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Changes of Total Water and Sucrose Space accompanying Induced Ion Uptake or Phosphate Swelling of Rat Liver Mitochondria

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1. Total water exchangeable with tritiated water and sucrose space were measured in rat liver mitochondria during the uptake of K^+ induced by valinomycin and the release caused by nigericin. The K^+ content and the sucrose-inaccessible water rose and fell together. 2. Swelling resulting from phosphate addition in a medium of high K^+ concentration was associated mainly with increased sucrose-accessible water, which carried dissolved K^+. This change was reversed by addition of ATP. 3. The response of the sucrose-inaccessible space to changed osmolarity was qualitatively that expected if the mitochondrial K^+ is assumed to be present in this space with a univalent anion. 4. It is brought out that the light-scattering method fails to distinguish between changes in sucrose space and in sucrose-inaccessible space, which in the present experiments could be altered respectively by phosphate (in high K^+ solution) and by cation uptake induced by antibiotic.

The compartmentation of mitochondrial water has long been accepted, though some doubt remains about the relative contributions of heterogeneity within the population and within each particle. Werkheiser & Bartley (1957), by a centrifugal method, found that about 70% of the total water was penetrated by sucrose, whereas only about 20% was penetrated by the larger carboxypolyglucose molecule, which is supposed to measure the extra-particulate water. It has been repeatedly observed (Malamed & Recknagel, 1959; Bartley, 1961; Tarr & Gamble, 1966) that, as the osmolarity of the medium is decreased, the volume of water inaccessible to sucrose increases. Taken with the observation (Amoore & Bartley, 1958) that the sucrose-inaccessible space and the K^+ content are correlated insofar as both fall during aging, the conclusion was drawn (Bartley, 1961; Tarr & Gamble, 1966) that the K^+ resides in an osmotically responsive space not penetrated by sucrose.

Tedeschi & Harris (1955) showed that there is an inverse correlation between extinction of the suspension and the mitochondrial volume estimated microscopically at different concentrations. One source of variability they and Bartley & Enser (1964) noted is the extraction of protein from the particles by the medium. This protein shift alters the refractive-index difference between the two phases and so changes the light-scattering properties. Another reason for the light-scattering being only qualitatively related to the swelling is the changes in shape that occur. Whittaker (1966) describes a succession of forms: rod, crescent and sphere during swelling, and reversion to a dense sphere on shrinkage.

At best the changes in light-scattering or opacity tell us that volume changes have taken place, but cannot tell us which compartment has altered. The swelling that results from induced K^+ uptake is associated with a decrease of light-scattering (at 150° to the incident beam) that is linearly related to the net K^+ uptake (Harris, Cockrell & Pressman, 1966; E. J. Harris and co-workers, unpublished work with gramicidin and dinactin). The K^+ uptake after addition of valinomycin can be reversed by nigericin (Graven, Estrada-O & Lardy, 1966) and the two agents applied in succession afford a convenient way to control cation-associated swelling. In the present studies we sought to relate the K^+ movements to both total water and sucrose spaces in samples taken sequentially from an incubation lasting a few minutes. Similar experiments were then made with another swelling–shrinkage method, namely the addition of phosphate followed by ATP (as studied extensively by Lehninger, 1959*a*,*b*, 1962). This latter system provides a contrast because the cation is not taken into the sucrose-inaccessible water.

In these experiments made on a labile system the separation of the particles without concomitant

secondary changes is a major problem. Millipore filtration is rapid but there is a large background held on the pad (Harris, Catlin & Pressman, 1967). If the particles are washed an indeterminate amount of material is removed. Conventional centrifugation takes many minutes to effect a separation and the material can become anaerobic during the process. For the purpose small centrifuges of low inertia are most useful. For example, the Coleman microcentrifuge brings down most of the particulate material in 15 sec. By adapting the Werkheiser & Bartley (1957) silicone method to a small scale we were able to maintain the K^+ associated with the centrifuged material at concentrations close to those deduced from the readings indicated by the K^+-selective glass electrode immersed in the suspension before and during the sampling. However, it is likely that under conditions of gross swelling an expulsion of water and solute occurs either at the time of swelling or during passage of the material from the aqueous to the silicone layer.

METHODS

Mitochondria were prepared from rat livers by Schneider's (1948) procedure, with 1 mM-EDTA in the 0·25 M-sucrose for the homogenization step. They were stored at 0° in 0·25 M-sucrose at a concentration of about 50 mg. of protein/ml. The protein concentration was determined by a biuret reaction (Layne, 1957), with serum albumin as a standard.

Reaction conditions. The mitochondrial suspension was incubated either (1) in the apparatus described by Pressman (1967) for simultaneous monitoring of light-scattering and K^+ and H^+ concentrations, from which samples could be taken, or (2) in a small vessel when only timed samples were required. Radioactive compounds were included in the medium and the reaction was started by the addition of mitochondria at a concentration of about 5 mg. of protein/ml. At recorded times, 0·2 ml. samples of suspension were taken and layered on top of a thin (3 mm.) layer of silicone [General Electric 1017 B158, density 1·058 for sucrose media, and Versilube (R) F50, density 1·038 for KCl media] itself layered on 40 μl. of 1·5 N-$HClO_4$ in a small plastic centrifuge tube. The tubes fitted a microcentrifuge (Coleman model 6-811). As each sample was taken it was promptly spun in one of a set of four centrifuges. When the mitochondria reached the acid their soluble constituents were released and samples of the acid were taken for analysis and assay of the radioactivity. Samples of the supernatant (now free from mitochondria) were taken for assay of the radioactivity.

Determinations of K^+ *and* Mg^{2+}. Samples from the acid extracts were diluted to provide K^+ or Mg^{2+} concentrations in the range 10–50 μM and were compared with standards in the Techtron atomic absorption spectrophotometer.

Radioactivity measurements. Samples (10–20 μl.) were dissolved in 15 ml. of fluid consisting of toluene–ethanol (3:1, v/v) with either 2 g. of 2,5-diphenyloxazole/l. plus 30 mg. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/l. or, in later work, with 2 g. of 2,5-bis-(5-*tert.*-butylbenzoxazol-2-yl)thiophen/l. The radioactivity was measured in a Packard scintillation spectrometer. This allowed simultaneous determination of either ^3H and ^{14}C or ^{131}I and ^{14}C radioactivities. The discrimination ratio between the activities of the two nuclides was usually about 200. The count rate was at least ten times the background (except when ^{131}I-labelled albumin was used) and sufficient counting time was allowed to provide statistical accuracy better than 3%.

Calculation of results. After subtraction of background the count rates in the two channels were converted into the radioactivities of the two nuclides being used by factors determined from the count rates of standard samples of the separate materials. The total amount of water carried by the mitochondria was found by calculating the dilution in the specific radioactivity of tritiated water in going from the incubation medium to the acid extract. For this calculation the concentration of exchangeable H^+/unit vol. of 0·25 M-sucrose was taken as equal to that in pure water, and the concentration of exchangeable H^+/unit vol. of 1·5 N-$HClO_4$ was taken as 0·93 times that of an equal volume of pure water. The amount of sucrose-permeable water was calculated by comparing the ^{14}C radioactivity in the medium with that in the mitochondrial extract.

RESULTS

Comparison between analytical results obtained by centrifugation and those observed with the K^+*-selective electrode.* The possibility of obtaining information about mitochondrial contents of ions after separation by conventional centrifugation has been questioned (Harris *et al.* 1966), but the present method provides so rapid and positive a separation that the ions are retained. This was shown by comparing the results of the K^+ analyses made on acid extracts with the K^+ contents estimated from the initial K^+ content found analytically and the changes recorded on the K^+-sensitive electrode. Fig. 1 shows the results of such an experiment. The line drawn is taken from the record of the K^+ concentration in the medium during K^+ uptake induced by valinomycin (added at the zero of the time-scale) and the subsequent release induced by nigericin. The changes in K^+ concentration in the medium, as sensed by the electrode, were calculated back to the equivalent changes that must have occurred in the contents of K^+ in the mitochondria. When this was done the changes deduced from the electrode record were exactly parallel to the differences found analytically. It is evident that there is a quantitative recovery in the acid extracts of the K^+ that the electrode shows has moved. From this we infer that the centrifugal method permits accurate recovery of the mitochondrial ions, though we shall question the retention of water and sucrose.

Included in Fig. 1 are analyses for acetate (labelled with ^{14}C). Throughout the whole cycle of uptake and release of K^+ there was an equivalent movement in the acetate carried by the mitochondria. Moreover, the initial sample had an

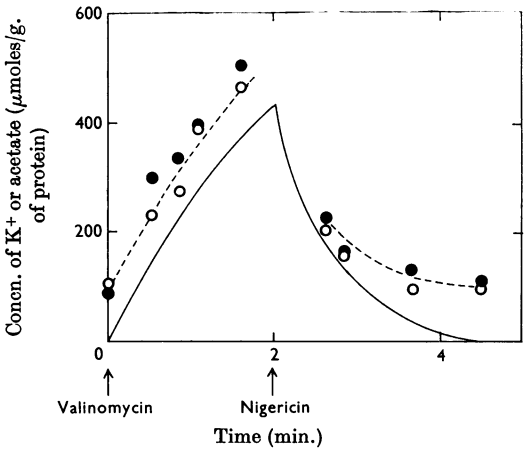

Fig. 1. Comparison between changes in K^+ measured with a selective electrode and analyses for K^+ (○) and for acetate (●) in acid extracts of mitochondria obtained from the centrifugal procedure. The continuous line is scaled from a tracing of the K^+ electrode record, which actually measures disappearance of K^+ from the medium; the ordinates are so chosen that the necessary factors have been applied to convert the record into changes of mitochondrial K^+. In this way it is possible to refer them immediately to the changes deduced by taking the differences between pairs of analytical results. The mitochondria (2·6 mg./ml.) were incubated at 20° in a medium containing 300 mM-sucrose, 20 mM-tris acetate, pH 6·5, 5 mM-KCl, 3 mM-tris glutamate, 3 mM-tris malate and trace amounts of [^{14}C]acetate and tritiated water. After approx. 3 min., at the first arrow, the valinomycin was added to a concentration of 84 μg./g. of protein, and at the second arrow nigericin was added to a concentration of 13 μg./g.

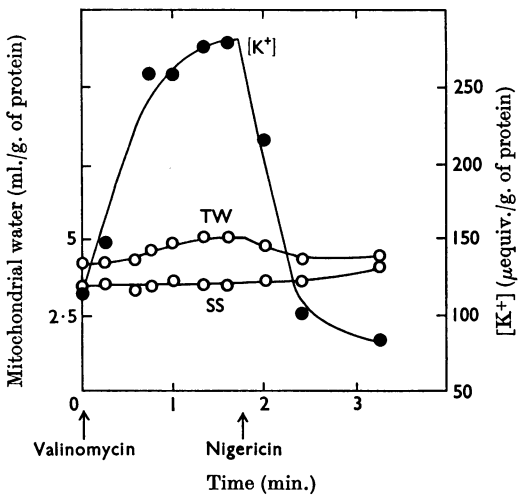

Fig. 2. Changes in total water and sucrose space associated with uptake and release of K^+ by mitochondria. Rat liver mitochondria (3 mg. of protein/ml.) were incubated at 18° in a medium containing 250 mM-sucrose, 20 mM-tris acetate, pH 7·0, 5 mM-KCl, 3 mM-tris glutamate, 3 mM-tris malate and trace amounts of [^{14}C]sucrose and tritiated water. After approx. 3 min., at the first arrow, valinomycin was added to a concentration of 10 μg./g. of protein, and at the second arrow nigericin was added to a concentration of 13 μg./g. of protein. The curves are labelled: TW, total water; SS, sucrose space; [K^+], K^+ concentration.

acetate content equivalent to the original K^+ content. During the incubation before the addition of valinomycin there must have been an exchange of endogenous anions (phosphate and substrate) for acetate.

Changes in tritium-exchangeable water and sucrose space during K^+ *uptake and release.* Fig. 2 shows an experiment similar in sequence to that in Fig. 1. In this sample the K^+, the water exchangeable with tritiated water and the sucrose space were measured. Along with the uptake of 170 μmoles of K^+/g, there was an increase of total water by 0·72 ml./g., equivalent to a K^+ concentration in the extra water of 235 mM. The sucrose space remained unaltered, so, by difference, the space inaccessible to sucrose must have increased with the K^+ content. The changes were reversed when the K^+ was lost.

This pattern of result was more often obtained when the K^+ uptake and hence the swelling was restricted, whether by time or by absence of a penetrating anion. However, with some preparations, and particularly if more time was allowed for K^+ uptake and swelling, a different result was obtained. In the experiment illustrated in Fig. 3, 310 μmoles of K^+/g. had been taken up at the time the movement was reversed by adding nigericin. The light-scattering record suggests that there was a large volume change. When the analyses of the separated material were made the surprising result was found that the total water increased by about 20% during the experiment without obvious relation to the K^+ content. When K^+ had been gained the sucrose space was decreased, and the change was reversed when K^+ was lost. In agreement with the previous result, the difference, i.e. the sucrose-inaccessible space, varied with the K^+ content. There seem two alternatives to explain the behaviour of the total water: either the light-scattering records primarily the changes in volume of the K^+-containing sucrose-inaccessible space, or the particles lose sucrose solution at the time of their separation through silicone. If the former supposition is correct the concentration at which K^+ with water moves into the space is remarkably high, about 300 mM. The explanation, moreover, is discounted by the fact that light-scattering responds to other conditions of swelling

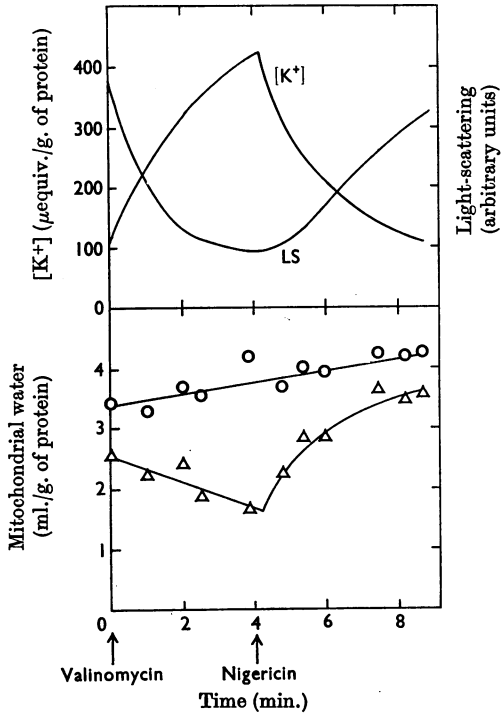

Fig. 3. Changes in sucrose space and total water content of mitochondria associated with the uptake and release of K^+ by mitochondria. Rat liver mitochondria (4·5 mg. of protein/ml.) were incubated under the same conditions as in Fig. 2 except that tris acetate, pH 6·5, was used. At the first arrow valinomycin was added to a concentration of 5 μg./g. of protein, and at the second arrow nigericin was added to a concentration of 2 μg./g. of protein. ○, Total water; △, sucrose space; LS, light-scattering; [K+], K^+ concentration. The changes in K^+ concentration in the medium sensed by the electrode have been related by suitable scaling to the equivalent changes in mitochondrial K^+ content.

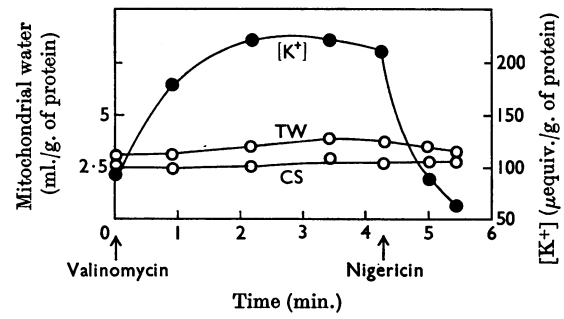

Fig. 4. Changes in Cl^--permeable space and total water content of mitochondria under conditions of K^+ uptake and release. Rat liver mitochondria (9 mg. of protein/ml.) were incubated at 23° in a medium containing 250 mM-sucrose, 20 mM-tris chloride, pH 7·4, 3 mM-tris glutamate, 3 mM-tris malate, 5 mM-KCl and trace amounts of [^{14}C]sucrose and $^{36}Cl^-$. Valinomycin was added to a concentration of 2·5 μg./g. of protein and nigericin to a concentration of 40 μg./g. of protein. The curves are labelled: TW, total water; CS, Cl^--permeable space; [K+], K^+ concentration.

in which K^+ is not involved. Perhaps the problem will be clarified by electron microscopy. A possible, though unlikely, reason for the constancy of the total water in such experiments could have been that the swollen particles carry down less adherent extraparticulate fluid than the unswollen particles. To test this, we used ^{131}I-labelled albumin, as supplied for blood-volume determination, along with [^{3}H]water. In experiments like those in which the sucrose-accessible space diminished by more than 1 ml./g. of protein as K^+ was gained, the extraparticulate water-bearing albumin carried down only varied by 0·15 ml./g. and the changes were not related to those of the sucrose space. Hence different amounts of adherent fluid can be dismissed as an explanation of the constancy of total water in these experiments.

In one experiment an alternative marker for the non-specific sucrose-accessible space was employed. With [^{14}C]EDTA as marker, and with added acetate to give a large K^+ shift, the non-specific space diminished when K^+ was gained and the total water remained nearly constant. The result confirms that the size of the sucrose- or EDTA-excluding space rises and falls with the K^+ content, and shows that the presumed loss of marker and water from the non-specific space occurs irrespective of the marker used.

Amoore & Bartley (1958) found that Cl^- occupies the same space as does sucrose. It seemed possible that under the influence of agents giving rise to massive K^+ uptake some Cl^- might be drawn into the sucrose-excluding space. This would be shown by an increase in Cl^- space when K^+ was gained. The experiment was run in a medium without acetate and the K^+ was gained along with water at a concentration of 157 mM. The Cl^--occupied space remained constant during the time K^+ was accumulated (Fig. 4). The total water increased with K^+ uptake as in the experiment illustrated in Fig. 2. According to previous results obtained with valinomycin (Harris *et al.* 1966) and some recently obtained with other agents inducing cation permeability, an uptake of 126 μmoles of K^+/g. will be accompanied by 19% decrease in the light-scattering. In the experiment illustrated in Fig. 4 the total volume found from tritiated-water exchange increased from 3·2 to 4·0 ml., i.e. by 25%, whereas the sucrose-inaccessible water was doubled.

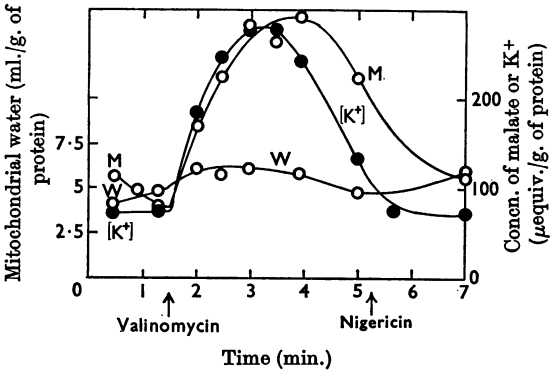

Fig. 5. Changes in total water, K^+ concentration and malate concentration in mitochondria. Rat liver mitochondria (9mg. of protein/ml.) were incubated at 23° in a medium containing 250mM-sucrose, 10mM-KCl, 20mM-tris chloride, pH7·4, 3mM-tris malate and trace amounts of [^{14}C]malate and tritiated water. At the first arrow valinomycin was added to a concentration of 2·5μg./g. of protein, and at the second arrow nigericin was added to a concentration of 40μg./g. of protein. The curves are labelled: W, total water; [K^+], K^+ concentration; M, malate concentration.

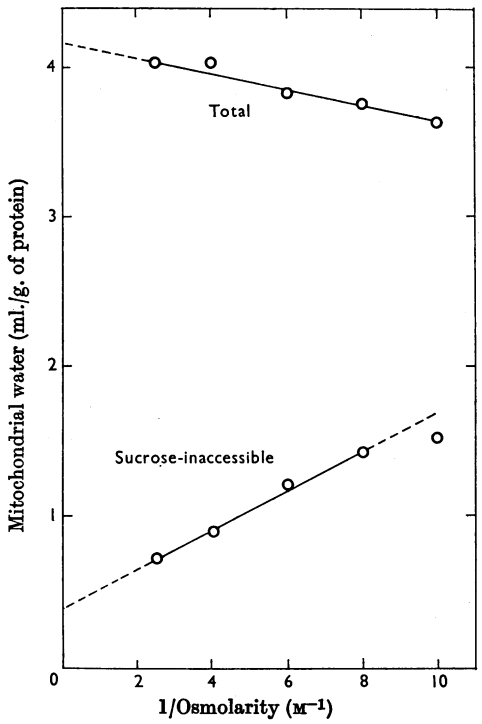

Fig. 6. Response of the total water content and the sucrose-impermeable water of mitochondria to changes in osmolarity. Rat liver mitochondria (6mg. of protein/ml.) were incubated at 23° in a medium containing 50mM-sucrose, 2·5mM-tris chloride, pH7·0, 5mM-phosphate buffer, pH7·0, 5mM-KCl, 1μg. of rotenone/ml., 2mM-EDTA, 10mM-$MgCl_2$ and trace amounts of [^{14}C]sucrose and tritiated water. The osmolarity of the medium was varied by further addition of KCl.

Without more information, however, this does not help to decide which change is responsible for the light-scattering decrease.

An increment in total water associated with gain of K^+ and malate is illustrated in Fig. 5. The K^+ and malate move with water at concentrations exceeding 100mM. The malate seems to lag behind the K^+, suggesting that the early part of K^+ uptake may be associated with internal alkalinity and that subsequent K^+ loss is associated with internal acid. The shedding of K^+ and malate started spontaneously in this experiment, perhaps because of accumulation of oxaloacetate, which would inhibit the progress of oxidation.

Response of the K^+ *concentration in the sucrose-inaccessible space to osmolarity.* Although the authors mentioned in the introduction, and others, have shown an osmometer-like response of the sucrose-inaccessible space to osmolarity, it was decided to compare the osmolarity of the internal space with that of the (sucrose) medium. The total water did not change appreciably in these conditions, but the sucrose space decreased in the hypo-osmotic media.

By difference the sucrose-inaccessible space increased; it is plotted in Fig. 6 against the inverse of the osmolarity of the sucrose solution. If we assume that the mitochondrial K^+ is associated with a univalent anion, the internal osmolarity can be calculated as the quotient of twice the K^+ content and the amount of sucrose-inaccessible water of the mitochondria. The numbers so obtained are compared in Table 1 with the external osmolarity. Except in the most swollen state there is a balance in this example between the osmolarity of the K^+ salt and that of the medium. This agreement may well be fortuitous, since other possible solutes (e.g. Mg^{2+}) have been ignored. Bartley & Enser (1964) found higher osmolarities inside than outside, and in some of our own experiments the same disparity was present. However, our results confirm that one factor in controlling the volume is the content of K^+.

Shifts of water and K^+ *accompanying phosphate swelling and ATP* ($\pm Mg^{2+}$) *contraction.* Although it is well known that addition of phosphate gives rise to swelling of mitochondria there is in fact little change in the water content or the sucrose space in 5min. at 22° either in a predominantly sucrose medium or in a sodium chloride medium.

If the addition is made to a medium with raised potassium chloride concentration (50mM or more) the swelling is rapid, and measurements by the centrifugal technique show that total water and sucrose space have increased after only a few minutes (Table 2).

Bartley & Enser (1963) showed that after 60min. many of the mitochondria were fragmented by this treatment. To minimize irreversible morphological changes, we used only short exposures, and then added ATP. This caused a loss of water from the sucrose space, which in some experiments could be further decreased by the addition of Mg^{2+} (not shown in Table 2). Even the unswollen mitochondria suspended in either sucrose or sodium chloride media underwent appreciable changes when ATP at 5mM was added; about 0·5ml. of water was lost from the sucrose space. The phosphate–ATP change seems to be primarily one of the volume of the sucrose space. Some confirmation of this could be obtained from the results of the potassium analyses. If the calculated amount of K^+ corresponding to the external concentration multiplied by the sucrose-accessible space is deducted from the total K^+, the difference will be constant so long as K^+ movements into the sucrose inaccessible space do not also take place. In the last column of Table 2, the corrected values so obtained are indeed reasonably constant, despite the changes in total K^+ accompanying the water movements. The increments of Mg^{2+} content after the Mg^{2+} addition were found to correspond to those expected for entry of the ion at its external concentration into the sucrose-accessible space.

There is no obvious explanation for phosphate and ATP bringing about changes in water and solute contents in the sucrose space, and the effects presumably reflect changes in the properties of the outer membrane itself (cf. Bartley & Enser, 1964).

Table 1. *Comparison between external osmolarity and the osmolarity calculated for a univalent potassium salt dissolved in the sucrose-inaccessible water*

External osmolarity (m-osmoles/l.)	Internal osmolarity (m-osmoles/l.)	Total mitochondrial water (ml./g. of protein)
90	120	3·34
120	130	3·24
180	174	2·95
250	248	3·19
350	334	3·35

DISCUSSION

The principal reason for performing these experiments was the fact that we had made at the same time measurements of movements of K^+ and substrate anions under similar conditions. Knowledge of the changes in sucrose-inaccessible water was a prerequisite for calculation of the concentrations. A subsidiary reason was that studies now in progress, with either electron-microscopic or microscopic techniques, have covered conditions similar to those used here.

The values that we find for total water and for sucrose space are compared in Table 3 with values obtained by other authors. We find rather more water/g. of protein than did Klingenberg & Pfaff (1966) and Bartley (1961), perhaps because the mitochondria were more swollen or lost less water at the time of separation. Klingenberg & Pfaff (1966) remark that the cristal space may collapse when there has been great swelling of the matrix space. Whether or not there is collapse, it remains true that the sucrose-inaccessible space varies with the K^+ content. The concentration of K^+ in this space appears to reach 250mM when acetate has been added to the system. If this concentration actually holds while the particles are in suspension, the energy demand for K^+ accumulation will exceed previous estimates (Cockrell, Harris & Pressman, 1966).

It is well known that gross increases in mitochondrial volume are accompanied by a raised

Table 2. *Changes in mitochondrial water, sucrose-accessible space and K^+ content under conditions of swelling induced by phosphate and subsequent shrinkage induced by ATP and magnesium sulphate*

The indicated additions were made successively on the same suspension of rat liver mitochondria at 22°. The mitochondria were incubated at a concentration of 15mg. of protein/ml. in a medium containing 83mM-KCl, 77mM-sucrose, 15mM-tris chloride, 3mM-tris glutamate and 3mM-tris malate.

Addition and its concn. (mM)	Time of addition (sec.)	Time of measurement (sec.)	Total water (ml./g. of protein)	Sucrose space (ml./g. of protein)	Total K^+ (μmoles/g. of protein)	K^+ in sucrose-inaccessible space (μmoles/g. of protein)
—	—	—	1·80	1·56	400	270
P_i (7·5)	0	165	2·25	1·92	423	263
ATP (5·0)	195	238	1·80	1·37	370	257
$MgSO_4$ (7·5)	265	375	1·78	1·36	353	240

Table 3. *Estimates of total water and sucrose-inaccessible water of mitochondria*

Source	Osmolarity of medium (osmole/l.)	Total water (ml./g. of protein)	Sucrose-inaccessible space
Bartley (1961, Table 2)	0·27	2·5*	0·36
Klingenberg & Pfaff (1966)	0·25	2·5	0·80
Present results	0·28	3·0	0·60
		3·1	0·80
		3·8	0·90
		4·1	0·80
		3·0	0·65
		2·7	0·40
	Mean	3·3	0·70

* Based on a sucrose-free dry wt./biuret protein ratio 1·2, a value that we commonly found.

permeability to substances of metabolic importance. Gutfreund & Jones (1964) obtained a fumarase activity only after swelling by exposure to Ca^{2+}, and Lehninger (1951) induced entry of NADH by hypo-osmolarity. Since the physiological system of phosphate in the presence of K^+ at a concentration similar to that in the cytoplasm causes swelling, and this is reversed by ATP in the presence of Mg^{2+}, which are other cytoplasmic components, it is possible that mitochondrial permeability may be modulated by the prevailing phosphate potential in the cytoplasm. Certainly it is important to study mitochondrial permeability in media approximating to the composition of the cytoplasm rather than in sucrose media. The requirement for phosphate noted in the penetration of some anions by Chappell & Haarhoff (1967) may have its origin in a swelling due to net gain of K^+ (or NH_4^+ in their experiments) and phosphate, which in turn increases the permeability to anions. The ready penetration of malate along with K^+, seen in Fig. 5 and in other similar experiments, bears out the importance of this substance as a carrier of reducing equivalents between mitochondrial interior and cytoplasm, as suggested by Racker (1961).

The demonstration that the sucrose space and the sucrose-inaccessible space are subject to independent variation exposes the weakness of the light-scattering or extinction methods of recording volume changes, and provides a clue to the complexities of the observations so made. For example, swelling accompanying induced K^+ uptake is an energy-linked process, and is reversed when the energy supply is interrupted (Moore & Pressman, 1964). The swelling described by Lehninger (1959*a*,*b*, 1962), and obtained by the addition of thyroxine, phosphate or Ca^{2+} ions, did not require substrate; it would take place with K^+-depleted mitochondria, and was to a considerable degree independent of the ions in the medium. The subsequent contraction with ATP + Mg^{2+} occurred equally readily in the presence of many inhibitors of respiration or phosphorylation. Accepting the thesis that the K^+-containing space provides osmotic responses, we are left with Lehninger's (1959*a*,*b*, 1962) suggestions that the volume changes of the sucrose space, as shown by our phosphate experiments, are related to cross-linkage effects, or to the state of polymerization of a colloid.

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