The intramitochondrial volume measured using sucrose as an extramitochondrial marker overestimates the true matrix volume determined with mannitol

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1. The matrix volume of isolated liver and heart mitochondria has been estimated at various osmolarities and in various osmotic supports using $^{36}\text{Cl}^-$ and $[^{14}\text{C}]\text{sucrose}$, D-mannitol, D-3-methoxyglucose and choline as extramitochondrial markers. 2. The use of 3-methoxyglucose was only possible at 0°C since it entered mitochondria at physiological temperatures. 3. All extramitochondrial markers used gave linear plots of apparent matrix volume against the reciprocal of the osmolarity, but the slope of this plot was greater when sucrose was used than with the other extramitochondrial markers. 4. When extrapolated to infinite osmolarity the mean matrix volume was zero when mannitol was used, but about $0.6\ \mu\text{l}/\text{mg}$ of protein for sucrose and Cl^- and $-0.4\ \mu\text{l}/\text{mg}$ of protein when choline was used. 5. At physiological osmolarity (about 330 m-osmol) the mean matrix volume of de-energized liver mitochondria in KCl medium estimated using mannitol was $0.46\ \mu\text{l}/\text{mg}$ of protein, whereas that obtained using sucrose was $1.68\ \mu\text{l}/\text{mg}$ of protein. Values in mannitol, choline and sucrose media were similar when mannitol but not sucrose was used as extramitochondrial marker. 6. It is argued that the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{mannitol}$ space more accurately reflects the true mitochondrial matrix volume than does the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose}$ space. 7. The consequences of this for measurements of the protonmotive force and the intramitochondrial concentration of metabolites are discussed.

An accurate estimate of the intramitochondrial volume is essential for the determination of the concentrations of metabolites within the mitochondria and for the measurement of the transmembrane pH and electrical gradients that comprise the protonmotive force (Nicholls, 1974; Akerboom *et al.*, 1979; Rottenburg, 1979; Ferguson & Sorigato, 1982). In addition we have recently suggested that changes in the mitochondrial volume may mediate some of the numerous effects of glucagon and α -agonists on the metabolism of liver mitochondria (Halestrap *et al.*, 1980; Halestrap, 1982; Armston *et al.*, 1982). To establish whether this may be the case, measurement of the mitochondrial volume within the intact cell under physiological conditions is essential. These considerations have led us to develop new techniques for measuring the mitochondrial volume *in situ*, which are reported in the following paper (Quinlan *et al.*, 1983). During the course of these studies we discovered that a variety of commonly used osmotic supports for mitochondria, such as sucrose, choline, mannitol and chloride, were not excluded from the same volume of the mitochondrial pellet obtained by centrifugation. Thus their use as extramitochondrial

markers in the measurement of intramitochondrial volumes using $^3\text{H}_2\text{O}$ yields quite different values for this parameter.

In the present paper we utilize changes in the matrix volume induced by altering both the nature of the osmotic support and its osmolarity in order to assess which of these extramitochondrial markers most accurately reflects the true matrix volume. We conclude that the conventional marker molecule, $[^{14}\text{C}]\text{sucrose}$, is unsuitable and is excluded from an additional compartment of the mitochondrion that is not within the matrix. In contrast $[^{14}\text{C}]\text{mannitol}$ would appear to be excluded only from the matrix, and is the extramitochondrial marker of choice. In the following paper (Quinlan *et al.*, 1983) we demonstrate that this molecule may also be used successfully to measure the intramitochondrial volume *in situ*.

Experimental

Materials

Mitochondria were prepared from livers of female albino Wistar rats (225–275 g) allowed free access to food and water as described previously (Halestrap,

1975) and were used within 2 h of preparation. The extraction medium contained 300 mM-sucrose, 10 mM-Tris/HCl and 2 mM-EGTA adjusted to pH 7.4 at 0°C. The control ratios for respiration with succinate were always greater than 4.

The sources of all chemicals and biochemicals were those given previously (Halestrap, 1975, 1982). Radiochemicals were purchased from Amersham International plc, Amersham, Bucks. HP7 9LL, U.K. The D-[1-¹⁴C]mannitol supplied by Amersham International contained about 1% [¹⁴C]mannose, but it proved unnecessary to purify the [¹⁴C]mannitol as described by Quinlan *et al.* (1983).

Methods

Measurement of mitochondrial volumes. Mitochondria (approx. 6 mg of protein/ml final concentration) were incubated for 2 min in the media indicated in the legends to the Figures, before sedimentation in an Eppendorf 5400 microfuge at 10000 *g* for 1 min and assay of the pellet for ³H and ¹⁴C as described previously (Halestrap & McGivan, 1979; Armston *et al.*, 1982). In all cases the media contained, in addition to the osmotic support indicated, 10 mM-Tris/HCl, 2 mM-EGTA, 2.5 mM-MgCl₂, 2.5 mM-potassium phosphate, 1 µg of rotenone/ml and 1 µg of antimycin A/ml and was adjusted to pH 7.4 at 0°C. ³H₂O and the ¹⁴C or ³⁶Cl⁻ extramitochondrial marker were present at 1 and 0.1 µCi/ml respectively. Unless otherwise stated the concentration of the extramitochondrial marker was 0.5 mM, although in the case of sucrose, carry-over of sucrose from the preparation medium raised this value to about 25 mM. All osmolarities were calculated from the total concentration of all components present in the final incubation. Calculations of the mitochondrial ³H₂O/¹⁴C marker space were made as described previously (Halestrap & McGivan, 1979) and are expressed as µl/mg of protein sedimented in the pellet. The protein in the pellet was measured in a parallel experiment using a modified biuret assay (Gornall *et al.*, 1949) and represents about 75% of the total mitochondrial protein added to the incubation. This may account for the slightly higher mitochondrial spaces that we measure compared with values determined by others who only measure the mitochondrial protein added to the incubation. However, values obtained by Wilson & Forman (1982) are in good agreement with those reported here. All values were measured in quadruplicate, each single observation including three determinations of the ³H and ¹⁴C content of both pellet and supernatant. Great care was taken to avoid ³H₂O loss by evaporation before and during counting radioactivity and to maintain constant quenching. Other refinements are documented elsewhere (Armston *et al.*, 1982).

Measurement of mitochondrial light scattering. Swelling of mitochondria upon their addition to various iso-osmotic media was measured by monitoring the decrease in light scattering at 520 nm as described by Chappell & Haarhoff (1967). Mitochondria (approx. 2 mg of protein/ml final concentration) were added to a solution of the compound to be investigated at a concentration giving an osmolarity of 300 m-osm. The solution was buffered at pH 7.4 with 5 mM-Tris/HCl and contained 1.0 mM-EGTA and 1 µg of both antimycin and rotenone/ml. The temperature was maintained at 37°C and the rate of change of absorbance at 520 nm was continuously recorded.

Results and discussion

The permeability of the mitochondrial inner membrane to sucrose, 3-methoxyglucose and mannitol

The early studies on mitochondrial permeability using light scattering to measure swelling of mitochondria in iso-osmotic media suggested that the inner membrane of the mitochondria was not permeable to glucose, xylitol or mannitol. However, smaller molecules did cause mitochondrial swelling (Chappell & Haarhoff, 1967). In Fig. 1 we confirm that iso-osmotic sucrose produces no significant decrease in light scattering of mitochondria compared with iso-osmotic glycerol, which is freely permeable. Iso-osmotic D-mannitol produced an extremely low rate of swelling, which was barely detectable, whereas D-3-methoxyglucose (a non-metabolizable analogue of glucose) gave a more

Fig. 1. *The light-scattering changes of mitochondria incubated in various iso-osmotic media*

Mitochondria (approx. 2 mg of protein/ml final concentration) were added to 300 mM solutions of sucrose (i), D-mannitol (ii), 3-methoxyglucose (iii) or glycerol (iv), all buffered at pH 7.4 with 5 mM-Tris/HCl and containing 1.0 mM-EGTA and 1 µg of both antimycin and rotenone/ml. The temperature was 37°C. The change in *A*₅₂₀ was continuously recorded as described in the Experimental section.

Fig. 2. Time-dependence of the values obtained for the matrix space using various extracellular markers

The mitochondrial matrix spaces were measured in 125 mM-KCl medium (300 m-osm) as described under 'Methods' at a temperature of 0°C (filled symbols) or 37°C (open symbols). The extramitochondrial markers were [^{14}C]sucrose (○ and ●), [^{14}C]mannitol (■ and □) or [^{14}C]3-methoxyglucose (▲ and △). Each value is the mean of four observations on the same batch of mitochondria with error bars indicating the s.e.m. The experiment is typical of three such experiments.

appreciable rate of swelling. However, the rate was still less than 1% of that seen with glycerol. These results suggest that mannitol might be a suitable extramitochondrial marker, but indicate that D-3-methoxyglucose may penetrate the mitochondrial matrix and so be unsuitable. To investigate this further the $^3\text{H}_2\text{O}/[^{14}\text{C}]$ sucrose space was compared with the $^3\text{H}_2\text{O}/\text{D}-[1-^{14}\text{C}]$ mannitol and $^3\text{H}_2\text{O}/\text{D}-3$ -methoxy[U- ^{14}C]glucose space at various times of incubation. These experiments were performed at 0.5 mM-D-mannitol or -D-3-methoxyglucose rather than at the 300 mM concentration used in the swelling studies. The results of such an experiment are shown in Fig. 2.

Several important features of the data of Fig. 2 are apparent. When measured at 0°C the $^3\text{H}_2\text{O}/[^{14}\text{C}]$ mannitol, -3-methoxyglucose and -sucrose spaces increased slightly over a 15 min period. However, the values using mannitol and 3-methoxyglucose were less than half those using sucrose. At 37°C the $^3\text{H}_2\text{O}/[^{14}\text{C}]$ sucrose space dropped only slightly over a 15 min period, whereas the $^3\text{H}_2\text{O}/[^{14}\text{C}]$ 3-methoxyglucose space dropped rapidly to almost zero, suggesting that 3-methoxyglucose was entering the mitochondria. The $^3\text{H}_2\text{O}/\text{D}-[^{14}\text{C}]$ mannitol space remained constant for about 5 min and then began to drop, suggesting that the mitochondria were becoming leaky to the mannitol during incubation. In eight separate experiments on different mitochondrial preparations the mean value ($\pm$ s.e.m.) for the $^3\text{H}_2\text{O}/[^{14}\text{C}]$ mannitol, -3-methoxyglucose and -sucrose spaces measured at 0°C after 2 min were 0.482 ± 0.037 , 0.543 ± 0.051 and $1.481 \pm 0.088 \mu\text{l}/\text{mg}$ of protein respectively. In a single experiment on heart mitochondria (mean for one preparation $\pm$ s.e.m.; four observations) the values were 0.385 ± 0.055 , 0.385 ± 0.055 and 0.907 ± 0.041 respectively. These data suggest that mannitol and 3-methoxyglucose enter a compartment of the mitochondria inaccessible to sucrose. At 37°C slow entry of 3-methoxyglucose into all the compartments accessible to water may occur, whereas mannitol only enters all compartments after a delay. We have also established that [^{14}C]urea enters all compartments that $^3\text{H}_2\text{O}$ does even at 0°C. The $^3\text{H}_2\text{O}/[^{14}\text{C}]$ sucrose space (mean $\pm$ s.e.m. of six observations) for one particular mitochondrial preparation was found to be $1.285 \pm 0.070 \mu\text{l}/\text{mg}$ of protein compared with $1.310 \pm 0.47 \mu\text{l}/\text{mg}$ of protein for the [^{14}C]urea/ ^3H]sucrose space. The important question posed by these results is whether the mannitol-inaccessible space or the sucrose-inaccessible space most accurately reflects the true matrix volume. In addition the nature of the compartment accessible to mannitol but not sucrose is of interest.

The effects of altering the osmolarity on the matrix volume measured using various extramitochondrial markers

Mitochondria have been reported to act as perfect osmometers, a change in the osmolarity of the suspending osmotic support being matched by a corresponding change in matrix volume to prevent an osmotic pressure gradient forming across the mitochondrial inner membrane. This is demonstrated by a linear plot of the reciprocal of the osmolarity against the measured matrix volume (Tedeschi & Harris, 1955; Rossi & Azzone, 1969; Massari & Azzone, 1972; Massari *et al.*, 1972; Azzone & Massari, 1973). One puzzling feature of these experiments is that when extrapolated to

infinite osmolarity the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose}$ space is not zero but varies from 0.2 to $0.8\mu\text{l}/\text{mg}$ of protein, depending on the experimental conditions used (Tedeschi & Harris, 1955; Harris & Van Dam, 1968; Rossi & Azzone, 1969; Nicholls *et al.*, 1972; Beavis, 1977; Armston *et al.*, 1982; Wilson & Forman, 1982). This is not expected for a perfect osmometer and is taken to represent hydration shells of proteins that are not compressible. Moreover, for this to represent 50% of the matrix volume and to be variable depending on the osmotic support used would not be predicted. However, the data shown in Figs. 3–7 suggest that in contrast with the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose}$ space, the $\text{H}_2\text{O}/\text{mannitol}$ space does behave exactly as predicted for a perfect osmometer in a variety of media.

The data of Fig. 3 demonstrate the effect of varying the osmolarity with KCl on the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose}$ and -D-mannitol spaces of de-energized mitochondria. Both spaces increase linearly

Fig. 3. The effects of varying the osmolarity with KCl on the mitochondrial matrix space measured using ^{14}C -sucrose (●) or ^{14}C -mannitol (■).

Incubation of mitochondria in 125 mM-KCl medium and subsequent measurement of the matrix space was performed as outlined in the Experimental section. Data are means ($\pm$ S.E.M. indicated as error bars) of six experiments on different preparations of mitochondria, each measured matrix space itself being the mean of four observations. The lines drawn were calculated by linear regression of the data. The slopes of the plots for the mannitol and sucrose data ($\pm$ S.E.) were 0.145 ± 0.010 and 0.324 ± 0.017 respectively. The ^{14}C -mannitol/ ^{14}C -sucrose space ($\blacktriangle$) was calculated as the difference of the two other spaces.

Fig. 4. The effects of varying the osmolarity with sucrose on the mitochondrial matrix space measured using ^{14}C -sucrose (●) or ^{14}C -mannitol (■).

Mitochondria were incubated in sucrose media and the matrix spaces measured as described in the Experimental section. Data are means of eight observations on mitochondria prepared from the livers of six rats. The S.E.M. of each observation is shown as error bars. The lines drawn were calculated by linear regression of the data. The slopes of the plots for the mannitol and sucrose data ($\pm$ S.E.) were 0.154 ± 0.011 and 0.217 ± 0.016 respectively. The ^{14}C -mannitol/ ^{14}C -sucrose space ($\blacktriangle$) was calculated as the difference of the two other spaces. The experiment is representative of three such experiments.

Fig. 5. The effects of varying the osmolarity with mannitol on the mitochondrial matrix space measured using ^{14}C -sucrose (●) or ^{14}C -mannitol (■).

The experiment was performed exactly as outlined in the legend to Fig. 4, except that the osmolarity was altered with mannitol. The lines drawn were calculated by linear regression of the data. The slopes of the plots for the mannitol and sucrose data ($\pm$ S.E.) were 0.111 ± 0.007 (for the first three points only) and 0.177 ± 0.002 (for the first four points only) respectively. The ^{14}C -mannitol/ ^{14}C -sucrose space ($\blacktriangle$) was calculated as the difference of the two other spaces.

with the reciprocal of osmolarity, although at the highest KCl concentration used (325 mM) some deviation was always observed with the water/mannitol space. The spaces were not affected by varying the carrier concentration of mannitol from 0.5 mM to 5 mM: nor was there an effect of phosphate concentration (0 to 2.5 mM). The use of [^{14}C]mannitol gave consistently lower spaces than did [^{14}C]sucrose, the discrepancy increasing with decreasing osmolarity. The water/mannitol spaces extrapolated to zero at infinite osmolarity, whereas the water/sucrose space extrapolated to about $0.7\ \mu\text{l}$ as discussed above. Fig. 4 shows similar data for mitochondria suspended in sucrose media as osmotic support and Fig. 5 data with mannitol as osmotic support. The use of mannitol medium gave a considerable deviation from linearity at the highest mannitol concentration used (750 mM) for both water/sucrose and water/mannitol space. The values for the water/mannitol spaces were similar in all three media and in all cases extrapolated to zero at infinite osmolarity. It would be predicted that de-energized mitochondria kept at 0°C for only 2 min would have the same intramitochondrial volume since the fluxes of K^+ that are thought to regulate mitochondrial volumes physiologically (Brierley, 1976; Garlid, 1980) should not occur at an appreciable rate in the absence of a membrane potential and pH gradient. However, the water/sucrose spaces varied in the three media and were considerably greater in KCl media than in sucrose or mannitol media as shown previously (Armstrong *et al.*, 1982). The extrapolated spaces at infinite osmolarity were also different in the three media, being about 0.7 to $0.8\ \mu\text{l}/\text{mg}$ of protein in KCl and mannitol media and about $0.2\ \mu\text{l}/\text{mg}$ of protein in sucrose media. This would be predicted if sucrose could not enter a compartment that KCl and mannitol could enter. This compartment would be osmotically active with respect to sucrose but not KCl and mannitol and so would shrink as the concentration of sucrose is increased, but remain a constant volume as the concentrations of KCl or mannitol are elevated. Since the water/sucrose space does not quite extrapolate to zero at infinite osmolarity even in sucrose medium it would appear that the space accessible to mannitol and K^+ but not to sucrose is not totally compressible.

The use of mannitol and sucrose as marker molecules has the advantage that they are uncharged and thus do not suffer the hazards of ion binding to proteins and phospholipids (Scarpa & Azzi, 1968; Beavis, 1977). However, we have also used $^{36}\text{Cl}^-$ and [^{14}C]choline as marker molecules since these are frequently used as ionic supports for mitochondria. The data are shown in Figs. 6 and 7. Linear plots for mitochondrial $^3\text{H}_2\text{O}/^{36}\text{Cl}^-$ or $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{choline}$ spaces are observed but in neither case

Fig. 6. The effects of varying the osmolarity with KCl on the mitochondrial matrix space measured using $^{36}\text{Cl}^-$. The experimental procedure was the same as indicated in the legend to Fig. 3, except that either [^{14}C]sucrose (●) or $^{36}\text{Cl}^-$ (■) was used as extramitochondrial marker. Data are means ($\pm$ S.E.M., indicated by error bars) of four observations on a single batch of mitochondria from six rat livers. The experiment is typical of two such experiments. The lines drawn were calculated by linear regression of the data and have slopes ($\pm$ S.E.) of 0.203 ± 0.008 and 0.320 ± 0.009 for the $^{36}\text{Cl}^-$ and [^{14}C]sucrose data respectively. The $^{36}\text{Cl}^-/[^{14}\text{C}]\text{sucrose}$ space (▲) was calculated as the difference of the two other spaces.

do they match those observed with mannitol or sucrose as extramitochondrial markers. When chloride was used (Fig. 6) the slope of the graph was similar to that observed with mannitol, although the intercept at infinite osmolarity gave a value of about $0.61\ \mu\text{l}/\text{mg}$ of protein, similar to that seen using [^{14}C]sucrose. This value is also the same as that reported by Rossi & Azzone (1969), who used $^{36}\text{Cl}^-$ as their extramitochondrial marker. In the case of [^{14}C]choline, once again the slope is similar to that obtained using mannitol but here the intercept at infinite osmolarity was $-0.42\ \mu\text{l}/\text{mg}$ of protein. These latter experiments were performed at constant choline chloride concentration (25 mM), the osmolarity being varied with sucrose. The rationale behind this is that choline is metabolized by mitochondria (Kimura & Singer, 1962) and can be bound by mitochondrial phospholipids (Beavis, 1977). By keeping the choline concentration constant and reasonably high it was hoped to minimize such effects and keep them the same at all osmolarities. However, the negative space at infinite osmolarity can be explained if a small amount of

Fig. 7. The effects of varying the osmolarity with sucrose on the mitochondrial matrix volume measured with [¹⁴C]choline

The experimental procedure was the same as that given for Figs. 3–6 except that the media contained 25 mM-choline chloride and the osmolarity was adjusted with sucrose. Data are shown for [¹⁴C]-choline (▲), [¹⁴C]sucrose (●) and [¹⁴C]mannitol (■) used as extramitochondrial markers in incubations of the same preparation of mitochondria. Values are means of four observations; the S.E.M. is omitted since in most cases it was less than the height of the symbols used. The lines drawn were calculated by linear regression of the data. The slopes of the plots for the mannitol, choline and sucrose data ($\pm$ S.E.) were 0.102 ± 0.010 , 0.087 ± 0.001 and 0.258 ± 0.006 respectively. The experiment is typical of two such experiments.

binding and metabolism is still occurring. We have no adequate explanation for the behaviour of the water/chloride space at infinite osmolarity. If ³⁶Cl⁻ were excluded from the same compartments as sucrose it would be expected that the same slope would be observed for the plot of ³H₂O/³⁶Cl⁻ and ³H₂O/[¹⁴C]sucrose spaces against the reciprocal of the osmolarity. If chloride behaved like mannitol and entered the same compartments as this molecule, the ³H₂O/³⁶Cl⁻ space should extrapolate to zero infinite osmolarity. It is possible that the anomalous behaviour of Cl⁻ may reflect fixed negative charges in the sucrose-impermeable mannitol-permeable space.

Conclusions

The data in the present paper demonstrate clearly that the measurement of the mitochondrial matrix volume with ³H₂O and [¹⁴C]sucrose yields values different from those obtained using ³H₂O and [¹⁴C]mannitol. In all media used (KCl, choline chloride, sucrose and mannitol) sucrose appears to be excluded from a compartment that mannitol may

enter. This compartment would appear to be responsible for the apparent ³H₂O/[¹⁴C]sucrose space at infinite osmolarity, determined by extrapolation of the plot of apparent matrix volume against the reciprocal of the osmolarity (Figs. 2–7). This compartment is permeable to mannitol and so a similar extrapolation of the ³H₂O/[¹⁴C]mannitol space against the reciprocal of osmolarity goes through the origin. We believe that this observation and other considerations argue strongly in favour of the ³H₂O/[¹⁴C]mannitol space rather than the ³H₂O/[¹⁴C]sucrose space being a true measure of the matrix volume. If mitochondria behave as perfect osmometers, as a linear plot of apparent matrix volume against the reciprocal of osmolarity suggests, several consequences can be predicted. First, the volume of mitochondria in all impenetrant media should be the same at the same osmolarity, and exhibit the same slope in the plot of volume against the reciprocal of the osmolarity. This is only true if the mitochondria are de-energized and unable to regulate their volume by transporting K⁺ (Brierley, 1976; Garlid, 1980). Secondly, at infinite osmolarity the volume should approach zero as all but tightly bound water in hydration shells would be lost. Both these predictions are fulfilled when mannitol is the extramitochondrial marker, but not when sucrose is used.

We have attempted to eliminate possible errors involved in the use of mannitol as an extra-mitochondrial marker. The data of Figs. 1 and 2 demonstrate that mannitol does not enter the mitochondria at low temperatures. Possible binding of mannitol to the mitochondria has been eliminated by varying the mannitol concentration in the medium in two ways. At constant osmolarity increasing the mannitol concentration from 0.5 to 5.0 mM had no effect on the measured [¹⁴C]mannitol space. Secondly, using mannitol as the osmotic support rather than sucrose or KCl gave the same measured ³H₂O/[¹⁴C]mannitol space as did the use of the other two osmotic supports (Figs. 3–5). The possibility that contaminants of the mitochondrial fraction, such as lysosomes or plasma-membrane vesicles, may affect the reliability of using the ³H₂O/[¹⁴C]mannitol space to measure the matrix volume may also be dismissed for the following reasons. Mannitol medium is often used in preference to sucrose medium in the preparation of mitochondria since it is thought to stabilize the mitochondria in some way that sucrose does not. Golgi membranes and plasma membranes are permeable to mannitol (White *et al.*, 1981; Quinlan *et al.*, 1983). Thus one explanation for this stabilization would be that mannitol enters the lysosomes, causing osmotic swelling and so rupturing them. This would release any degradative enzymes into the media, to be washed away in subsequent washing of the mitochondria. If mannitol does enter lysosomes

and other vesicles contaminating the mitochondrial fraction, it would still make it a more reliable extramitochondrial marker molecule than sucrose since it would eliminate unwanted spaces from the measured value of the mitochondrial volume. In fact two observations suggest that contamination by lysosomes is not a major source of the difference between the mannitol and sucrose spaces. First, heart mitochondria show a similar discrepancy between the mannitol and sucrose spaces and yet heart contains fewer lysosomes than liver. Secondly, lysosomes would be expected to act as osmometers and shrink to almost zero volume at infinite osmolarity. Thus the measured $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose}$ space would be expected to extrapolate to zero at infinite osmolarity if lysosomes were the cause of the discrepancy. The data of Figs. 3–7 show this is not the case.

If it is accepted that the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{mannitol}$ space is a true measure of the matrix volume, then there are two important consequences that must be considered. First, all measurements of the components of the protonmotive force that require the use of the matrix volume in their calculations will underestimate the true values if the $^3\text{H}_2\text{O}/[^{14}\text{C}]\text{sucrose}$ space is used to estimate the mitochondrial volume. The use of both membrane-potential and pH-gradient-sensitive marker molecules for this purpose requires the intramitochondrial concentration of these compounds to be known before the values of the membrane potential and pH gradients can be calculated. In the calculation of the intramitochondrial concentration of these molecules uptake of a radioactive marker into the mitochondria is divided by the matrix volume and the final calculation to give the membrane potential and pH gradient in mV involves multiplication of the logarithm of this ratio by 60 (see Rottenburg, 1975, 1979; Ferguson & Sorgato, 1982). Thus if a true mitochondrial volume of about $0.5\ \mu\text{l}/\text{mg}$ of protein rather than $1\ \mu\text{l}/\text{mg}$ of protein is used in the calculations, the values of both components of the calculated protonmotive force will increase by about 18 mV. Thus the final protonmotive force will be about 36 mV higher than previously calculated using the higher matrix volume. Our own data for State-4 liver mitochondria in sucrose medium give a value for the true protonmotive force (mean $\pm$ S.E.M. of eight separate experiments on different batches of mitochondria) of $211 \pm 3.2\ \text{mV}$. The pH gradient measured using D-lactate was $30.1 \pm 2.3\ \text{mV}$, whereas the membrane potential measured using $^{86}\text{Rb}^+$ was $181 \pm 1.4\ \text{mV}$. Previous studies have reported values in this range (Mitchell & Moyle, 1969; Nicholls, 1974; Prpic *et al.*, 1978) and it is of interest that these workers assumed (rather than measured) a mitochondrial volume of about $0.4\ \mu\text{l}/\text{mg}$ of protein.

The second consequence of a smaller matrix volume is that measured concentrations of metabolites within the mitochondria and their rela-

tionship to the control of enzyme activity will need to be re-assessed.

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