The Swelling of Rat Liver Mitochondria by Thyroxine and its Reversal*

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(Received for publication, May 22, 1958)

ABSTRACT

The in vitro swelling action of L-thyroxine on rat liver mitochondria as examined photometrically represents an acceleration of a process which the mitochondria are already inherently capable of undergoing spontaneously, as indicated by the identical kinetic characteristics and the extent of thyroxine-induced and spontaneous swelling, the nearly identical pH dependence, and the fact that sucrose has a specific inhibitory action on both types of swelling. However, thyroxine does not appear to be a "catalyst" or coenzyme since it does not decrease the temperature coefficient of spontaneous swelling. The temperature coefficient is very high, approximately 6.0 near 20°.

Aging of mitochondria at 0° causes loss of thyroxine sensitivity which correlates closely with the loss of bound DPN from the mitochondria, but not with loss of activity of the respiratory chain or with the efficiency of oxidative phosphorylation. Tests with various respiratory chain inhibitors showed that the oxidation state of bound DPN may be a major determinant of thyroxine sensitivity; the oxidation state of the other respiratory carriers does not appear to influence sensitivity to thyroxine. These facts and other considerations suggest that a bound form of mitochondrial DPN is the "target" of the action of thyroxine.

The thyroxine-induced swelling is not reversed by increasing the osmolar concentration of external sucrose, but can be "passively" or osmotically reversed by adding the high-particle weight solute polyvinylpyrrolidone. The mitochondrial membrane becomes more permeable to sucrose during the swelling reaction. On the other hand, thyroxine-induced swelling can be "actively" reversed by ATP in a medium of 0.15 M KCl or NaCl but not in a 0.30 M sucrose medium. The action of ATP is specific; ADP, Mn++, and ethylenediaminetetraacetate are not active. It is concluded that sucrose is an inhibitor of the enzymatic relationship between oxidative phosphorylation and the contractility and permeability properties of the mitochondrial membrane.

Occurrence of different types of mitochondrial swelling, the intracellular factors affecting the swelling and shrinking of mitochondria, as well as the physiological significance of thyroxine-induced swelling are discussed.

Earlier work in this laboratory has shown that the action of thyroxine on phosphorylating respiration in liver mitochondria is quite different in nature from the action of the classical uncoupling agent 2,4-dinitrophenol, with which thyroxine has often been compared. Although thyroxine may uncouple oxidative phosphorylation in intact mitochondria (1-4), it was found not to uncouple phosphorylation occurring in submitochondrial fragments (5-10), suggesting its action is indirect and dependent on some aspect of mitochondrial structure. Phosphorylation in submitochondrial fragments is, on the other hand, readily uncoupled.

* Supported by grants from the National Science Foundation, the National Institutes of Health, The Nutrition Foundation, Inc., and the Whitehall Foundation. Preliminary communications of portions of this work have been presented (Lehninger, A. L., and Ray, B. L., Science, 1957, 125, 748, and Lehninger, A. L., in International Symposium on Enzyme Chemistry, Tokyo, 1958.)
This paper presents a more detailed study of some swelling. An accompanying paper (18) shows that swelling is fundamentally similar to spontaneous.

These experiments represent an approach to factors concerned in the thyroxine-induced swelling in an earlier publication from this laboratory (12). Reduced glutathione also causes a swelling of mitochondria, which appears to be quite different in nature from that induced by thyroxine.

Experimental Details

Rat liver mitochondria were isolated by the method of Schneider (19), employing 0.25 M sucrose, without added EDTA. Care was taken to avoid contamination by the nuclear fraction, the microsomes, and the so-called "fluffy layer." The mitochondria were washed three times with cold 0.25 M sucrose and then gently suspended in 0.25 M sucrose to make a stock suspension which was kept at 0°, containing the mitochondria derived from 1.0 gm. of fresh rat liver per ml. The rats (Carworth Farms, Wistar) were always fed ad libitum.

The standard test system for mitochondrial swelling experiments consisted of 5.0 ml. 0.5 M sucrose—0.02 M tris (hydroxymethyl)-aminomethane-HCl (tris) buffer, pH 7.4, made up in 15 X 100 mm. test tube cuvettes suitable for use in the tube holder of the Beckman model B spectrophotometer. Temperature control was very critical (see below) and tubes were incubated in constant temperature water baths. Absorbancy changes were read at 520 m/z over specified time intervals. The reaction was begun by adding mitochondria from the stock suspension to the otherwise complete test medium. Approximately 0.05 to 0.10 ml. of such a suspension (40 to 100 mg. tissue equivalent) was added to each tube, yielding an initial absorbancy between 0.50 and 0.70. Absorbancy of such suspensions at 520 m/z in the range 0.15 to 0.70 was approximately linear with the concentration of mitochondria; all experiments were carried out in this range. It has been established that the absorbancy of rat liver mitochondria in this range is quantitatively related, in an inverse manner, to the water content of the mitochondria; all experiments were conducted under all of the great number of different experimental conditions employed in this study. However it has not been established that such proportionality existed under all of the great number of different experimental conditions employed in this study. Theoretically, changes in light transmission could be caused not only by changes in volume of the mitochondria, but also by aggregation, by loss of refractile or absorbing material from the mitochondria.

L-Thyroxine, of high purity, made available through the generosity of Dr. Arthur E. Heming of Smith, Kline, and French, Inc., Philadelphia, was used in all experiments and was made up in a clear stock solution of 1 X 10^-2 M at 30° with the aid of NaOH to yield a final pH of about 8.0.

Experimental Results

Preparative History of Mitochondria and the Thyroxine Swelling Effect.—Mitochondria prepared in 0.88 M sucrose are relatively intact with rod-like morphology (22), but they are not highly active in fatty acid oxidation and oxidative phosphoryla-
tion because of the inhibitory action of high concentrations of sucrose (19, 23, 24). Such mitochondria are relatively insensitive to the swelling action of thyroxine. A procedure employing 0.44 M sucrose has been found to show excellent preservation of both morphology and enzymatic organization (25); this medium was used in an earlier study of the thyroxine-induced swelling of mitochondria in this laboratory (12). However it has been found on further study that mitochondria prepared in 0.25 M sucrose (19) are actually more sensitive to thyroxine than those prepared in higher sucrose concentrations, even though they have already undergone some swelling and morphological disorganization (19, 25-28) and have a higher rate of spontaneous swelling than those prepared in 0.44 M or 0.88 M sucrose. Mitochondria prepared in 0.25 M sucrose and stored in this medium at 0° ordinarily gave maximum swelling response to thyroxine for several hours, and some response could usually be detected 24 hours after preparation. Addition of EDTA to the 0.25 M sucrose isolation medium was found unnecessary to obtain mitochondria yielding maximum swelling response, contrary to the finding with 0.44 M sucrose (12), and it was never used.

Mitochondria prepared with media containing polyvinylpyrrolidone (PVP), which are morphologically relatively intact (26), do not swell when exposed to thyroxine unless the PVP is first removed by washing with 0.25 M sucrose.

Effect of Sucrose Concentration in the Swelling Test Medium.—In Fig. 1 are shown comparisons of the rates of spontaneous swelling and thyroxine-induced swelling as a function of sucrose concentration. The spontaneous swelling rate is greater at low sucrose concentration and approaches zero at sucrose concentrations above 0.3 to 0.4 M at 20°, as shown earlier (11, 12). However, it is seen that the thyroxine-induced swelling is similarly dependent on sucrose concentration. Actually the action of even very high concentrations of L-thyroxine could be completely abolished as sucrose concentration was increased substantially above 0.5 M. It is evident that spontaneous and thyroxine-induced swelling are affected in a very similar way by variations in sucrose concentration. Other experiments reported below indicate that sucrose, in addition to affecting osmotic relationships, also may have an inhibitory action on some enzyme systems concerned in swelling and its reversal.

The inhibition of the thyroxine swelling effect by high sucrose concentrations has been found to be reversible if the time of exposure is relatively short. Mitochondria kept in a medium of 1.0 M sucrose for 30 minutes at 20°, conditions under which no swelling whatsoever can be induced by 1 × 10⁻⁵ M thyroxine, and then re-isolated by centrifugation and suspended in 0.3 M sucrose—0.02 M tris pH 7.4, were found to swell rapidly in the latter medium when 1 × 10⁻⁵ M thyroxine was added.

Effect of Other Solutes on Thyroxine-Induced Swelling.—The thyroxine-induced swelling of rat liver mitochondria could also be observed in media containing glucose, raffinose, KCl, or NaCl as major solutes. The behavior of mitochondria in glucose and raffinose media is qualitatively similar to that in sucrose. The swelling
behavior in isotonic KCl was of special interest, since K⁺ is the major intracellular cation. K⁺ is freely and rapidly permeable into mitochondria and is actively accumulated during respiration and phosphorylation, presumably in a bound form (29-33).

Fig. 2 shows that rat liver mitochondria have a higher spontaneous swelling rate in 0.15 M KCl than in 0.3 M sucrose. However, thyroxine always caused a substantial increment in the rate of swelling. In fact it has been found that rat liver mitochondria in 0.15 M KCl will respond to significantly lower concentrations of thyroxine than in a medium of 0.3 M sucrose, despite the fact that they are already spherical and swollen. As is shown below, reversal of thyroxine-induced swelling by ATP can be readily demonstrated in a KCl medium, but not in a sucrose medium.

High molecular weight solutes which are presumably not diffusible through the mitochondrial membrane, such as serum albumin, serum γ-globulin, and polyvinylpyrrolidone (PVP) (26, 27), inhibit both the spontaneous and thyroxine-induced swelling. As little as 1.0 mg per ml of crystallized bovine serum albumin completely prevents the swelling normally induced by 1 × 10⁻⁵ M L-thyroxine in 0.3 M sucrose. Similarly, swelling is prevented by PVP, but at higher concentrations. Presumably these solutes preserve a "colloid" osmotic pressure difference across the membranes (cf. 32). Undoubtedly part of the effect of serum albumin is due to binding of thyroxine.

Kinetics and Extent of the Swelling Reaction.—The findings in Fig. 3 show that the spontaneous swelling of freshly prepared mitochondria in sucrose proceeds to a stable and characteristic end value which represents about 30 to 40 per cent of the initial optical absorbancy of the suspension under the geometrical and optical conditions employed. This change corresponds to about a threefold increase in volume, or a reduction in per cent dry weight to about 10 to 12 per cent (cf. 11, 32). It is also seen that the identical terminal absorbancy value is reached in the presence of high concentrations of thyroxine, but much more rapidly. At higher temperatures (37°) swelling in the presence of thyroxine begins immediately without a detectable lag period and proceeds rapidly to the same terminal value as at lower temperatures (Fig. 3). At lower temperatures a lag period is evident which is followed by a sharp break into the normal swelling curve. The lag period is also often evident in the spontaneous swelling (see also reference 11) and is followed by a more rapid decline to a plateau which also ultimately approximates that attained in the presence of thyroxine. These findings suggest that thyroxine hastens a process which the mitochondria are already inherently capable of undergoing spontaneously, and that thyroxine-induced swelling may not differ in kind from spontaneous swelling. On the other hand, swelling induced by other agents such as glutathione is characterized by sharply different properties and kinetics and presumably has a different chemical and morphological basis (18).

Temperature Coefficient of the Swelling Reaction.—Spontaneous and thyroxine-induced swelling have extraordinarily high temperature coefficients, as is shown from the experiments in Table I. In order
TABLE I

Effect of Temperature on Thyroxine-Induced and Spontaneous Swelling

Standard test system of 0.3 M sucrose--0.02 M tris pH 7.4, plus 30 mg. tissue equivalent of rat liver mitochondria and incubations carried out at temperatures noted, with frequent readings of light absorbancy at 520 nm. Swelling rate expressed as reciprocal of "quarter-time" of swelling.

<table>
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<th>T °C</th>
<th>1/tq</th>
<th>Q10</th>
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<tr>
<td>15</td>
<td>0.10</td>
<td>4.8</td>
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<tr>
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<tr>
<td>25</td>
<td>0.50</td>
<td>5.0</td>
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<tr>
<td>30</td>
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<tr>
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Spontaneous swelling

<table>
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<th>1/tq</th>
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<tr>
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<td>0.022</td>
<td>4.8</td>
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<tr>
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<td>3.0</td>
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<tr>
<td>40</td>
<td>0.250</td>
<td>3.8</td>
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to use the kinetic data on rates of swelling for calculation of temperature coefficients (Q10) and the energies of activation, the initial rates were taken as the time required to reach one-fourth the maximal extent of swelling as determined by absorbancy measurements (so called "quarter-times," tl/4). The calculated temperature coefficients (Q10) are given for adjacent temperature intervals. It is seen that Q10 at all temperature ranges tested, for both spontaneous and thyroxine-induced swelling, is higher than that of most chemical and enzymatic reactions and much higher than for a simple diffusion process. An apparent energy of activation for thyroxine-induced swelling may be calculated from the Arrhenius equation. For the interval 15°–20° it is some 33,400 calories per "mole." It is pertinent that a number of membrane-dependent phenomena are known to have high temperature coefficients. A recent example is provided by the studies of Crane et al. on the rate of penetration of sugars into Ehrlich ascites tumor cells (34), which shows Q10 values of about 4.0 at 20°.

These findings indicate that thyroxine is not acting as a catalyst in the ordinary sense (i.e., by lowering the activation energy of the spontaneous swelling process), since if anything, the Q10 of the thyroxine-induced swelling is somewhat larger than that of spontaneous swelling. However the process measured by the absorbancy changes is undoubtedly very complex and as yet difficult to interpret precisely.

Inadequate recognition of the very critical dependence of swelling rate on temperature may account for some fluctuations in behavior noted in earlier work and some discrepancies between findings in different laboratories.

Effect of pH.—The swelling effect of thyroxine is strikingly dependent on pH, as is shown in Fig. 4. The increment in swelling rate produced by thyroxine is maximum at pH 7.5 in a medium of 0.3 M sucrose buffered with 0.02 M tris-histidine mixtures. The thyroxine effect is completely absent above pH 8.5, is still pronounced at pH 7.0, but absent at pH 6.5. In this experiment the thyroxine-induced swelling was measured at 20°, at which the rate of spontaneous swelling was insignificant. The spontaneous swelling was measured at 30° at which it proceeds at an appreciable rate, and was found to have approximately the same pH dependence (cf. reference 11) as the thyroxine-induced swelling, again suggesting similarity of the mechanism of spontaneous and thyroxine-induced swelling. The optimum pH for glutathione-induced swelling is substantially different (18).

Sensitivity of Mitochondria to Thyroxine.—Earlier experiments showed measurable swelling responses with about 10^{-6} M thyroxine (12), which

![Fig. 4. Effect of pH on thyroxine-induced and spontaneous swelling of rat liver mitochondria. Standard test system contained 0.3 M sucrose, 0.02 M tris, and 0.02 M histidine, brought to the pH values shown; 50 mg. tissue equivalent rat liver mitochondria were added. Thyroxine was added at 1 \times 10^{-5} M and the rate of swelling at 20° observed. The spontaneous swelling rate was measured at 30°.](image)
is about 100 times the concentration of “thyroxine” in the blood and tissues of euthyroid animals, but considerably lower than that usually required (>2 × 10⁻⁵ M) to uncouple phosphorylation from respiration in in vitro experiments with mitochondria (1-4, 8). On closer study, using the standard test system of 0.3 M sucrose-0.02 M tris buffer pH 7.4, and temperatures of 20°-25°, thyroxine was often found to cause significant increases in the rate of swelling at concentrations of 1 × 10⁻⁷ M and occasionally at 1 × 10⁻⁸ M. However, as mentioned above, mitochondria are consistently more sensitive to thyroxine in a medium of 0.15 M KCl; response to 1 × 10⁻⁵ M thyroxine was often seen. The sensitivity of rat liver mitochondria to thyroxine-induced swelling can be expected to be greater at temperatures approaching 37°. Physiological concentrations of thyroxine thus are capable of producing detectable mitochondrial swelling.

Effect of Aging of Mitochondria.—Sensitivity of mitochondria to thyroxine-induced swelling is lost on aging at 0°. Experiments were carried out to determine to what extent loss of thyroxine-sensitivity correlated with some characteristic degenerative changes in the mitochondria measured by enzymatic tests. In experiments summarized in Fig. 5 samples were taken at different time intervals from a stock suspension of mitochondria held at 0° (1 ml. = 1 gm. equivalent). Measurements were then made of the thyroxine-induced swelling rate in a standard test medium of 0.3 M sucrose-0.02 M tris pH 7.4. At the same time intervals, samples of the stock suspension were tested for the rate of oxidation of L-β-hydroxybutyrate and the P:2e ratio of the oxidative phosphorylation linked to this oxidation in the absence of added DPN, as well as the rate of oxidation of β-β-hydroxybutyrate in the presence of added DPN.

It is seen from the curves that the thyroxine-induced swelling of the mitochondria in the standard test system was maxima! during about 6 hours of storage of the stock suspension at 0°, but fell off sharply thereafter; only one-fourth the original sensitivity remained at 24 hours. Over the same time interval the rate of oxidation of β-β-hydroxybutyrate by the mitochondria in the absence of added DPN, which is a test of the content of bound DPN (48), also remained maximum for about 6 to 9 hours of aging and then decreased markedly. On the other hand, when the oxidative test system was supplemented with “external” DPN, the rate of oxidation of β-β-hydroxybutyrate remained high and constant over a 50-hour period of aging of the mitochondria at 0°. The efficiency of oxidative phosphorylation, expressed as the P:2e ratio, coupled to oxidation via bound DPN.
DPN remained nearly maximal and did not start to decline until about 30 hours of aging at 0°, despite the already significant drop in the bound DPN and consequent decline in oxidation rate. Loss of sensitivity to thyroxine thus correlates very well with the loss of bound, functional DPN during aging of the mitochondria at 0°, but does not correlate well with the relatively constant efficiency of oxidative phosphorylation and the constant rate of oxidation when excess DPN is added. These findings with thyroxine are therefore consistent with those of Hunter and Ford (35), who have shown that swelling of mitochondria induced by inorganic phosphate is concomitant with loss or destruction of bound pyridine nucleotides. This similarity was confirmed by our finding that phosphate-treated mitochondria do not swell further in the presence of thyroxine.

These findings and others to be enumerated below suggest that mitochondrial DPN may be involved in the action of thyroxine. However, it is also known that spontaneous swelling is accompanied by destruction of intramitochondrial ATP (17, 36).

**Effect of Respiratory Inhibitors on the Susceptibility of Mitochondria to Thyroxine-Induced Swelling.**—The action of the respiratory carriers, which are embedded in the membranes and/or cristae (37–39), appears to be a determinant of the responsiveness of the mitochondria to the swelling action of thyroxine. Lehninger and Ray (40) have demonstrated that thyroxine does not cause swelling of rat liver mitochondria in the presence of cyanide or under anaerobic conditions; such conditions also prevent the action of other swelling agents (40, 41). It was suggested that swelling induced by thyroxine and other agents does not occur when the respiratory carriers are maintained in the reduced state (40). Experiments in Fig. 6 show that thyroxine-induced swelling is also prevented in the presence of excess β-hydroxybutyrate as chain reductant, by antimycin A, which blocks respiration between cytochromes b and c (42) and also by sodium amytal, which blocks respiration between DPN and flavoprotein (42, 43). The mitochondrial DPN is in the fully reduced form in the presence of each of the three respiratory inhibitors when substrate is in excess, whereas the oxidation-reduction state of the other carriers varies depending on the inhibitor used. These circumstances are consistent with the hypothesis that bound DPN is the site of action of thyroxine and that the mitochondria are susceptible to thyroxine-induced swelling only when at least some of the DPN is in the oxidized state; the oxidation-reduction state of the other carriers appears to be irrelevant to the action of thyroxine.

An alternative explanation is that thyroxine-induced or spontaneous swelling can occur only if respiration is proceeding so that any respiratory inhibitor, regardless of site of action, would result in prevention of swelling (44). However, other observations on the relation of respiration to swelling and its reversal (16, 17) are not all easily explained on this basis, nor are the findings on glutathione-induced swelling (18). Whatever the basis for inhibition of swelling by agents which block the respiratory chain, it is clear that the oxidation-reduction state of the respiratory carriers other than DPN is not a factor in sensitivity to thyroxine-induced swelling.

**"Passive" or Osmotic Reversal of Thyroxine-Induced Swelling.**—Experiments were carried out to determine whether thyroxine-induced swelling could be reversed by introduction of high concentrations of solutes such as sucrose, serum albumin, and PVP into the test medium in order to increase the extramitochondrial osmotic pressure. When thyroxine-induced swelling was first allowed to take place in the normal medium of 0.3 μ sucrose and an addition of concentrated sucrose solution then made to the system to bring the concentration...
Effects of hypertonic sucrose and PVP in reversal of swelling. Tests carried out at 20° and pH 7.4. Top curve (in left figure) shows that 0.87 M sucrose completely prevents swelling induced by $1 \times 10^{-5}$ M thyroxine. The bottom curve (triangles) shows the usual swelling produced by $1 \times 10^{-4}$ M thyroxine in 0.3 M sucrose medium. At 5 minutes, sucrose concentration in duplicate tube was raised to 0.87 M; the dotted line shows that delayed addition neither stopped nor reversed the thyroxine-induced swelling.

In experiments shown in right figure, effect of PVP (7.7 per cent final concentration, in standard sucrose-tris medium) on swelling is shown. Top curve shows prevention of swelling induced by $1 \times 10^{-4}$ M thyroxine by PVP added at zero time. Curve labelled T4 (triangles) shows normal swelling, in absence of PVP. When PVP was added to duplicate tube at 10 minutes after T4-induced swelling began, the absorbancy increased as shown in dotted line.

To 0.87 M (a concentration which can prevent thyroxine-induced swelling when added initially (see Fig. 1)), it was found, as shown in Fig. 7, that such a delayed addition of a high sucrose concentration after swelling had begun had absolutely no retarding or reversing effect on the swelling. From such experiments it may be concluded that the permeability of the mitochondrial membrane (s) to sucrose has undergone a change following thyroxine-induced swelling, so that sucrose permeates more readily following swelling, allowing no significant osmotic pressure gradient to be set up by increasing external sucrose concentration. Also, it appears probable that a small amount of swelling must first occur before thyroxine can become effective in altering the permeability to sucrose, otherwise it could be expected that thyroxine would cause an immediate swelling even in the presence of 0.87 M sucrose. The definite lag period (Fig. 3) supports this explanation.

On the other hand, it was possible to reverse thyroxine-induced mitochondrial swelling almost completely by addition of a solute of much higher particle weight, namely polyvinylpyrrolidone (particle weight 60,000 to 70,000). Experiments in Fig. 7 show that PVP not only completely prevents swelling by thyroxine but also can reverse it almost completely after it has nearly reached the terminal absorbancy plateau. Although permeability to sucrose has increased strikingly following thyroxine-induced swelling, the mitochondrial membranes apparently remain relatively impermeable to PVP and thus permit maintenance of a high osmotic pressure gradient when PVP is added to the medium.

"Active" Reversal of Thyroxine-Induced Swelling.—Although Dickens and Salmony (13) have reported the reversal of thyroxine-induced swelling by simple addition of ATP to a sucrose test medium similar to that used in this study, in our hands ATP or the combination of ATP and Mg++ (or Mn++) did not prove to be capable of reversing the swelling action of thyroxine on rat liver mitochondria in the sucrose system. A variety of ATP and thyroxine samples were employed; also, ATP was added at different points in the swelling curve. Mitochondria from Sprague-Dawley, Wistar, and mongrel white rats were tested, without any difference in action. This failure suggested that reversal of thyroxine-induced swelling by ATP is dependent on factors not yet fully understood.

On further investigation it was found that when a medium of 0.15 M-0.02 M tris pH 7.4 was used instead of the standard 0.3 M sucrose-0.02 M tris, then reversal of thyroxine-induced swelling by ATP could be readily and reproducibly observed.
as is shown by the experiments in Fig. 8, which compare the effect of ATP in sucrose and KCl media. When sucrose was added to the KCl medium, the reversing effect of ATP was inhibited.

Further experiments showed that neither EDTA nor ADP were able to replace ATP in reversing thyroxine-induced swelling, indicating that ATP is acting specifically, possibly as a phosphate donor, rather than as a non-specific metal chelating agent. Neither Mn++ nor Mg++ could reverse swelling added alone, nor did they improve the reversal by ATP.

There appears to be no specific requirement for high concentrations of K+ per se in the reversal of swelling by ATP, since reversal also occurred readily in a medium of 0.15 M NaCl-0.02 M tris pH 7.4.

**DISCUSSION**

Existence of Different Types and Stages of Mitochondrial Swelling.—It is clear that mitochondria may undergo different types and stages of swelling, depending on their initial chemical and morphological condition and the nature of the chemical stimulus, and it is probable that these various modalities of swelling result in different morphological configurations. Thyroxine-induced swelling is very similar in a number of characteristics to the spontaneous swelling process, but appears to be quite different from swelling induced by glutathione and cysteine (18) and also from that produced by β-chloromercuribenzoate (unpublished observations). Also to be distinguished is the simple osmotic swelling in response to variations in the external osmotic pressure which occurs almost instantaneously following exposure to osmotic gradients, such as has been described by Tedeschi and Harris (21). Simple osmotic swelling and shrinking take place in the first seconds or minutes of exposure (21) and preceede and fuse into the slower, presumably non-osmotic phase of swelling as observed in photometric experiments such as those described here (cf. reference 21). Furthermore it must be pointed out that thyroxine-induced swelling may be observed in mitochondria that are already swollen and in which disorganization of the cristae is evident, as in mitochondria prepared in 0.25 M sucrose (25-28). On the other hand, mitochondria which have undergone swelling in the presence of phosphate, do not respond further in the presence of thyroxine, presumably because they have already lost DPN (35), which experiments described in this paper indicate to be the sensitive component with which thyroxine may interact.

The existence of different types and stages of mitochondrial swelling is also indicated by the fact that different contraction or shrinking responses may be observed, following addition of "active" agents such as ATP, or by increasing osmotic pressure of the external medium through introduction of solute molecules of differing particle weight. Thus thyroxine-induced swelling can be reversed with ATP in a saline medium, but glutathione-induced swelling cannot (18). Thyroxine-induced swelling can be reversed osmotically by PVP but not by sucrose, whereas neither solute can reverse glutathione-induced swelling (18).

The complex structure of the mitochondrion, with its outer and inner membranes and the characteristic arrangement of cristae (45) could obviously permit different types and stages of mitochondrial swelling to occur, particularly if there is internal compartimentation of the mitochondrion and if the permeability of the outer and inner membranes may differ. The membranes contain the enzymes of electron transport and coupled phosphorylation in ordered arrangement and it now appears probable that the permeability and contractility of the membranes is conditioned by the activity of these enzyme systems (9, 11, 16). Since respiration and phosphorylation are very complex and have a number of control points (cf. reference 46), a variety of chemical and morphological responses can be expected when the mitochondria are exposed to different agents.

Any attempt to analyze in morphological and physical terms the swelling action of thyroxine on mitochondria must be qualified by the fact that many of the swelling experiments have been carried out with already swollen mitochondria placed in an unphysiological medium containing a solute, sucrose, which is in all probability inhibitory and unfavorable, as pointed out below. Furthermore, the recent work of Amoore and Bartley indicates that mitochondria as ordinarily prepared contain at least two general types of structure, the aqueous phases of which are differently penetrated by sucrose, KCl, and phosphate (47). This fact provides little assurance of a mitochondrial population which is homogeneous with respect to compartmentation and permeability.

Nevertheless it appears possible to correlate these findings with the important studies of
of the mitochondrial membrane, in which respira-
tion and phosphorylation occur. Earlier work has
already demonstrated that sucrose inhibits respiration
(24), uncouples oxidative phosphorylation
(5), inhibits the ATP-Pi exchange reaction, and
also the ATPase activity in subfragments of
the mitochondrial membranes (48). The site of in-
hibition of sucrose in the coupling mechanism has
been tentatively identified as being on a reaction
between the terminal transphosphorylation (49)
and the site of DNP action, presumably the point
where inorganic phosphate is taken up (46). These
findings suggest that the apparent suitability of
sucrose for isolation of mitochondria with preserva-
tion of their morphology is not primarily oc-
casioned by its osmotic effects, but is the result of
a reversible inhibition or "fixation" of an enzymic
step in the coupling of respiration with membrane
contractility and permeability. On the other hand,
it is quite evident that sucrose is an unfavorable
medium for studies of mitochondrial swelling and
its reversal.

Site of Action of Thyroxine.—A number of con-
siderations point to a bound form of mitochondrial
DPN as the site of action of thyroxine in causing
swelling. These may be enumerated: (1) Sensitivity
of liver mitochondria to thyroxine-induced swelling
correlates closely with their content of functional
DPN, as shown in the aging experiment (Fig. 5).
This correlation is closer than the correlation be-
 tween spontaneous swelling and intramitochon-
drial ATP (36), which falls to one-fifth its normal
value before spontaneous swelling ensues. There
is also no correlation with oxidative phosphoryla-
tion. (2) Phosphate-treated mitochondria, which
have lost DPN (35), no longer respond to thyrox-
ine. (3) Bound DPN is lost from thyroxine-
treated mitochondria (15), a finding which we have
confirmed in unpublished experiments. (4) PVP,
ATP, and Mg++
, which prevent thyroxine-induced
swelling, also prevent loss of bound DPN in the
presence of thyroxine (15). (5) Conditions in which
mitochondrial DPN is in the reduced state prevent
the swelling action of thyroxine, whereas the
oxidation-reduction state of the other respiratory
carriers appears to be irrelevant. (6) Thyroxine
and related thyroactive compounds are capable of
inhibiting a number of DPN-linked dehydro-
genases (50).

It has been postulated that specifically bound
forms of certain respiratory carriers are involved
in the coupling of phosphorylation to electron
transport (cf. references 6, 42). A bound form of
DPN reactive in phosphorylating respiration with ω-hydroxybutyric dehydrogenase appears to be present in phosphorylating submitochondrial fragments (51). Recently Purvis has demonstrated the occurrence of a "masked" or bound form of DPN in intact liver mitochondria, believed to be concerned in oxidative phosphorylation (52). A functional connection between a bound form of DPN and the condition of the mitochondrial membrane thus appears very likely.

Displacement or alteration of a bound form of DPN in the mitochondrial membrane by thyroxine may not suffice to explain massive uncoupling of oxidative phosphorylation at all three sites in the chain by very high and possibly toxic doses or concentrations of thyroxine (1, 2). It must be recalled, however, that mitochondria isolated from livers of animals made hyperthyroid by minimal deficiency in respiratory control by ADP (3). The effect of thyroxine-induced swelling of mitochondria on respiratory control by ADP is being investigated further.

Physiological Significance of Thyroxine-Induced Swelling.—Mitochondrial swelling is readily promoted by inorganic phosphate, reduced glutathione, Ca++, and thyroxine, and its analogs in concentrations in which they are present in the cell. On the other hand, the swelling induced by these agents, thyroxine included, can be prevented or opposed by ATP, Mg++, Mn++, and probably also the soluble cytoplasmic proteins, which may be presumed to behave like serum albumin and PVP. Actually the known intracellular factors opposing swelling appear to be ample to prevent completely the swelling caused by the intracellular concentrations of the known swelling agents. Although liver mitochondria were often observed to swell in vitro in the presence of $1 \times 10^{-8}$ M thyroxine, approximately the thyroxine concentration in the cell, this concentration, or even much higher concentrations of thyroxine would cause no overt swelling if mitochondria were placed in a medium resembling the intracellular fluid with respect to concentrations of protein, ATP, and Mg++. For this reason it appears necessary to postulate that the intracellular swelling and contractile factors are normally in a state of dynamic balance, with frank swelling appearing only in case of outright or long maintained imbalance of these factors.

Physiological Significance of Thyroxine-Induced Swelling.—The swelling of liver mitochondria induced by thyroxine in vitro has in vivo significance, since (a) mitochondria isolated from livers of hypo- and hyperthyroid rats differ in the expected directions with respect to the rate of spontaneous swelling in sucrose solutions (12), (b) microscopic examination of liver sections of hyperthyroid rats shows the mitochondria to be swollen (53), and (c) electron micrographs of hyperthyroid rat liver mitochondria show disorganization of fine structure (54).

Although mitochondrial swelling induced by thyroxine thus occurs in vitro, it is probably not a general consequence of the action of thyroxine on mitochondria of all cell types. Although liver and kidney mitochondria swell in response to thyroxine, those of skeletal and cardiac muscle show little or no response and those of spleen, brain, and testis none (55). However, respiration of skeletal and cardiac muscle is increased in hyperthyroidism, and uncoupling of phosphorylation in heart mitochondria is brought about by thyroxine either in vitro or in vivo (56-58). Swelling of mitochondria is therefore not a necessary first stage in the uncoupling of phosphorylation. It is conceivable and likely, however, that thyroxine interacts with the same general type of chemical receptors or "targets" in heart mitochondria as in liver mitochondria, to produce changes in the relationship of the respiratory chain enzymes to the ultrastructure and properties of the mitochondrial membrane, which are not necessarily detectable optically as swelling, but which can lead to significant alteration of rates of respiration and phosphorylation, as well as changes in permeability or contractility of the membranes or changes in function of the active transport systems in these bodies. However, the characteristic action of thyroxine in vitro must also be dictated by organ distribution and intracellular distribution of the hormone. The importance of this factor is shown by the fact that phloridzin mimics the swelling action of thyroxine in all respects when tested in vitro (59), but the symptoms following phloridzin administration to an intact animal are of course very different from those of hyperthyroidism.

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