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

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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

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by such agents as DNP, dicumarol, and gramicidin (5, 10).

A different experimental approach to the action of thyroxine was afforded by the finding that low concentrations of the hormone cause very great acceleration of the rate of passive swelling of mitochondria, measured by the optical method of Raaflaub (11), whereas DNP actually prevents thyroxine-induced swelling (7, 9, 12). The swelling action of thyroxine was briefly remarked on independently by Klemperer (4) and has since been confirmed by Dickens and Salmony (13), Beyer, Löw, and Ernster (14) and Emmelot and Bos (15). That the *in vitro* swelling action of thyroxine on isolated mitochondria may be relevant to the action of thyroxine *in vivo* was shown by the finding that mitochondria isolated from the livers of hyperthyroid rats have a much higher spontaneous swelling rate, and those from hypothyroid rats a much lower swelling rate than those from livers of euthyroid rats (12).

From such experiments it was postulated (7, 9) that thyroxine interacts with certain receptors in the mitochondrial membranes to produce configurational changes in their molecular organization. Uncoupling of phosphorylation by thyroxine may therefore be a result of "dislocation" of active centers of the respiratory or coupling enzymes in the membrane fabric. Such a disturbance in the organization of the membranes could also be expected to produce changes in the dynamic steady state configuration of the mitochondrion, in which passive swelling is counterbalanced by active contraction (16, 17). Thus, in addition to producing changes in respiration and phosphorylation, thyroxine may also cause changes in permeability and active binding or accumulation of substrates, phosphate, nucleotides, and simple electrolytes (9).

The swelling action of thyroxine and other agents on rat liver mitochondria has been surveyed in an earlier publication from this laboratory (12). This paper presents a more detailed study of some factors concerned in the thyroxine-induced swelling of rat liver mitochondria and its reversal by ATP. These experiments represent an approach to identification of the molecular "targets" in the mitochondrial structure with which thyroxine reacts and also show that thyroxine-induced swelling is fundamentally similar to spontaneous swelling. An accompanying paper (18) shows that 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. 0.3 M sucrose–0.02 M tris (hydroxymethyl)-aminomethane-HCl (tris) buffer, pH 7.4, made up in 15 × 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 mμ 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 mμ 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 (11, 16, 17, 20, 21). 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 lysis to smaller particles, or 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 1×10^{-3} 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-

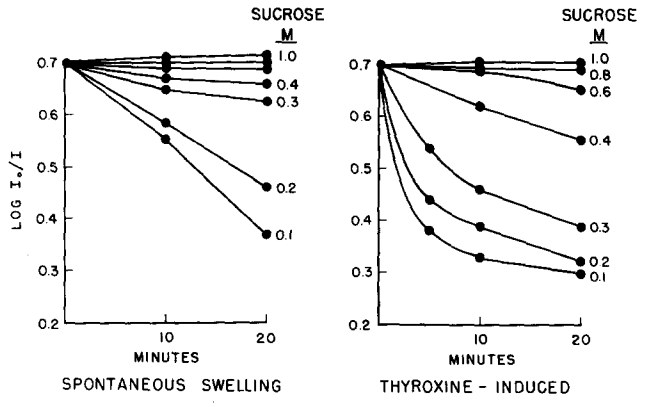

FIG. 1. Effect of sucrose concentration on spontaneous and thyroxine-induced swelling. Medium was 0.02 M tris pH 7.4 and sucrose concentrations shown. L-Thyroxine was 1×10^{-5} M; temperature, 20°.

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

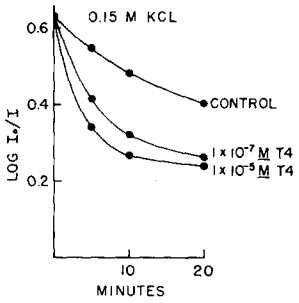

FIG. 2. Effect of KCl on thyroxine-induced swelling. The test medium was 0.15 M KCl–0.02 M tris pH 7.4. Temperature, 20°.

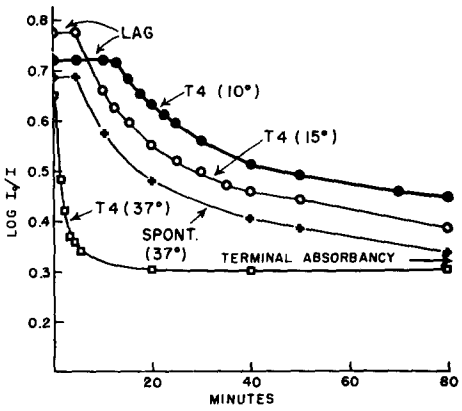

FIG. 3. Kinetics of spontaneous and thyroxine-induced swelling. Standard test system of 5.0 ml. 0.3 M sucrose–0.02 M tris pH 7.4 plus 50 mg. tissue equivalent mitochondria at temperatures shown. L-thyroxine was added in concentration of 1.0×10^{-5} M.

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 50 mg. tissue equivalent of rat liver mitochondria and incubations carried out at temperatures noted, with frequent readings of light absorbancy at 520 mμ. Swelling rate expressed as reciprocal of "quarter-time" of swelling.

Thyroxine-induced swelling

T	$1/t_{\frac{1}{4}}$	Q_{10}
°C	*min.*$^{-1}$	
0	0.013	—
10	0.045	3.5
15	0.10	4.8
20	0.27	6.0
25	0.50	5.0
30	0.83	3.1
35	1.40	2.8

Spontaneous swelling

T	$1/t_{\frac{1}{4}}$	Q_{10}
°C	*min.*$^{-1}$	
10	0.0045	—
20	0.022	4.8
30	0.066	3.0
40	0.250	3.8

to use the kinetic data on rates of swelling for calculation of temperature coefficients (Q_{10}) 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," $t_{1/4}$). The calculated temperature coefficients (Q_{10}) are given for adjacent temperature intervals. It is seen that Q_{10} 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 Q_{10} 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 Q_{10} of the thyroxine-induced swelling is somewhat larger than that of spontaneous swelling. However the process measured by the aborbancy 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

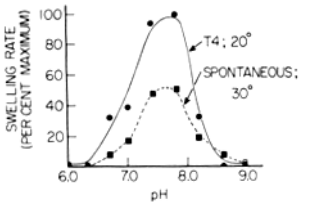

FIG. 4. Effect of pH on thyroxine-induced and spontaneous swelling of rat liver mitochondria. Standard test system contained 0.3 M sucrose, 0.02 M tris, and 0.02 M histidine, brought to the pH values shown; 50 mg. tissue equivalent rat liver mitochondria were added. Thyroxine was added at 1×10^{-5} M and the rate of swelling at 20° observed. The spontaneous swelling rate was measured at 30°.

is about 100 times the concentration of "thyroxine" in the blood and tissues of euthyroid animals, but considerably lower than that usually required ($>2 \times 10^{-5}$ 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^{-8} 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 D-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 D-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 maximal 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 D-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

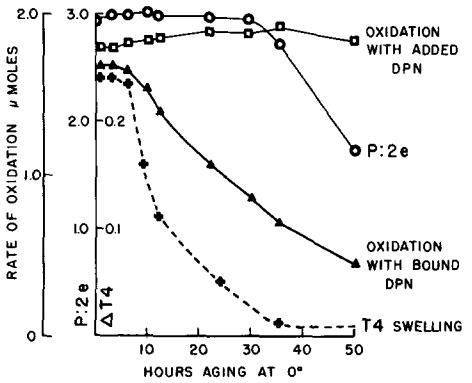

FIG. 5. Relationship of bound DPN to thyroxine-sensitivity. Freshly prepared mitochondria were suspended in 0.25 M sucrose (1 ml. contained 1 gm. tissue equivalent), and held at 0°. At different time intervals samples were removed and tested in the following systems:

a. Thyroxine-Induced Swelling.—Standard test system of 0.3 M sucrose–0.02 M tris pH 7.4 at 20°, in absence and presence of 1×10^{-5} M L-thyroxine. The reaction was started by adding 0.05 ml. mitochondria. The sensitivity to thyroxine recorded is arbitrarily designated as the optical density increment at 520 mμ produced by thyroxine above the control swelling, in absence of thyroxine, observed at the 5.0 minute interval.

b. Oxidation via "Bound" DPN.—The mitochondrial sample, taken from the stock suspension undergoing aging, and equivalent to 300 mg. fresh liver, was added at zero time to a test system containing 0.02 M DL-β-hydroxybutyrate, 0.0024 M ADP, 0.025 M phosphate labelled with P^{32}, 0.005 M $MgCl_2$, 0.001 M EDTA, 0.025 M glucose, and 5.0 mg. purified hexokinase (Sigma). The reaction was stopped, after 20 minutes incubation at 20°, with trichloroacetic acid and the acetoacetate formed and phosphate taken up determined as described previously (11). Rate of oxidation given in terms of μmoles acetoacetate formed in above test system in 20 minutes.

c. Efficiency of Oxidative Phosphorylation.—The P:2e ratios were calculated from data collected under (*b*).

d. Oxidation in Presence of Excess DPN.—The basic test system described under (*b*) was supplemented with 0.003 M DPN and the rate of oxidation expressed in the same terms.

test system was supplemented with "external" DPN, the rate of oxidation of D-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 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.

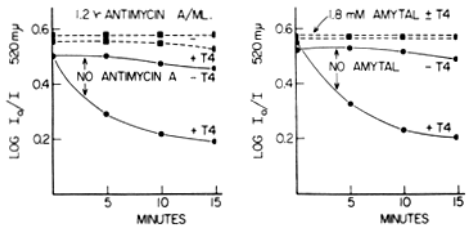

FIG. 6. Action of antimycin A and amytal on thyroxine-induced swelling. Standard test system of 5.0 ml. 0.3 M sucrose–0.02 M tris pH 7.4 at 20°. Thyroxine was added at 1×10^{-5} M. Antimycin A added as freshly prepared solution in 50 per cent ethanol, yielding final concentration of 0.12 micrograms antimycin A per ml. Sodium amytal was added as a freshly prepared 0.18 M solution in 50 per cent ethanol, yielding a final concentration of 0.0018 M sodium amytal and 0.5 per cent ethanol. Antimycin and amytal tubes also contained 0.001 M D-β-hydroxybutyrate to ensure reduction of carriers to point of inhibition. All tubes contained 50 mg. tissue equivalent of rat liver mitochondria, added last.

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 M sucrose and an addition of concentrated sucrose solution then made to the system to bring the concentration

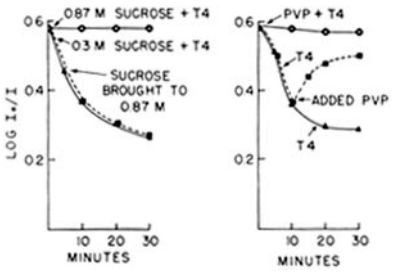

FIG. 7. Effects of hypertonic sucrose and PVP in reversal of swelling. Tests carried out at 20° and pH 7.4. Top curve (in left figure) shows that 0.87 M sucrose completely prevents swelling induced by 1×10^{-5} M thyroxine. The bottom curve (triangles) shows the usual swelling produced by 1×10^{-5} M 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 1×10^{-5} 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,

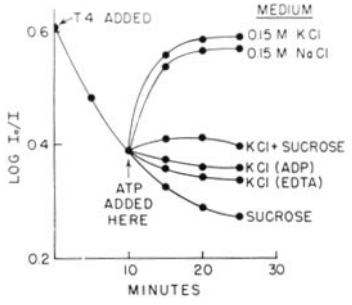

FIG. 8. Reversal of thyroxine-induced swelling by ATP in KCl medium. Thyroxine (1×10^{-5} M) was added at zero time to tubes containing medium of 0.30 M sucrose–0.02 M tris pH 7.4 or 0.15 M KCl–0.02 M tris pH 7.4 as shown; 0.02 M sucrose was added to 0.15 M KCl–0.02 M tris medium as shown. At time shown, 0.003 M ATP (or 0.003 M ADP or 0.001 M EDTA) was added in small volume to each tube. Data normalized for volume changes. Temperature, 20°.

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 *p*-chloromercuribenzoate (unpublished observations). Also to be distingushed 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 precede 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 compartmentation 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

Tedeschi and Harris (21) and of Bartley and his colleagues (32, 47). The studies of Tedeschi and Harris indicate the mitochondrion to be surrounded by a semipermeable membrane and that the mitochondria, over very short time intervals, behave as ideal osmometers if a large "osmotic dead space" of some 40 to 50 per cent of the mitochondrial volume is assumed (21). Werkheiser and Bartley have found, however, that mitochondria isolated from 0.25 M sucrose are already about 60 per cent penetrated by sucrose, and this value is constant enough to suggest the occurrence of an internal compartment which is not readily penetrated by sucrose at 0° and an outer compartment which is readily penetrated (32). On longer exposure to sucrose solutions at higher temperatures, more nearly approximating those used in this study, sucrose is able to penetrate the inner space (31, 32, 47). From these considerations it may be suggested that the early, rapid, purely osmotic changes observed by Tedeschi and Harris may involve temporary osmotic gradients across the inner membrane and the slower phase of swelling, in which the inner space is penetrated by sucrose, may be equivalent to the changes studied in the type of experiment carried out in this study. Furthermore, it may be suggested that the permeability of the inner membrane to sucrose may be increased during swelling, particularly in the presence of thyroxine, since high osmotic gradients of sucrose will not reverse thyroxine-induced swelling although large solute molecules such as PVP will. The reversal of swelling by ATP may be the result of a contractile process as has been postulated (11, 16), possibly with restoration of the relative impermeability of the inner membrane to sucrose.

This picture of the swelling process suggests that the outer membrane is of less significance than the inner membrane and is consistent with the view of Palade that an inner compartment and the cristae are formed by invagination of the inner membrane and that the respiratory enzymes may be largely present in the inner membrane, particularly the cristae (45).

Sucrose as Suspending Medium.—The finding that sucrose can inhibit swelling and also inhibits the reversal of swelling by ATP may be the consequence of the inhibition by sucrose of some intermediate reaction in the coupling of phosphorylation to electron transport and thus the contractility of the mitochondrial membrane, in which respiration and phosphorylation occur. Earlier work has already demonstrated that sucrose inhibits respiration (24), uncouples oxidative phosphorylation (5), inhibits the ATP-P_i^{32} exchange reaction, and also the ATPase activity in subfragments of the mitochondrial membranes (48). The site of inhibition 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 preservation of their morphology is not primarily occasioned 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 considerations 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 between spontaneous swelling and intramitochondrial ATP (36), which falls to one-fifth its normal value before spontaneous swelling ensues. There is also no correlation with oxidative phosphorylation. (2) Phosphate-treated mitochondria, which have lost DPN (35), no longer respond to thyroxine. (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 dehydrogenases (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 D-β-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 doses of thyroxine do not always show lowered P:O ratios; however such mitochondria do show deficiency in respiratory control by ADP (3). The effect of thyroxine-induced swelling of mitochondria on respiratory control by ADP is being investigated further.

The Intracellular Environment and Mitochondrial 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 1×10^{-8} M thyroxine, approximately the tyroxine 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 unbalance 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 vivo*, 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 vivo* 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.

Bibliography

1. Maley, G. F., and Lardy, H. A., *J. Biol. Chem.*, 1955, **215,** 377; 1954, **204,** 435.
2. Martius, C., and Hess, B., *Biochem. Z.*, 1955, **326,** 191.
3. Hoch, F. L., and Lipmann, F., *Proc. Nat. Acad. Sc.*, 1955, **40,** 909.
4. Klemperer, H., *Biochem. J.*, 1955, **60,** 122.

5. Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, 1956, **219,** 489.
6. Lehninger, A. L., Wadkins, C. L., Cooper, C., Devlin, T. M., and Gamble, J. L., Jr., *Science*, 1958, **128,** 450.
7. Tapley, D. F., Cooper, C., and Lehninger, A. L., *Biochim. et Biophysica Acta*, 1955, **18,** 597.
8. Tapley, D. F., and Cooper, C., *J. Biol. Chem.*, 1956, **222,** 341.
9. Lehninger, A. L., *in*, Enzymes: Units of Biological Structure and Function, (O. H. Gaebler, editor), New York, Academic Press, Inc., 1956, 217.
10. MacMurray, W. C., Maley, G. F., and Lardy, H. A., *J. Biol. Chem.*, 1958, **230,** 219.
11. Raaflaub, J., *Helv. physiol. et pharmacol. Acta*, 1953, **11,** 142, 157.
12. Tapley, D. F., *J. Biol. Chem.*, 1956, **222,** 325.
13. Dickens, F., and Salmony, D., *Biochem. J.*, 1956, **64,** 645.
14. Beyer, R. E., Löw, H., and Ernster, L., *Acta Chem. Scand.*, 1956, **10,** 1039.
15. Emmelot, P., and Bos, C. J., *Exp. Cell Research*, 1957, **12,** 191; 1958, **14,** 132.
16. Price, C. A., Fonnesu, A., and Davies, R. E., *Biochem. J.*, 1956, **64,** 754. Fonnesu, A., and Davies, R. E., *Biochem. J.*, 1956, **64,** 769.
17. Ernster, L., and Lindberg, O., *Ann. Rev. Physiol.*, 1958, **20,** 13.
18. Lehninger, A. L., and Schneider, M. S., *J. Biophysic. and Biochem. Cytol.* **5,** 1959, 109.
19. Schneider, W. C., *J. Biol. Chem.*, 1948, **176,** 259.
20. Witter, R. F., and Cottone, M. A., *Biochim. et Biophysica Acta*, 1956, **22,** 364.
21. Tedeschi, H., and Harris, D. L., *Arch. Biochem. and Biophysics*, 1955, **58,** 52; *Biochim. et Biophysica Acta*, 1958, **28,** 392.
22. Hogeboom, G. H., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, 1948, **172,** 619.
23. Lehninger, A. L., and Kennedy, E. P., *J. Biol. Chem.*, 1948, **173,** 753.
24. Johnson, D., and Lardy, H. A., *Nature*, 1958, **181,** 701.
25. Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S., and Cottone, M. A., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 139.
26. Novikoff, A. B., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 65.
27. Dalton, A. J., *Symp. Soc. Exp. Biol.*, Mitochondria and Other Cytoplasmic Inclusions, No. 10, New York, Academic Press, Inc., 1955, 148.
28. Birbeck, M. S. C., and Reid, E., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 609.
29. Stanbury, S. W., and Mudge, H. G., *Proc. Soc. Exp. Biol. and Med.*, 1953, **82,** 675.
30. Davies, R. E., *Symp. Soc. Exp. Biol.*, Active Transport and Secretion, No. 8, New York, Academic Press, Inc., 1954, 453.
31. Jackson, K. L., and Pace, N., *J. Gen. Physiol.*, 1956, **40,** 47.
32. Werkheiser, W. C., and Bartley, W., *Biochem. J.*, 1957, **66,** 79.
33. Gamble, J. L., Jr., *J. Biol. Chem.*, 1957, **228,** 955.
34. Crane, R. K., Field, R. A., Cori, C. F., and Roberts, M. L., *J. Biol. Chem.*, 1957, **224,** 649.
35. Hunter, F. E., Jr., and Ford, L., *J. Biol. Chem.*, 1955, **216,** 357.
36. Brenner-Holzach, O., and Raaflaub, J., *Helv. physiol. et pharmacol. Acta*, 1954, **12,** 242.
37. Watson, M. L., and Siekevitz, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 379.
38. Ball, E. G., and Barrnett, R. J., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 1023.
39. Lehninger, A. L., *in* Current Research in Molecular Biology, (R. E. Zirkle, editor), Chicago, University of Chicago Press, in press.
40. Lehninger, A. L., and Ray, B. L., *Biochim. et Biophysica Acta*, 1957, **26,** 643.
41. Hunter, F. E., Jr., Davis, J., and Carlat, L., *Biochim. et Biophysica Acta*, 1956, **20,** 237.
42. Chance, B., and Williams, G. R., *Advances Enzymol.*, 1956, **17,** 65.
43. Estabrook, R. W., *J. Biol. Chem.*, 1957, **227,** 1093.
44. Chappell, J. B., and Greville, G. D., *Nature*, 1958, **182,** 813.
45. Palade, G. E., *in* Enzymes: Units of Biological Structure and Function, (O. H. Gaebler, editor), New York, Academic Press, Inc., 1956, 185.
46. Lehninger, A. L., Remmert, L. F., and Wadkins, C. L., Ciba Symposium on Metabolic Control Mechanisms, July, 1958, in press.
47. Amoore, J. E., and Bartley, W., *Biochem. J.*, 1958, **69,** 223.
48. Cooper, C., and Lehninger, A. L., *J. Biol. Chem.*, 1957, **224,** 547.
49. Wadkins, C. L., and Lehninger, A. L., *J. Biol. Chem.*, in press.
50. Wolff, J., and Wolff, E. C., *Biochim. et Biophysica Acta*, 1957, **26,** 389.
51. Gamble, J. L., Jr., and Lehninger, A. L., *J. Biol. Chem.*, 1956, **223,** 921.
52. Purvis, J. L., *Nature*, 1958, **182,** 711.
53. Aebi, H., and Abelin, I., *Biochem. Z.*, 1953, **324,** 364.
54. Schulz, H., Löw, H., Ernster, L., and Sjöstrand, F. S., Electron Microscopy, New York, Academic Press, Inc., 1957, 134.
55. Tapley, D. F., and Cooper, C., *Nature*, 1956, **178,** 1119.
56. Vitale, J. J., Hegsted, D. M., Nakamura, M., and Connors, P., *J. Biol. Chem.*, 1957, **226,** 597.
57. Vitale, J. J., Nakamura, M., and Hegsted, D. M., *J. Biol. Chem.*, 1957, **228,** 573.
58. Wiswell, J. G., and Braverman, M., *Endocrinology*, 1957, **61,** 153.
59. Lehninger, A. L., and Schneider, M., *Z. physiol. Chem.*, in press.