 1983).

We have observed that the measured volume of each of the mitochondrial spaces varies substantially as a function of the amount of mitochondria used for the determination. This finding has important implications for many studies of mitochondria.

MATERIALS AND METHODS

Animals and reagents

Male rats of the Sprague–Dawley strain, weighing 150–250 g, were obtained from Simonsen Laboratories, Gilroy, CA, U.S.A.; they were fed *ad libitum* on standard laboratory chow, and killed by cervical dislocation.

The following radioactive materials were obtained from ICN, Irvine, CA, U.S.A.: $^3\text{H}_2\text{O}$ (sp. radioactivity 100 Ci/mol), ^{14}C -sucrose (sp. radioactivity 350 Ci/mol), ^{14}C -mannitol (sp. radioactivity 42 Ci/mol), ^{14}C -inulin (sp. radioactivity 2.26 mCi/g) and ^3H -adenine-2,8- ^3H -NAD $^+$ (sp. radioactivity 21 Ci/mmol). ^{14}C -Dextran (sp. radioactivity 1.24 mCi/g; M_r 70000) was from New England Nuclear, Boston, MA, U.S.A. Hydrofluor was from National Diagnostics, Somerville, NJ, U.S.A., and Dow Corning silicones 200 (1.5 cSt) and 550 were from

William F. Nye, New Bedford, MA, U.S.A. All other reagents were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Experimental methods

Liver mitochondria were prepared in 0.3 M-mannitol/2 mM-Hepes/KOH, pH 7.4, as described by Cohen *et al.* (1985); this method yields 70–75% recovery of mitochondria, with minimal contamination with lysosomes and endoplasmic reticulum. Incubations were performed in duplicate in 5 ml or 20 ml glass vials in a Dubnoff shaking bath at 30 °C; the final incubation volume was 0.5–1.2 ml, and the mitochondrial protein concentration was 3–4 mg/ml. The standard incubation medium contained (final concns.) 50 mM-Tris/HCl, pH 7.4, 15 mM-KCl, 15 mM-KHCO $_3$, 5 mM-NH $_4$ Cl, 10 mM-ornithine, 5 mM-MgCl $_2$, 5 mM-KH $_2$ PO $_4$, 2 mM-EDTA, 0.1% defatted bovine serum albumin, 10 mM-succinate and 60 mM-mannitol. This medium supports high rates of citrulline synthesis (30–50 nmol/min per mg) in liver mitochondria from rats fed on a normal diet (Cohen *et al.*, 1982); the rate of citrulline synthesis was measured in every experiment to assess the metabolic integrity of the mitochondrial preparations used. For the measurement of mitochondrial spaces, the standard medium contained, in addition to the above components, one of the following radioactive tracers: 1.2–7.2 μCi of $^3\text{H}_2\text{O}/\text{ml}$; 0.85–3.4 μCi of ^{14}C -sucrose/ml; 1.2–2 μCi of ^{14}C -mannitol/ml; 1.2 μCi of ^{14}C -inulin/ml; 1.4–2.5 μCi of ^{14}C -dextran/ml; 1.4–2.4 μCi of ^3H -NAD $^+$ /ml. The amount of tracer used in a given incubation was chosen to provide at least 1000 c.p.m. in the mitochondrial pellet samples used for scintillation counting (see below). We normally measure the total mitochondrial and sucrose space simultaneously by using $^3\text{H}_2\text{O}$ and ^{14}C -sucrose, but we used single labels in the present work in order to avoid corrections for spill-over (see Cohen & Cheung, 1984); control experiments in which both methods were used in parallel showed that they yield identical results.

The incubation mixtures were preincubated at 30 °C for 2 min, and the mitochondria, also prewarmed to

30 °C (for reasons discussed by Cohen *et al.*, 1985), were added to start the incubations. Samples of the mixtures were removed, and the mitochondria were immediately separated from the medium and deproteinized by centrifugation for 1 min through silicone oil (Rajiman & Bartulis, 1979) into 0.1 ml of 14% (w/v) HClO_4 in 1.5 ml tubes and Eppendorf 5412 and 5414 micro-centrifuges (average maximum centrifugal force 8000 and 15600 *g* respectively). Identical results were obtained with the two centrifuges. The total time elapsed between the start of incubations and centrifugation was 30 s. Samples of the supernatants were deproteinized with HClO_4 (final concn. 1.7 M). Duplicate 10 μl portions of the protein-free supernatants and of the mitochondrial extracts were counted for radioactivity in 4 ml of Hydrofluor in a Beckman LS 100 C liquid-scintillation counter. The manner in which samples were prepared and counted is important; necessary precautions are described by Cohen & Cheung (1984) and Armston *et al.* (1982).

For the measurement of citrulline synthesis, incubations were terminated by adding HClO_4 (final concn. 1.7 M). Citrulline was measured as described in Cohen *et al.* (1980) and protein as in Lowry *et al.* (1951), with bovine serum albumin standards.

RESULTS AND DISCUSSION

The following nomenclature will be used: total water space is that measured by $^3\text{H}_2\text{O}$; sucrose space is the volume of water accessible to [^{14}C]sucrose; dextran space, measured with [^{14}C]dextran, represents extraparticulate water that is carried down with the mitochondria. Intermembrane space is the sucrose space minus the dextran space; matrix space is the total water space minus the sucrose space. All spaces are expressed in μl of water/mg of mitochondrial protein. It should be noted that the matrix space reflects only the volume of water present in the matrix; the total matrix volume is likely to be about twice as large, owing to the large amount of protein present in this compartment (Srere, 1980).

Effect of the volume of mitochondrial suspension centrifuged

We have found that, in any given experiment, the measured total water, sucrose and matrix spaces are inversely proportional to the volume of incubation mixture centrifuged (Fig. 1*a*). The same observation was made when the separations were carried out in 0.5 ml micro-centrifuge tubes, which enabled us to use 0.05–0.3 ml of incubation mixture (results not shown).

In order to distinguish extraparticulate water from water in the intermembrane space, incubations were done with [^{14}C]dextran. Amoore & Bartley (1958) showed that a polyglucose of M_r 50000 neither penetrated nor adsorbed to isolated mitochondria. The [^{14}C]dextran of M_r 70000 used in the present experiments can be expected to reflect only extramitochondrial water. Preliminary experiments with unlabelled dextran showed that, at the concentrations used, it had no effect on the total water and sucrose spaces or the rate of citrulline synthesis.

Fig. 1(*b*) shows that the dextran space varies similarly to the water and sucrose spaces. The volume of the dextran space decreases from 35% to 18% of that of the total water space when the volume of incubation mixture centrifuged is increased from 0.3 ml to 1.0 ml; the

Fig. 1. Effect of the volume of incubation medium centrifuged on mitochondrial spaces

Mitochondria were incubated and separated from the incubation medium as described in the Materials and methods section. Measured total water (●), sucrose (○), mannitol (◆), inulin (△) and dextran (■) spaces are plotted versus the volume of incubation mixture centrifuged. The mitochondrial protein concentration was 3.24 and 4.45 mg/ml of incubation mixture in (a) and (b) respectively.

changes observed in the total water and the sucrose spaces are primarily the result of changes in the extraparticulate water. However, the calculated matrix and intermembrane spaces of the mitochondria used in the experiments of Fig. 1 also decrease substantially (16% and 38% respectively) over the same volume range (also see below).

Similar results were obtained when [^{14}C]inulin and [^{14}C]mannitol were used instead of labelled sucrose (Fig. 1*a*), except that, for each volume of incubation mixture centrifuged, the mitochondrial spaces permeable to mannitol and inulin were approx. 109% and 86% respectively of that permeable to sucrose.

NAD^+ is thought not to permeate into the matrix, and might thus be useful to measure the space outside the matrix. This proved not to be the case; when [$\text{adenine-2,8-}^3\text{H}$]NAD $^+$ was incubated with mitochondria

Fig. 2. Effect of the amount of mitochondria centrifuged on mitochondrial spaces

Mitochondria were incubated and separated from the incubation medium as described in the Materials and methods section, except that three different protein concentrations, 1.25, 2.72 and 5.88 mg/ml of incubation medium, were used; 0.7 ml portions of the mixtures were centrifuged for each determination. (a) Measured total water (●), sucrose (○) and dextran (■) spaces. (b) Calculated matrix (▲) and intermembrane (□) spaces.

for 5 s, 30 s and 5 min, the ratio of ^3H d.p.m./μl of matrix space (measured with ^{14}C sucrose and $^3\text{H}_2\text{O}$) to ^3H d.p.m./μl of medium was 10, 23 and 76 respectively. We do not know whether NAD^+ or metabolites of it accumulated in the matrix.

The sucrose, mannitol and dextran spaces measured at 30 °C after 15 s–5 min of exposure to these markers were constant (results not shown), indicating that the compounds did not penetrate into the matrix under those conditions. Halestrap & Quinlan (1983) have reported that mannitol does not permeate into the matrix during the first 5 min of incubation at 37 °C, but does thereafter.

Several experiments were done in which the volume of silicone oil added to the centrifuge tubes was varied from 0.1 to 0.9 ml, at a constant volume of mitochondrial suspension. In contrast with the preceding results, the mitochondrial water spaces did not change as a function of the volume of the silicone-oil layer (results not shown).

Effect of the amount of mitochondria centrifuged

In the two experiments of Fig. 1, both the volume of incubation mixture and the amount of mitochondria sedimented were varied simultaneously. In Fig. 2 are shown the results of one of several experiments in which the volume centrifuged was constant, but the amount of

Fig. 3. Mitochondrial spaces measured at a constant amount of mitochondria; lack of effect of the volume of incubation mixture centrifuged

Mitochondria were incubated and separated from the incubation medium as described in the Materials and methods section, except that the protein concentration in the incubations was varied so that the volume centrifuged for each determination would contain 1.9 mg of mitochondrial protein. (a) Measured total water (●), sucrose (○) and dextran (■) spaces. (b) Calculated matrix (▲) and intermembrane (□) spaces.

mitochondria (expressed as mg of mitochondrial protein) was varied. The results were similar to those of the experiments in Fig. 1: the volume of each of the spaces decreased substantially as the amount of protein in the incubation mixture was increased (Fig. 2); the matrix and intermembrane spaces decreased by a smaller, yet still substantial, amount (Fig. 2b).

In converse experiments, a constant amount of mitochondria present in different volumes was centrifuged. Under these conditions the measured volume of each space was constant (Fig. 3). These results show that the critical variable in the experiments of Figs. 1 and 2 was the total amount of mitochondria centrifuged. [We have established that, under our conditions, 95–98% of the protein applied to the centrifugation tubes is recovered in the pellets. Our observations are valid, therefore, whether expressed per mg of protein applied to the tube or recovered in the pellet; see Armston *et al.* (1982).]

Total water and sucrose spaces measured in six experiments with six different mitochondrial preparations were plotted versus the protein contained in the various volumes centrifuged. The data ($n = 25$) fit straight lines with correlation coefficients (r) of -0.960 for water and -0.959 for sucrose, and slopes of -0.617 and -0.495 respectively (Figs. 4a and 4b). The corresponding matrix spaces also fit a straight line with

Fig. 4. Relationship of mitochondrial spaces to the amount of mitochondrial protein centrifuged

Mitochondrial spaces obtained in either six (a) or three (b) different experiments, and expressed as $\mu\text{l}/\text{mg}$ of mitochondrial protein, are plotted versus the amount of

$r = -0.712$, which is significantly different from zero at the 0.001 level; the slope is -0.121 (Fig. 4c). In three of the six experiments the intermembrane space was also determined. The data ($n = 14$) fit a straight line of $r = -0.844$ (significant at the 0.001 level) and a slope of -0.159 (Fig. 4d). The scatter of the matrix- and intermembrane-space values around the lines is greater than that of the total water- and sucrose-space values, undoubtedly reflecting the fact that the first two are calculated from two measured values, and may contain additive errors. It is clear from these data that, whereas the greatest effect of varying the amount of mitochondria centrifuged is on the dextran space, the matrix and intermembrane spaces are also significantly affected.

Similar experiments were done by the centrifugation procedure of Armston *et al.* (1982) and Whipps & Halestrap (1984), which does not use a non-aqueous layer. Linear-regression analysis of individual experiments indicated that the measured matrix space did not vary as a function of the amount of protein centrifuged. The day-to-day variation in the measured matrix space was large ($0.85\text{--}1.36 \mu\text{l}/\text{mg}$ in three experiments). Day-to-day variation in matrix space measured without a silicone-oil layer, as well as variation from one group of experiments to another, have previously been reported (Armston *et al.*, 1982; Whipps & Halestrap, 1984). In one experiment in the present studies, measurements were done by both methods; the matrix space measured without oil was the same as that measured by centrifugal filtration of 2.4 mg of protein, i.e. about the mid-point of the range of protein studied. If one wishes to know the measured matrix space in a given experiment, centrifugation without oil may be preferable, because it gives values which appear to be independent of the amount of mitochondria used. If, however, one needs to measure mitochondrial spaces in order to calculate the concentrations or amounts of metabolites in the intermembrane or matrix space, then it is essential to prevent any uncontrolled utilization of metabolites, and therefore the reactions must be quenched. For this purpose the centrifugal-filtration method is required. It is clear that the values of the mitochondrial matrix space obtained with the latter method are relative. For a given amount of mitochondrial preparation, however, the values obtained are very reproducible and should make it possible to compare results from different experiments and even from different laboratories.

In all experiments, centrifugal filtration was done at $8000 g$ and at $15600 g$; no differences were observed in the experimental values obtained under the two conditions.

It has been stated that sucrose permeates into the matrix as a result of the pressure applied to mitochondria during centrifugation (Sitaramam & Sarma, 1981; Sambasivarao & Sitaramam, 1983). The latter authors also point out that values of the sucrose space reported in the literature are 'unduly large and varied', and conclude that 'the sucrose space hypothesis is now

mitochondrial protein centrifuged to obtain the measurement. The amount of protein ranged from 0.42 to 4.45 mg , in volumes ranging from 0.1 to 1.0 ml . (a) Total water space; (b) sucrose space; (c) matrix space; (d) inter-membrane space (IMS).

obsolete' (Sambasivarao & Sitaramam, 1985). In our experiments, doubling the centrifugal force applied had no effect on any of the spaces measured in a given amount of mitochondria. This argues against the proposed permeation of sucrose. Moreover, the inulin space differed from the sucrose space by less than 10%. If sucrose permeates the inner membrane during centrifugation, then one must argue that inulin, with an M_r of about 5000, permeates to the same extent; this seems unlikely. Our findings are consistent with those of Whipps & Halestrap (1984), which indicated that neither the sucrose nor the mannitol space changes on repeated centrifugation of mitochondria with [^{14}C]sucrose or [^{14}C]mannitol at about 10000 g, or on application of pressures as high as 16 MPa (150 atm), i.e. greater than the maximal pressures exerted during centrifugation at 10000 g (measurements of mitochondrial volumes are generally made by using 8000–10000 g for very short times). Sitaramam & Sarma (1981) and Sambasivarao & Sitaramam (1983) used mitochondria suspended in grossly hyperosmolar sucrose solutions, and centrifuged them at 25000 g for 30 min; it is conceivable that sucrose permeates into the matrix under those conditions. However that may be, the evidence obtained with conventional methods indicates that the sucrose and the intermembrane spaces do exist in isolated mitochondria. The variability in the reported volumes of the sucrose space mentioned by Sambasivarao & Sitaramam (1983) is likely to be related to the fact that different amounts of mitochondria have been used in various studies.

Halestrap & Quinlan (1983) found that, at 0 °C, the matrix space measured with mannitol was much smaller than that measured with sucrose; they interpreted this to indicate that mannitol enters a compartment outside the matrix which is inaccessible to sucrose, and proposed that mannitol is the better agent to use to measure matrix volumes. In our experiments, which were carried out at 30 °C, the volumes of the mannitol and sucrose spaces were very similar (Fig. 1a); the matrix spaces measured with mannitol and sucrose differed by about 20%. The discrepancy between our findings and those of Halestrap & Quinlan (1983) may result from the temperature difference, or from the use of centrifugation, rather than centrifugal filtration, to separate the mitochondria from the medium.

The volume of the mitochondrial spaces must be known in order to calculate the concentration of compounds in matrix water. Errors in the measurements

can give rise to large errors in the calculation of derived quantities, such as protonmotive force (Halestrap & Quinlan, 1983), and transport rates. Some sources of random error have been discussed by Armston *et al.* (1982) and Cohen & Cheung (1984). In the present work, we show that measurements of mitochondrial spaces by the centrifugal-filtration method are affected by the amount of mitochondria used for the determinations. To the extent that different amounts are used in different work, the measured volumes will differ, thus limiting the validity of comparisons among experiments. For a given experiment, since the effect of this variable on the sucrose and dextran spaces is large, substantial errors in the calculated concentration of compounds in the matrix may arise unless all pertinent measurements are made with the same amount of mitochondria.

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