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THE OSMOTIC NATURE OF THE ION-INDUCED SWELLING OF RAT-LIVER MITOCHONDRIA

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SUMMARY

Analytical techniques have been developed to measure the relation between water uptake and cation uptake in rat-liver mitochondria. The water content of inner and outer mitochondrial compartments were separately measured using [^{131}I]albumin and [^{14}C]sucrose. The cation contents of the same mitochondrial pellets were measured using flame photometry. These data were used to measure the water content of each compartment which was necessary, since the basic osmotic relationships are only revealed when movements into the inner and outer compartments are separately examined. The relation between the water uptake and K^+ uptake, either spontaneous or valinomycin-induced, was dependent on medium osmolality. The concentration of the K^+ solution taken up in the inner compartment was compatible with the hypothesis that transport-linked mitochondrial swelling is driven by osmotic pressure differences which have been induced by solute movement. The changes in volume of the water compartment and in ion content during phosphate-induced swelling and during incubation in the absence of substrate were analyzed using the same methods. Under these experimental conditions also, the mitochondrial inner compartment appears to be in osmotic equilibrium with the external medium.

INTRODUCTION

The swelling of mitochondria has been the subject of numerous investigations (see ref. 1). Despite the detailed knowledge of the conditions that induce or inhibit swelling, the mechanism of the process has still not been clearly explained. Either of two clearly distinguishable processes could account for the volume change of mitochondria: (1) the change of volume could be primarily due to transport of solutes which cause osmotic gradients and thus lead to water transport; (2) the change in volume could be initiated primarily by a mechanochemical transformation or conformational change so that both water and solutes would flow as a result of the hydrostatic pressure that develops in the system. The first explanation has been advocated by CHAPPELL AND CROFTS[2], RASMUSSEN *et al.*[3] and AZZI AND AZZONE[4,5], while the second explanation has been supported by HACKENBROCK[6,7] and BLONDIN AND GREEN[8]. In both cases the mitochondria are expected to reach osmotic equilibrium regardless

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of the initial step. Thus even though a process such as ATP-induced contraction may be initiated by a conformational transformation, no appreciable shrinking would be possible unless transport of water and solutes were to follow to maintain osmotic equilibrium[9]. If a swelling process is driven by hydrostatic pressure, the composition of the solution taken up by mitochondria will be the same as that of the medium, modified somewhat by the filtration through the membrane. If the process is driven by ion movements, the composition of the transported fluid should differ from that of the medium.

The link between swelling and transport has often been used to measure the magnitude of ion movements, considering them to be expressed by the change in the light scattering of the mitochondrial suspension[10]. However, no data has previously been available to correlate the swelling quantitatively with the ion uptake, and there has been no experimental verification of the belief that the ion-induced swelling is an isosmolal process[11,12]. It is known[13,14] that only the sucrose-impermeable compartment is osmotically active, and therefore it is the relation between ion and water accumulation by the inner compartment that should confirm that the process is isosmolal. Consequently, we have undertaken to determine the role played by each compartment in the relation between K^+ transport and water transport in rat-liver mitochondria.

EXPERIMENTAL METHODS

Rat-liver mitochondria are prepared and washed once in 0.25 M sucrose as previously described[15]; after incubation in the test medium, the mitochondrial suspension is spun in cytocrit tubes[16]. The pellet is then analyzed for water content (gravimetrically), for ion content (flame photometry) and for water compartments by the use of tracers*. The volume of the extramitochondrial compartment is determined as the [^{131}I]albumin space, that of the outer mitochondrial compartment as the [^{14}C]sucrose space in the mitochondrial water, and the inner compartment volume is then obtained as the difference between the total mitochondrial water and the outer compartment water. The radioactivity of ^{131}I and ^{14}C were determined simultaneously in a scintillation counter (Nuclear Chicago, Des Plaines, Ill., model 6801). The sample was prepared for counting by digestion with 1 M hyamine hydroxide in methanol and dissolved in Bray's scintillation liquid[18]. The quenching was determined by adding known amounts of isotope to the samples.

RESULTS AND DISCUSSION

The effect of medium osmolality on water and K^+ uptake

When mitochondria are incubated in the standard medium (detailed composition of standard medium given in legend to Fig. 1), that is in the presence of 10 mM KCl, 2.5 mM ATP and 5 mM succinate with sucrose added to produce a total osmolality of 250 mosM, a slow increase in the total K^+ and water contents is observed[15] as shown in Fig. 1A. If valinomycin is added, both the rate and the amount of the K^+ uptake and water uptake increase, as shown in Fig. 1B. The K^+ concentration

* A detailed description and evaluation of the methods of analysis of the mitochondrial pellet is given elsewhere[17].

of the transferred solution can be calculated by dividing the gain in K^+ content by the gain in water content. In a typical experiment (Fig. 1) in the standard medium, the K^+ concentration of the transferred solution is 200 mM in the first 10-min and 36 mM in the second 10-min period, while in the presence of valinomycin the K^+ concentration is 165 mM in the first period and 143 mM in the second period. Thus in the absence of valinomycin, there is an apparent lag in the increase in the total mitochondrial water content as compared to the increase in the K^+ content. This

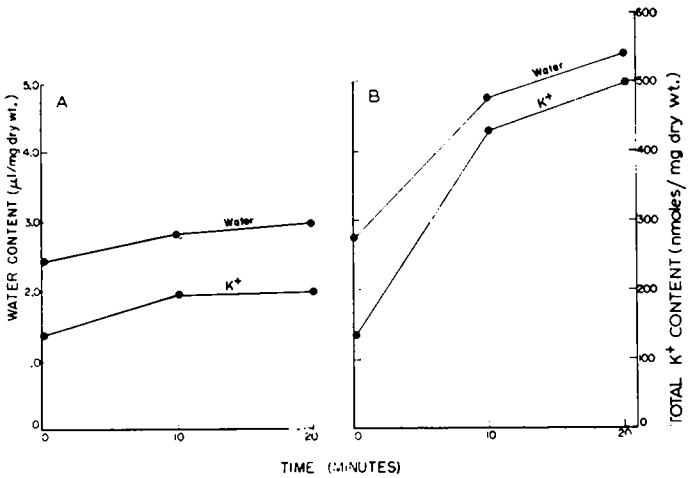

Fig. 1. Total water and K^+ content of rat-liver mitochondria. Mitochondria were incubated in the standard medium that contains 10 mM KCl, 2.5 mM disodium ATP, 5 mM disodium succinate and 208 mM sucrose; pH 6.6, gas O_2– CO_2 (95:5, v/v), $T = 30°$. A. Shows the results in the absence of valinomycin (standard). B. Gives the results in the presence of 0.015 µg/mg protein of valinomycin (concentrations are expressed in terms of µg/mg of protein; the valinomycin is added immediately after the zero time sample is taken). The protein content is 3 mg/ml suspension.

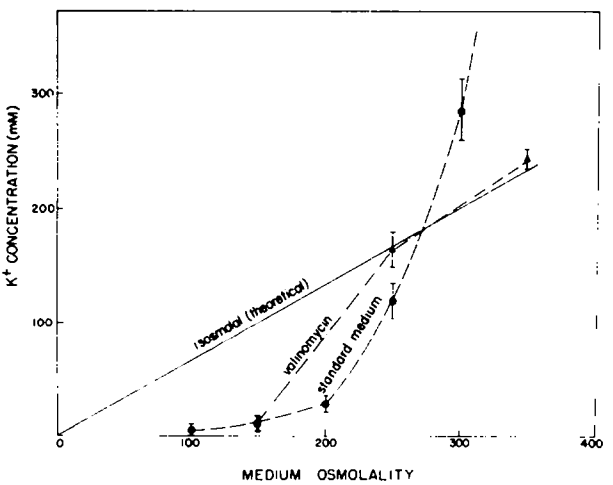

Fig. 2. K^+ concentration of the solution taken up by the total mitochondrial pellet as a function of medium osmolality. The experimental values are calculated from the results in Table I (total K^+ gain/total water gain). The theoretical curve is based on the assumption that the transport process is isosmolal.

lag is much smaller in the presence of valinomycin. Table I summarizes a series of experiments on the 20-min uptake at different osmolalities (two experiments for each value) identical in design with the experiment reported in Fig. 1, except for sucrose concentration. K^+ transport depends on the osmolality of the medium at low osmolalities and increases slowly with increasing osmolality up to about 250 mosM, whereas the water uptake decreases with increases in osmolality over the whole experimental range. From these results the K^+ concentration of the transferred solution has been calculated as a function of medium osmolality, as shown in Fig. 2. Since the salt that is entering the mitochondria under these conditions is mostly dipotassium succinate[17], the predicted K^+ concentration for isosmolal swelling may also be computed. Although the concentration of the transferred solution does indeed depend on medium osmolality, the deviation from isosmolal transport is considerable. Only the experiments in the presence of valinomycin at high osmolality show good agreement with the calculated values. Inspection of Table I reveals that these are experiments in which massive transport of K^+ occurred. If one assumed that the swelling is the result of an isosmolal transfer of salt into the inner compartment, the total swelling would reflect the change in the water content of this compartment only when the changes in the other compartments are negligible in comparison, as is probably the case in the presence of valinomycin. When the total transport is relatively small, however, the relation between the total water and K^+ uptake is not an index of changes in the internal compartment. For this purpose a careful determination of water compartmentation during swelling is needed.

TABLE I

THE EFFECT OF MEDIUM OSMOLALITY ON K^+ AND WATER UPTAKE

Each value is an average of two experiments. The experimental conditions are the same as given in Fig. 1 except that the sucrose concentrations have been varied to provide the indicated osmolality. The incubation time is 20 min.

Osmolality (mosM)	*Standard*		*+ Valinomycin*	
	K^+ uptake (nmoles/mg dry wt.)	*Water uptake (µl/mg dry wt.)*	*K^+ uptake (nmoles/mg dry wt.)*	*Water uptake (µl/mg dry wt.)*
100	17	3.50	—	—
150	35	2.70	80	6.00
200	42	1.45	—	—
250	57	0.47	510	3.13
300	58	0.20	—	—
350	61	0.11	475	1.93

Compartmentation of the mitochondrial water after swelling

The volume of the separate mitochondrial compartments has been determined using a modification of the conventional tracer method[14]. It was found that variations in the parameters of the system (such as total pellet water, extramitochondrial water, *etc.*) are considerable not only between experiments but also between duplicate samples and indeed even between portions of the same pellet. For that reason, in contrast to the procedure of BENTZEL AND SOLOMON[14], no predetermined factors or average values are used for the calculation, and all the necessary parameters are

determined on the same sample: water content, ^{14}C and ^{131}I activity and counting efficiency. Identical determinations are made for the supernatant from the same cytocrit tube as for the pellet being analyzed. The ion content is determined from another portion of the same pellet. In order to minimize variability due to position of the mitochondria in the pellet, duplicates were taken. The samples for each type of determination were alternatively switched and the duplicates were averaged after the calculation of compartment volumes. The water compartment most dependent on position in the pellet column is the extramitochondrial water which is often 4 times greater in the upper portion of the pellet than at the bottom. This is to be expected, since the mitochondria at the bottom should be better packed than those at the top; presumably the more swollen mitochondria are in the upper portion of the pellet because they are less dense than the shrunken mitochondria and sediment more slowly. Such differences can be observed by the naked eye, since the bottom of the pellet always has a higher absorbance than the top. Even though the spread of some variables (such as total pellet water and extramitochondrial water) covers a considerable range, the parameters derived from these measurements, as mitochondrial water in the above instance, cover a much smaller range, thus indicating that these two variables are not independent. For this reason, the determination of all the necessary parameters on the same pellet causes a considerable increase in overall accuracy.

Fig. 3 shows the average results of seven experiments on cation content, water content and water compartments when the mitochondria are incubated in the standard medium. The rate of K^+ uptake is very similar to the water uptake by the inner compartment. The total change in mitochondrial pellet water content arises from two contributions: a decrease in the outer compartment over the entire 20 min and an increase in the extramitochondrial water during the second 10 min. These results clearly demonstrate that the changes in the total water under these conditions can only accidently reflect the changes in the inner compartment. It is interesting to

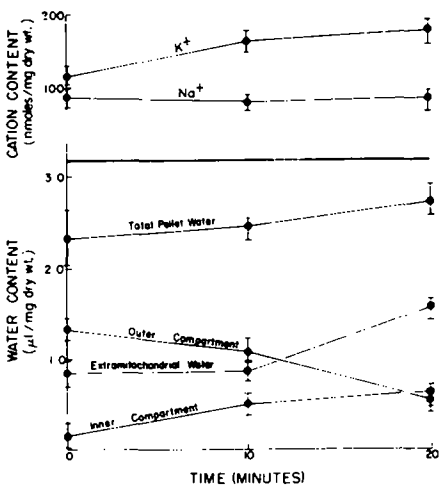

Fig. 3. Cation content and water content of the mitochondrial compartments. The medium is the same as described in the legend to Fig. 1 except that 1 mg/ml albumin, and 1 μC each of [^{14}C]-sucrose, and [^{131}I]albumin have been added.

note the relation between the inner compartment and the outer compartment: for the first 10 min while the extramitochondrial water does not change, most of the inner compartment swelling is at the expense of the volume of the outer compartment. Later both the inner compartment and the extramitochondrial water increase, while the outer compartment shrinks further. From these data, we may calculate the concentration of the solution that enters the inner compartment. Assuming that all the K^+ gain occurs in the inner compartment, the K^+ concentration of the solution that has penetrated the inner compartment is 144 $\pm$ 23 (S.D.) mM in the first 10 min and 141 $\pm$ 27 (S.D.) mM in the total 20-min period. These figures are in fair agreement with the theoretical value of 166 $\pm$ 20 mM calculated for the transfer of potassium succinate at 250 mosM. The theoretical calculation is based on the following assumptions: ideal solutions, completely ionized succinate, no ion binding or cation exchanges and no net movement of cations other than K^+ and Na^+ or anions other than succinate. Therefore we have taken the accuracy of the predicted value to be no better than $\pm$ 20 mM.

In this calculation we assume that all the water that penetrates the inner compartment is solvent water. BENTZEL AND SOLOMON[14] interpreted their results as evidence that some of the water initially present in the inner compartment was "nonsolvent" water, a quantity that was not expected to change appreciably with changes in mitochondrial volume. In the present study the concentrations are calculated on the basis of the quantity of solution transferred, thus avoiding the question of the apparent solvent properties of the water initially present.

Fig. 4 shows the average results of four such experiments in the presence of valinomycin. In this case also the changes in the inner compartment correspond to those of the K^+ content. The changes in the other compartments are similar to those observed in the preceding experiments. The size of the inner compartment in this case is so large in comparison with the other compartment that the changes in

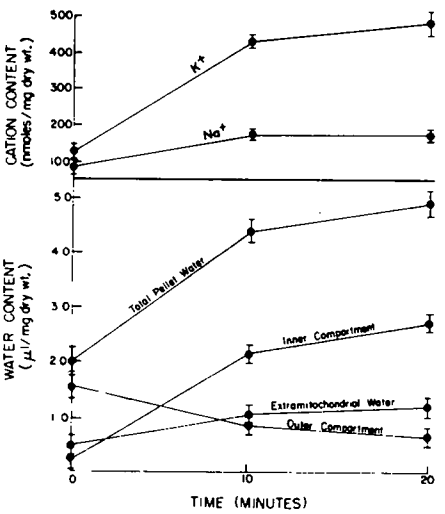

Fig. 4. Cation and water content of the mitochondrial compartments after addition of valinomycin. Conditions are the same as in Fig. 3 except for the addition of valinomycin (0.015 μg/mg protein, added immediately after the zero time sample is taken).

the total water content primarily reflect the increase in the inner compartment as already discussed in connection with Fig. 2. The K^+ concentration in the solution transferred into the inner compartment is found to be 152 mM for the first 10 min and 133 mM for the 20-min period. Appreciable amounts of Na^+ are also taken up by the mitochondria during experiments of this type*. If the total concentration of Na^+ *plus* K^+ is calculated, the inner compartment uptake is 197 ± 12 (S.D.) mM and 168 ± 14 (S.D.) mM for the 10-min and 20-min periods, respectively. The first value appears to be higher than the theoretical value of 166 ± 20 mM and higher than the values observed in the absence of valinomycin (144 mM and 141 mM for the 10-min and 20-min periods, respectively). Perhaps the reason for the high value at the 10-min period is that the initial phase of uptake in the presence of valinomycin is a K^+-H^+ exchange which does not change the osmolality of the mitochondria. The 168 mM cation concentration of the transferred solution that is found for the 20-min period is in remarkable agreement with the expected value of 166 mM.

These experiments may also be used to verify the assumption that the potassium salt is transported into the inner compartment as has been postulated by many (see ref. 19). A similar conclusion was also recently reached by WENNER AND HAGA[20] using electron microscopy. The experiments at various osmolalities (Table I) show an inverse relation between the medium osmolality and the total water gain. In view of the small magnitude of the K^+ uptake in the inner compartment, calculations based on total pellet water as made for Fig. 2 indicate a significant deviation from isosmolal transport. This apparent deviation may be attributed to changes in the outer and the extramitochondrial compartments as shown in Figs. 3 and 4. In both instances, the outer compartment reduces its volume, while the inner compartment volume increases. This finding indicates that the outer compartment is freely permeable to small solutes, and its volume is determined mostly by the state of concentration of the inner compartment. The initial volume of the inner compartment is found to be very small (between 10 and 20% of total water). These values are significantly smaller than those of BENTZEL AND SOLOMON[14]. This may be due in part to the difference in technique but probably is the result of the very different incubation medium. Our initial values are determined immediately after suspension of the freshly prepared mitochondria at 4° in the test medium, which is very different from that of BENTZEL AND SOLOMON[14]. They incubated their mitochondria at room temperature in a medium devoid of K^+ or substrate for long periods.

When the mitochondria swell, the unfolding inner membrane pushes against the outer membrane and reduces the volume of the outer compartment. In some cases of very swollen mitochondria, the outer compartment seems to disappear altogether as if the outer membrane were broken (H. ROTTENBERG, unpublished data). It should be stressed that the increase in the extramitochondrial volume is mainly due to the increase in the size of the mitochondria. If the extramitochondrial volume is expressed as a percentage of the total pellet water, there is no significant

* The Na^+ uptake in these experiments is about 25% of the K^+ uptake. However, in earlier experiments with a freshly prepared valinomycin solution, such stimulation of Na^+ uptake was not observed. In the later experiments, the external Na^+ concentration was about twice as great as the K^+ concentration, while the initial internal Na^+ concentration was about half that of K^+; thus the selectivity for K^+ was still very high, even though the valinomycin solution used in these later experiments was more than a year old. It is possible that aging could have caused a loss in ion selectivity.

change. Before swelling the extramitochondrial water constitutes 27%, while after 20 min it is 26% (Fig. 4); at the same time the inner compartment increases from 12 to 55%, while the outer compartment decreases from 61 to 19%.

The fact that the potassium succinate concentration in the transferred solution is much greater than that of the medium, and indeed even greater than that of the mitochondrial internal compartment[17], clearly indicates that the primary cause for the swelling is the salt transport. The observation that the osmolality of the transferred solution is close to that of the medium is an indication that water follows the salt to maintain osmotic equilibrium.

Phosphate swelling

We have found[17] that phosphate swelling is associated with loss of K^+. The mechanism of this process is not clear, and it is disturbing that swelling which is expected to result in net salt uptake is instead accompanied by loss of K^+. Fig. 5 shows the average results of three experiments on mitochondria incubated in the presence of 10 mM inorganic phosphate. K^+ is lost so that after 20 min, the 11 mM K^+ concentration of the pellet is almost equal to the 10.5 mM K^+ in the medium. At the same time the Na^+ content of the pellet increases due to the increase of the sucrose-accessible water space, which also contains Na^+; the pellet concentration and the external medium concentration are both 35 mM after 20 min. The Na^+ movement should not be mistaken for transport against a concentration gradient. From the very beginning of the experiment the sucrose space which includes the extramitochondrial water and the mitochondrial sucrose-accessible compartment is almost equal to the total water space. It is known that phosphate induces increased permeability of the mitochondrial membrane[4] and it is evident that the inner membrane in these experiments becomes permeable to sucrose. These changes also impair the ability of the mitochondria to maintain a concentration gradient of K^+ which accounts for the net loss of K^+. The movement of sucrose and Na^+ into the mitochondria and the loss of

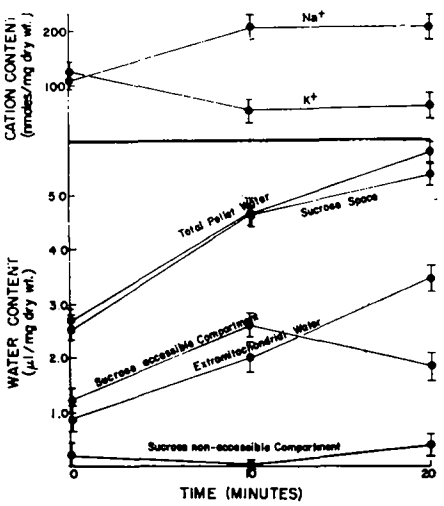

Fig. 5. Phosphate-induced swelling of the mitochondrial pellet. Conditions are the same as in Fig. 3 except that 10 mM P_i (NaH_2PO_4–Na_2HPO_4, pH 7.0) has been added to the medium.

K^+ leads to equilibration of the small solutes, but the proteins and other organic salts still retained by the mitochondria can cause a considerable swelling. Thus, regardless of the initial cause of the phosphate-induced changes, the data agree with the assumption that the mitochondria maintain osmotic equilibrium.

Although [^{14}C]sucrose was used throughout this study as a marker for the outer compartment, it is evident from this experiment on the phosphate-induced swelling that a better marker is needed in order to define this compartment under all conditions.

Volume changes in the absence of succinate

If the swelling induced by ion uptake is an isosmolar process, the shrinkage induced by losses of ion should also be isosmolar. It is well known that inhibition of respiration causes loss of water as well as loss of ions[17]. In this instance also the use of total water as an index is misleading and the isosmolar relation only becomes apparent when the water change in the inner compartment is measured, particularly for losses of ions from freshly prepared mitochondria. As shown in Table II there are extensive losses of K^+ in the absence of substrate though the pellet is more swollen.

TABLE II

CHANGES IN ION CONTENT AND WATER COMPARTMENTS OF THE MITOCHONDRIAL PELLET AFTER INCUBATION IN THE ABSENCE OF SUCCINATE

Average of three experiments; conditions the same as given in the legend to Fig. 3 except for the omission of disodium succinate. The incubation time is 20 min.

	Δ 20 min
K^+ (nmoles/mg dry wt.)	−38
Na^+ (nmoles/mg dry wt.)	+48
Total pellet water (μl/mg dry wt.)	+ 1.95
Extramitochondrial water (μl/mg dry wt.)	+ 2.05
Inner compartment (μl/mg dry wt.)	− 0.34
Outer compartment (μl/mg dry wt.)	− 0.24

The increase in total pellet water arises from a large increase in the extramitochondrial water together with a slight increase in the outer compartment. The inner compartment shrinks, and the average K^+ concentration of the extruded solution from this compartment is 112 mM (147, 92, 97 mM) of K^+ alone compared with the theoretical value of 166 mM. Considering the small magnitude of these changes and the possibility of additional leaks in the absence of metabolism, the results in the absence of succinate are consistent with the view that water transport is driven by solute transport.

The results of the present study support the view that the swelling associated with K^+ transport is an osmotic process in which water moves following a gradient produced by ion transport. The crucial point is the determination of inner compartment water movement. Thus, at 250 mosM, the transport of dipotassium succinate causes the inner compartment to take up water to maintain its osmolality equal to that of the medium. The experiments on swelling in the presence of phosphate, or in the absence of succinate, were performed since the analysis of total water and K^+ transport in these cases appeared, at first sight, to refute the notion that such swelling

is osmotically induced. However when movements into individual compartments are taken into account, the results are in agreement with the osmotic gradient hypothesis. Many cases which seem to agree with this hypothesis on the basis of total water and K^+ analysis, such as the loss of water and K^+ on inhibition of respiration, remain to be investigated using this method. In particular, the phenomenon of ATP-induced contraction could benefit from such a detailed analysis since it has been strongly suggested that this process involves contractile proteins[21].

In summation, all the evidence presented in this study fits the notion that water movement follows solute movement in accordance with conventional physical forces, so that there is no need to invoke any other physical causes for mitochondrial volume changes which are linked to solute transport. If there are, indeed, cases of water movement primarily induced by contractile processes in the mitochondria, convincing quantitative analytical evidence will be required to demonstrate that the contractile process is the physical cause of water movement.

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