OSMOTIC AND METABOLIC ALTERATIONS OF MITOCHONDRIAL SIZE

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ABSTRACT

Sustained contraction (dehydration) of rat liver mitochondria can be readily produced by increasing the tonicity of the outside media, provided Ca^{++} is removed by EDTA, fatty acids are removed by albumin, and a source of chemical energy (mitochondrial substrate or ATP) is present. This was demonstrated both gravimetrically and turbidimetrically. It was also demonstrated that the net movement of sucrose and H_2O under altered conditions of tonicity in mitochondria was dependent on the state of the mitochondria; *e.g.*, in the presence of EDTA, diffusion was blocked, both into and out of mitochondria, whereas, in the presence of EDTA and electron-transport substrates, movement of sucrose and water out of mitochondria was increased. In the presence of Ca^{++}, gramicidin, or fatty acids, diffusion of sucrose into and out of mitochondria is very rapid. Mitochondria obey osmotic law only after Ca^{++} and fatty acids are removed from them.

INTRODUCTION

Tedeschi *et al.* have shown that liver mitochondria, prepared in isotonic sucrose-EDTA (ethylenediamino-tetraacetic acid), obey osmotic (Boyle-van't Hoff) law (1). Tedeschi (2) has also shown that mitochondria prepared in sucrose without EDTA tend to obey osmotic law. However, obedience to this law is short-lived, especially under hypotonic conditions. If mitochondria were contracted by raising the osmotic or ionic strength, contraction lasted only 1 minute, and this was followed by a very rapid swelling rate. No explanation of this rapid swelling was apparent, but presumably could not be the result of osmotic changes. It is also known (3) that mitochondria can be made to contract maximally in solutions of essentially pure water. Thus, it appears that although mitochondria do obey osmotic law under some conditions, it seems unlikely that all mitochondrial size changes can be entirely explained in osmotic terms.

Data in this report indicate that mitochondria can be made to obey osmotic law under a wide variety of osmotic conditions, provided liberated Ca^{++} and fatty acids are removed from the media, and, under some conditions, provided a source of chemical energy is present. The rapid swelling, observed by Tedeschi (2), which followed osmotic contraction, can be completely eliminated if EDTA and serum albumin are present in the buffer. Furthermore, the diffusibility of sucrose, salts, and water, both into and out of mitochondria, under conditions of altered concentrations, is shown to be differentially controlled by EDTA, serum albumin, and chemical energy. For example, Ca^{++} and fatty acids markedly increase the permeability of mitochondria to sucrose in both directions (into and out of the mitochondria). EDTA decreases permeability in both directions. However, EDTA plus chemical energy decreases diffusion of sucrose into mitochondria, but in-

creases diffusion of sucrose out of mitochondria. Likewise, it is shown that, in the presence of Ca^{++} or fatty acids, osmotic contraction of mitochondria does not occur. If Ca^{++} and fatty acids are removed by EDTA and albumin, osmotic contraction returns. Thus, Ca^{++} and fatty acids are not causing lysis of mitochondria, but rather are reversibly changing the semipermeable mitochondrial membranes from a semipermeable membrane into ones that rapidly equilibrate with sucrose and salts. It is further demonstrated that, for metabolic contraction of mitochondria to occur, the mitochondrial membranes must be maintained in a semipermeable state.

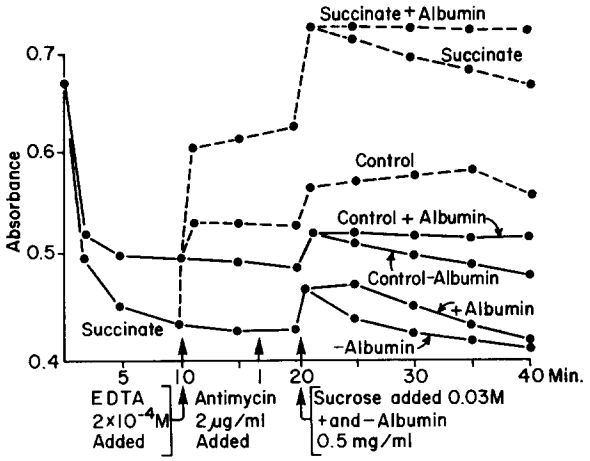

FIGURE 1 *Contraction of Mitochondria by EDTA and sucrose.* Fresh mitochondria, prepared according to Hogeboom (4), from liver obtained from 200-gram rats were incubated in 3 ml of 0.03 Tris HCl pH 7.3, with and without Tris-succinate, 5×10^{-4} M, at 25°. At 10 minutes, Mg^{++} EDTA (2×10^{-4} M pH 7.3) was added. (----- = EDTA addition). At 20 minutes, 0.07 ml of 1 M sucrose (with and without serum albumin, 0.05 ml of a solution containing 30 mg/ml) was added. Absorbance was followed at 520 mμ. All solutions were buffered at pH 7.3 with Tris. Total volume of each incubation was kept constant by addition of appropriate amounts of water. Similar experiments were done containing more mitochondria in larger volumes of buffer, and mitochondrial weights were obtained by centrifugation at 10 minutes, 15 minutes, and 30 minutes. In all cases, the change in weight of the mitochondrial pellets was linearly proportional to the change in absorbance. Antimycin, 2 μg/ml, was added to duplicates of all incubations at 17 minutes. No difference in osmotic contraction was observed in the presence or absence of antimycin. Therefore, only one set of data is plotted in the figures. Absorbance is plotted on the ordinate and time on the abscissa. Time of addition is indicated by the arrows.

METHODS

Mitochondria were isolated from the livers of Osborne-Mendel rats, weighing 180 to 200 grams, according to the method of Schneider and Hogeboom (4). Two washings with 0.25 M sucrose, 0.02 M Tris pH 7.3, were done.

Water movement in mitochondria was followed both turbidimetrically at 520 mμ, using a Bausch and Lomb colorimeter No. 340, and gravimetrically, using the methods of Amoore and Bartley (5).

For the gravimetric assays, mitochondria were sedimented at 5° at 12,000 *g* for 10 minutes in the No. 40 rotor of a Spinco Model L centrifuge. The centrifuge tubes were then drained by inversion for 5 minutes, and sides of tubes wiped dry with absorbent tissue. The tubes were weighed before and after sedimentation of the mitochondrial pellet. Extramitochondrial content of buffer (space) was assayed in all conditions of these experiments, using EDTA-C^{14}, and found to be about 17.5 per cent of the water space. The variation observed was from 16.2 to 19.3 per cent. This agrees well with the results of Werkheiser *et al.* (6), who used polyglucose to assay the extramitochondrial space, and with Jackson and Pace (7). Under the conditions of these experiments, good agreement between the turbidimetric and the gravimetric methods was obtained in all conditions.

MEASUREMENTS: Soluble protein was measured both turbidimetrically (8) and colorimetrically (9); Ca^{++}, by flame photometry at 620 mμ in nitric acid digests, using the Zeiss spectrophotometer; and sucrose by the method of Kulka (10).

RESULTS

The data in Fig. 1 indicate that mitochondria, prepared in the absence of EDTA and incubated, either isotonically (11), or in very low osmotic and ionic strength buffer, can be contracted by succinate and EDTA. Addition of 0.03 M sucrose causes further contraction, and this contraction is sustained if serum albumin is present. Osmotic contraction also occurs in the presence of succinate without EDTA. However, this contraction is completely overcomes the inhibition due to Ca^{++}. Addition of EDTA alone causes some contraction, but this is amplified by addition of sucrose (Fig. 1). However, if mitochondria are repeatedly washed with sucrose or aged for 12 hours at 0°, no contraction by EDTA occurs, unless substrate is added. Substrate-EDTA contraction is readily blocked by antimycin, but not by oligomycin (11).

If oleic or palmitic acid, 2×10^{-5} M, are added under the conditions of Fig. 2, no contraction en-

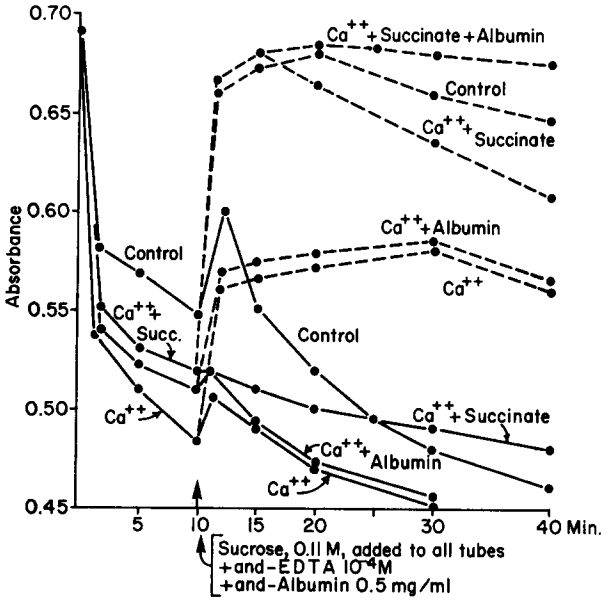

FIGURE 2 *Effect of Ca^{++}, albumin, succinate, and EDTA on sucrose contraction.* Buffer, 0.025 M sucrose, 0.02 M Tris pH 7.3. Incubations were performed as in Fig. 1. The Ca^{++} concentration was 10^{-4} M and succinate 5×10^{-4} M. At 10 minutes, enough sucrose was added to make the final concentration of sucrose 0.11 M. Mg^{++} EDTA, 10^{-4} M (final concentration), and albumin 0.5 mg/ml (final concentration) was added at 10 minutes to the tubes indicated. - - - - - = Succinate and EDTA, or EDTA + albumin + sucrose addition at 10 minutes. —— = sucrose addition only at 10 minutes to each of the 4 incubations.

small and is followed by swelling, both in the presence and absence of albumin. Contraction by sucrose without substrate and EDTA is minimal and not sustained.

Osmotic contraction is not inhibited by antimycin, oligomycin, or both, if the inhibitors are added after the addition of substrate-EDTA (Fig. 1). However, addition of antimycin prior to addition of succinate (5 minutes) completely blocked the effects of succinate-EDTA contraction, but not osmotic contractions (11). Osmotic contraction can also be obtained by addition of 0.03 M KCl or NaCl. (These results are not presented, but are the same as those presented in Fig. 1 for sucrose).

Osmotic contraction by sucrose or salts is blocked by the previous addition of excess Ca^{++} to the medium despite the fact that EDTA, in excess of Ca^{++}, is added after swelling has occurred (Fig. 2). However, addition of substrate sues after the addition of substrate, EDTA, and sucrose. Addition of serum albumin, 1 mg/ml, completely eliminates the fatty acid inhibition. These data are not presented since they are identical to those presented in Fig. 2 for Ca^{++} and EDTA. It can be seen (Fig. 2) that albumin is necessary to maintain maximal contraction when no fatty acids are added. This is expected, since it is known that mitochondria do liberate fatty acids during incubation, especially under hypotonic conditions or in the absence of sufficient ATP to reesterify the liberated fatty acids (11, 3).

In Table I are listed a number of mitochondrial swelling agents which also inhibit sucrose contraction. However, inhibition of osmotic contraction by these agents, except gramicidin, tyrocidine, and sucrose, can be at least partially overcome by addition of EDTA along with the sucrose. Addition of albumin as well as EDTA is necessary for sustained contraction (Figs. 1 and 2).

Since high concentrations of sucrose inhibit osmotic contraction (Table I), as well as ATP or substrate contraction (3), the apparent KI's for sucrose inhibition for these two types of contraction were compared. Mitochondria were swollen with succinate in various concentrations of sucrose, and the rates of contraction in the presence

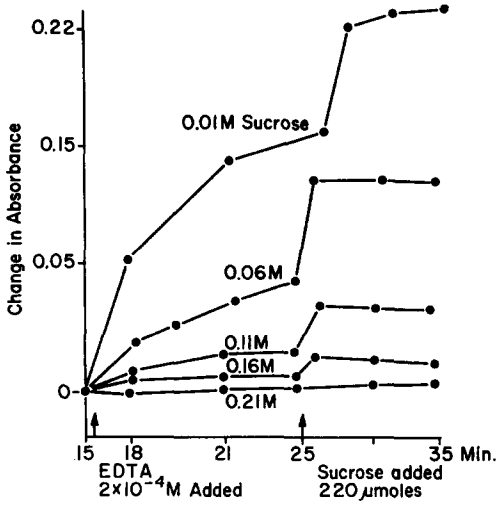

FIGURE 3 *Contraction of mitochondria in sucrose — succinate.* Rat liver mitochondria were preincubated for 15 minutes in varying concentrations of sucrose in 3 ml of Tris buffer, containing 5×10^{-4} M succinate as indicated. At 15 minutes, all mitochondria had swollen approximately the same, from an initial absorbance of 0.7 to 0.45. At this point, Mg^{++} EDTA, 2×10^{-4} M, was added. Shrinkage was expressed as absorbance at 520 mμ. At 25 minutes, 220 μmoles of sucrose was added to each tube in a volume of 0.04 ml. Absorbance at 520 mμ was plotted from 15 to 35 minutes.

of EDTA and EDTA plus sucrose were compared. A concentration of 0.08 M sucrose inhibited both substrate and osmotic contraction by approximately 50 per cent (Fig. 3). However, contraction by sucrose is only slightly inhibited in the presence of other osmotic conditions, such as isotonic KCl or NaCl (Table I). The inhibition of osmotic contraction by sucrose is, therefore, not related to the initial osmolality of the buffer, but to sucrose itself. This inhibition of contraction by sucrose is strikingly temperature dependent. If incubations are done at 23° in the presence of a high concentration of sucrose, and then the temperature quickly raised to 38°, metabolic contraction promptly occurs (Table II). This contraction requires either substrate and EDTA, or ATP. For comparison, similar data on contraction obtained from mitochondria incubated in water are presented in Table II. The degree of contraction obtained in sucrose in the presence of ATP at 38° is essentially equal to that obtained in water.

In an effort to study further the role of the mitochondrial membrane on changes in size of mitochondria, the movement of sucrose, protein, Ca^{++}, and water into and out of mitochondria during extreme osmotic changes was assayed (Table III). Mitochondria were first equilibrated with buffer of low osmotic activity at 5° (Table III *A*) and then quickly exposed to buffer of high osmotic activity (sucrose, Table III *B*). Since 10 minutes was required to isolate the mitochondria from the buffer, initial rates could not be obtained. However, the data clearly indicate that mitochondria under differing conditions do not obey osmotic law. EDTA slows the rate at which sucrose diffuses out of mitochondria. Likewise, swelling by buffer of low osmotic activity is blocked by EDTA. Contraction by buffer of high osmotic activity also requires EDTA (Table III *B*). These effects of EDTA on mitochondrial water movement are amplified by either succinate or ATP. However, whereas EDTA impedes the outflow of sucrose under hypotonic conditions, the addition of succinate or ATP with EDTA overcomes this inhibition by EDTA. Also, under both conditions of osmotic stress, either ATP or succinate blocks the removal of Ca^{++} from the mitochondria by EDTA. EDTA also blocks the loss of protein from mitochondria when the outside osmotic pressure is quickly raised. This effect of EDTA is further accentuated by succinate or ATP. The data in Table III *B* further indicate that, although sucrose rapidly equilibrates with mitochondrial water when the concentration of sucrose is quickly increased in the buffer, a source of energy, as well as EDTA, is required for maximal osmotic contraction. In the presence of succinate alone, increasing the outside osmotic pressure actually increases swelling, and also increases the loss of Ca^{++} and protein from the mitochondria. Thus, it is clear that not only can swelling of mitochondria under extreme conditions of hypotonicity be blocked by EDTA and an energy source, but also that maximum osmotic contraction of mitochondria requires the presence of energy and EDTA.

DISCUSSION

Osmotic contraction of mitochondria at 23° does not occur in the presence of Ca^{++}, fatty acids, gramicidin, or high concentrations of sucrose. These agents, likewise, block metabolic contraction. The data in this paper indicate that freshly isolated mitochondria do possess a semipermeable membrane. However, during incubation, Ca^{++} slowly leaks from mitochondria and fatty acids accumulate in mitochondria in the absence of energy, and the semipermeable membranes of the mitochondria become more and more leaky. This process is reversible, but requires both the removal of Ca^{++} and fatty acids, as well as a source of chemical energy, for maximal reversal of the membranes to a semipermeable state. Likewise, metabolic contraction of mitochondria (either by ATP or succinate-EDTA) also requires that mitochondrial membranes be maintained in a semipermeable state.

It is highly unlikely that metabolic contraction is the result of the differential movement of ions through these semipermeable membranes, since this contraction can occur in the almost complete absence of ions, other than a small concentration of Tris buffer (0.02 M). Osmotic or metabolic contraction can also be obtained in water which contains no buffer or ions other than albumin and EDTA (unpublished data).

No explanation of how chemical energy mimics

TABLE I

Additions	Absorbance × 1000			
	0 to 5 min.	5 to 6 min.		6 to 15 min.
	−EDTA	−EDTA	+EDTA	−EDTA
Buffer–0.015 M Sucrose				
1. Control	−60	+90	+100	−170
2. Succinate 2×10^{-4} M	−130	+40	+250	−90
3. Ca^{++} 3×10^{-4} M	−210	+10	+45	−40
4. Cysteine 5×10^{-4} M	−180	+85	+110	−145
5. Tyrocidine 3 μg/ml	−170	0	0	−70
6. Gramacidin 2 μg/ml	−190	0	0	−50
7. Thyroxine 5×10^{-5} M	−120	+15	+65	−80
Buffer–0.05 M Sucrose				
8. —	−95	+15	+25	−80
Buffer—0.25 M Sucrose				
9. Control	−20	0	0	−30
10. Succinate 2×10^{-4} M	−95	+10	+15	−15
Buffer—0.025 M KCl				
11. Control	−100	+70	+85	−140
Buffer–0.125 M KCl				
12. Control	−20	+60	+95	−70
13. Succinate	−110	+30	+195	−80

0.11 M Sucrose added + and − EDTA, 2×10^{-4} M after 5 min. to each tube.
Liver mitochondria obtained from 0.15 gm of liver were incubated for 5 minutes in the buffer indicated, at 25°. All tubes contained 0.02 M Tris pH 7.3. Swelling is recorded as −absorbance × 1,000 from 0 to 5 minutes, 5 to 6 minutes, and 6 to 15 minutes. Enough sucrose, or sucrose plus Mg^{++}EDTA, was added at 5 minutes to increase the final sucrose concentration by 0.11 M, and contraction is expressed as +absorbance × 1,000.

osmotic dehydration of mitochondria is apparent from the data in this report. Ohnishi and Ohnishi (12) have implied that the "contractile protein" of mitochondria may be the source of the energy for dehydration. This is a possible explanation. However, if the contractile protein is involved, one must conclude from these data either that this contractile protein is what is responsible for agents is not to liberate fatty acids or Ca^{++} from mitochondria, but must act by altering the semipermeable properties of the membranes in some way, since osmotic contraction is also blocked by these agents.

The nature of inhibition of both osmotic and metabolic contraction by high concentrations of sucrose is not clear from these data. However,

TABLE II

Contraction of Mitochondria by Heat in Sucrose and H_2O

Additions	Buffer–0.22 M Sucrose 0.02 M Tris		Buffer – 0.02 M Tris	
	ΔAbs. × 1000 0 to 10 min. 23°	ΔAbs. × 1000 10 to 15 min. 38°	ΔAbs. 0 to 10 min. 23°	ΔAbs. 10 to 15 min. 38°
Control	−60	−220	−190	−70
Succinate 2×10^{-4} M	−190	−80	−270	+40
Succinate 2×10^{-4} M + Mg^{++} 10^{-3} M	−150	−60	−180	+80
EDTA 2×10^{-4} M	−40	−25	−100	−60
Succinate 2×10^{-4} M EDTA 2×10^{-4} M	−135	+25	−180	+105
ATP 10^{-3} M	−40	+55	−100	+40
ATP 10^{-3} M + Antimycin 3 μg/ml	−40	+60	−80	+50
Succinate 2×10^{-4} M + Antimycin 3 μg/ml	−140	−140	−230	−35

Aliquots of fresh mitochondria were incubated as in Table I, either in 0.22 M sucrose or in water for 10 minutes at 23°. Change in absorbance at 520 mμ was followed, and the change in absorbance recorded in column 1 for each buffer. The temperature of the incubation mixture was quickly raised to 38° by shaking in a water bath for 5 minutes, and the change in absorbance recorded at the end of this 5 minutes. This information is in column 2 for each buffer. All additions were at 0 time, except EDTA and antimycin, which were added at 5 minutes. When incubated in 0.02 M Tris, the contraction produced by addition of EDTA was maximal and had plateaued before the temperature was elevated. The numbers in the graph represent only the additional contraction produced by the increase in temperature.

the maintenance of the semipermeable properties of mitochondrial membranes, or else that Ca^{++}, fatty acids, and basic polypeptides block both the osmotic as well as the contractile factors in mitochondria.

The basic polypeptides, tyrocidin and gramicidin, completely block metabolic or osmotic contraction (Lehninger, 3, and Table I), and this inhibition is not affected by either albumin or EDTA. Thus, the role of these types of swelling since both osmotic contraction as well as metabolic contraction are inhibited by sucrose, the mode of action of sucrose must be to alter the semipermeable property of the membranes. The data in Table I indicate that addition of sucrose to the outside of the mitochondria, under either isotonic or hypotonic conditions (KCl), does produce a marked contraction of the mitochondria. Thus, inhibition of contraction by sucrose is not related to the initial osmolality of the solution.

TABLE III

Net Flux of Sucrose, Protein, Ca^{++}, and H_2O in Mitochondria during Osmotic Stress

Additions	Sucrose content mg	Sucrose conc. in/out	H_2O content mg	Protein in buffer mg	Ca^{++} % Bound
A Buffer 0.025 M Sucrose–0.02 M Tris pH 7.3					
0 Time	5.05	0.91	65.0	1.25	56
None	1.11	1.45	77.2	1.45	78
Succinate 5×10^{-4} M	1.22	1.60	75.2	1.41	95
EDTA 2×10^{-4} M	1.92	2.7	71.1	1.62	26
EDTA-2×10^{-4} M + Succinate 5×10^{-4} M	1.01	1.43	69.8	1.72	57
ATP-2×10^{-4} M + EDTA-2×10^{-4} M	1.34	1.78	68.1	1.53	55
B Sucrose added—Final conc.-0.23 M					
Addition					
None	6.81	1.17	76.2	2.32	57
Succinate 5×10^{-4} M	8.12	1.24	83.4	2.75	48
EDTA 2×10^{-4} M	5.83	1.22	61.1	2.15	0
EDTA + Succinate	4.87	1.09	56.6	1.89	15
ATP-2×10^{-4} M + EDTA-2×10^{-4} M	4.95	1.15	55.5	1.85	32

0.5 ml of mitochondria in 0.25 M sucrose, obtained from 1 gm of rat liver, was added to 4.5 ml of 0.02 M Tris pH 7.3 at 5°, containing the additions as indicated. The column, labeled 0 time, indicates mitochondria which were diluted to 5 ml of 0.25 M sucrose, and centrifuged immediately. These data represent the state of the mitochondria immediately before they were placed in the hypotonic buffer. Six duplicate incubations were performed simultaneously. Three incubations were centrifuged after 1 minute at 10,000 *g* at 0° for 10 minutes. Sucrose, enough to make the final concentration of sucrose 0.23 M, was added to the other three incubations, after incubation for 1 minute at 5°, and then centrifuged immediately. The centrifuge tubes were wiped dry and the mitochondrial pellets weighed immediately after centrifugation. The pellets were then sonicated at 40,000 kc with a Branson Sonifer in 2 ml of 0.01 M Na acetate pH 4.5 for 30 seconds. Sucrose, Ca^{++}, and protein were measured as stated. Dry weight of mitochondria was obtained by desiccating similar pellets over P_2O_5 in a vacuum until constant weights were obtained. Water content of mitochondria was expressed as the difference between wet weight and dry weight. Ca^{++} content was expressed as that per cent of total Ca^{++} in the incubation recovered in the mitochondrial pellets. The initial average concentration of Ca^{++} within the mitochondria at 0 time was 2.1×10^{-2} M. All triplicate determinations did not vary by more than 12 per cent. Data in Table III *A* were those obtained from mitochondria in which sucrose was diffusing out. Table III *B* shows data on mitochondria in which sucrose was diffusing into the mitochondria. The column labeled "Sucrose conc. in/out" represents the ratio of the concentration of sucrose in the mitochondrial pellet to that of the outside buffer. No correction was made for the extramitochondrial space, since this space was constant (15 to 20%) in all cases.

High concentrations of sucrose must, therefore, in some way stabilize or "freeze" mitochondrial membranes, in such a way that they no longer can respond to osmotic changes. The data in Table II offer some support to this concept, since, by simply raising the temperature of the incubations to 38° (thawing), contraction of mitochondria in the presence of a high concentration of sucrose can be readily demonstrated.

Thus, it is concluded that mitochondrial membranes do behave like semipermeable membranes, and the permeability of the membrane can be strikingly altered by small amounts of Ca^{++} or fatty acids. Since Ca^{++} and fatty acids are substances which are constantly liberated from these mitochondria (11), incubation of isolated mitochondria rapidly leads to alterations of mitochondrial membrane permeability. Furthermore, it has been shown that metabolic contraction of mitochondria, in the absence of added ions, can occur only if the membranes are maintained in a semipermeable state.

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