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# Thyroid Status Influences Calcium Ion Accumulation and Retention by Rat Liver Mitochondria (42777)

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*Abstract.* $Ca^{2+}$ accumulation and retention by isolated rat liver mitochondria (RLM), measured with a $Ca^{2+}$ electrode as $a_{Ca}$, are markedly influenced by thyroid status. RLM from propylthiouracil (PTU)-fed rats took up $Ca^{2+}$ from a suspending medium (SM) until 10.26 ± 2.51 (SD) × $10^{-5}$ *M* $Ca^{2+}$ had been added ($n$ = 5). RLM from PTU rats given $T_3$ (100 μg/kg daily × 6) showed uptake only until 2.37 ± 0.59 × $10^{-5}$ *M* $Ca^{2+}$ had been added ($n$ = 6) and RLM from normal rats showed uptake until 3.69 ± 0.53 × $10^{-5}$ *M* ($n$ = 9) $Ca^{2+}$ was reached. RLM from the three animal groups lowered the $a_{Ca}$ in the SM from 9.13 ± 1.69 × $10^{-6}$ to 4.96 ± 2.08 × $10^{-6}$ *M* regardless of hormonal status. The time in minutes that the $a_{Ca}$ remained below the initial level in the rat groups was PTU 94.8 ± 26.2, PTU + $T_3$, 11.5 ± 3.9, and normal 26.7 ± 3.8. All differences were significant at the 0.001 level (ANOVA). bGH did not affect $Ca^{2+}$ handling by RLM from PTU rats. Administered $T_3$ increased RLM α-glycerophosphate dehydrogenase activity 24–36 hr before normalizing $Ca^{2+}$ handling. The thyroid hormone-sensitive system described here adjusts the SM $Ca^{2+}$ concentration to a level far above cytosolic so that its function may be to regulate intramitochondrial [$Ca^{2+}$]. The level of intramitochondrial $Ca^{2+}$ may be of importance in the mechanism of action of thyroid hormone. 

The tissue responses to a variety of hormonal and other stimuli involve extracellular and cytosolic ionized calcium ($Ca^{2+}$) (1). One of the intracellular targets for the action of thyroid hormones is the mitochondrion (2), and $T_3$ administered to hypothyroid rats accelerates efflux of $Ca^{2+}$ from isolated liver mitochondria incubated with inhibitors of part of the electron transport chain (3). Here it is shown that in an inhibitor-free medium resembling cytosol, the thyroid status of the rat also has a profound effect on $Ca^{2+}$ handling by isolated liver mitochondria. Mitochondria from hypothyroid animals show a markedly prolonged retention of the traces of $Ca^{2+}$ found in the medium and an increased capacity to take up added $Ca^{2+}$. Portions of this work have been presented briefly elsewhere (4).

**Methods.** Male Charles River CD rats weighing 50 g when received were kept up to 14 months on a diet of powdered Purina Chows containing 0.1% propylthiouracil (PTU), with unlimited access to water. Serum $T_4$ levels of PTU rats were all below 10 ng/ml (normal above 40 ng/ml). Normal rats were age-matched and fed unmodified Purina Chows. Unless otherwise stated, $T_3$, 100 μg/kg body wt was given to PTU-treated rats once daily ip for 6 days, and saline was given to PTU controls. Where indicated, bGH 1 IU was given daily for 5 days ip.

After an overnight fast, rats were decapitated, and trunk blood collected. Liver mitochondria were isolated in 0.25 *M* sucrose with two washes of the resuspended pellet. Thyroids were dissected and weighed during mitochondrial isolation. State 4 oxygen consumption and respiratory control ratios (RCR) were measured at 25°C with a Clark-type oxygen electrode (Yellow Springs Instruments). The medium (Solution A) contained (in m*M*) 125 sucrose, 60 KCl, 3 Hepes, 1 $KH_2PO_4$, 0.5 EGTA, 5 succinate, and 4 μ*M* rotenone, pH 7.4. Aliquots of $Na_2$ ADP (0.25 μmole) were added to the medium, which contained 1.5 mg mitochondrial protein per milliliter, and all RCR exceeded 3.0 (5).

$Ca^{2+}$ ion activity ($a_{Ca}$) was measured at 25°C in Solution B containing (in m*M*) 115 KCl, 25 Hepes, 5 $KH_2PO_4$, 1 $MgCl_2$, 5 NaCl, 5 glutamate, 5 malate, and 0.125 ATP at pH 7.0. A commercially available calcium electrode (Ionetics or W.P.I. Corp.) was immersed in the solution together with a salt bridge leading to a calomel electrode as reference. The electrodes were connected to a



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Beckman Model 76A Expandomatic pH meter the output of which was recorded on one channel of a Grass Model 5 Polygraph with 5Pl preamplifier. Before adding 1 mg mitochondrial protein/milliliter solution, $CaCl_2$ was added to a separate aliquot of solution increasing the $[Ca^{2+}]$ by steps of $5 \times 10^{-6}$ *M* and assuring a linear response to changes in $pCa^{2+}$. In medium B the electrodes are capable of detecting $2 \times 10^{-7}$ *M* added $Ca^{2+}$ and show a linear response to log added $Ca^{2+}$ above $6 \times 10^{-7}$ *M*. The $[Ca^{2+}]$ contaminating the reagents in the solution prior to mitochondrial addition and remaining in the supernatant fluid after removal of mitochondria by centrifugation was measured with the Quin 2 method (6).

α-Glycerophosphate dehydrogenase activity was expressed as $\Delta A$ per minute per milligram protein at 37°C (7). Total mitochondrial calcium was measured by atomic absorption after extraction in the solution of Tew (8), and protein was determined by the Lowry technique using BSA standards (9).

Sucrose, oligomycin, antimycin, α-glycerophosphate, BSA, $Na_2$ ATP, $Na_2$ ADP, and ruthenium red were purchased from Sigma, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) from Aldrich. bGH was a gift from Dr. M. Sonenberg.

The Quin 2 measurements were made in the laboratory of Dr. M. Gershengorn, and the $T_4$ levels determined in the laboratory of Dr. J. Hurley.

**Results.** Table I shows the effects of thyroid status on body weights, thyroid weights, total mitochondrial calcium content, State 4 mitochondrial oxygen consumption, and mitochondrial α-glycerophosphate dehydrogenase activity (EC 1.1.99.5). All measurements varied in the expected direction except for total mitochondrial calcium, which was unaffected.

Mitochondrial handling of $Ca^{2+}$ was tested in retention and loading types of experiments. After the output from the electrodes immersed in 1 ml of solution B (see Methods) had become constant, 1 mg of mitochondrial protein was added.

The $a_{Ca}$ dropped rapidly, and remained low for a variable period of time before returning to the initial level and above. The *retention time* is the time in minutes between mitochondrial addition and the return of the $a_{Ca}$ in the medium to the initial level. Figure 1 shows that the retention time of liver mito-

TABLE I. THE EFFECTS OF VARIATIONS IN HORMONAL STATUS OF THE RATS ON BODY WEIGHT, THYROID WEIGHT, AND PROPERTIES OF ISOLATED LIVER MITOCHONDRIA

| | | | Mitochondria | |
|---|---|---|---|---|
| Experimental group | Body weight (g) | mg Thyroid (weight/100 g[d]) | Total calcium[c] (nmole/mg protein) | State four[e] oxygen consumption (ng atoms/min/mg protein) | α-glycerophosphate dehydrogenase (ΔA/min/mg protein) |
| Normal (8)[a] | 410 ± 92[b] (5) | 4.3 ± 0.9 (10) | 15.8 ± 2.0 (11) | 28.8 ± 11.2 (5) | 0.0513 ± 0.0071 |
| PTU up to 14 months (6) | 124 ± 21 (5) | 41 ± 12 (5) | 12.9 ± 4.3 (6) | 22.1 ± 7.2 (5) | 0.00505 ± 0.00127 |
| PTU + $T_3$ 100 μg/kg × 6 (9) | 145 ± 35 (8) | 38 ± 27 (4) | 14.9 ± 0.8 (6) | 61.3 ± 15[f] (6) | 0.957 ± 0.278 |
| PTU + bGH 1 IU/day × 5 (2) | 122[g] (2) | 67 (2) | 14.2 (2) | 21.6 (2) | 0.0092 |

[a] Number of animals in parentheses.
[b] Standard deviation.
[c] Measured by atomic absorption.
[d] Body weight.
[e] The oxygen consumption after the first aliquot of added ADP has been phosphorylated (5).
[f] Differs from value for rats receiving PTU alone $P = <0.001$.
[g] Mean of two animals.

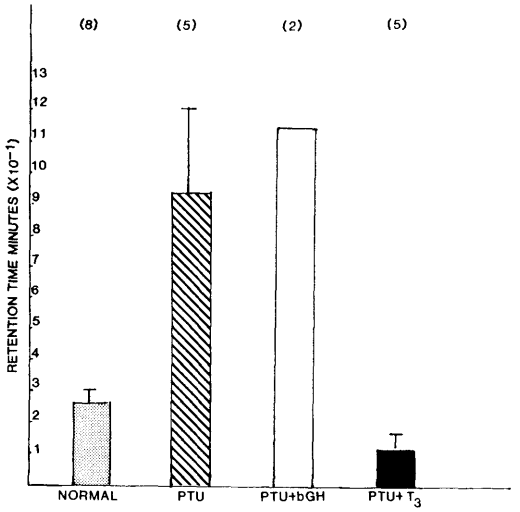


FIG. 1. The effect of thyroid status, noted below each bar, on rat liver mitochondrial $Ca^{2+}$ retention time. The $Ca^{2+}$ electrode trace is lowered by the addition of 1 mg mitochondrial protein to 1 ml solution B. The time in minutes $\times 10^{-1}$ for the return of the trace to the initial level is shown on the vertical axis. Parentheses enclose the number of rats in each group. The standard deviation of each mean is indicated by the line above each bar, and the means of the PTU group, the PTU + $T_3$ group, and the normal group all differ from each other at a $P$ level of <0.001 (ANOVA) (temperature 25°C).

chondria from hypothyroid animals is markedly prolonged. Administered $T_3$ produces a striking fall in retention time in PTU rats which does not appear to be secondary to the well-known $T_3$-induced increase in GH levels (10), since bGH administered to two animals did not reproduce the effect. The mean retention time for mitochondria from normal animals lies between the value for PTU rats treated with $T_3$ and rats given PTU alone.

The *loading* type of experiment also started with the addition of 1 mg mitochondrial protein to 1 ml of medium. When the $a_{Ca}$ had fallen to a constant low level, additions of 5 $\mu$l of 1 m$M$ $CaCl_2$ were made at 1 min intervals. After each addition the $a_{Ca}$ in the medium rose, but fell again as some of the $Ca^{2+}$ was taken up by the mitochondria. Additions were continued until a fall no longer occurred, as illustrated diagramatically in Fig. 2. The mean concentration of $Ca^{2+}$ added to the solution at the overload point is shown on the vertical axis of Fig. 3. Liver mitochondria from hypothyroid rats accumulated $Ca^{2+}$ from solutions containing far more $Ca^{2+}$ than those from normal animals, and $T_3$ administration led to a striking decrease in this property which also could not be attributed to the effect of GH (mean value for two animals given). Neither of these types of experiments depends upon knowing the exact concentration of $Ca^{2+}$ in the solution, which requires careful adjustment of the standards used for electrode calibration (11). The overload point correlated with retention time in each mitochondrial preparation regardless of hormonal status of the rat with a regression coefficient of 0.92 ($n$ = 21, $P < 0.001$). The control rats gained weight throughout the experimental period and an inverse correlation was seen between body weight and the time $Ca^{2+}$ was retained by their liver mitochondria ($r$ = 0.673, $P$ = <0.05, $n$ = 9).

The medium without added mitochondria contained 9.13 ± 1.69 (SD) $\mu M$ total calcium ($n$ = 7), and added mitochondria lowered the level to 4.96 ± 2.08 $\mu M$ ($n$ = 8). The level to which the mitochondria adjusted the medium could not be related to the thyroid status of the animal from which they were prepared. Duplicate determinations both of retention time and uptake of added $CaCl_2$ at overload agreed within 10%. $Ca^{2+}$ uptake was blocked by 5 $\mu M$ ruthenium red, and both retention time and uptake of added $Ca^{2+}$ were decreased by the omission of ATP and increased by the addition of either ATP or ADP to the medium. Oligomycin 15 $\mu M$ increased retention, and both antimycin 15 $\mu M$ and CCCP 10 $\mu M$ abolished it, showing that an intact electron transport system and ATP are important in this system. Added $T_3$ ranging in concentration from $10^{-11}$ to $10^{-7}$ $M$ did not affect the $Ca^{2+}$ overload point of mitochondria from normal or PTU treated rats. Sonication of control mitochondria for 20 min in a MSE 3000 tissue disintegrator completely abolished $Ca^{2+}$ uptake, as did overnight freezing at −20°C. Gentler disruption of mitochondria by two 5 min exposures to a Polytron Model PT 10 homogenizer reduced the RCR to between two and three. This procedure did not abolish mitochon-

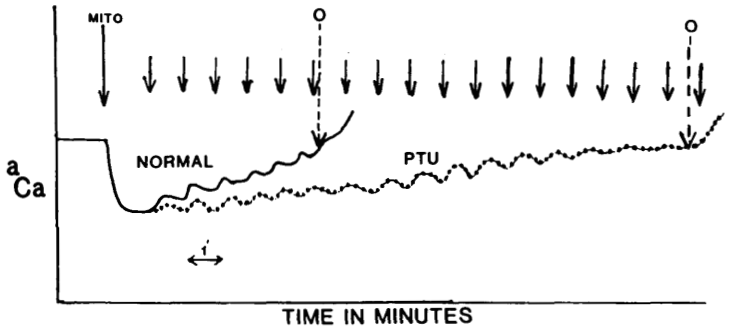


FIG. 2. Diagram of an accumulation experiment, in which the $Ca^{2+}$ electrode trace of data from liver mitochondria of a normal rat (broad solid line) is compared on the same chart to data from a PTU rat (dotted line). Vertical axis is calcium ion activity ($a_{Ca}$). Mitochondria are added at the first arrow, and after the trace has stabilized, 5 $\mu$l aliquots of 1 m$M$ $CaCl_2$ are added at the small arrows. In this illustrative example, 30 $\mu$l, 1 m$M$ $CaCl_2$ are added to bring the $a_{Ca}$ in the medium back to the initial level ($2.9 \times 10^{-5}$ $M$ concentration of added $Ca^{2+}$) in the preparation from the normal animal (first large arrow marked "O" indicating overload). To bring the medium back to the initial level in the case of the PTU preparation (second large arrow marked "O") the final concentration of added $Ca^{2+}$ was $7.8 \times 10^{-5}$ $M$. One milliliter of medium B, 1 mg mitochondrial protein, temperature 25°C.

drial $Ca^{2+}$ uptake but reduced both overload point and retention time.

The time interval between hormone administration and detectable mitochondrial calcium response was tested by giving a single injection of $T_3$, 2 mg/kg body wt, to PTU-treated rats. This large dose is sufficient to saturate hepatic nuclear $T_3$ receptors (12). Mitochondrial $\alpha$-glycerophosphate dehydrogenase activity, expressed as $\Delta A$ per minute per milliliter protein at 37°C, increased from the untreated hypothyroid value of 0.00505 $\pm$ 0.00127 SD ($n$ = 5) to 0.0794 $\pm$ 0.0363 ($n$ = 4, $P < 0.005$) 18–24 hr later. This 10-fold increase in enzyme activity occurred without change in retention time (117.4 $\pm$ 32 min) or overload point ($10.46 \pm 2.52 \times 10^{-5}$ $M$).

Injections of $T_3$, 100 $\mu$g/kg body wt, were given daily for 2 days to PTU-treated rats. Enzyme activity increased 150-fold to 0.392 24 hr after the second injection. Retention time remained elevated (70.6 min) and overload point fell only slightly to $6.5 \times 10^{-5}$ $M$. These observations, the mean results from two animals, show that the enzyme response precedes changes in mitochondrial $Ca^{2+}$ handling.

**Discussion.** The experiments reported here show that the thyroid status of the rat has a marked effect upon the ability of isolated liver mitochondria to accumulate $Ca^{2+}$ from solutions of increasing $Ca^{2+}$ concentration. In addition, mitochondria take up $Ca^{2+}$ from the suspending medium without added $Ca^{2+}$ and the time during which it is retained is also thyroid-hormone dependent. These

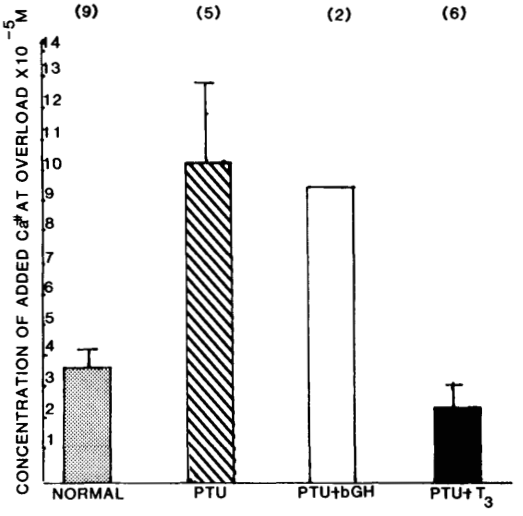


FIG. 3. Comparison of amount of $Ca^{2+}$ added to produce mitochondrial overload (final concentration of added $Ca^{2+}$ on vertical axis) for groups of animals with different thyroid status (horizontal axis). The standard deviation of each mean is shown by the line above the bar, and the means of the PTU, the PTU + $T_3$, and normal groups differ from each other at a $P$ level of <0.001 (ANOVA). Experiments done as outlined in the legend to Fig. 2.

two processes are so closely correlated in the same preparation that they may well represent different aspects of the same mitochondrial $Ca^{2+}$ transport system. No consistent thyroid hormone effects on net uptake of $Ca^{2+}$ were observed, hence efflux is probably the responsive process. $Ca^{2+}$ efflux was also the thyroid and growth hormone responsive process when uptake was blocked in earlier experiments under very different conditions (3). The failure to show a growth hormone response in the present report remains unexplained.

Liver mitochondria adjusted the suspending medium to a $Ca^{2+}$ concentration of $5 \times 10^{-6}$ *M* in these experiments, a high level which was independent of the hormone status of the rat. By contrast, others have observed that the cytosolic $Ca^{2+}$ concentration of *cells isolated* from the livers of hypothyroid rats is decreased to $1.7 \times 10^{-7}$ *M*, below the $2.7 \times 10^{-7}$ *M* levels found in the cytosol of cells isolated from normal or hormone-replaced animals. The same investigators described a rapid rise in the cytosolic $Ca^{2+}$ of isolated hepatocytes produced by administered vasopressin or catecholamines. This rapid hormone-responsive calcium-signaling, although also affected by thyroid status, adjusts the cytosol to well below the $5 \times 10^{-6}$*M* observed here and may be produced, at least in part, by nonmitochondrial structures (13).

Electron-probe X-ray microanalysis of liver cells shows the total Ca content of mitochondria *in situ* to be about 1.1 nmole/mg protein, and the $Ca^{2+}$ concentration in the matrix is estimated to be 0.3 $\mu M$ (14). The high total mitochondrial Ca content (nmole/mg protein) found in this and many other reports (13–16) point to accumulation during conventional isolation procedures. The thyroid hormone effects described here therefore occur in mitochondria with partially filled Ca pools. If they can also be shown in mitochondria with low total Ca content, the system described here may regulate *intramitochondrial* $Ca^{2+}$ concentration and be involved in activation of mitochondrial enzymes (15).

Swelling of rat liver mitochondria has been produced both by added and by administered thyroid hormone (16). Added $Ca^{2+}$ has been shown to regulate both intramitochondrial volume and metabolite content (17). Administered thyroid hormone has recently been reported to accelerate *t*-butylhydroperoxide-induced $Ca^{2+}$ release from liver mitochondria of hypophysectomized rats, and mitochondrial reduced glutathione (GSH) has been implicated (18). Further study of the state of Ca within mitochondria and of the many systems for $Ca^{2+}$ translocation may reveal a close relationship between mitochondrial $Ca^{2+}$ regulation and the late metabolic effects of thyroid hormone.


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