Reversal of Thyroxine-induced Swelling of Rat Liver Mitochondria by Adenosine Triphosphate*

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L-Thyroxine and related thyroactive compounds cause rapid swelling of liver and kidney mitochondria by adenosine triphosphate were not successful (2, 5, 6), a recent communication from this laboratory showed that ATP readily reverses thyroxine-induced swelling in a medium of buffered isotonic KCl but is not effective in the buffered sucrose medium which has usually been used in swelling experiments (5). The inhibitory action of sucrose on mitochondrial contraction was suggested to be related to the finding that sucrose inhibits an intermediate reaction of oxidative phosphorylation (5, 12-15). In an abstract, Chappell et al. (10, 11) as refined by Werkheiser and Bartley (20) it was considered necessary to establish more directly that the increase in optical density occurring when ATP is added to thyroxine-swollen mitochondria is in fact caused by extrusion of water from the mitochondria. Although earlier efforts to demonstrate reversal of thyroxine-induced swelling of rat liver mitochondria by adenosine triphosphate were not successful (2-5, 9), the possibility that thyroxine-induced swelling of mitochondria can be reversed by enzymatic reactions, particularly those associated with respiration and phosphorylation, is a matter of some interest since a growing body of evidence now indicates that the volume and morphological configuration of the mitochondrion is a reflection of a dynamic balance between uptake and extrusion of water (5, 9-11).

Although earlier efforts to demonstrate reversal of thyroxine-induced swelling of rat liver mitochondria by adenosine triphosphate were not successful (2-5, 9), a recent communication from this laboratory showed that ATP readily reverses thyroxine-induced swelling in a medium of buffered isotonic KCl but is not effective in the buffered sucrose medium which has usually been used in swelling experiments (5). The inhibitory action of sucrose on mitochondrial contraction was suggested to be related to the finding that sucrose inhibits an intermediate reaction of oxidative phosphorylation (5, 12-15). In an abstract, Chappell et al. (10, 11) have independently noted reversal of thyroxine-induced swelling in a KCl medium.

This paper reports a more detailed study of the action of ATP in causing reversal of thyroxine-induced swelling of rat liver mitochondria, the quantitative relationships between water extruded and ATP utilized, and the relationship of intermediary reactions of the energy-coupling mechanism to the contractile process.

EXPERIMENTAL

Methods—Mitochondria were isolated by the methods of Schenider (17) from the livers of Wistar strain male albino rats weighing from 150 to 250 gm. which were fed ad libitum. The homogenizing medium was 0.25 M sucrose and a Potter-Elvehjem type homogenizer with smooth glass tube and Teflon pestle was used. The mitochondria were washed 3 times with cold 0.25 M sucrose and then made up in a stock suspension in 0.25 M sucrose so that 1.0 ml. contained the mitochondria derived from 1.0 gm. of whole liver. The stock suspension was kept in ice and used within 3 to 4 hours after preparation.

The swelling and reversal experiments were carried out in matched 15 x 100-mm. tubes containing ordinarily 5.0 ml. of 0.125 M KCl-0.02 M Tris buffer, pH 7.4, with various other additions as shown in the legends of Figs. 1 to 12 and Table 1. The swelling was usually induced by the presence of 1 X 10^-4 M L-thyroxine (gift of Dr. A. E. Heming, Smith, Kline and French Laboratories, Philadelphia) in the medium. Additions of the solution suspension of mitochondria were made last, the aliquot being chosen to give an initial absorbancy of 0.5 to 0.6 at 520 mμ. The tubes were incubated at 20° in a water bath; an earlier study showed the critical dependence of swelling rate on temperature (5). At given times tubes were quickly removed from the bath, wiped dry, and the absorbancy read at 520 mμ in a Beckman Model B spectrophotometer. When colored reagents were used appropriate wave lengths were chosen to avoid their absorption maxima. Additions of ATP and other reagents during the course of incubation were made in very small volumes (~0.05 ml.) of concentrated solutions to minimize absorbancy changes caused by dilution. The optical absorbancy of mitochondrial suspensions has been shown to bear definite relationships to the water content of the mitochondria (10, 11, 18-20). Direct gravimetric measurements of changes in intramitochondrial water were made by the general procedure of Price et al. (10, 11) as refined by Werkheiser and Bartley (20). All reagents used were of highest commercial quality.

Experimental Findings

Direct Gravimetric Measurement of Water Extrusion from Thyroxine-swollen Mitochondria by ATP—In a preceding study (5) the reversal of thyroxine-induced swelling of rat liver mitochondria by ATP was followed by simple photometric measurement of optical absorbancy of the mitochondrial suspensions. Although the photometric method has been found to correlate well with actual measurements of mitochondrial diameters (18, 19) and with direct gravimetric measurement of water content (10, 11, 20) it was considered necessary to establish more directly that the increase in optical density occurring when ATP is added to thyroxine-swollen mitochondria is in fact caused by extrusion of water from the mitochondria.

The typical data in Table I compare the optical method with the direct gravimetric measurement of water content (10, 11, 20). It is seen that the decrease in optical absorbancy of the mitochondrial suspension on exposure to thyroxine corresponds to a substantial gain in water content of the mitochondria. On addition of ATP to the thyroxine-swollen mitochondria, the optical absorbancy increases again, approaching the original level. Correspondingly, the directly measured water content of the mitochondria also decreases to approach the original water content before thyroxine-induced swelling. It may, therefore, be con-
The mitochondria of one tube were centrifuged and the mitochondria were washed free of the centrifuge tube and incubated at 20°C. Simultaneously, the optical absorbancy changes at 520 nm on an aliquot of the system were measured using 1.0-cm. cells equipped with quartz "spacers" to produce a light path of 1.0 mm. When the absorbancy changes indicated thyroxine-induced swelling was substantially complete, 0.001 M ATP was then added. When the optical absorbancy had returned toward the zero time value before thyroxine-induced swelling was essentially complete, 0.02 M Tris buffer pH 7.4 containing 3 x 10^-5 M L-thyroxine was added. Mitochondrial wet weight and dry weight determined (10, 11, 20). To the other tubes and to the optical system a final concentration of 0.01 M ATP was then added. When the optical absorbancy had returned toward the zero time value before thyroxine-induced swelling, the mitochondria were centrifuged out and wet weight and dry weight determined. Mitochondrial weights given were corrected for adhering extramitochondrial water. The increases in optical absorbancy of suspensions of thyroxine-swollen mitochondria brought about by ATP represented actual extrusion of intramitochondrial water. Such measurements also permitted direct calculation of the amount of water extruded and its relation to the amount of ATP added. For example, in the experiment of Table I, addition of 60 μmoles of ATP to the thyroxine-swollen mitochondria caused extrusion of as much as 650 μmoles of H₂O from the mitochondria. Experiments described below correlate quantitatively the magnitude of the water extrusion with the enzymatic hydrolysis of ATP.

**Effect of Sucrose on Contraction and Swelling**—The following experiments examine in a more quantitative manner the effect of graded sucrose additions to a medium of 0.15 M NaCl as in 0.15 M KCl. However, the rate is much lower in 0.15 M NaCl. In 0.15 M LiCl, thyroxine-induced swelling is very slow and addition of ATP fails to halt it, but after some 10 minutes exposure to ATP, reversal occurs suddenly. The experiments suggest some specificity among the alkali metal ions as supporting solutes for the reversal reaction.

**Fig. 1.** Effect of KCl concentration on mitochondrial contraction by ATP. The test system contained 0.02 M Tris-HCl pH 7.4 and KCl concentrations shown, and 1.0 x 10^-5 M L-thyroxine. Mitochondria added last at time zero. At time shown by arrow ATP was stirred in rapidly to make final concentration of 1 x 10^-5 M. Further readings were made at time intervals shown.

**Table 1**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Condition of mitochondria</th>
<th>Optical absorbancy at 520 nm</th>
<th>Corrected wet weight mitochondria</th>
<th>Dry weight</th>
<th>H₂O absorbed (+) or extracted (→)</th>
<th>μmoles</th>
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<tbody>
<tr>
<td>1</td>
<td>Initial</td>
<td>0.540</td>
<td>11.0</td>
<td>30.0</td>
<td>480</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Swollen by thyroxine</td>
<td>0.230</td>
<td>19.8</td>
<td>16.6</td>
<td>+480</td>
<td></td>
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<tr>
<td></td>
<td>Contracted by ATP</td>
<td>0.430</td>
<td>11.8</td>
<td>27.9</td>
<td>-444</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Initial</td>
<td>0.655</td>
<td>9.1</td>
<td>26.4</td>
<td>872</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Swollen by thyroxine</td>
<td>0.195</td>
<td>24.8</td>
<td>11.7</td>
<td>+872</td>
<td></td>
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<tr>
<td></td>
<td>Contracted by ATP</td>
<td>0.395</td>
<td>14.0</td>
<td>20.7</td>
<td>-600</td>
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<tr>
<td>3</td>
<td>Initial</td>
<td>0.615</td>
<td>17.8</td>
<td>24.5</td>
<td>483</td>
<td></td>
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<tr>
<td></td>
<td>Swollen by thyroxine</td>
<td>0.220</td>
<td>26.5</td>
<td>16.5</td>
<td>+483</td>
<td></td>
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<tr>
<td></td>
<td>Contracted by ATP</td>
<td>0.510</td>
<td>20.3</td>
<td>21.5</td>
<td>-344</td>
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<tr>
<td>4</td>
<td>Initial</td>
<td>0.575</td>
<td>22.0</td>
<td>25.2</td>
<td>678</td>
<td></td>
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<tr>
<td></td>
<td>Swollen by thyroxine</td>
<td>0.210</td>
<td>34.2</td>
<td>16.2</td>
<td>+678</td>
<td></td>
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<tr>
<td></td>
<td>Contracted by ATP</td>
<td>0.455</td>
<td>22.5</td>
<td>24.6</td>
<td>-650</td>
<td></td>
</tr>
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</table>
Fig. 3 shows that sucrose added to the basal medium inhibits thyroxine-induced swelling, with 0.1 M sucrose causing some 60 per cent inhibition. It is of interest that 0.3 M sucrose inhibits almost completely the swelling induced by $1 \times 10^{-4}$ M thyroxine, in view of the fact that media containing 0.3 M sucrose (5) or even higher concentrations (2), have been used in the past in experiments on thyroxine-induced swelling, although ordinarily with much higher concentrations of thyroxine ($1 \times 10^{-3}$ M).

The inhibition of thyroxine-induced swelling by sucrose in a KCl medium shown here thus provides an explanation for the finding that mitochondria are far more sensitive to the swelling action of thyroxine in saline media than in sucrose media (5).

In Fig. 4 is shown the effect of adding sucrose, together with ATP, to mitochondria swollen by thyroxine in a medium of 0.125 M KCl-0.02 M Tris. It is seen that all concentrations of sucrose added are inhibitory to the contraction effect produced by ATP; about 50 to 60 per cent inhibition was produced by 0.1 M sucrose. The swelling and contraction of mitochondria thus seem to be about equally sensitive to the inhibitory action of sucrose, suggesting a common denominator in these two events.

The inhibition of ATP-induced water extrusion by sucrose is evidently reversible, as is the inhibition of mitochondrial swelling by sucrose (5). When mitochondria are suspended in 0.8 M sucrose, a medium which completely prevents thyroxine-induced swelling or ATP-induced contraction, and then recovered by centrifugation and resuspended in 0.125 M KCl-0.02 M Tris pH 7.4, they swell in the presence of added thyroxine and the swelling is reversed by ATP.

Effect of ATP Concentration—Reversal of thyroxine-induced swelling readily occurs with concentrations of ATP between 0.0005 M and 0.05 M (Fig. 5); at $1 \times 10^{-4}$ M ATP contraction was only occasionally observable. The rate of contraction increases with ATP concentration and appears to be maximum at about 0.01 M. After the rapid contraction produced by the higher concentrations of ATP, the optical density is maintained constant for 30 to 60 minutes. At lower concentrations of ATP the contracted state often is not maintained, and the mitochondria "reswell." Experiments described below indicate that "reswelling" occurring with low concentration of ATP may be due to removal of ATP by ATPase activity.

Failure of ATP to Reverse Spontaneous Swelling—ATP is able to reverse swelling caused by thyroxine up to about $3 \times 10^{-4}$ M, a concentration which produces a maximal swelling rate, as is shown by the experiments in Fig. 6. There was no reversal of swelling produced by higher thyroxine concentrations. However, the rather unexpected finding was made that ATP does not cause reversal of mitochondrial swelling when thyroxine is omitted entirely, i.e., ATP does not reverse the spontaneous swelling of mitochondria which occurs in a medium of 0.125 M KCl. The typical experiment in Fig. 7 shows this important event.

**Fig. 3.** Inhibition of thyroxine-induced swelling by sucrose. The medium was 0.125 M KCl-0.02 M Tris pH 7.4 containing $1 \times 10^{-4}$ M L-thyroxine to which the indicated additions of sucrose were made.

**Fig. 4.** Inhibition of ATP reversal by sucrose. The medium was 0.125 M KCl-0.02 M Tris pH 7.4 containing $1 \times 10^{-4}$ M L-thyroxine. At time shown, 0.005 M ATP was added, together with final concentrations of sucrose shown.

**Fig. 2.** Effect of alkali metal cations. Test system contained 0.02 M Tris-HCl pH 7.4 with 0.15 M concentrations of NaCl, KCl, LiCl, and NH$_4$Cl as shown. Thyroxine was added at $1.0 \times 10^{-5}$ M; ATP at $1 \times 10^{-4}$ M.
difference clearly. It may be suggested that thyroxine-induced and spontaneous swelling of mitochondria are not similar in origin or kind in the KCl medium, contrary to an earlier suggestion based on their behavior in sucrose media (5). It will be shown elsewhere that reversal of spontaneous swelling and also swelling produced by agents other than thyroxine (5, 21, 22) have somewhat different properties and requirements than reversal of thyroxine-induced swelling.

**Stability of Contractile System**—In the standard test medium at 20° the presence of $1 \times 10^{-4}$ M thyroxine ordinarily causes very rapid swelling of mitochondria which approaches a limiting absorbancy value within about 10 to 15 minutes (5). In the experiment of Fig. 8 the time of addition of ATP to mitochondria completely swollen by thyroxine was varied to determine how long the mitochondria could remain in the swollen state at 20° and still contract on addition of ATP. Rapid reversal of the swelling, after addition of ATP, took place at all times tested, for as long as 3 hours at room temperature following institution of swelling by thyroxine. It is remarkable that the ability to contract with ATP was retained for such a long interval, since thyroxine-treated mitochondria kept under these conditions have completely lost their ability to oxidize glutamate and $\beta$-hydroxybutyrate and no longer catalyze the exchange reaction between ATP and labeled inorganic orthophosphate (5, 6).

**Specificity of ATP**—Experiments in Fig. 9 show that ATP appears to have absolute specificity in causing reversal of mitochondrial swelling. No activity was shown by cytidine triphosphate, uridine triphosphate, guanosine triphosphate, or inosine triphosphate. Earlier experiments had shown that neither ADP nor adenosine 5'-phosphate are effective (5). No other substances pertinent to respiration and phosphorylation have been found to cause reversal of swelling when tested singly. These included 0.01 M ethylenediaminetetraacetate, 0.005 M MnCl$_2$, $5 \times 10^{-5}$ M 2,4 dinitrophenol, $1 \times 10^{-5}$ M gramicidin, 0.003 M DPN, 0.001 M cyanide, 0.0018 M sodium amytyl, 1.0 $\mu$g. of antimycin A, serum albumin (1 mg. per ml.), 0.001 M coenzyme A, 0.001 M phosphate, $1 \times 10^{-5}$ M cytochrome c, 0.01 M glutamate, and 0.01 M $\beta$-hydroxybutyrate.

**Effect of Uncoupling Agents and Respiratory Inhibitors on ATP-induced Contraction**—Several agents were found capable of inhibiting the reversal of swelling by ATP. In Fig. 10 are shown the effects of some agents known to uncouple oxidative phos-
phosphorylation. It is seen that ATP-induced reversal is inhibited by azide, Dicumarol, arsenate, p-chloromercuribenzoate, and gramicidin, at concentrations which are known to uncouple oxidative phosphorylation essentially completely. On the other hand, it is highly significant that 2,4-dinitrophenol does not inhibit ATP-induced reversal of thyroxine swelling significantly at concentrations which would uncouple oxidative phosphorylation essentially completely. This result has been observed repeatedly; it contrasts with the inhibitory action of dinitrophenol in respiration-induced reversal of mitochondrial swelling observed by Price et al. (10, 11).

In Fig. 11 are shown experiments on three respiratory inhibitors tested at concentrations known to produce complete inhibition of the respiratory chain (cf. (5)). It is seen that cyanide

Fig. 8. Time of addition of ATP to swollen mitochondria. Test systems contained 0.15 M KCl-0.02 M Tris pH 7.4, 1 X 10^-8 M l-thyroxine, and mitochondria. ATP was added to separate but identical tubes at the points shown in the swelling curve, producing at each time tested a prompt contraction. The continuous curve shows the course of swelling induced by thyroxine (no ATP added); the dotted line shows the rate of spontaneous swelling in the absence of thyroxine and ATP.

Fig. 9. Specificity of ATP. Test systems of 0.125 M KCl-0.02 M Tris-HCl pH 7.4 contained 1.0 X 10^-8 M l-thyroxine. Nucleoside 5'-triphosphates indicated added at time shown to give final concentration of 2.5 X 10^-8 M.

Fig. 10. Effect of uncoupling agents on contraction by ATP. Test medium was 0.125 M KCl-0.02 M Tris-HCl pH 7.4, containing 1.0 X 10^-8 M l-thyroxine. At time shown 5 X 10^-8 M (final) ATP was added, either alone, or with 5 X 10^-8 M dinitrophenol (DNP), 5 X 10^-2 M sodium arsenate, 1 X 10^-2 M Dicumarol, 0.002 M sodium azide, 1 X 10^-6 M gramicidin, or 1 X 10^-4 M p-chloromercuribenzoate (PCMB) as shown (concentrations given are those in complete medium).

Fig. 11. Effect of respiratory inhibitors on contraction by ATP. Medium was 0.125 M KCl-0.02 M Tris-HCl pH 7.4, containing 1 X 10^-8 M l-thyroxine. At time shown, 5 X 10^-8 M ATP was added either alone or with 1 X 10^-2 M NaCN, 0.0018 M sodium amytal, or 0.12 µg. of m antiminycin A (anti-A) per ml., as shown (concentrations are those in complete medium).
thyroxine in sucrose media (5, 23).
ail three respiratory inhibitors prevent the swelling action of freshly prepared sodium amytal repeatedly caused inhibition of independently of respiration and oxidation. On the other hand, indicates that mitochondrial contraction by ATP can proceed contraction of thyroxine-swollen mitochondria, and this finding of DPN was necessary to observe maximal mitochondrial con-

Optical changes at 520 rnp were made in a cell with l&mm. light path to give the record shown; the mitochondrial system was from ATP determined colorimetrically. Calculations of amounts of ATP split which are shown on figure were based on the as-

At time shown, 60 µmoles of ATP were added. Gravimetric estimations of water content were made at times indicated by points A, B, C, and D. Simultaneously, measurements of the optical changes at 520 m were made in a cell with 1.0-mm. light path to give the record shown; the mitochondrial system was exactly that used in the tubes for the gravimetric analysis. After addition of ATP, aliquots of another identical tube were removed, fixed with trichloroacetic acid and inorganic phosphate formation from ATP determined colorimetrically. Calculations of amounts of ATP split which are shown on figure were based on the assumption that 1 mole of ATP was hydrolyzed per mole of inorganic phosphate formed.

and antimycin A have no significant effect on ATP-induced contraction of thyroxine-swollen mitochondria, and this finding indicates that mitochondrial contraction by ATP can proceed independently of respiration and oxidation. On the other hand, freshly prepared sodium neymal repeatedly caused inhibition of the ATP-induced reversal. This finding is of some interest since all three respiratory inhibitors prevent the swelling action of thyroxine in sucrose media (5, 23).

**Effect of Other Agents on ATP Reversal**—A number of agents other than uncouplers or respiratory inhibitors were tested for their ability to affect the rate and extent of the reversal of thyroxine-induced swelling by ATP. The following substances were found to inhibit mitochondrial contraction when added simultaneously with ATP in experimental conditions such as shown in Figs. 10 and 11: 0.005 m CaCl₂ (completely), 0.002 m DPN (50 per cent), 1 X 10⁻⁴ m sodium oleate (100 per cent), and 5 X 10⁻⁶ m stilbestrol (100 per cent). The inhibition by Ca++ is not surprising since Ca++ is known to cause swelling itself (2, 21). Stilbestrol, on the other hand, prevents thyroxine-induced swelling (8). The somewhat inhibitory action of DPN in this system contrasts with the finding of Price et al. (10, 11) that the presence of DPN was necessary to observe maximal mitochondrial con-

**Fig. 12. ATPase activity and water extrusion.** For gravimetric estimations of water exchanges, rat liver mitochondria were added at zero time to tared centrifuge tubes containing 6.0 ml of 0.125 m KCl-0.02 m Tris pH 7.4 and 3 X 10⁻⁶ m L-thyroxine. At time shown, 60 µmoles of ATP were added. Gravimetric estimations of water content were made at times indicated by points A, B, C, and D. Simultaneously, measurements of the optical changes at 520 m were made in a cell with 1.0-mm. light path to give the record shown; the mitochondrial system was exactly that used in the tubes for the gravimetric analysis. After addition of ATP, aliquots of another identical tube were removed, fixed with trichloroacetic acid and inorganic phosphate formation from ATP determined colorimetrically. Calculations of amounts of ATP split which are shown on figure were based on the assumption that 1 mole of ATP was hydrolyzed per mole of inorganic phosphate formed.

Neither 0.005 m Mn++ nor Mg++ enhanced the action of ATP; in fact Mn++ was definitely inhibitory, a surprising result in view of the stabilizing action of Mn++ on mitochondria (26). On the other hand, bovine serum albumin (1 mg. per ml.) and ethylenediaminetetraacetate (0.001 m) were found occasionally to enhance the action of ATP.

**Quantitative Relationship between Water Extrusion and ATPase Activity**—The preceding experiments demonstrate a definite relationship between extrusion of water from thyroxine-swollen mitochondria and the presence of ATP. In the following experiment the absolute amount of the water exchange was measured by the direct gravimetric method in experiments where mitochondria were swollen by the action of thyroxine and then contracted again by ATP. Simultaneously, the amount of inorganic phosphate formed from the added ATP was measured during the active contraction and also during the following stationary phase in which the mitochondria remained contracted. Data of a typical experiment are summarized in Fig. 12, which shows that during thyroxine-induced swelling, some 780 µmoles of water entered the mitochondria from the medium. Following addition of 60 µmoles of ATP, the mitochondria contracted with the extrusion of about 650 µmoles of water. Simultaneously with the extrusion of water, inorganic phosphate was formed from ATP at a rate which paralleled the rate of extrusion of water as measured optically or gravimetrically. When the mitochondria reached the contraction plateau, 1.65 µmoles of inorganic phosphate had been formed from the ATP during the extrusion of 650 µmoles of H₂O, or a ratio of 390 moles of H₂O extruded per mole of inorganic phosphate arising by hydrolysis of ATP. In a series of such experiments this ratio varied from 205 to 460 moles of water extruded per mole of inorganic phosphate appearing from ATP.

The findings in Fig. 12 show that hydrolysis of ATP occurred during the contraction phase but that no further hydrolysis occurred after the mitochondria had reached a stationary contracted state. This cessation of ATPase activity following contraction of the mitochondria to an approximately stationary state was seen in most of the nine experiments of this type carried out; in others, however, ATPase activity continued during the stationary state at about the same rate as during the active contraction phase.

**DISCUSSION**

**Significance of Composition of Medium in Studies of Mitochondrial Swelling and Contraction**—Most studies of mitochondrial swelling and contraction have been carried out in media containing sucrose or similar solutes such as mannitol (27), on the presumed basis that these solutes are relatively impermeant through the mitochondrial membrane and provide osmotic maintenance of mitochondrial structure. This supposition does not seem tenable, since Werkheiser and Bartley (20) have shown that the mito-
mitochondrial water is already 60 per cent penetrated by sucrose during centrifugal isolation of mitochondria from sucrose homogenate at 0°. The finding that sucrose is inhibitory to both mitochondrial swelling and contraction, as well as to an intermediate step in oxidative phosphorylation (12-15) has suggested an alternative rationale (5) for the efficacy of sucrose solutions in preserving mitochondrial morphology during isolation, namely that sucrose acts as a reversible "fixative" or inhibitor of the enzyme(s) concerned in shape and volume changes. Although it is at present difficult to exclude completely the possibility that these effects of sucrose are osmotic in nature, it is significant that sucrose at 0.3 M and above is inhibitory to intermediate reactions of oxidative phosphorylation in submitochondrial fragments which are presumably much less susceptible, if at all, to osmotic effects.

This suggests that the inhibition may be caused by some specific chemical feature of sucrose and other polyhydroxylic molecules rather than by its action as a nonspecific solute. Furthermore, other experiments show that inhibition of contraction is not given by isoosmotic concentrations of simple alcohols and other neutral molecules; the results of these studies will be published.

These effects of sucrose, which are reversible, thus make it an ideal supporting solute for isolation and preservation of mitochondria, but less favorable for studies of mitochondrial swelling and contraction.

Relationship to Earlier Studies—The observations described here also provide some indication as to why earlier studies of water extrusion in mitochondria indicated it to be very labile and evanescent in nature. The comprehensive and painstaking studies of Price et al. (10, 11), in which water extrusion from mitochondria was observed to take place during respiration, used very complex media containing a mixture of agents, some of which are now known to contribute to swelling, some to contraction, and others to the inhibition of these processes. For example, the medium most often used in their study contained inorganic phosphate, Mg++, nucleotides, oxidizable substrates, and sucrose. Of these agents, the Mg++, nucleotides, and substrates would contribute to contraction by making possible roxiphoto-reactivation of synthesis of ATP. On the other hand, inorganic phosphate would promote swelling and sucrose would be inhibitory to both swelling and contraction. Thus it is evident that reversal of swelling in such a system would depend critically on a complex balance of factors.

In contrast, the present study indicates that in a KCl-Tris medium neither oxidative phosphorylation nor respiration is necessary for contraction of thyroxine-swollen mitochondria if ATP specifically is present; no other agent seems necessary in the medium or accelerates the action of ATP. Although one or more of the intermediate reactions of energy-coupling may be involved in the contraction of thyroxine swollen mitochondria, the complete coupling mechanism certainly is not involved in view of the fact that dinitrophenol does not affect the contraction by ATP. It is significant that these nonrespiring conditions resemble those under which Chappell and Perry (27) and Price et al. (10, 11) were able to observe a limited degree of contraction of pigeon breast muscle mitochondria.

Various Types of Mitochondrial Swelling and Requirements for Reversal—It is now known that a number of agents in addition to thyroxine cause mitochondrial swelling; these include inorganic phosphate (25, 28), hypotonicity of the medium (2, 5, 18, 19, 28), Ca++ (2, 24, 28), reduced glutathione (22), as well as unphysiological agents such as heavy metals (2), phloridzin (21), some detergents (29), and carbon tetrachloride (30). There is now evidence that at least some of these agents may differ in the mechanism by which they initiate swelling and in the morphological changes produced (5, 22). Similarly, it seems very probable that the cofactor requirements for producing active water extrusion and its mechanism may differ depending on the agent causing the swelling. For example, a comparison of thyroxine-induced and glutathione-induced swelling showed that ATP alone could not reverse swelling produced by glutathione (22). In addition, in this paper it is shown that spontaneous swelling in an isotonic medium is not reversed by addition of ATP alone, whereas thyroxine-induced swelling in the same medium is readily and rapidly reversed by ATP. An extensive survey of the requirements in the test medium for reversal of other types of mitochondrial swelling has been carried out in this laboratory and will be presented for publication shortly; it provides additional evidence for the multiplicity and specificity of factors involved in swelling and its reversal.

Nature of Water Extrusion Mechanism—It has been suggested before that water extrusion from mitochondria may be the result of a contractile process (10, 11, 28, 31). The data on the stoichiometric relationships between the masses of water moved and ATP utilized, which are presented in this paper, clearly demonstrate that there is no simple, stoichiometric mole-for-mole relationship between H2O transported and ATP utilized; rather, it is shown that over 400 moles of H2O may be extruded per mole of inorganic phosphate formed from ATP. This finding therefore excludes transport by a chemical or enzymatic interaction between water and ATP, or between water and some carrier generated or activated at the expense of the bond energy of ATP, in which 1 mole of ATP is ultimately required to move 1 mole of H2O. The evidence is compatible, however, with a contractile mechanism in the membrane(s) activated by ATP, possibly by phosphorylation of acceptor functions in the membrane, which is capable of shrinking the volume of the mitochondria and thus extruding many molecules of water into the medium. Such a process thus would have a striking physical and chemical similarity to the action of the actomyosin system of muscle.

It is not possible to conclude, however, that the hydrolysis of ATP occurring during the contraction is related to the contraction per se although it ceases in most experiments after the mitochondria have contracted maximally. An alternative explanation is that ATPase activity is shown by swollen mitochondria and not by contracted mitochondria. On the other hand, the swollen state is not requisite for ATPase activity since dinitrophenol inhibits swelling of rat liver mitochondria (2) under conditions in which ATPase is greatly stimulated. Activation of mitochondrial ATPase activity by thyroxine has already been reported (32, 34); it differs in some respects from dinitrophenol-stimulated ATPase (33).

Although a contractile mechanism is strongly suggested by the findings reported here, it does not represent a unique explanation for the available evidence. It is also conceivable, for example, that the water extrusion is caused by a reversible, ATP-driven polymerization of some internal solute molecule not capable of traversing the membrane. A great reduction of the concentration of solute molecules in the internal phase by a polymerization initiated by ATP (as in the G-actin → F-actin conversion, for example) could cause loss of internal water through a decrease...
in osmotic pressure of the internal phase. For the present, however, the contraction hypothesis seems best able to account for known facts.

At this time it does not seem possible to determine whether it is the inner or outer mitochondrial membrane or both, which participates in the contractile phenomenon. Recent findings on the permeability of the membranes and compartmentation of intramitochondrial water (5, 19) suggest that the outer membrane is very freely permeable to most solutes tested and that the inner membrane is the more likely site of the swelling and contraction phenomena; the inner membrane is also presumably the site of the respiratory enzyme assemblies. The cristae, which have been shown to be invaginations of the inner membrane (35), are geometrically suggestive of a mechanism of swelling and contraction, in which reversible “unpleating” and “repleating” of the cristae through side-by-side cross-linking attachments could take place without necessarily involving any contractility changes in the dimensions of the inner membranes. On the other hand, the contractile process could be envisioned as being evenly distributed in two dimensions over the entire membrane sheet. The apparently uniform distribution of respiratory enzyme assemblies in the membrane is suggestive in this connection (36).

Enzymatic Relationships between Contraction and Mechanism of Oxidative Phosphorylation—Although water extrusion from thyroxine-swollen mitochondria by ATP does not require respiration, it may involve more or less directly at least one enzymatic reaction intermediate in the coupling of phosphorylation to respiration. The specificity of the contractile system for the triphosphate of adenosine and the inhibition of the contraction by azide, Dicumarol, arsenate, p-chloromercuribenzoate, gemicidin, and sucrose, which are also characteristic inhibitors of coupled phosphorylation, suggests the participation in the contraction of one or more reaction(s) shared with the mechanism of oxidative phosphorylation (14, 15, 36). On the other hand, the failure of dinitrophenol to affect the contraction is puzzling and unexpected. Since dinitrophenol can be regarded as the most specific inhibitor known for oxidative phosphorylation it is possible that its failure to affect the contraction excludes all reactions of the coupling sequence as participants in the contractile mechanism. Certainly at the least it indicates that not all of the coupling mechanism is concerned in contraction.

In view of these findings with dinitrophenol an alternative formulation for the contractile mechanism must be considered in which the energy-coupling reactions do not participate except as a means of producing ATP during respiration. An attractive possibility is afforded by the fact that mitochondria contain considerable phosphoprotein, the phosphate of which becomes labeled during respiration in media containing P32-labeled inorganic phosphate (37). Rat liver mitochondria also contain a protein phosphokinase (38). It seems conceivable that the protein phosphokinase and the phosphoprotein of mitochondria are elements in an ATP-driven contractile phenomenon acting independently of the coupling mechanism. The possible relationship of the protein phosphokinase of mitochondria to the contraction is under study.

Lastly, it may be pointed out that these experiments show thyroxine-induced swelling to be reversible by ATP, providing further support for a possible physiological role of thyroxine in adjusting the intracellular volume and state of the mitochondrion as well as its diverse and complex enzymatic mechanisms of respiration, phosphorylation, and active transport (3-5).

SUMMARY

Through optical and also direct gravimetric methods it has been demonstrated that adenosine triphosphate (ATP) causes extrusion of water from mitochondria swollen by contact with L-thyroxine. The extrusion reaction proceeds best in a medium of 0.125 M KCl or 0.02 M tris(hydroxymethyl)aminomethane buffer, and occurs also in NaCl or distilled water, but does not occur in sucrose media, which inhibit both mitochondrial swelling and contraction.

The contractile mechanism is relatively stable, since addition of ATP will rapidly contract mitochondria kept as long as 3 hours at 20° following induction of swelling by addition of thyroxine. ATP is specific; no other nucleoside 5’-triphosphate is active nor is any other substance tested. The contraction by ATP is not affected by inhibiting respiration with cyanide or antimycin A, nor by the presence of 2,4-dinitrophenol, and is thus independent of coupled respiration. On the other hand, Dicumarol, azide, arsenate, and gemicidin do inhibit the contraction, suggesting the possibility that one or more intermediate reactions of oxidative phosphorylation may be involved in contraction.

ATP undergoes hydrolysis during the contraction of thyroxine-swollen mitochondria, but the hydrolysis ceases again after the mitochondria have reached a stable, contracted state. Over 400 moles of water may be extruded per mole of inorganic phosphate formed from ATP, a finding which excludes a simple mole-for-mole mechanism for transport of water and which suggests participation of a contractile process similar to the actomyosin system. A “mechano-enzyme” function is postulated to account for these findings and their relationship to the intermediate reactions of oxidative phosphorylation which occur in the mitochondrial membrane.

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