Biochem. J. (1987) 247, 315–320 (Printed in Great Britain)

The influence of nanomolar calcium ions and physiological levels of thyroid hormone on oxidative phosphorylation in rat liver mitochondria

A possible signal amplification control mechanism

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Using different conditions mitochondria from hypothyroid rats can show both unchanged ADP/O ratios and lowered ADP/O ratios without evidence of uncoupling when compared with euthyroid controls. Raising the free Ca^{2+} concentration to around 25 nM progressively lowered the ADP/O ratio in hypothyroid but not in euthyroid mitochondria. Ruthenium Red did not alter this behaviour and further increasing the Ca^{2+} concentration to levels below those which stimulate State 3 respiration had no additional effect. Measurements of the free Ca^{2+} concentration in the mitochondrial suspending medium using a Quin 2 fluorescence assay showed that the mitochondria did not buffer the free Ca^{2+} at these low concentrations. At 25 nM-free Ca^{2+}, addition of 10^{-13} M-T_3 to hypothyroid mitochondria produced an immediate and significant increase in the ADP/O ratio without altering the free Ca^{2+} concentration. The hormone effect was maximal by 10^{-11} M. The concentration of ATP synthetase can be estimated to lie at about 10 nM in these experiments. Hence it appears possible that a substantial amplification of the hormone signal may have taken place. Comparison with binding studies suggests that T_3 may have been maximally stimulating when somewhat less than half its receptor sites had been filled. The possible mechanisms by which this receptor mediated alteration of the ADP/O ratio might be achieved are discussed.

INTRODUCTION

Evidence has begun to accumulate in recent years that in addition to the proposed nuclear mechanism for thyroid hormone action, tri-iodo-L-thyronine (T_3) can regulate mitochondrial metabolism in a rapid direct fashion [for review see 1]. There is however some disagreement between different laboratories about the nature of the effect of hypothyroidism and of subsequent hormone addition on the ADP/O ratio. We have found that liver mitochondria from thyroidectomized rats show ADP/O ratios of about 60 % of normal with no evidence of uncoupling or loss of respiratory control and that hormone addition both *in vivo* and *in vitro* can restore this ratio to normal [2–5]. Similar findings have been presented by Sterling and his co-workers who reported that nanomolar concentrations of T_3 stimulated ATP formation by submitochondrial vesicles prepared from livers of hypothyroid rats [6] and that intravenous injection of near-physiological amounts of T_3 significantly stimulated ATP production more than oxygen consumption in mitochondria prepared 30 min later [7]. More recently saturation-transfer ^{31}P nuclear magnetic resonance studies of ATP synthesis compared with oxygen uptake in intact perfused hearts from hypothyroid rats suggested that the ADP/O ratio had been substantially reduced [8].

By contrast, Bronk & Bronk [9], Hoch [10] and Tager and his co-workers [11] although finding other changes in oxidative phosphorylation in hypothyroid livers which could be relatively rapidly altered by thyroid hormone, all reported no change in the ADP/O ratios in their experiments.

Here we demonstrate that the likely reason for this discrepancy is the presence in our preparations of calcium ions at resting cell concentrations which are generally regarded as much too low to affect mitochondrial metabolism. Furthermore, under these conditions the ADP/O ratio can be significantly and very rapidly increased by 10^{-13} M-T_3 demonstrating the presence of a signal amplification mechanism.

MATERIALS AND METHODS

Materials

Quin 2 was purchased from Aldrich. Adenosine 5′-diphosphate (di-monocyclohexylammonium salt) 3,3′,5-tri-iodo-L-thyronine (free acid) and Chelex 100 chelating resin were supplied by Sigma. The purity of the ADP (< 0.5 mol % AMP, ATP) was monitored by h.p.l.c. on a Micropak AX-5, 15 cm × 4 mm column (Varian Mat, Bremen, Federal Republic of Germany), equilibrated with 20 mM-KH_2PO_4 (pH 3.0) and developed at room temperature at 1.5 ml · min^{-1} with a linear gradient rising to 0.75 M-KH_2PO_4 over 25 min. Ruthenium Red and all other chemicals were obtained from BDH and were of the highest grade available.

Animals and mitochondria

Male rats (130–150 g) of a Sprague–Dawley strain bred in the department were thyroidectomized and maintained on normal diet except that their drinking water contained 0.5 mM-calcium lactate. Animals were used after at least 6 weeks when their weight was constant at 180–220 g (weight of the normal control litter

Abbreviations used: T_3, tri-iodo-L-thyronine; BSA, bovine serum albumin.
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mates > 350 g). Rats were killed by cervical dislocation and liver mitochondria prepared as previously described [5].

Oxygen electrode experiments

The ADP/O ratios were estimated [2] using 0.35 mg of mitochondrial protein/ml in oxygen electrode buffer (130 mM-KCl/2 mM-$MgCl_2$/2 mM-EGTA/5 mM-Tris/HCl with and without 2% defatted albumin), pH 7.2 at 37 °C in a Clarke-type electrode with 2 mM-phosphate, 3.3 mM-succinate as substrates in the presence of 3.3 $\mu g \cdot ml^{-1}$ rotenone and in 0.2 μmol of ADP. Neither the ADP/O ratio nor State-4 respiration changed significantly with three successive additions of ADP to the same incubation.

Tri-iodo-L-thyronine titration

A stock solution of 3×10^{-5} M-T_3, pH 7.2, was serially diluted with oxygen electrode buffer. The ADP/O ratios were estimated in oxygen electrode buffer containing 33 μM-Ca^{2+} (3.98 nM-Ca^{2+} free). The appropriate amount of T_3 was added to mitochondria in State 4 and the ADP/O ratio measurements began 2 min later.

Protein estimation

Protein concentration was determined by the method of Markwell *et al.* [12], using BSA as a standard. Typically the oxygen electrode chamber contained 0.35 mg of mitochondrial protein $\cdot ml^{-1}$.

Calcium titration

Mitochondria were suspended in oxygen electrode buffer and brought to State 4 by the addition of 0.05 μmol of ADP and, where appropriate, Ruthenium Red (2 μM) was added. Oxygen electrode (EGTA) buffer containing the required amount of Ca^{2+} and previously readjusted to pH 7.2 if necessary was then added. The free Ca^{2+} concentration (Ca_f) was calculated as follows:

$$K_1 = \frac{[ECa]}{[E_f][Ca_f]},\ K_2 = \frac{[EMg]}{[E_f][Mg_f]}$$

where E_f, Ca_f, Mg_f represent the free molar concentrations of EGTA, Ca^{2+} and Mg^{2+} respectively.

$$\begin{aligned} Ca_0 &= ECa + Ca_f \\ Mg_0 &= EMg + Mg_f \\ E_0 &= ECa + EMg + E_f \end{aligned}$$

where E_0, Ca_0, Mg_0 represent the total molar concentrations of EGTA, Ca^{2+} and Mg^{2+} respectively. Solving for Ca_f gives:

$$(K_1^2 - K_1K_2)\, Ca_f^3 + [K_1 - K_2 + K_1^2 (E_0 - Ca_0) + 2K_1K_2Ca_0 + K_1K_2Mg_0 - K_1K_2E_0]\, Ca_f^2 + (2K_2Ca_0 + K_1K_2Ca_0E_0 - K_1Ca_0 - K_1K_2Ca_0Mg_0 - K_1K_2Ca_0^2)\, Ca_f - K_2Ca_0^2 = 0 \quad (1)$$

The values for the apparent association constants K_1 and K_2 at pH 7.2 can be calculated from the true constants [13] to be $4.9 \times 10^6\ M^{-1}$ and 86.7 M^{-1} respectively. The roots of eqn. (1) can be evaluated using the Newton–Raphson iterative procedure. The binding of these ions to 0.1 mM-ATP ($K_a \simeq 10^4$) does not significantly alter their concentrations.

Quin 2

The fluorescent quinoline Ca^{2+} indicator, quin 2, was used to measure the extra-mitochondrial [Ca^{2+}] after various experimental protocols. Calcium buffers for calibration were made up with oxygen electrode buffer and $CaCl_2,2H_2O$, the pH of each being 7.2. Fluorescences were recorded on a Perkin-Elmer 3000 fluorescence spectrometer. Standard monochromator settings were 340 nm excitation and 490 nm emission with 10 nm slits.

Mitochondrial preparations from the oxygen electrode chamber were centrifuged for 2 min in an Eppendorf Zentrifuge 3200 and the supernatants decanted ensuring no disturbance of the mitochondrial pellet. All mitochondrial preparations for centrifugation were taken at the same time after two successive additions of ADP (0.05 μmol and 0.2 μmol, respectively) and were in State-4 respiration, which also allowed ADP/O ratios to be estimated.

Samples for measurement of Ca^{2+} fluorescence contained 0.1 ml of mitochondrial supernatant or standard Ca^{2+} buffer, 2.885 ml of oxygen electrode buffer and 15 μl of 10 mM-quin 2 in dimethylsulphoxide. All readings were carried out in quartz cuvettes at room temperature against a blank using 0.1 ml of oxygen electrode buffer containing no added $CaCl_2$.

One problem with this procedure is that using the EGTA incubation buffer as the zero calcium does not establish what the true base content of Ca^{2+} is. This was estimated by comparing the fluorescence of the buffer in the absence of EGTA with that of a series of identical buffers containing increased concentrations of EGTA. Using association constants for quin 2 of $8.7 \times 10^6\ M^{-1}$ [14] and for EGTA, $4.9 \times 10^6\ M^{-1}$ (see above) it can be calculated that, at 10 mM-EGTA, more than 99% of the bound Ca^{2+} will chelate with EGTA. The actual fluorescence values (not shown) did not change much between 5 and 30 mM-EGTA (when quin 2 will chelate less than 0.3% of the bound Ca^{2+}) and the mean value was taken as the true calcium-free quin 2. At much higher EGTA concentrations (100 mM–200 mM) there was a significant increase in the quin 2 fluorescence which may imply that some quenching ion which binds much less well to EGTA than to quin 2 was being removed. Using the zero calcium baseline, the total Ca^{2+} concentration derived from the added buffer salts was estimated to be about 70 μM: in the presence of the 2 mM-EGTA this is equivalent to a free [Ca^{2+}] of 9 nM before any further addition of calcium salt and this concentration has been added to all estimates of the free Ca^{2+} concentration.

Preparation of calcium ion-free, fatty acid-free bovine serum albumin

Bovine serum albumin (2.8 g; fraction V) was rendered fatty acid-free by the method of Chen [15]. This preparation still contains significant quantities of bound Ca^{2+} most of which can be removed by a chelating resin (M. Crompton, personal communications). The albumin was fully dissolved in double distilled water and added to 20 g of Chelex 100 resin which had previously been washed with 0.1 M-KCl (Aristar) and then three times

Table 1. The effect of the presence of defatted bovine serum albumin (BSA) and of tri-iodothyronine (T_3) on ADP/O ratios of liver mitochondria from euthyroid and from thyroidectomized rats

The standard buffer conditions in the oxygen electrode were 130 mM-KCl/2 mM-$MgCl_2$/2 mM-EGTA/5 mM-Tris/2% BSA/2 mM-phosphate/3.3 mM-succinate/3.3 $\mu g \cdot ml^{-1}$ rotenone, pH 7.2. Other experimental details are given in the Materials and methods section. The values are the means of the number of preparations shown ±S.E.M. *$P > 0.15$, †$P < 0.0025$, ‡$P < 0.0005$ versus euthyroid: §$P < 0.025$ versus in the absence of added T_3.

Preparation	Medium changes	*n*	ADP/O	State 3 respiration (ng-atom of O/min per mg)	Respiratory control ratio
Euthyroid	–	3	1.81±0.03	205±10	4.1±0.03
Euthyroid	$+3\times10^{-7}$ M T_3	2	1.79	156	3.0
Euthyroid	BSA-free	3	1.79±0.07	198±10	3.6±0.03
Thyroidectomized	–	7	†1.21±0.09	‡75±12	3.5±0.1
Thyroidectomized	$+10^{-7}$ M T_3	7	*§1.59±0.12	80±10	3.5±0.1
Thyroidectomized	BSA-free	8	1.61±0.17	95±10	3.8±0.2
Thyroidectomized	BSA-free $+10^{-7}$ M T_3	3	1.53±0.06	88±10	3.7±0.3
Thyroidectomized	Chelex-treated BSA	3	*1.58±0.13	84±10	3.6±0.2

with water. The mixture was stirred overnight at 4 °C and then packed into a column and eluted with water. The fractions which absorbed light at 280 nm were collected, freeze-dried and stored at −20 °C.

RESULTS AND DISCUSSION

The effects of bovine serum albumin on the ADP/O ratio

The experiments reported in Table 1 show, as we have reported before [2,4], that the ADP/O found with liver mitochondria from an animal thyroidectomized 6 weeks previously is significantly lower than preparations from euthyroid animals without any sign of increased uncoupling: moreover addition of 10^{-7} M-T_3 to the electrode suspension significantly raised the ratio to a value near to the euthyroid value. (Erratum: respiratory rates in [2] were, in error, quoted at 10 times their true values.) By contrast, when the hypothyroid mitochondria were tested in the same buffer, except that it contained no BSA, the ADP/O was not different from that found either in the presence or absence of BSA with normal mitochondria (Table 1). This result would agree with those who have reported no change in the ratio in hypothyroid preparations [9–11]. These findings suggest that the BSA or something added with it may somehow be responsible for the lowered ADP/O ratios in hypothyroid mitochondria while having no effect on normal preparations. Although the BSA had been defatted with activated charcoal [15] and dialysed, no attempt had been made to remove adhering cations. When this was done using Chelex 100 resin (see the Materials and methods section), the BSA lost its ability to affect the ratio (Table 1) pointing to a role for an adhering cation. One obvious candidate could be calcium ions which would be expected to be present in serum protein. However, 2 mM-EGTA had been included in the buffer to ensure that the free calcium ion concentrations should remain substantially more than 10^2 times lower than that reported to be needed to stimulate respiration in hypothyroid [16] or euthyroid [17] mitochondria. Nevertheless we examined the effect of adding Ca^{2+} to our oxygen electrode buffer.

The effect of calcium ions on the ADP/O ratio

Fig. 1 shows the effect of increasing the calcium ion content of the oxygen electrode buffer on the measured ADP/O ratio for both hypothyroid and euthyroid preparations. Care was taken to control the pH, and the free Ca^{2+} ion concentration in the Mg^{2+} and EGTA buffer was calculated using the apparent stability constants for the chelator–ion complexes as detailed in the Materials and methods section.

For hypothyroid preparations the ADP/O ratio dropped rapidly to reach a value of about 2 nM added free Ca^{2+} which was significantly lower than in the basal calcium ion concentration: thereafter increasing additions of Ca^{2+} up to a calculated free concentration of 250 nM, although producing a slight trend towards higher ratios (Fig. 1), had no statistically significant effect (e.g. 13 nM versus 56 nM; $P > 0.05$, 21 degrees of freedom). The resting level of Ca^{2+} in cell cytosol is generally accepted to lie at about 10^{-8} M or a little higher [18] so this titration would aim to cover the range in an unstimulated cell. The addition of Ruthenium Red had no influence on the Ca^{2+}-induced drop in ADP/O (Fig. 1) demonstrating that it appears to be the extra-mitochondrial presence of the ion which is effective. When the relative effects of 13 nM-Ca^{2+} and BSA are compared on a number of preparations it is clear (Fig. 1) that the ratio was lowered significantly further ($P < 0.005$) by the albumin. This implies that there are factors in addition to Ca^{2+} ions which BSA brings to the medium. However the clear sensitivity which the hypothyroid mitochondria shows to Ca^{2+} alone has allowed us to simplify the medium by replacing BSA by increasing the free [Ca^{2+}] to 13 nM (105 μM total) and thereby exclude a protein whose T_3 binding made estimation of true hormone concentrations very difficult.

One problem in using these very low free Ca^{2+} concentrations is that while one can control the ion content of the buffer it is unclear how much Ca^{2+} may be contributed, or indeed removed, by the mitochondrial preparation. Accordingly mitochondria were centrifuged from suspension in oxygen electrode buffer and the medium content of Ca^{2+} assayed using the quin 2 fluorescence assay (see the Materials and methods

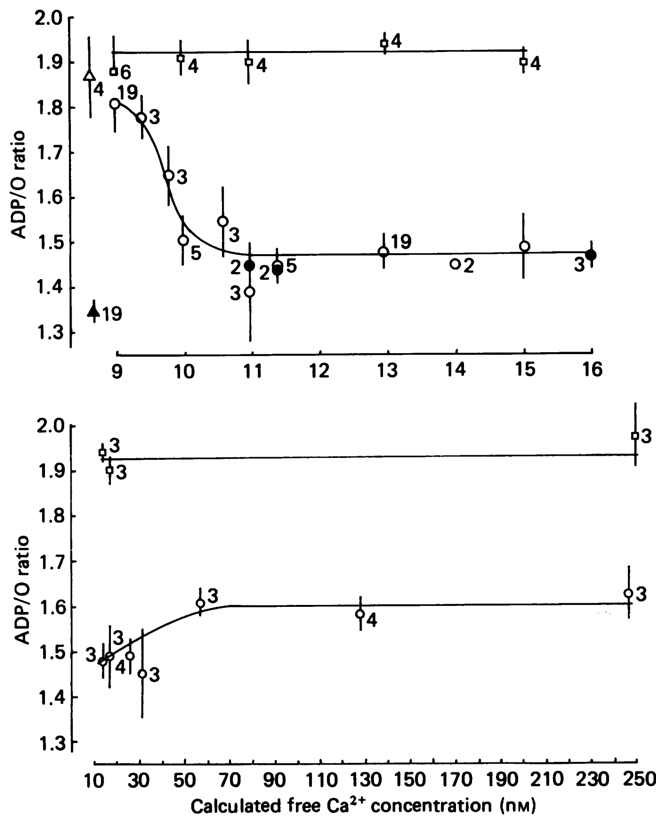

Fig. 1. Effect of increasing the Ca^{2+} concentration on the ADP/O ratio

The values are the means±S.E.M. of the number of preparations shown taken from euthyroid (□) or hypothyroid (○) animals. The oxygen electrode conditions, the calculation of free Ca^{2+} concentrations and the estimation of the basal calcium content of the buffer are described in the Materials and methods section. Hypothyroid preparations in which the medium contained (●) 2 μM-Ruthenium Red or (▲) 2% BSA and euthyroid preparations tested in the presence of (△) 2% BSA are also shown.

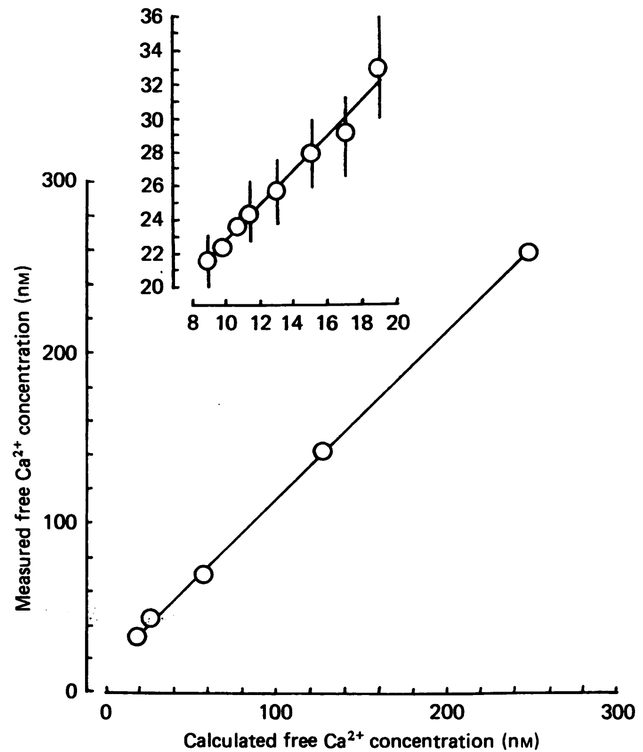

Fig. 2. Influence of hypothyroid mitochondrial preparations on the calculated free Ca^{2+} concentrations

Samples from oxygen electrode buffer suspensions used for measuring ADP/O ratios (i.e. after 9 min incubation, see Fig. 1) were centrifuged to remove the mitochondria and the free concentration of Ca^{2+} measured using a fluorescence assay as detailed in the Methods section. The values are the means±S.E.M. of the same number of preparations shown in Fig. 1; the values shown in the inset figure without error bars were from two samples only.

section). The results presented in Fig. 2 show that the mitochondrial suspension contributes additional Ca^{2+} which raises the free concentration by 12–13 nM to around 25 nM and that, except for this, the calculated and measured free Ca^{2+} concentrations agree very well at least up to 250 nM. This finding suggests that the affinity of the Ca^{2+} uptake system in these hypothyroid mitochondria is not appreciably increased over that reported for euthyroid mitochondria in the presence of Mg^{2+} (K_m apparent about 50 μM [19]). In three experiments, the addition of normal mitochondria to oxygen electrode buffer increased the free Ca^{2+} concentration by 13±2 nM.

The effect of T_3 *in vitro* on the ADP/O ratio in hypothyroid mitochondria

The effect of adding a range of T_3 concentrations to oxygen electrode medium containing 13 nM free Ca^{2+} ions and mitochondria from thyroidectomized rats is shown in Fig. 3. While 10^{-14} M hormone had no effect, 10^{-13} M significantly raised the ADP/O ratio ($P < 0.025$) and the restoration became maximal by 10^{-11} M.

Saturable T_3 binding sites with a K_a of 2×10^{-11} M^{-1} have been reported in inner mitochondrial membranes from a number of tissues which respond to thyroid hormone (e.g. liver, kidney, heart) and to be absent from those (e.g. adult brain, spleen and testis) which are unresponsive [20]. Two other groups have described similar binding sites in liver [21] and kidney [22] mitochondria where the lower affinity constants found (5–0.3×10^{10} M^{-1}) may have resulted from use of the T_3-binding detergent Triton X-100. Partial purification of a T_3 binding component (K_a 2–6×10^{11} M^{-1}) from beef heart mitochondria has been reported [23]. Tight-binding constants of this order are to be expected given that 99.5% of T_3 is bound to plasma protein and its free concentration is estimated to be about 3×10^{-12} M [see 24].

Comparing these binding studies with our findings of the effectiveness of T_3 in stimulating oxidative phosphorylation (Fig. 3) suggests that, as with plasma membrane hormone–receptor systems, T_3 can exert its maximal effect on mitochondria when somewhat less than half its receptor sites are occupied. Fig. 3 also shows that T_3 addition had no influence on the free Ca^{2+} concentration so that the increased ADP/O cannot be attributed to increased sequestration of Ca^{2+}.

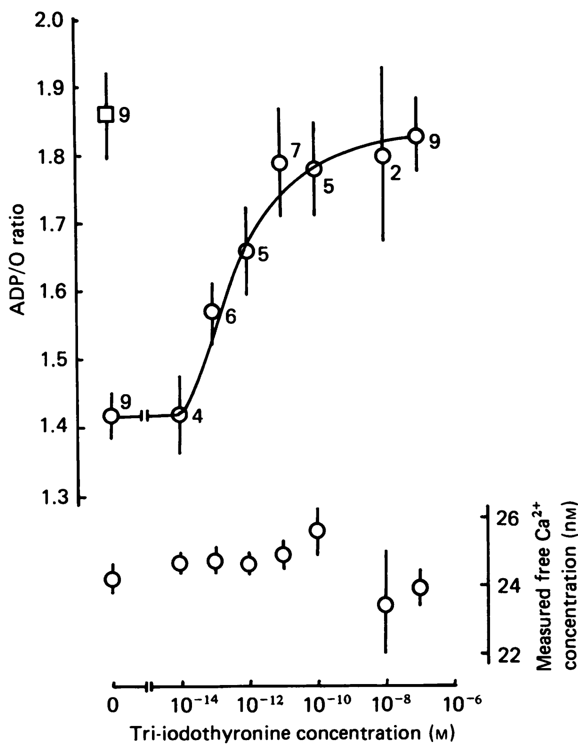

Fig. 3. Rapid stimulation of ADP/O ratio in hypothyroid mitochondria by a range of concentrations of tri-iodothyronine

A stock solution of T_3 was serially diluted with oxygen electrode buffer free of BSA but containing 13 nM free Ca^{2+} and added to an oxygen electrode vessel containing the same buffer with added succinate (3.3 mM) phosphate (2 mM), rotenone (3.3 μg/ml) and hypothyroid mitochondria (1.0 mg of protein) which had been brought to State 4 by the prior addition of 0.05 μmol ADP. The ADP/O ratio was estimated as the mean of three successive additions of 0.2 μmol ADP. The points (○) are the means ± S.E.M. (in one case the range) of the number of preparations shown. The ADP/O ratio obtained with the same preparations in the absence of added Ca^{2+} ions (□) is also shown. Samples from some of these electrode suspensions were used to estimate the free Ca^{2+} concentration (see Fig. 2 and the Methods section) and these (○) are given with reference to the right-hand scale.

GENERAL DISCUSSION

Part of the difficulty in studying the mechanism of the rapid effect of T_3 on mitochondrial respiration has been the discrepant findings reported by different laboratories. One reason for differences over the effect of thyroidectomy on the ADP/O ratio appears to reside in what could have been regarded as an insignificant level of free Ca^{2+} in the buffer. In agreement with findings that mitochondria do not take up Ca^{2+} or increase their respiration in response to Ca^{2+} at nM concentrations [16,17,19], is the observation that Ruthenium Red, an inhibitor of Ca^{2+} uptake, does not impede the effectiveness of Ca^{2+} in lowering the ADP/O ratio in hypothyroid preparations. Whether this signifies the presence of a low capacity, very high affinity uptake system for Ca^{2+} which is Ruthenium Red insensitive or that Ca^{2+} exerts its effect at the external face of the inner membrane is unclear.

Munn [25] gives the content of F_1ATPase to be 0.03 nmol/mg of protein, about one third of the cytochrome *b* content: so at 0.35 mg of protein/ml suspension there are present around 10 nM-F_1ATPase units. Comparing the concentration of the components of the respiratory chain with the concentration of T_3 required to stimulate the ADP/O ratio (Fig. 3) suggests that some amplification process may be involved in the hormone mechanism. A maximal effect at 10^{-11} M-T_3 implies around 100-fold enhancement even if all the hormone sites are filled which they may not be (see above).

The mechanism whereby T_3 receptor-mediated regulation of oxidative phosphorylation is achieved is as yet unclear. Alteration of the balance between ATP synthesis and the energy-linked transhydrogenase has been shown to be of minor importance [4] and while T_3 stimulation of the adenine nucleotide translocator is sufficiently rapid [3], estimates of the flux control coefficient showed that it has a lower measure of control after thyroidectomy and that this was unaltered at short times after hormone replacement [5]. Measurements of the H^+/O ratio by the oxygen pulse technique have shown that this was reduced by exactly the same fraction as the ADP/O ratio after thyroidectomy and was restored in parallel with the ADP/O ratio by T_3 [26]. While this suggests some direct effect on the respiratory chain-linked proton pump, the kinetic behaviour of the H^+ fluxes leaves open to question whether the medium protons measured were indeed directly representative of those driving the proton translocating ATP synthetase [26]. Clearly the establishment of a relatively simple receptor-linked system capable of rapidly altering the ADP/O ratio will improve markedly the prospects of defining more closely the mechanism of rapid T_3 action on mitochondria.

This work was carried out with project grant support from The Wellcome Trust. A. Crespo-Armas' salary was provided by the Consejo de Desarrollo, Universidad Central de Venezuala, Caracas. We are indebted to Dr. Martin Crompton of this department for helpful advice and discussion.

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Received 20 May 1987/9 July 1987; accepted 16 July 1987