CONTRACTION OF HYPOTONICALLY SWOLLEN MITOCHONDRIA BY OXIDIZABLE SUBSTRATES

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The shrinkage of liver mitochondria hypotonically swollen by adenosine triphosphate (ATP), Mg^{++} and bovine serum albumin has been reported (Lehninger, 1959). However, it was not shown that oxidizable substrates would promote contraction of mitochondria excessively swollen in a hypotonic medium. Other investigators (Crofts and Chappell, 1965; Chappell and Crofts, 1965) have "reversed" calcium-ion-induced swelling by substrates of electron transfer, but only after removal of Ca^{++} by chelation.

The present experiments demonstrate shrinkage of hypotonically swollen beef heart mitochondria by oxidizable substrates, e.g. succinate where terminal phosphorylation is inhibited by oligomycin or aurovertin. The "long term" contraction (that which requires several minutes to reach completion) is inhibited by electron transfer inhibitors, such as malonate and antimycin.

Mitochondria swollen in hypotonic medium will shrink to a limited extent when the medium is made isotonic with respect to a given salt, but will shortly begin to swell again in the absence of oxidizable substrates. The effect of substrates will depend to a large extent on the anionic composition of the medium. In a carboxylate medium substrate promotes swelling, and in a chloride medium substrate promotes shrinking. The volume changes in either case are arrested by electron transfer inhibitors.

Methods and Materials

Prior to separating into "light" and "heavy" fractions (Hatefi and Lester, 1958) beef heart mitochondria were prepared by a modification of the

method described by Crane et al., (1956). Only heavy mitochondria which exhibited high respiratory control ratios were used to demonstrate substrate induced contraction. The mitochondria were kept at 0-4° in a sucrose (0.25M)-Tris (0.02M, pH 7.5) medium at a protein concentration of approximately 40 mg/ml. This stock solution was diluted to 5 mg/ml just prior to use in the experiments. One-tenth ml of this suspension (5 mg/ml) was diluted thirty-fold in 2 mM imidazol-HCl (pH 6.9) which gave a final protein concentration of 0.17 mg/ml. The optical density (OD) of the swollen mitochondria was immediately determined in a Beckman DU spectrophotometer at 520 mμ. Other experimental details are given in the legends of the tables.

Proteins were determined by the biuret method with bovine serum albumin (BSA) serving as the standard (Gornall et al., 1949).

Results and Discussion

Substrate induced contraction of hypotonically swollen beef heart mitochondria (Table I) is promoted by systems which generate NADH, by NADH itself and by succinate. NADH contraction is less than that of the generating systems (pyruvate + malate) probably because once contraction begins, NADH penetration into the mitochondrion becomes limiting as the contracted state is approached. Contraction by either system is inhibited to a limited extent by both rotenone and antimycin.

Succinate promotes shrinkage of swollen mitochondria more rapidly and to a greater extent when accompanied by glutamate, or glutamate plus arsenite. This has been interpreted to indicate the removal of oxaloacetate, a potent inhibitor of succinate oxidation, from the medium via a transamination reaction that yields α-ketoglutarate and asparate (Chappell, 1964). Further α-ketoglutarate oxidation is stopped by arsenite; thus substrate level phosphorylation has been eliminated as a source of energy for enhancement of mitochondrial shrinkage.

The maximum difference between the swollen and contracted states is dependent upon the ionic strength (or osmotic force) and composition of the suspending medium (Table II). It was determined that maximal changes in optical density were recorded in a slightly hypotonic medium (ionic strength = 0.08). Very small changes were observed at low ionic strength (0.002) and high ionic strength (0.30). When mitochondria were swollen at 25° in 2 mM imidazol-HCl (pH 6.9), the OD decreased (from approximately 0.650 in isotonic

TABLE I

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Conditions: Beef heart mitochondria hypotonically swollen in 2 mM imidazol-HCl (pH 6.9) were incubated at 25° for 5 minutes prior to raising the molarity of imidazol-HCl to 7 mM. This suspension was incubated for 10 minutes before the addition of oxidizable substrate to a concentration of 6 mM. Optical densities (OD) were followed at 520 mμ for 15 minutes after the addition of substrate. The protein concentration in each case was 0.17 mg/ml. Changes in optical density (ΔOD) were calculated by subtracting the OD obtained prior to adding substrate (initial OD) from that obtained following incubation in the presence of substrate for 15 minutes. Maleate and incubation medium (imidazol-HCl) were used as "non-oxidizable substrates" to allow for evaluation of any purely osmotic effects. Values given are averages of three replications.

Substrate	Initial OD	ΔOD
Pyruvate + malate	0.304	+0.149
Succinate + glutamate + arsenite	0.310	+0.240
NADH	0.303	+0.111
Succinate	0.311	+0.200
Incubation medium	0.305	-0.028
Maleate	0.311	-0.046

TABLE II

EFFECTS OF VARIOUS SALTS ON SUBSTRATE INDUCED MITOCHONDRIAL VOLUME CHANGES

Conditions: Same as those given in Table I, except that after hypotonically swelling mitochondria in 2 mM imidazol-HCl (pH 6.9) for 5 minutes, the appropriate salt was added to bring the molarity to 0.14. Values listed are averages of two replications.

Salt	Initial OD	ΔOD
KCl	0.468	+0.188
NaCl	0.462	+0.196
NH_4Cl	0.416	+0.206
Tris-HCl	0.527	+0.164
Imidazol-HCl	0.335	+0.228
K-acetate	0.483	-0.204
Na-acetate	0.465	-0.198
NH_4-acetate	0.302	-0.020
K-succinate	0.360	-0.086
K-maleate	0.442	-0.019
K-fumarate	0.470	-0.006

sucrose to 0.250 in 2 mM imidazol-HCl) almost instantaneously, and when sufficient imidazol-HCl was added to change the molarity to 7 mM, very little if any increase in OD was noted, especially if the mitochondria were depleted of endogenous substrate by a short incubation period in the 2 mM buffer. However, when succinate was added, an increase in OD ensued which plateaued in less than 15 minutes. The date in Table II illustrate the dependence of mitochondrial volume changes on the composition of the suspending medium. In media containing chloride under isotonic conditions, an increase in OD ensued upon the addition of succinate, whereas in media containing acetate, swelling was promoted.

Hypotonically swollen mitochondria decrease in volume (from an OD of approximately 0.250 to 0.550 with all gradations between) if salt is added to raise the molarity to 0.14 (approximately isotonic); but this is an unstable condition at 25°, and a slow swelling commences which is either enhanced (acetate medium) or reversed (chloride medium) when an oxidizable substrate is introduced. The succinate itself at high molarity (0.14M) prompts swelling, probably due to the carboxylate moiety of the anion. Irrespective of the direction of the volume change promoted by oxidizable substrate (succinate), the change was inhibited by antimycin.

Malonate, a competitive inhibitor of succinate oxidation, inhibits succinate induced contraction in a competitive manner (Table III). This would not be expected if osmotic work was the sole contributor driving the volume change since the addition of malonate would increase the osmotic force exerted on the external surface of the mitochondrion. If the latter were true, this should result in a decrease in volume, but the opposite effect has been observed - an inhibition of volume change. Therefore, further evidence has been supplied for the concept that mitochondrial volume and conformational changes can not be explained on purely osmotic grounds (Tedeschi and Hegarty, 1966) but probably encompass at least three (chemical, mechanical and osmotic) processes. Evidence for the involvement of a mechanical process has been supplied by electron microscopy (Blair and Tan, 1967) in addition to "light scattering" and volume changes presented in this paper.

TABLE III

INHIBITION OF SUCCINATE INDUCED CONTRACTION OF HYPOTONICALLY SWOLLEN MITOCHONDRIA AS A FUNCTION OF MALONATE CONCENTRATION

Conditions: Same as those given in Table I, except that malonate was introduced prior to making the suspension 7 mM imidazol-HCl (pH 6.9). Values listed are averages of three replications.

Malonate concentration (mM)	Initial OD	+ΔOD
0.0	0.291	0.215
2.5	0.289	0.172
5.0	0.289	0.137
10.0	0.291	0.105
20.0	0.289	0.045
50.0	0.287	0.001

Mitochondrial contraction in the presence of an oxidizable substrate is arrested when electron transfer inhibitors are added before the addition of substrate (Table IV) but is not inhibited by reagents capable of preventing terminal phosphorylation (oligomycin, aurovertin, and atractyloside). This evidence indicates the involvement of intermediates prior to the sites of action of these phosphorylation inhibitors, but after the electrons have traversed the electron transfer chain. It was also observed that electron transfer (succinate was the original donor) caused an increase in the OD, even in

TABLE IV

EFFECT OF INHIBITORS ON SUCCINATE INDUCED CONTRACTION OF HYPOTONICALLY SWOLLEN MITOCHONDRIA

Conditions: Concentration of malonate was 50 mM; concentrations of antimycin, oligomycin, aurovertin and atractyloside were 1 μg/mg protein. Inhibitors were added prior to increasing the imidazol-HCl concentration to 7 mM. Other conditions and calculations are the same as those given in Table I. Values are averages of three replications.

Inhibitor	Initial OD	+ΔOD
None	0.289	0.204
Malonate	0.287	0.001
Antimycin	0.291	0.040
Oligomycin	0.280	0.212
Aurovertin	0.290	0.213
Atractyloside	0.295	0.190

the presence of antimycin. This effect was immediate and transient. In most cases this immediate increase gradually subsided and after an hour of incubation the mitochondria were more swollen than mitochondria which were kept under the same conditions in the absence of antimycin and succinate. This observation may be cited as evidence that reduction of electron transfer components elicits "conformational" changes in the membrane components themselves; but these changes lead to an unstable conformation which can not be maintained, and dissociation or destruction of the succinate oxidase chain ensues.

Gravimetric evidence for a decrease in mitochondrial volume (extrusion of water) is presented in Table V. The data substantiate the relationship between mitochondrial volume and optical density (Tedeschi and Harris, 1958) in addition to demonstrating that succinate has induced a volumetric change in the mitochondria. However, these data do not identify the "primary" effect of the energy derived from oxidation of substrate; i.e. does electron transfer effect a mechanical change directly, or does it cause extrusion of water? If the latter is true, contraction would be identified as a secondary effect, but the data in Tables IV and V tend to support the hypothesis that contraction or conformational changes precede extrusion of water.

TABLE V

GRAVIMETRIC DETERMINATION OF WATER EXTRUDED FROM MITOCHONDRIA BY SUCCINATE

Conditions: Mitochondria were incubated under conditions similar to those given in Table I; however, measurements were made only upon completion of contraction with the samples which were exposed to succinate. Control samples were run simultaneously. Each value represents three replications with three observations per replication.

Condition	OD	mg water
Without succinate	0.582	11.6
With succinate	0.833	7.9
With antimycin + succinate	0.563	11.7

The data presented in this paper demonstrate that mitochondrial volume change induced by hypotonicity can be "reversed" by an oxidizable substrate.

This reversal is inhibited by electron transfer inhibitors but not by inhibitors of terminal phosphorylation. Based on the available evidence it is postulated that mitochondrial volume changes implicate three forces derived from chemical, osmotic and mechanical processes. These three processes are indistinguishable as independent entities while the mitochondria are undergoing volume changes. However, the primary supply of energy and the initiator of volume change is the chemical event - the oxidation of substrate.

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