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# Ion Transport in Liver Mitochondria from Normal and Thyroxine-Treated Rats

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## Abstract

Liver mitochondria isolated from rats 24 h after a single subcutaneous injection of 8 mg thyroxine per kilogram body weight were compared with those isolated from control rats that received injections of isotonic saline at the same time. The mitochondria isolated from the thyroxine-treated rats show higher rates of energy-dependent $K^+$ and phosphate accumulation than those from control animals. It was also found that mitochondria from the hormone-treated animals required a larger addition of $Ca^{2+}$/mg mitochondrial protein in order to uncouple oxidative phosphorylation, and showed smaller tendency to swell *in vitro* under energizing conditions. The data obtained on ion movements support previous observations that the stimulation of the basal rate of mitochondrial respiration by thyroxine is associated with an increase in the transmembrane protonic electrochemical potential difference, and indicate that *in vivo* the hormone raises the intramitochondrial concentration of $K^+$ and phosphate.

## Introduction

In a recent paper [1] we confirmed earlier studies [2, 3] demonstrating that liver mitochondria isolated from rats which had been treated with thyroxine oxidized succinate at a rate of over 40% faster than euthyroid mitochondria. We also made measurements of the mitochondrial protonic electrochemical difference ($\Delta\mu H^+$) and found that in state 4 [4] this was 16 mV greater in organelles from thyroxine-treated rats than in those from controls. We

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proposed that the hormone-induced rise in $\Delta\mu H^+$ resulted in an increase in the ionic conductance of the respiratory membrane. This change enhanced state 4 oxidative rates and increased respiration-dependent ion uptake.

Our data [1] emphasized a fundamental difference between the *in vivo* action of thyroxine upon mitochondria and some of its *in vitro* effects. For example, when it is added to mitochondria *in vitro* the hormone can stimulate respiration by uncoupling oxidative phosphorylation from electron transfer [5], which will tend to diminish $\Delta\mu H^+$ [6]. In this communication we report evidence which has a bearing on significance of another *in vitro* effect of thyroxine, namely the stimulation of mitochondrial matrix swelling [7, 8]. The data presented below provide the first demonstration that following *in vivo* thyroxine administration, state 4 swelling is inhibited rather than stimulated. This result was particularly surprising since our previous report indicated that after a 15-min state 4 incubation, thyroxine-treated mitochondria contained more $K^+$ and phosphate than the controls. Other work in this laboratory [9, 10] confirmed early reports [11, 12] that state 4 matrix swelling is an osmotic response to ion uptake, and consequently we had expected thyroxine treatment to result in increased matrix swelling. One possible explanation for this apparent paradox is that the hormone treatment increases the proportion of mitochondrial ions adsorbed to membranes or held in some other form that does not contribute to the osmotically active contents of the matrix. Such a phenomenon could invalidate the earlier interpretation of our data because when we measured thyroxine-stimulated ion uptake we did not take account of any possible differences in $K^+$-binding between our two populations of mitochondria, and consequently we have now made estimates of $K^+$-binding.

Furthermore, significant quantities of phosphate can be accumulated by mitochondria *in vitro* in an energy-independent exchange with endogenous anions [13]. If thyroxine treatment were to enhance this exchange process, the hormonal stimulation of state 4 phosphate uptake [1] could be explained in this way rather than as a consequence of the elevation of $\Delta\mu H^+$. We report further phosphate-uptake data in this paper.

Our conclusions are that thyroxine does not significantly increase mitochondrial $K^+$ binding, nor the energy-independent phosphate uptake. Thus we confirm our original proposal that the differences in ion content are a consequence of the enhancement of $\Delta\mu H^+$ in state 4 by thyroxine. Moreover, we support this conclusion with evidence of additional consequences of the difference in $\Delta\mu H^+$ between our two populations of mitochondria in state 4. Finally, we discuss the possible significance of the thyroxine-mediated inhibition of state 4 mitochondrial swelling.

## Materials and Methods

For each experiment four male Wistar rats (200–250 g) were used: two were injected subcutaneously with 8 mg thyroxine[2] per kilogram body weight, and two received injections of isotonic saline. Injections were performed under light ether anesthesia 24 h before isolation of the mitochondria in 0.25 M sucrose by the method of Kielley and Kielley [14]. The final mitochondrial pellets from control and treated animals were each resuspended in 2 ml sucrose and following a protein assay [15] were usually diluted to a concentration of 95 mg protein/ml with more sucrose. This was occasionally supplemented with 0.1 $\mu$Ci/ml [$^{14}$C]-sucrose and 0.2 $\mu$Ci/ml $^3H_2O$ in order to determine matrix volume [16].

When determining matrix volume and ion content, 0.5 ml mitochondrial suspension was layered upon 0.5 ml silicon oil (Wacker Ltd., London, U.K.) which in turn was placed over 0.5 ml 2M $HClO_4$ in Eppendorf centrifuge tubes. The mitochondria separated into the acid during centrifugation at 10,000 $\times$ g in an Eppendorf microfuge. Efficient separation of the mitochondria requires that the specific gravity of the silicon oil be less than that of the mitochondria but more than that of the suspending medium. For separation of state 4 mitochondria the oil was a 2:1 (v/v) mixture of AP100:AR20 (specific gravity 1.04), and a 20-sec centrifugation was sufficient. Because the nonincubated mitochondria were contained in a denser medium than the incubated organelles, they had to be separated through oil at a higher density (AP100, specific gravity 1.07). However, the nonincubated mitochondria from thyroxine-treated rats were somewhat denser than the controls (see Table III) and were therefore separated most efficiently through a mixture of oil that was less dense [14:1 (v/v) AP100:AR20, specific gravity 1.066]. After centrifugation for 3 min there were no signs of any mitochondria in the original layer of the suspension medium.

$K^+$ was assayed by flame photometry [17], phosphate was measured using the technique of Murphy and Riley [18], while $Mg^{2+}$ and $Ca^{2+}$ were assayed by atomic absorption spectrometry with 60 mM $LaCl_3$ to reduce interference from phosphate. When present, radioisotopes (Radiochemical Centre, Amersham, Bucks., U.K.) were assayed by liquid scintillation counting in a Beckman LS-230 counter. Aqueous samples were transferred to Patterson and Greene's Solution [19] (9% v/v). Background was always corrected for, as was spillover during dual isotope counting [20]. When

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mitochondrial volumes were calculated [16], a nonsolvent volume of 0.3 μl/mg protein was employed, as in our earlier work [1].

Matrix volume changes were determined by monitoring the decrease in light scattering by mitochondrial suspensions at 610 nm with a Unicam SP1800 spectrophotometer. The incubations were stirred by a magnetically driven Teflon-coated follower.

A Rank oxygen-sensitive electrode was used to record respiratory rates as described by Bronk and Parsons [21], unless otherwise indicated.

FCCP, ADP, and Antimycin A were supplied by Boehringer.

## Results

### *The Effect of $K^+$ on the Stimulation of Respiration by Thyroxine*

Table I indicates that raising the $K^+$ concentration from 0.1 to 20 mM caused a small (17%) but significant ($p < 0.05$) increase in the state 4 respiratory rate of control mitochondria although no such change occurred in mitochondria from thyroxine-treated rats. Since our previous measurements of $\Delta\mu H^+$ were necessarily made in 0.1 mM $K^+$ [1], it could be argued that they are not strictly comparable with the experiments reported in this paper where 20 mM $K^+$ is employed. However, we believe that this is not the case since the data in Table I show that the stimulatory effect of thyroxine treatment on state 4 respiration, which is still observed at the higher $K^+$ level, is 3 times as large as the increase caused by raising the $K^+$ concentration from 0.1 to 20 mM. It should be appreciated that to avoid any osmotic perturbation of mitochondrial function the sucrose concentration was increased from 80 to 120 mM in the media containing 0.1 mM $K^+$.

**Table I.** The Effect of $K^+$ upon Respiratory Rates of Mitochondria from Control and Thyroxine-Treated Rats[a]

| Animals | $[K^+]$ (mM) | Respiratory rate (natoms O/mg protein/min) State 4 | % cont. | State 3 | % cont. | ADP:O |
|---|---|---|---|---|---|---|
| Control | 0.1 | 18 ± 0.8 | — | 93 ± 2.8 | — | 1.89 ± 0.03 |
| Thyroxine-treated | 0.1 | 28 ± 1.1 | 156 | 133 ± 3.5 | 143 | 1.89 ± 0.05 |
| Control | 20 | 21 ± 0.6 | — | 106 ± 3.3 | — | 1.95 ± 0.08 |
| Tyroxine-treated | 20 | 30 ± 0.8 | 143 | 139 ± 4.0 | 131 | 1.90 ± 0.06 |

[a]Means ± standard errors from eight paired preparations. Incubation conditions: 10 mM Tris-phosphate pH 7.0, 5 mM $MgCl_2$, 5mM Tris-succinate, 2–3 mg mitochondrial protein/ml; 120 mM sucrose in incubations with 0.1 mM $K^+$, and 80 mM sucrose in incubations with 20 mM KCl added. ADP (300 nmol/mg protein) added at 2 min. Final volume 3.15 ml; temperature, 30°C.

Table I also shows that in euthyroid mitochondria the respiratory rate in the presence of ADP (i.e., state 3 [4]) was increased by 14% ($p < 0.01$) in the high-$K^+$ Medium. This is in agreement with Höfer and Pressman [22], who attributed the increase to an improved rate of phosphorylation of ADP. No such effect was apparent in the organelles from thyroxine-treated rats.

In both the high- and low-$K^+$ media thyroxine did not significantly affect the efficiency of ATP synthesis as measured through ADP:O ratios. This is in accordance with earlier studies [2, 3].

### *State 4 Matrix Swelling by Control Mitochondria and Those from Thyroxine-Treated Rats*

Over a wide range of wavelengths a decrease in absorbance of a mitochondrial suspension is a measure of matrix swelling [23]. Figure 1 clearly demonstrates that in the presence of 20 mM $K^+$ and 10 mM phosphate mitochondria from thyroxine-treated rats swell significantly less than the controls. This result was independently verified by analyzing the changes in matrix volume with the dual radioisotope technique over the same time course. This method depends on comparing the distribution of [$^{14}$C]-sucrose which is excluded from the matrix with that of $^3H_2O$ which is not. In these experiments, the mitochondrial protein concentration was raised to 3.4 mg/ml, and the media were gassed with oxygen to prevent anaerobosis. Between 30 sec and 15 min the matrix compartments of the mitochondria from thyroxine-treated rats increased in volume by 0.17 ± 0.01 $\mu$l/mg protein ($n = 5$). This increase was significantly ($p < 0.001$) less than that in the controls (0.43 ± 0.04 $\mu$l/mg protein). When an oxygen electrode was inserted in the top of the cuvette, the "peaks" at the culmination of each incubation (Fig. 1) were found to coincide with the suspension becoming anaerobic.

Figure 1 also shows that hormonal inhibition of state 4 swelling occurred in incubations without added $K^+$. However, total matrix swelling in both

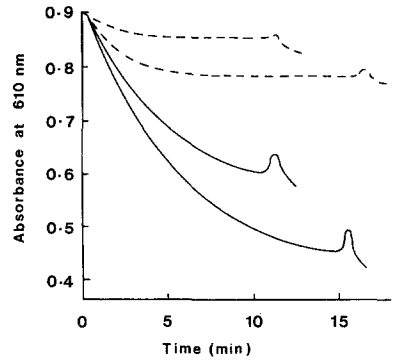


**Fig. 1.** Decrease in absorbance of mitochondria from control and thyroxine-treated rats during state 4. Incubation conditions: as in Table I, except that the broken lines show incubations in the absence of 20 mM KCl and in all cases 1.5 mg mitochondrial protein/ml was present. The upper curve of each pair shows the absorbance changes of mitochondria from thyroxine-treated rats and the lower curve that of control mitochondria.

populations of mitochondria was much less than that in 20 mM $K^+$, and below the limits of accuracy of the dual isotope technique. These data confirm a previous report [9] that mitochondrial swelling requires permeable cations.

Thus, in marked contrast to the thyroxine-mediated increase in mitochondrial volume following thyroxine treatment *in vitro* [7, 8], the *in vivo* hormone treatment inhibits state 4 matrix swelling. In view of firm evidence from this laboratory [9, 10] and others [11, 12] that increases in mitochondrial volume are an osmotic response to ion accumulation, it would seem that thyroxine treatment either reduces total ion uptake or decreases the proportion of ions which contribute to the osmolarity of the matrix.

### *$K^+$ and Phosphate Uptake by Control Mitochondria and Those from Thyroxine-Treated Rats*

Within 10 sec of the addition of mitochondria to the incubation medium there is often a substantial uptake of cations [24] and anions [13]. The former is usually largely attributed [28] to adsorption to membranes, and the latter to exchange with endogenous anions. These processes occur independently of any energy supply and are thus separate from the subsequent slower uptake of ions which is dependent on $\Delta\mu H^+$ [25, 26]. We observed ion accumulation by mitochondria when $\Delta\mu H^+$ would be expected to be maximal (i.e., state 4), and compared these results with those obtained when mitochondria were

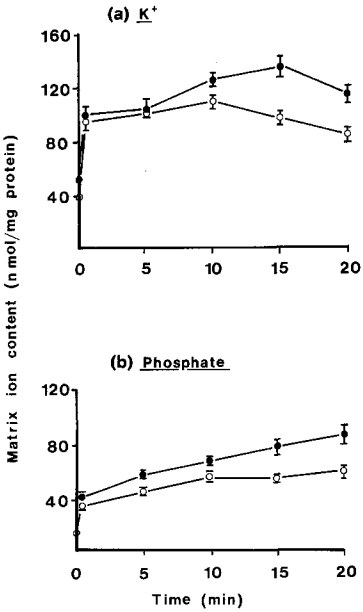


**Fig. 2.** Influence of thyroxine treatment on the accumulation of $K^+$ and phosphate by mitochondria in state 4. Each point shows the mean and standard error from five paired preparations; O, mitochondria from controls; ●, mitochondria from thyroxine-treated rats; (a) $K^+$ accumulation; (b) phosphate accumulation. Incubation conditions: 80 mM sucrose, 20 mM KCl, 10 mM Tris-phosphate at pH 7.0, 5 mM $MgCl_2$, 5 mM Tris-succinate, [$^{14}$C]-sucrose (0.1 $\mu$Ci/ml), $^3H_2O$ (0.2 $\mu$Ci/ml), 3.4 mg mitochondrial protein/ml, final volume 5.6 ml, temperature 30°C.

incubated in the presence of antimycin A and FCCP (i.e., when $\Delta\mu H^+ \rightarrow 0$ [27].

During the first 0.5 min of state 4 incubations there was rapid accumulation of about 50 nmol $K^+$ and 25–30 nmol phosphate/mg protein by both populations of mitochondria (Fig. 2). During the initial 0.5 min, thyroxine treatment did not alter the extent of accumulation ($p > 0.05$), and significant differences in $K^+$ and phosphate content only arose in the slower, secondary phase. The organelles from thyroxine-treated animals accumulated more phosphate than the controls after 5 min ($p < 0.02$). Differences in $K^+$ content did not become significant ($p < 0.01$) until the mitochondria had been incubated for 15 min, probably because the rates of $K^+$ flux are relatively low in respiring liver mitochondria [28].

Figure 3 demonstrates that when $\Delta\mu H^+$ was abolished by antimycin A plus FCCP, the amounts of $K^+$ and phosphate associated with the mitochondria after 0.5 min were only 20% less than the corresponding values in Fig. 2. However, as shown in Fig. 3 the secondary phase of ion uptake was completely absent, and there was no significant difference in the quantities of $K^+$ and phosphate retained by the mitochondria from the two groups of animals. These results indicate that the hormone does not increase energy-independent adsorption of $K^+$ to mitochondrial membranes, nor does it stimulate energy-independent exchange of phosphate with endogenous anions. Thus, the data in Figs. 2 and 3 are consistent with the view that thyroxine produces its effects by raising $\Delta\mu H^+$ in state 4 [1], although they do not preclude the possibility that thyroxine treatment enhances a slower energy-*dependent* binding of $K^+$. However, this is unlikely to provide an

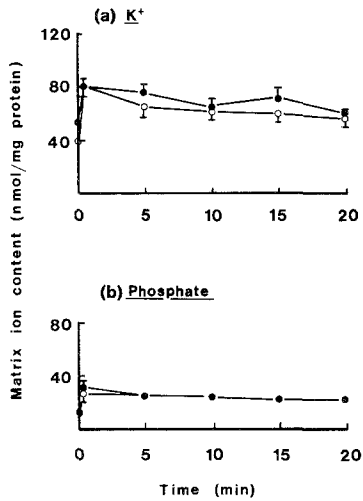


**Fig. 3.** Influence of thyroxine treatment on the accumulation of $K^+$ and phosphate by de-energized mitochondria. Each point shows the mean and standard error from three paired preparations; ○, mitochondria from controls, ●, mitochondria from thyroxine-treated rats; (a) $K^+$ accumulation; (b) phosphate accumulation. Incubation conditions as in Fig. 2 except that 0.2 μM FCCP and 400 ng of antimycin A per milligram mitochondrial protein were also present.

explanation for the hormone-effected decrease in state 4 swelling (Fig. 1) since the swelling inhibition is evident in incubations without added $K^+$.

### *Uptake of $Mg^{2+}$ by Mitochondria from Controls and Thyroxine-Treated Animals*

We also investigated the possibility that thyroxine treatment may increase energy-dependent binding of $Mg^{2+}$ at the expense of unbound $Mg^{2+}$ in the matrix. This could explain the inhibition of state 4 swelling by the hormone. Table II shows that mitochondrial $Mg^{2+}$ uptake within 0.5 min, which may be taken as the amount passively adsorbed to the membranes [28], was unaffected by our hormone treatment. This result was independent of the $K^+$ concentration. Table II also illustrates that the total energy-dependent accumulation of $Mg^{2+}$ is unaffected by thyroxine treatment, in accordance with our earlier data. It should be emphasized, however, that a significant proportion of the endogenous $Mg^{2+}$ may become bound to the mitochondria under the influence of $\Delta\mu H^+$ [28]. Consequently our data do not preclude the possibility that thyroxine treatment results in a redistribution of $Mg^{2+}$ so that a greater proportion is bound.

### *Uncoupling of Mitochondrial Oxidative Phosphorylation by $Ca^{2+}$*

In view of the thyroxine-effected increase in $\Delta\mu H^+$ we have also investigated whether thyroxine treatment causes any change in the amount of $Ca^{2+}$ required to uncouple mitochondria. This has been done by characterizing, to the nearest 5 nmoles $Ca^{2+}$/mg protein, the amount of $Ca^{2+}$ that is necessary to stimulate state 4 respiration to the extent that it cannot be increased by FCCP and ADP. The amount of $Ca^{2+}$ required for uncoupling

**Table II.** Influence of Thyroxine Treatment on the Quantity of $Mg^{2+}$ Accumulated by Mitochondria after 0.5 and 20 min[a]

| $K^+$ concentration | Status | $Mg^{2+}$ accumulated (nmol/mg protein) | |
|---|---|---|---|
| | | 0.5 min | 20 min |
| 0.1 mM | Control | 20 | 45 |
| | Thyroxine-treated | 19 | 43 |
| 20 mM | Control | 19 | 36 |
| | Thyroxine-treated | 20 | 36 |

[a]Incubation conditions: 10 mM Tris-phosphate pH 7.0, 5 mM $MgCl_2$, 5 mM Tris-succinate, 3.4 mg mitochondrial protein/ml. 120 mM sucrose in incubations with 0.1 mM $K^+$, and 80 mM sucrose in incubations with 20 mM KCl added. [$^{14}$C]-sucrose (0.1 $\mu$Ci/ml), $^3H_2O$ (0.2 $\mu$Ci/ml). Final volume 5.6 ml, temperature 30°C. $Mg^{2+}$ accumulated is calculated as (total matrix content) − (matrix content of nonincubated mitochondria). Values are means of two paired preparations.

would be expected to be related to the value of $\Delta\mu H^+$ in state 4 as well as to the inherent structural integrity of the mitochondria. Since the effect of $Ca^{2+}$ upon mitochondrial respiration is time-dependent [29] we always added it 0.5 min after the mitochondria, followed by the addition of FCCP and ADP 1.5 min later. Under these conditions the mitochondria from control rats required the addition of 123 ± 8.3 nmol $Ca^{2+}$/mg protein ($n = 4$) before they became uncoupled. By contrast, mitochondria from thyroxine-treated rats required the addition of 207 ± 10 nmol $Ca^{2+}$/mg protein ($n = 4$). This 70% difference ($p < 0.001$) is even larger if endogenous $Ca^{2+}$ is taken into account since Table II shows that organelles from the treated animals have approximately twice the $Ca^{2+}$ content of the controls.

### *Calcium and Phosphate Uptake by Control Mitochondria and Those from Thyroxine-Treated Animals*

Calcium phosphate uptake by mitochondria is an energy-dependent process [30] and as such would be expected to be influenced by the magnitude of $\Delta\mu H^+$. We observed the mitochondrial uptake of phosphate and $^{45}Ca^{2+}$ (corrected for the dilution of specific activity by endogenous $Ca^{2+}$) following an addition of $Ca^{2+}$ (50 nmol/mg protein) that was not sufficient to uncouple the mitochondria. Figure 4 clearly indicates that under these conditions the organelles from the treated animals accumulated more phosphate than the controls. The extent of $Ca^{2+}$ uptake did not seem to be significantly affected by thyroxine treatment, but the increased phosphate uptake by mitochondria from thyroxine-treated animals is again consistent with the enhancement of state 4 $\Delta\mu H^+$ by the hormone.

### *Ion Content of Nonincubated Mitochondria*

Table III shows that the matrix of nonincubated mitochondria from thyroxine-treated rats contained more $K^+$ and $Ca^{2+}$ than the controls

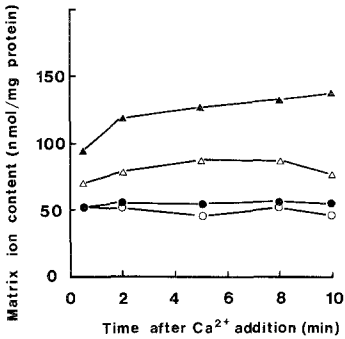


**Fig. 4.** Influence of thyroxine treatment on the $Ca^{2+}$ and phosphate content of the mitochondrial matrix following the addition of $Ca^{2+}$. Each point shows the mean of two paired preparations; ○, $Ca^{2+}$ content of control mitochondria; ●, $Ca^{2+}$ content of thyroxine-treated mitochondria; △, phosphate content of control mitochondria; ▲, phosphate content of thyroxine-treated mitochondria. Incubation conditions: 80 mM sucrose, 20 mM KCl, 10 mM Tris-phosphate pH 7.0, 5 mM $MgCl_2$, 5 mM Tris-succinate, 3.5 mg mitochondrial protein/ml 50 nmol $Ca^{2+}$/mg mitochondrial protein added at zero time, final volume 5.45 ml, temperature, 30°C.

**Table III.** Quantities of Ions Observed in Suspensions of Nonincubated Mitochondria 3–5 h Following Their Isolation from Rat Liver[a]

| | Matrix concentration (nmol/mg protein) | | | | Extramitochondrial concentration (mM) | | | | Matrix volume (μl/mg protein) |
|---|---|---|---|---|---|---|---|---|---|
| | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | Phosphate | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | Phosphate | |
| Control | 38 ± 2.2 (6) | 6.6 ± 1.0 (4) | 14 ± 1.7 (2) | 11 ± 0.9 (6) | 6.2 ± 0.9 (6) | 0.3 ± 0.06 (4) | nd | 1.5 ± 0.25 (6) | 0.57 ± 0.29 (6) |
| Thyroxine-treated | 52 ± 4.6 (6) | 12.5 ± 0.5 (4) | 18.6 ± 1.6 (2) | 12 ± 1.7 (6) | 7.3 ± 1.0 (6) | 0.2 ± 0.04 (4) | nd | 2.6 ± 0.4 (6) | 0.53 ± 0.23 (6) |
| | $p < 0.005$ | $p < 0.005$ | | $p > 0.1$ | $p > 0.1$ | $p > 0.1$ | — | $p < 0.05$ | $p > 0.1$ |

[a]Means ± SEM are given for the number of paired preparations shown in parentheses. Probabilities refer to differences between control and thyroxine-treated mitochondria. nd = not determined. Matrix volume is corrected for nonsolvent water.

although the phosphate content was unaffected by the hormone treatment. The differences in $K^+$ and $Ca^{2+}$ content suggest that thyroxine may increase uptake of these cations *in vivo*. A possible alternative explanation is that the mitochondria from the thyroxine-treated animals lose these ions less readily during the isolation procedure, but we found no evidence for this when we measured ion content of the supernatants saved from the isolation procedure (data not shown). However, our data do not rule out the possibility that there is some redistribution of ions between the various cellular fractions in the earlier stages of the isolation procedure.

The higher matrix content of $Ca^{2+}$ in the mitochondria from thyroxine-treated rats (Table III) is consistent with an earlier study by Magalhaes [41], who demonstrated that thyroidectomy reduced the $Ca^{2+}$ content of nonincubated mitochondria. Magalhaes also showed that thyroidectomy increased the mitochondrial $Mg^{2+}$ content and that this could be reduced by the administration of thyroid hormones *in vivo*. On the basis we might have predicted that our thyroxine treatment of normal rats would result in a reduced concentration of $Mg^{2+}$ in the mitochondria. However, this expectation was not confirmed by our limited data on $Mg^{2+}$ content (Table III).

Table III also shows that compared with the controls there was a significantly greater quantity of phosphate in the sucrose suspension medium containing the organelles from the thyroxine-treated rats. It seems that following isolation, phosphate leaches out the mitochondria [31], and when this is taken into account it appears that the mitochondria from treated animals contained more phosphate than the controls *in vivo*. Thus, the data in Table III indicate that the thyroxine treatment enhanced the uptake of $K^+$, $Ca^{2+}$, and phosphate *in vivo*.

It is also clear from Table III that there was no significant difference in the matrix volume of the two populations of nonincubated mitochondria. These results suggest that the inhibition of swelling in the mitochondria from the thyroxine-treated rats (Fig. 1) is not a consequence of any difference in the extent to which the organelles were swollen upon isolation.

## Discussion

The results reported in this paper show that the changes in mitochondrial ion transport that result from thyroxine treatment of rats *in vivo* can all be considered as consequences of the hormone-induced enhancement of $\Delta\mu H^+$ reported in a previous paper [1]. Thus the thyroxine treatment increases the energy-dependent uptake of $K^+$ and phosphate, although it has no effect on the rapid energy-independent phase [13, 24] of uptake of these two ions (Figs. 2 and 3). Furthermore, the quantity of $Ca^{2+}$ that is required to

uncouple oxidative phosphorylation is also markedly increased by the hormone treatment, as would be expected in view of the higher $\Delta\mu H^+$.

When $Ca^{2+}$ was added to our incubation media at concentrations below those which induce uncoupling, virtually all of the $Ca^{2+}$ was accumulated by both control and thyroxine-treated mitochondria (Fig. 4). In these experiments we did not attempt to determine the matrix $Ca^{2+}$ concentration, and hence the transmembrane distribution ratio, since the latter is a poor indication of $\Delta\mu H^+$, due to the unknown and variable amounts of $Ca^{2+}$-binding [16]. On the other hand, we did find that freshly isolated mitochondria from thyroxine-treated rats contained nearly twice the amount of $Ca^{2+}$ found in the corresponding control organelles (Table III). These data may indicate that thyroxine treatment increases mitochondrial $Ca^{2+}$ uptake *in vivo,* in agreement with Magalhaes [41]. Nevertheless, we cannot rule out the alternative possibility that thyroxine treatment modifies any redistribution of $Ca^{2+}$ that may occur between the various cellular fractions during our mitochondrial isolation procedure. We note that some workers [42] have included $La^{3+}$ in their isolation medium in order to attempt to inhibit such redistribution. However, we felt that since mitochondrial $Ca^{2+}$ efflux is insensitive to $La^{3+}$ [43], such a procedure would not produce definitive results.

In view of the increased uptake of $K^+$, $Ca^{2+}$, and phosphate as a consequence of thyroxine treatment *in vivo,* it was surprising to find that the hormone inhibited state 4 swelling (Fig. 1). These observations do not support the hypothesis [7, 8] that was recently revived [33], that an increase in matrix swelling is associated with the stimulation of respiration by the hormone. State 4 swelling is an osmotic response to ion uptake [9, 10], and consequently it seems likely that the decrease in swelling caused by the hormone can only result from a decreased osmolarity of the mitochondrial matrix. Since our results show that the uptake of $K^+$ and phosphate, at least, is increased by thyroxine treatment, it seems likely the hormone increases the extent to which matrix ions are held in as osmotically inactive form. This could either be due to increased binding or to precipitation of the phosphate salts of the divalent cations, $Mg^{2+}$ and $Ca^{2+}$. We have no direct evidence to support either of these possibilities, but the low solubility of the various calcium and magnesium phosphates makes it apparent that only a small proportion of the divalent cations can be free in the mitochondrial matrix. Our data show that thyroxine treatment *in vivo* increases phosphate uptake during incubation of mitochondria *in vitro* (Fig. 2 and 4), and in view of the higher initial $Ca^{2+}$ content of the mitochondria from hormone-treated rats, it seems highly likely that there would be increased precipitation of insoluble calcium phosphate salts. This would tend to reduce the extent of state 4 swelling.

Magnesium phosphates are more soluble than the corresponding calcium

salts so that they are less likely to form precipitates in the matrix. However, it is possible that thyroxine increases the binding of $Mg^{2+}$ by mitochondrial membranes. We have found (Table II) that energy-independent binding of $Mg^{2+}$ was unaffected by hormone treatment, but our data do not preclude the possibility that thyroxine increases the energy-dependent binding of $Mg^{2+}$ at the expense of soluble $Mg^{2+}$, and others have shown [28] that this may be a significant process in mitochondria. Unfortunately, we were unable to observe state 4 swelling in the absence of $Mg^{2+}$ because under these conditions the mitochondria swell rapidly and become uncoupled [37]. An energy-dependent increase in the binding of $Mg^{2+}$ would have important consequences. One effect which might be expected would be an increase in the rigidity of the membrane [38]. Such a phenomenon might help to explain the finding that mitochondria from thyroxine-treated rats are much more resistant to the uncoupling effects of $Ca^{2+}$. A decreased *ohmic* conductace of the respiratory membrane would also be expected following an increased binding of $Mg^{2+}$ [39, 40]. This is likely to result in an elevated $\Delta\mu H^+$. Thus, $\Delta\mu H^+$ may rise to the point where Nicholls [27] has suggested that an apparent dielectric breakdown of the respiratory membrane may occur and lead to *nonohmic* increases in transmembrane ionic current, and to respiratory stimulation.

In this paper we also show that the stimulatory effects of thyroxine treatment *in vivo* on mitochondrial respiration are not prevented by raising the level of $K^+$ in the incubation medium to 20 mM. This is an important point because in our previous publication [1] we demonstrated that the hormonal stimulation of respiratory rate was associated with an enhancement of $\Delta\mu H^+$ in state 4. The measurements of $\Delta\mu H^+$ can only be made with low levels of $K^+$ in the incubation medium, but Table I shows that the state 4 respiratory rate was still increased by the hormone at the higher $K^+$ concentration. Also it is clear from the table that the increases in state 3 and state 4 respiration are achieved without any reduction in phosphorylation efficiency. This indicates that the thyroxine effect is not due to any uncoupling of oxidative phosphorylation, in agreement with our observation that the hormone elevates $\Delta\mu H^+$ [1]. Although there were small increases in the state 3 and 4 respiratory rates of control mitochondria when the $K^+$ concentration was raised, there were no significant changes in the respiratory rates of mitochondria from thyroxine-treated rats. This suggests that the effects of $K^+$ and thyroxine on mitochondrial respiration are not additive.

We would like to emphasize the distinction between the mechanisms by which thyroxine stimulates respiration in state 4 and in state 3. In state 4, the respiratory rate is limited by the ionic current back into the mitochondrial matrix [32], and we believe thyroxine enhances this current following the elevation of $\Delta\mu H^+$ [1]. State 3 rates are limited by the maximum activity of

the respiratory chain, and so the mechanism of action of the hormone in this case must be different. A consequence of this duality of action is that thyroxine treatment causes a small reduction in the respiratory control ratio (state 3/state 4). However, this must not be taken as indicative of any significant change in the coupling of phosphorylation to electron transfer since the phosphorylation efficiency is unaffected.

In our earlier paper [1] we proposed that the increased matrix phosphate content found in the mitochondria from thyroxine-treated rats may contribute to an increased rate of ATP synthesis by lowering the phosphorylation potential. Our present data indicate that if the liver contains significant quantities of $Ca^{2+}$ the matrix phosphate content is likely to be even higher following thyroxine treatment (Fig. 4) than when $K^+$ is the only major cation (Fig. 2), although the additional phosphate may be largely in the form of insoluble salts. The elevated matrix $K^+$ concentration in the treated animals may be important in reducing the depolarizing influence of a rise in cytosolic ADP concentrations. This is because $K^+$ efflux during ATP synthesis may balance electrogenic exchange of $ADP^{3-}$ for $ATP^{4-}$ [9]. Thus, as a consequence of the increases in state 4 $\Delta\mu H^+$ and respiratory rate caused by thyroxine treatment, liver mitochondria have a greater capacity to respond to a sudden increase in the demand for ATP in the cytoplasm.

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