A Heat-stable Factor Required for Contraction of Pretreated Mitochondria*

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Fresh rat liver mitochondria allowed to swell under a variety of conditions readily contracts on addition of adenosine triphosphate, MgCl₂, and serum albumin (1, 2). However, in particular cases, additional factors are required. For instance, catalase and glutathione peroxidase (3) are indispensable for the adenosine triphosphate-induced contraction of mitochondria swollen in the presence of reduced glutathione. In addition, the role of unsaturated fatty acids and α-glycerophosphate in the swelling-contraction cycle has been pointed out by Wojtczak and Lehninger (4) and Wojtczak, Wlodawer, and Zborowski (5).

We have recently reported in preliminary communications (6-8) that rat liver mitochondria pre-aged in 0.25 M sucrose or extracted with 0.6 M KC1, and then allowed to swell, do not contract again on addition of ATP, BSA, and MgCl₂ at pH below 7.4. A factor which restores contraction in these pretreated mitochondria was subsequently isolated from KC1 extracts of fresh mitochondria. These extracts contain the so called “contractile protein” of Ohnishi and Ohnishi (9, 10). The contraction-supporting factor is, however, heat-stable. The experiments reported in detail in this paper demonstrate that this factor is a lipid and describe the characteristics of the contraction restored in “aged” mitochondria. In the following paper, the identity and the properties of the active lipid(s) are described (11).

EXPERIMENTAL PROCEDURE

Rat liver mitochondria were prepared according to Hogeboom (12). After isolation, the mitochondria were diluted with cold 0.25 M sucrose so that 0.05 ml of the suspension added to 3 ml of 0.125 M KC1-0.02 M Tris-HCl medium at pH 7.4 in a 1-cm path cuvette gave an absorbance reading in the range of 0.500 to 0.600 at 520 mμ. This amount corresponded to about 0.40 to 0.50 mg of protein. Digitonin fragments of mitochondria were prepared from liver mitochondria by the method of Devlin and Lehninger (13).

Mitochondria used in the experiments to be described were either pre-aged in isotonic sucrose or extracted with 0.6 M KC1 to elicit dependence of the contraction on the heat-stable factor. In the first case, the suspension of mitochondria in 0.25 M sucrose (10 mg of protein per ml) was aged at 2°C for periods of hours or days, as indicated. In the other case, an exactly measured volume of the mitochondrial suspension at the same concentra-

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\* The abbreviation used is: BSA, bovine serum albumin.

tion was centrifuged and the pellet was extracted with 1 volume of a mixture of 0.6 M KC1-0.01 M Tris-HCl, pH 7.6, for 2 hours at 2°C. After centrifugation, the KC1-extracted mitochondria were finally suspended in the same volume of 0.25 M sucrose as initially; this suspension was immediately assayed.

Preparation of soluble extracts from fresh mitochondria was usually carried out on a large scale. Mitochondria from 50 g of rat liver were extracted with 50 ml of 0.6 M KC1-0.01 M Tris, pH 7.6, for 2 hours at 2°C with occasional stirring. The clarified extracts were then dialyzed overnight at 2°C against 5 volumes of water. The precipitate which appeared was collected by centrifugation; it corresponds to the contractile protein of mitochondria described briefly by Ohnishi and Ohnishi (9, 10). The supernatant fluid was concentrated by freeze-drying to a small volume and again dialyzed against 100 to 200 volumes of water for 16 hours at 2°C. The protein fractions recovered in the precipitate and in the supernatant fluid will be referred to as the water-insoluble and water-soluble fractions, respectively. Subfractions were obtained from the soluble protein fraction by ammonium sulfate precipitation at +2°C as described below.

Uptake and extrusion of water by suspended mitochondria was usually measured by following the change in absorbance in a Beckman model B spectrophotometer at 530 mμ (2, 14). Cuvettes of 1-cm path were used; the temperature was 20°-22°C. Gravimetric measurements were often performed to check the optical data (15). In the optical tests, the initial absorbance of the mitochondrial suspension was adjusted to 0.500 to 0.600; the mitochondria were then allowed to swell until the absorbance reached a value of about 0.180. Contraction was then initiated by addition of a mixture of ATP, MgCl₂, and bovine serum albumin to yield final concentrations in the complete medium of 5 mM, 3 mM, and 2 mg per ml, respectively (1). In some experiments, ATP and BSA were added first, followed by addition of MgCl₂ after a 5-minute delay (7). In the gravimetric tests, 25-ml aliquots were centrifuged in tared polypropylene Servall centrifuge tubes for 5 minutes at 20,000 × g in the cold room. The supernatant fluids were decanted and the interior of the tubes carefully blotted with filter paper strips and Q-tips before weighing. The wet weights were usually obtained within 30 minutes after the aliquots had been taken.

A “phosphatido-peptide” fraction from rat liver was prepared following the extraction procedure described by Huggins and Cohn (16). The lipids present in saline extracts of mitochondria were extracted with 20 volumes of chloroform-methanol (2:1) and washed with 0.12 M KC1 according to Folch, Lees, and Sloane Stanley (17). The total lipids, redissolved in pure chloroform,
were applied to a silicic acid column (2 g of Mallinckrodt A. R. grade reagent (100 mesh)) in chloroform. After removal of the neutral lipids by pure chloroform, the phospholipids were eluted by the method of Fiske and SubbaRow (28). Pretreated mitochondria were either extracted for 2 hours in 0.6 M KC1 and after centrifugation resuspended in 0.25 M sucrose, crease of absorbance at 520 mp is recorded on the figure. Temperature was 22°C. The extent of contraction at pH 7.4 (at which there is no contraction) to 8.0, by addition of a small volume of a Tris buffer, pH 9.5, no significant contraction was observed after addition of ATP, BSA, and MgCl2. This experiment indicates that the alteration of the contractile mechanism of mitochondria which occurred at pH 7.4 is not reversed simply by raising the pH. It must be noted that the time required for the swelling to proceed to the standard absorbance of 0.180 varied with the pH of the medium; it is shorter at alkaline pH. However, the duration of the swelling period did not seem to affect directly the extent of the ATP-induced contraction of aged mitochondria. Fig. 2 shows the result of an experiment where ATP was added

**RESULTS**

*Effect of pH of Swelling Medium on Contraction of Pre-aged or Extracted Mitochondria.* Freshly prepared or pretreated mitochondria were allowed to swell in the presence of 10^-5 M sodium olate, in media in which the pH ranged from 6.8 to 9.1, from an initial absorbance of 0.600 at 520 mp to a terminal absorbance of about 0.180. Contraction was then initiated by addition of a mixture of ATP, BSA, and MgCl2 adjusted to the pH of the swelling medium. The extent of contraction observed 15 minutes after addition of ATP as an increase in absorbance was used as standard end point. As shown in Fig. 1A, the contraction of mitochondria previously extracted with 0.6 M KC1 was lower than that of fresh untreated mitochondria over the whole range of pH tested. On the other hand, the contraction of mitochondria which had been pre-aged before test showed a different behavior, in that contraction was severely or completely impaired at pH values below 7.4 but not significantly impaired over 7.7 (Fig. 1B). Aging of mitochondria in a cold sucrose medium, thus, severely limits the ATP-linked contraction of mitochondria below pH 7.4.

When the pH at the end of the swelling phase was raised from pH 7.4 (at which there is no contraction) to 8.0, by addition of a small volume of a Tris buffer, pH 9.5, no significant contraction was observed after addition of ATP, BSA, and MgCl2. This experiment indicates that the alteration of the contractile mechanism of mitochondria which occurred at pH 7.4 is not reversed simply by raising the pH.
Effect of time of addition of ATP during swelling phase on mitochondrial contraction. Mitochondria were pre-aged for 3 days in cold 0.25 M sucrose. Other conditions were the same as in Fig. 1, A and B, except that the pH of the swelling medium was 7.4.

![Graph showing effect of time of addition of ATP during swelling phase on mitochondrial contraction.](image)

**Fig. 2.**

Effect of pH of contraction medium on contraction of pre-aged mitochondria. The swelling medium consisted of 0.125 M KCl, 0.0017 M Tris buffer, pH 7.7, and 3.10^-5 M sodium oleate. Mitochondria pre-aged for 1 day in 0.25 M sucrose were added to this medium in order that the absorbance of the suspension at 520 mp in a 1-cm pathway cuvette was 0.600. Swelling was allowed to proceed until the absorbance reached a value of 0.180. The suspension was then immediately distributed in different cuvettes and mitochondrial contraction was initiated by addition of 0.005 M ATP, 2 mg per ml of BSA, and 0.003 M MgCl₂ at different pH values. The final pH resulting from the addition of ATP to the medium is recorded on the figure.

![Graph showing effect of pH of contraction medium on contraction of pre-aged mitochondria.](image)

**Fig. 3.**

Effect of Concentration of Mitochondria—The experiments reported above were carried out with amounts of mitochondria corresponding to about 0.40 mg of protein in 3 ml of swelling medium. However, with a 3-fold increase in concentration of the mitochondria, there was little or no impairment of contraction since significant reversal of the swelling occurred at pH 7.4 after addition of ATP, BSA, and MgCl₂ (Fig. 4). This observation suggests that a factor necessary for contraction might be released into the medium from pre-aged mitochondria and that in dilute suspensions of mitochondria its concentration in the medium becomes critical for the ATP-induced contraction. A similar concentration-dependence of contraction observed by Lehninger (29) following glutathione-induced swelling led to the finding that catalase and glutathione peroxidase can restore contraction of GSH-swollen mitochondria (3).

Restoration of Contraction by Extracts from Fresh Mitochondria—The preceding experiments have shown that pre-aged or extracted mitochondria are unable to contract on addition of ATP, BSA, and Mg⁺⁺ after swelling has been carried out at pH equal to or lower than 7.4. However, if extracts of freshly prepared mitochondria were added to the swelling medium together with the pretreated mitochondria, significant contraction occurred, indicating that the extract of mitochondria contained factor(s) capable of replacing or restoring the factor(s) lost or damaged during pretreatment. Water-soluble and water-insoluble protein fractions prepared from 0.6 M KCl extracts of fresh mitochondria (Tables I and II) were found to contain contraction-restoring activity. A typical gravimetric experiment showing the contraction-restoring activity of the water-soluble protein fraction is illustrated in Fig. 5.

![Graph showing restoration of contraction by extracts from fresh mitochondria.](image)

**Fig. 4.**

After mitochondria were allowed to swell for the same length of time (20 minutes), either at pH 8.0 or at pH 7.4. Contraction readily occurred at pH 8.0, but failed completely at pH 7.4. The pH of the swelling medium is the important factor, whereas the pH of the contraction medium is of only minor importance. This is illustrated by the following experiment, in which swelling was allowed to proceed at pH 7.7 in all tubes, but the pH of the medium was adjusted after swelling to different pH values in the zone 6.7 to 8.7. It is seen (Fig. 3) that contraction is maximal between pH 7.0 and pH 8.0. It may be concluded that the impairment of the contractile mechanism by low pH occurs during the swelling phase.

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In the experiment described in Table II, restoration of half-maximum contraction required from 0.06 to 0.12 mg of the various protein fractions when the amount of pre-aged mitochondria used corresponded to about 0.40 mg of protein. Contraction of pre-aged mitochondria in the presence of water-soluble and water-insoluble protein fractions were only observed when these frac-
**TABLE I**

Protein and lipid content of various fractions obtained from mitochondrial extracts

Mitochondria from 50 g of rat liver were extracted for 2 hours at 2° with 50 ml of 0.6 M KCl-0.02 M Tris, pH 7.6.

**Extract diluted 6 times with water**

**Insoluble fraction***

- Protein: 4.5 mg
- Lipid: acyl esters, 2.9 μeq
- Phosphorus, 1.4 μatoms

**Soluble fraction**

- Protein: 13.2 mg
- Lipid: acyl esters, 3.0 μeq
- Phosphorus, 1.3 μatoms

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* This corresponds to “contractile protein” of Ohnishi and Ohnishi (9, 10).

**TABLE II**

Effect of various mitochondrial protein fractions on ATP-induced contraction of pre-aged mitochondria

The protein fractions were obtained as shown in Table I. The experimental conditions were as in Fig. 1. The pH of the swelling medium was 7.4. In Experiment 1, the mitochondria were pre-aged for 2 days in cold 0.25 M sucrose. The absorbance was adjusted to 0.680 and swelling proceeded to a value of 0.180. In Experiment 2, mitochondria were pre-aged for 3 days. The initial absorbance was 0.670, the final, 0.180. Reversal of the swelling was initiated by addition of 0.005 M ATP, 2 mg per ml of BSA, and 0.003 M MgCl₂ (final concentrations).

<table>
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<th>Additions at the beginning of the swelling phase</th>
<th>Protein</th>
<th>ATP-induced contraction</th>
<th>ΔAme X 1000</th>
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<td></td>
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<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Insoluble protein fraction</td>
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<td>130</td>
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<td></td>
<td>600</td>
<td>240</td>
<td>120</td>
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<tr>
<td>Soluble protein fraction</td>
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<td>600</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
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<td></td>
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<tr>
<td>None</td>
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<td></td>
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</tr>
<tr>
<td>Soluble protein fraction</td>
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<td>70</td>
<td>240</td>
</tr>
<tr>
<td>Subfraction, 0-20% (NH₄)₂SO₄</td>
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<td>105</td>
<td>240</td>
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<td></td>
<td>240</td>
<td>205</td>
<td>120</td>
</tr>
<tr>
<td>Subfraction, 20-60% (NH₄)₂SO₄</td>
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<td>130</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>240</td>
<td>185</td>
<td>130</td>
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<td>Subfraction, 60-100% (NH₄)₂SO₄</td>
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<td>240</td>
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<td></td>
<td>240</td>
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It has also been found that 0.6 M extracts of digitonin fragments of rat liver mitochondrial membranes have the same con-

**Fig. 5.** Contraction and water extrusion in pre-aged mitochondria swollen at pH 7.4. Effect of water-soluble protein fraction obtained from KCl extracts of fresh mitochondria. Two swelling media were prepared. They contained: 0.125 M KCl; 0.02 M Tris, pH 7.4; and 10⁻³ M oleate. One of them was supplemented with the water-soluble protein fraction obtained from mitochondrial extracts (0.18 mg of protein per ml) (cf. “Experimental Procedure”). Rat liver mitochondria pre-aged for 3 days in cold 0.25 M sucrose were added to both media (0.17 mg of mitochondrial protein per ml) and swelling was allowed to proceed. Contraction was induced by addition of fra soluble protein fraction and BSA, and then, 0.003 M MgCl₂. Swelling and contraction were followed by changes in absorbance at 520 nm and by gravimetric measurements. In the last procedure, 25 ml aliquots were removed at different times, centrifuged at 20,000 × g for 5 minutes at 2°, and the wet weight determined (the amount of water gained or extruded is indicated on the figure).
traction-restoring activity as extracts of whole mitochondria. Presumably the contraction-restoring factor is actually present in the mitochondrial membrane.

Properties of Contraction Restored by Extracts from Fresh Mitochondria—Addition of KCl extracts of fresh mitochondria to mitochondria which had been previously extracted with 0.6 M KCl resulted in a significant stimulation of contraction over a wide range of pH (Fig. 7A). With the same addition, contraction of mitochondria pre-aged in 0.25 M sucrose was stimulated below pH 7.7 (Fig. 7B).

Aging in sucrose or extraction by KC1 are not the only factors responsible for the loss of contraction of mitochondria. As shown in Fig. 8, addition of mitochondrial KC1 extract to fresh untreated mitochondria during the swelling phase also brought about a significant stimulation of the normal contraction of pH below 7.8. This observation indicates that the swelling of untreated mitochondria under specific conditions of pH leads to an immediate alteration of the mitochondrial membrane which is reflected by a partial loss of its normal contractile properties.

The 0.6 M KCl extracts of fresh mitochondria are able to restore contraction of pretreated mitochondria whether they are swollen spontaneously or in the presence of oleate, Ca++, thyroxine, or phosphate. However, such extracts did not stimulate reversal of hypotonic swelling or of swelling initiated by glutathione (Fig. 9). A complementary test was made to assess comparatively the effects of a mitochondrial KCl extract and of a preparation of purified catalase (i.e. C factor II, required for reversal of GSH swelling (3)) on the reversal of swelling initiated by oleate, phosphate, and glutathione. As is seen in Fig. 10, catalase restored fully the ATP-induced contraction after GSH swelling but was without effect on swelling initiated by the other agents. It is interesting to note that catalase is a necessary factor for the reversal of GSH swelling not only in fresh mitochondria (3) but also in aged mitochondria. On the other hand,
the factor extracted by 0.6 M KCl restored the ATP-induced contraction after oleate- or phosphate-induced swelling, but not after GSH swelling. The contraction factor present in the KCl extract is, therefore, not identical with either glutathione peroxidase or catalase (3).

Oligomycin, which inhibits phosphorylating respiration (30, 31) has been found by Neubert and Lehninger (32) to be a potent inhibitor of ATP-induced reversal of mitochondrial swelling caused by reduced glutathione or thyroxine. As shown in Fig. 11, oligomycin inhibits also the ATP-induced contraction restored by mitochondrial extracts.

**Effect of Various Treatments on Activity of Mitochondrial Extracts**—Boiling for 5 minutes did not affect the activity of the mitochondrial extracts (Fig. 12); the active factor is evidently heat-stable although associated with proteins. Removal of the lipid components from these extracts by treatment with chloroform-methanol (2:1) was found to abolish their activity completely. However, the extracted lipids alone failed to restore contracting activity. On the other hand, partial and transitory reconstitution of the activity was obtained when the lipids which had been extracted into chloroform-methanol were added back to the extracted protein fraction (Fig. 12). This property will be discussed in the following paper (11).

Addition of a small amount of serum albumin together with the extracts at the beginning of the swelling phase slightly potentiated the restoring activity of the extracts (Fig. 13). Nevertheless, a higher concentration of serum albumin had an inhibitory effect; serum albumin by itself could not replace the mitochondrial extracts in supporting contraction.

**Fractionation of Proteins of Mitochondrial Extracts**—Attempts were made to isolate a more active contraction-restoring fraction from extracts of fresh mitochondria by ammonia sulfate fractionation. Three subfractions were prepared from the water-soluble fraction of mitochondrial extracts according to the flow sheet given in Table I. When tested on the contraction of pre-aged mitochondria, every fraction obtained by this procedure was found to have about the same activity per mg of protein (Table II). This result indicates that the restoring factor is neither a specific protein nor is it associated with a specific protein, but is associated with all proteins of the mitochondrial extract. The different protein fractions (Table I) were found to contain lipids. Most of the bound lipids were phospholipids as indicated by the determination of acyl ester and phosphorus (Table I). However, a specific and typical distribution of phospholipids in such fractions was not revealed by application of thin layer chro-
Fig. 12. Effect of boiling or of chloroform-methanol extraction on ability of protein fractions to restore contraction. Mitochondria were pre-aged in cold 0.25 M sucrose for 1 day (Experiments 1 (left) and 2 (middle)) or for 3 days (Experiment 3 (right)). Conditions of swelling and contraction were the same as in Fig. 2. The insoluble protein fraction (0.20 mg) (Experiment 1) and the soluble protein fraction (0.70 mg) (Experiments 2 and 3) were added at the beginning of the swelling phase.

Fig. 13. Effect of serum albumin on contraction of pre-aged mitochondria in absence or in presence of soluble protein fraction. Mitochondria were pre-aged for 2 days in cold 0.25 M sucrose. Conditions of swelling and contraction were the same as in Fig. 2. Water-soluble protein fraction (0.60 mg) and serum albumin at the indicated concentrations were added at the beginning of the swelling phase. The increase in absorbance at 15 minutes after the addition of ATP is recorded on the figure.

matography (18). Quantitative determination of phospholipids present in the soluble protein fraction extracted from freshly prepared mitochondria was carried out. The total phospholipids were then subjected to mild alkaline hydrolysis (20-22); the water-soluble phosphate esters resulting were identified by paper chromatography (cf. "Experimental Procedure"). Their distribution calculated from the phosphate content was the following: phosphatidylethanolamine, 47.5%; phosphatidylcholine, 22.5%; phosphatidylinositol, 12%; phosphatidylserine + cardiolipin, 3.4%; phosphatidylglycerol, 1.5%; and unidentified, 13%. The presence of phosphatidylglycerol has already been observed in rat liver mitochondria by Gray (23).

It is noteworthy that the phosphatidyl-peptide fraction prepared from rat liver according to the procedure by Huggins and Cohn (16) could also restore contraction in aged mitochondria.

Relationship between Effects of Mitochondrial Extracts on Contraction, ATPase, and ATP-Pi Exchange Activities—As shown in Table III, swelling of pre-aged mitochondria causes loss of their ATP-Pi exchange activity, the development of a significantly high Mg²⁺-stimulated ATPase activity, and the loss of ability to contract on addition of ATP, BSA, and Mg²⁺. Addition of mitochondrial KCl extracts to the swelling medium restored the contraction and partially prevented the development of Mg²⁺-stimulated ATPase in pre-aged mitochondria. However, the extracts did not restore the ATP-Pi exchange reaction. Boiling the extracts did not interfere with their activity on the contraction and on the Mg²⁺ ATPase activity of pre-aged mitochondria but treatment with chloroform-methanol abolished both their action on contraction as well as on ATP hydrolysis. It is therefore, probable that the effects of the extract on the ATPase activity and on the contraction are caused by the same factor.

DISCUSSION

The experiments described here show that mitochondria after aging in 0.25 M sucrose or extraction with 0.6 M KCl lose part or all of their contractile properties and release into the medium a heat-stable factor of lipid nature, attached to the extracted protein fraction.
The heat-stable factor restores contraction after spontaneous swelling or swelling initiated by oleate, Ca++, thyroxine, and, to a lesser extent, after swelling initiated by phosphate. Mitochondrial swelling induced by these specific agents has been shown to give rise to production of "U factor" (i.e., free unsaturated fatty acids) in mitochondria. Hypotonic swelling, or swelling induced by GSH, which do not proceed with production of U factor, are not reversed by the heat-stable factor. As illustrated in Table IV, the specificity of the relationship between restoration of contraction by the new factor and the formation of U factor is noteworthy in view of the lipid nature of the heat-stable contraction factor.

The heat-stable contraction factor is present in different fractions obtained from extracts of rat liver mitochondria. These fractions differ in their water-solubility and their relative content of phospholipids. On the basis of lipid phosphorus and acyl coter content, and an average molecular weight of 750 for phospholipids, it can be calculated that the water-insoluble fraction contained about 23% of phospholipids by weight and the water-soluble fraction only 9%. It has been found by Green, Tisdale, Criddle, and Beck that the "structural protein" which consists obtained from mitochondria extracted with 0.6 M KCl work. The properties of the insoluble and soluble protein fractions obtained from mitochondria extracted with 0.6 M KCl appear to be similar to those of structural protein with respect to lipid binding.

Ohnishi and Ohnishi (9, 10) have recently isolated from rabbit and chicken liver mitochondria a protein-containing fraction soluble only in KCl solutions of high ionic strength which has ATPase activity and physical properties similar to those of muscle actomyosin. Neifakh and Kazakova (34) have also prepared an actomyosin-like protein from mouse liver mitochondria. The experiments in this paper show that this fraction (i.e., the mitochondrial protein fraction insoluble in water but soluble in 0.6 M KCl) stimulates contraction of mitochondria. However, the contraction-supporting activity is evidently not a function of the specific protein(s) formed in this fraction, since other mitochondrial protein fractions also support contraction and since the ability in any case is heat-stable and given by a specific lipid which is bound to the proteins. We (6, 7) have been able to confirm some of the findings of Ohnishi and Ohnishi on the properties of their protein fraction; however, we have not observed changes in viscosity of the contractile protein fraction on adding ATP. Although our findings exclude the protein of Ohnishi and Ohnishi as being responsible for supporting contraction under the conditions described, it is of course possible that the protein plays some other significant role in the mitochondrion.

As shown by Lehninger (35), mitochondria lose their ability to couple phosphorylation to respiration after swelling. Similarly, it was found here that pre-aged and swollen mitochondria no longer catalyze the ATP-Pi exchange reaction and furthermore exhibit a large Mg++-stimulated ATPase activity which is characteristic of nonphosphorylating mitochondrial preparations. However, since contraction by ATP supported by the heat-stable factor is completely inhibited by oligomycin (2, 32), the formation of some high energy intermediate of oxidative phosphorylation appears to be necessary for the over-all contraction process (36). It is possible that the heat-stable factor repairs some specific damage in the sequence of reactions involved in oxidative phosphorylation, rather than a defect in the contractile process per se.

Some evidence for this view is provided by the finding that the factor prevents the unmasking of Mg++-stimulated ATPase in aged mitochondria; on the other hand, it is certain that the factor does not repair all the damaged steps in the oxidative phosphorylation sequence since the ATP-Pi exchange activity is not restored, nor is oxidative phosphorylation per se (unpublished observations).

**SUMMARY**

1. Mitochondria pre-aged for hours or days at 2°C in 0.25 M sucrose and swollen under a variety of conditions lose partially or totally their ability to contract on addition of adenosine triphosphate + Mg++ + bovine serum albumin. The loss of ability to contract occurs at or below pH 7.4 after the mitochondria have been pre-aged in 0.25 M sucrose; the impairment occurs during the swelling phase, and is most severe in dilute suspensions.

2. After swelling, pre-aged mitochondria can contract again on addition of ATP + Mg++ + bovine serum albumin when a 0.6 M KCl extract of fresh mitochondria is added to the swelling medium. The contraction-restoring activity is found in a protein fraction precipitating on dialysis against distilled water as well as in the protein which remains soluble.

3. The protein fractions restore ATP-induced contraction in mitochondria swollen spontaneously or in the presence of oleate, Ca++, thyroxine, or phosphate. They are ineffective with hypotonic swelling or glutathione-induced swelling.

4. Catalase (C factor II), which is required to restore contraction of glutathione-swollen mitochondria, will not replace the KCl-extracted protein fraction in supporting contraction of pre-aged mitochondria; conversely, the KCl extracts will not replace catalase in reversing glutathione swelling.

5. The ATP-induced contraction restored by KCl extracts from fresh mitochondria is inhibited by oligomycin.

6. The active factor contained in the KCl-extracted mitochondrial protein fractions is a heat-stable compound of lipid nature bound to protein.

7. Contraction restored in pretreated mitochondria by the addition of the heat-stable factor is accompanied by a decrease of the Mg++-stimulated ATPase activity of these mitochondria.
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