In conclusion we can say that with respect to deoxyadenine nucleotides, the specificity of the overall process of the oxidative phosphorylation also resides at the level of the translocase. The transport of the deoxyadenine nucleotides demonstrated previously[2,3] must be one or two orders of magnitude slower than that of the adenine nucleotides.

The authors are indebted to Professor E. C. SLATER and Dr. P. BORST for many helpful suggestions and to Miss. J. J. KETTING and Miss J. H. DE KONING for excellent assistance. This work was supported in part by grants from the U.S. Public Health Service (Grant No. AM 08690) and the Life Insurance Medical Research Fund.

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Received July 7th, 1967

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BBA 43172

Electron microscopic evidence for volumetric changes in heart mitochondria

Electron microscopy of mitochondria in various metabolic states has recently been reported[1,2], and changes in contractile states induced by ATP have been observed[3,4]. CHAPPELL AND CROFTS[5,6] have shown that oxidation of electron transfer substrates will cause the contraction of mitochondria swollen with Ca^{2+} provided the Ca^{2+} is previously removed. We have recently reported[7] that oxidizable substrates (*e.g.* succinate) will contract beef heart mitochondria which have been swollen by exposure to hypotonic conditions. The substrate-induced contraction is blocked by electron transfer inhibitors (antimycin), but is not affected by inhibitors of terminal phosphorylation (oligomycin), and is prevented by 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone[8].

The spectrophotometric and gravimetric evidence for substrate-induced contraction of heart mitochondria is now well documented. This evidence substantiates the inclusion or requirement of a "contractile" process in energy transducing functions

of mitochondrial preparations. However, it does not distinguish among three postulated sites of involvement of a contractile process in mitochondrial energy metabolism.

These studies on substrate-induced contraction can not differentiate among these postulates, but can differentiate between bivalent cation accumulation and volumetric changes without the added complication of metal ion precipitation. This communication describes simple volumetric changes which occur in beef heart mitochondria that have been exposed to hypotonic imidazole–HCl (pH 6.9), when succinate is introduced.

Although oxidation reactions, volumetric changes, and light scattering studies have been correlated in recent reports[7,8] on substrate-induced contraction of mitochondria, electron micrographs were not presented. It seemed important to provide electron microscopic evidence for changes in mitochondrial size, shape and structure under the various conditions used to demonstrate substrate-induced shrinking of beef heart mitochondria.

Beef heart mitochondria were prepared as reported previously, utilizing a modification of the method described by CRANE, GLENN AND GREEN[9]. The heavy beef heart mitochondria were stored and treated as reported earlier[7]. Protein was estimated by a biuret method after solubilization with deoxycholate.

Table I shows the results of an experiment carried out with mitochondria treated so as to demonstrate contraction with oxidizable substrate. When mitochondria are swollen in hypotonic imidazole–HCl the volume increases considerably over that of the control in isotonic sucrose. However, the introduction of succinate promotes a decrease in the volume of the swollen mitochondria which is clearly dependent on the oxidation of the substrate. Antimycin completely inhibits both contraction and succinate oxidation. This observation does not support the proposition that mitochondrial contraction is a purely osmotic process[10].

TABLE I

WATER EXTRUSION FROM SWOLLEN MITOCHONDRIA INDUCED BY OXIDATION OF SUCCINATE

Beef heart mitochondria were diluted to a protein concn. of 0.17 mg/ml in either 0.25 M sucrose–0.02 M Tris–HCl (pH 6.9) or 2 mM imidazole–HCl (pH 6.9). The diluted mitochondria were incubated at 25° for 5 min before increasing the concn. of imidazole–HCl to 7 mM. After incubation for 10 min succinate was added to a concn. of 6 mM. Absorbances at 520 mμ (A_{520}) were measured on a Beckman DU spectrophotometer after incubating the reaction mixture for another 15 min. Antimycin A (1 μg/mg protein) was introduced where indicated prior to making the suspension 7 mM imidazole–HCl; succinate was added 10 min later. Gravimetric measurements were made on wet and dry pellets after centrifuging in a Spinco SW39 rotor for 10 min at 25000 rev./min. The total vol. of the suspension was 5 ml containing 0.85 mg of mitochondrial protein. Oxidation rates (mμatoms of oxygen/min per mg protein) were determined polarigraphically in a Gilson medical electronics oxygraph under conditions identical to those used for volumetric studies. The values listed are averages of three replications.

Treatment of mitochondria	A_{520}	H_2O (*mg*)	*Oxidation rates*
Isotonic sucrose	0.943	7.1	—
Hypotonically swollen	0.582	11.6	—
Succinate contraction	0.833	7.9	235
Antimycin A inhibition	0.563	11.8	7

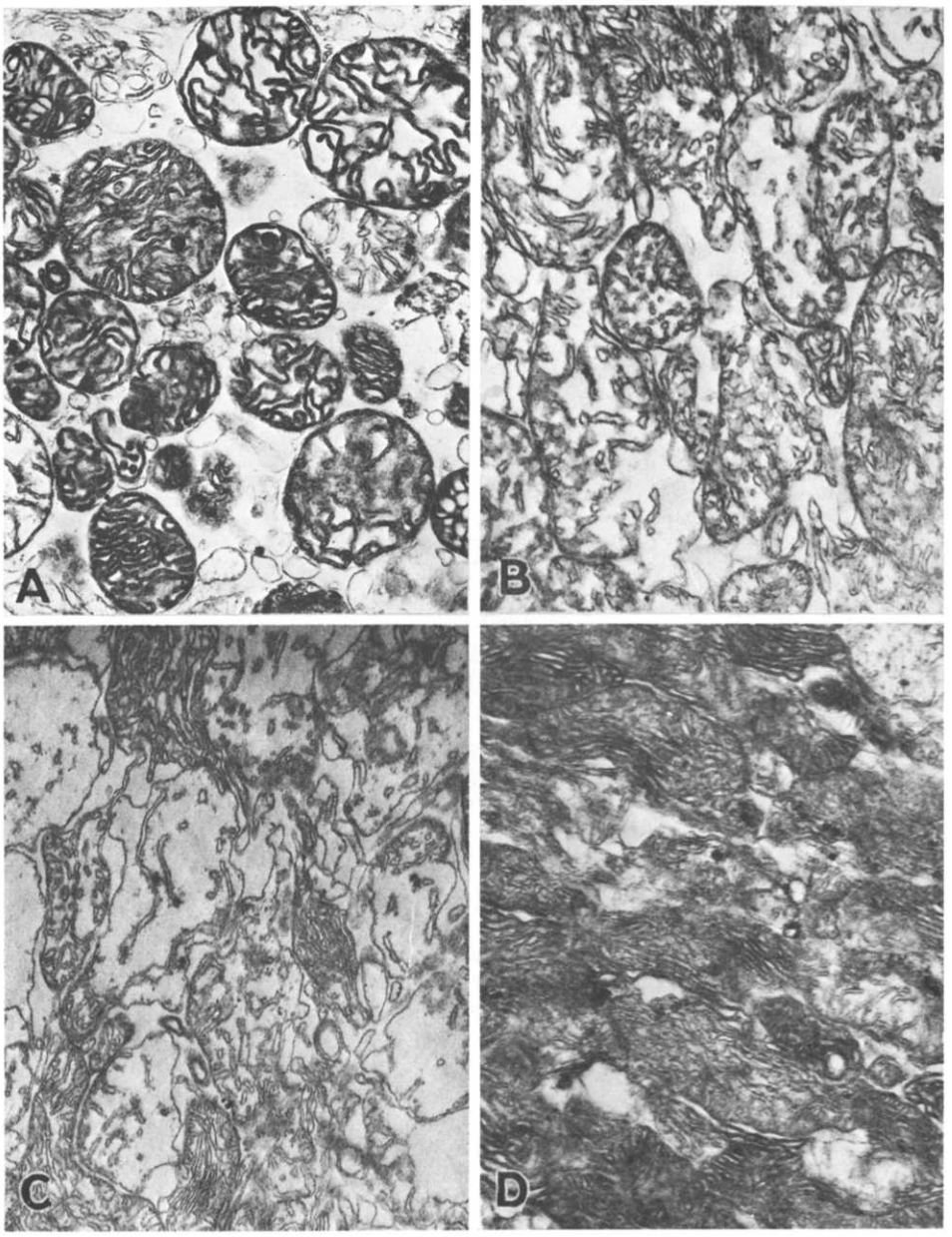

Fig. 1. Conditions and methods were the same as those given in the legend to Table I for obtaining gravimetric measurements. The wet pellets were fixed with 1% osmium tetroxide in phosphate buffer (pH 7.4, ref. 11). The fixed pellets were dehydrated with ethanol before embedding in Epon 812 (ref. 12). Specimens were sectioned (thickness of approximately 500 Å) and then stained with lead citrate[13] prior to examination with an RCA EMU-2 electron microscope. Magnification 15000 × . (A) Mitochondria were diluted in isotonic sucrose and incubated for 30 min at 0° before centrifuging to obtain the pellet. (B) Mitochondria were swollen in 2 mM imidazole–HCl and incubated for 30 min at 25° before centrifuging. (C) Mitochondria were swollen in 2 mM imidazole–HCl, incubated for 5 min at 25° before adding antimycin A, brought to 7 mM imidazole–HCl, incubated for 10 min prior to introducing succinate (6 mM), and then incubated an additional 15 min before centrifuging. (D) Same as C except antimycin A was not added.

In Fig. 1 are shown representative photographs of the mitochondria described in Table I. The untreated mitochondria (not swollen) present a classical view of isolated heart sarcosomes (A). The outer membrane is apparently intact and constrains or surrounds the convoluted inner membrane network. The mitochondria swollen in 2 mM imidazole–HCl (B) show a very different membranous organization, and in most cases, the outer membrane appears to be ruptured, though not always fully dissociated from the inner membrane. In some photographs (not shown) the outer membrane appears to be attached to the inner membrane by connecting bridges of unknown chemical and morphological structure. However, these connecting bridges are infrequent and not clearly reproducible in all mitochondrial preparations. When the swollen mitochondria are exposed to succinate oxidation for 15 min, shrinking ensues (D), and the inner membrane appears to be reorganized into mitochondrial forms that are normally observed only *in situ*. These contracted mitochondria have various shapes and rarely exhibit a spherical form. In a few instances, the rudiments of the original outer membrane are detectable. Fig. C illustrates the requirement for oxidation of substrate to promote volume changes of mitochondria. Antimycin blocks oxidation of succinate and volume change, but not the membrane reorganization. The change in structural appearance of the membranes may be due to reduction of electron transfer components.

The observations presented in this communication illustrate that mitochondrial volume changes are not completely reversible. Isolated mitochondria induced to contract with substrate do not revert to a spherical form but reorganize into a form similar to that seen *in situ*, except the original intact outer membrane is not readily observable.

This investigation was supported by a Heart Research Center Grant (HE 06308) of the National Heart Institute and an American Heart Association Grant (62G139).

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Received May 16th, 1967