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Hypothyroidism in rats does not lower mitochondrial ADP/O and H^+/O ratios

Roderick P. HAFNER and Martin D. BRAND
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K.

We investigated reports that mitochondria isolated from hypothyroid rats have decreased ADP/O and H^+/O ratios. We observed no decrease in the H^+/O ratio in mitochondria from hypothyroid rats, in the presence of either 2% (w/v) fatty-acid-free bovine serum albumin or 100 nM free Ca^{2+}. The ADP/O ratio in mitochondria isolated from hypothyroid rats in the presence of 2% fatty-acid-free bovine serum albumin was measured. Under normal experimental conditions we found no decrease in the ADP/O ratio, relative to that measured for littermate controls. At the low concentrations of mitochondrial protein used in the previously reported studies, the ADP/O ratio of mitochondria from hypothyroid rats was decreased, whereas that for control rats was only slightly decreased. The difference between the ADP/O ratios measured for mitochondria from hypothyroid rats and from control rats under these conditions was eliminated by inhibition of endogenous adenylate kinase. We suggest that the lowering of the apparent ADP/O ratio in mitochondria from hypothyroid rats at low concentrations of mitochondrial protein is an experimental artefact resulting from the breakdown of ADP to AMP.

INTRODUCTION

The role of the mitochondria in cellular energy metabolism and the possibility of direct control of mitochondrial function by thyroid hormone has been the subject of much study (for a recent review see Brand & Murphy, 1987). Mitochondria isolated from hypothyroid rats characteristically show respiration rates substantially lower than those of mitochondria isolated from euthyroid rats. Various workers have shown that injection of nanomolar quantities of T_3 into hypothyroid rats in the presence of inhibitors of DNA transcription and protein synthesis can rapidly increase the respiration rate of the subsequently isolated mitochondria (Bronk, 1966; Sterling *et al.*, 1980). The possibility that there is a T_3 receptor in the mitochondrial inner membrane is a matter of dispute (Samuels & Tsai, 1973; Greif & Sloane, 1978; Sterling *et al.*, 1978; Hashizume & Ichikawa, 1982), but it is accepted that the mitochondria are a primary site of T_3 accumulation (see also Palacios-Romero & Mowbray, 1979; Sterling *et al.*, 1984). The theory that T_3 acts on mitochondria by physiological uncoupling has now been largely rejected (for a review see Guernsey & Edelman, 1983).

The possibility that thyroid hormone might control the ADP/O ratio was suggested by Palacios-Romero & Mowbray (1979), who reported a decreased ADP/O ratio in liver mitochondria isolated from rats that had been thyroidectomized 6 weeks previously. They further demonstrated that this decreased ADP/O ratio could be restored to normal values 15 min after the injection of a 'near-physiological' dose of T_3. Subsequently it has been suggested, by using an unorthodox extrapolation procedure, that there is also a decreased H^+/O ratio in mitochondria from hypothyroid rats (Crespo-Armas & Mowbray, 1987). In contrast, most other workers report no decrease in ADP/O ratio in hypothyroid-rat liver mitochondria (Bronk & Bronk, 1962; Hoch, 1967; Katsuyuki *et al.*, 1978; Sterling *et al.*, 1980; Ezawa *et al.*, 1984; Verhoeven *et al.*, 1985). Thomas & Mowbray (1987) have suggested that the failure of other workers to observe such an effect is due to a necessity for the presence of nanomolar concentrations of free Ca^{2+} in the assay, which were supplied by the fatty-acid-free BSA in the original reports from their group. However, Herd (1978) has measured the Ca^{2+}/O ratio in mitochondria from hypothyroid rats as the T_3 concentration *in vitro* was titrated from zero to 10 μM. Assuming that the Ca^{2+}/O ratio can be used as an indicator of the H^+/O ratio (see Brand *et al.*, 1976*a*), Herd's (1978) study indicates that T_3 has no effect on the H^+/O ratio in mitochondria from hypothyroid rats in the presence of Ca^{2+}. This directly contrasts with the observations by Thomas & Mowbray (1987). Others have suggested that saturation-transfer ^{31}P-n.m.r. studies in intact perfused hypothyroid-rat hearts (Seymour *et al.*, 1983) can be interpreted in terms of a decreased ADP/O ratio in hypothyroid animals (Hassinen, 1986; Thomas & Mowbray, 1987). It is not possible to make such a calculation from the data of Seymour *et al.* (1983) without assuming that the flux through the ATP synthase is unidirectional, for which there is no evidence.

There is growing indirect evidence that the mitochondrial proton pumps may not have a fixed stoichiometry, but rather may slip at high membrane potential (Pietrobon *et al.*, 1981, 1983; Zoratti *et al.*, 1986). A direct measurement of the q^+ (charge translocated)/O ratio in mitochondria at high membrane potential, in our

Abbreviations used: BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; PTU, 6-n-propyl-2-thiouracil; T_3, 3,3',5-tri-iodo-L-thyronine; T_4, L-thyroxine.

laboratory, has shown this to be true (Murphy & Brand, 1987). It might be that thyroid hormones modify the slip in the proton pumps, and so alter the ADP/O ratio. This has led us to examine more closely the reported decreased H^+/O and ADP/O ratios in hypothyroid-rat mitochondria in the presence of nanomolar concentrations of free Ca^{2+} or 2% (w/v) fatty-acid-free BSA. The results given in the present paper show that there is no decrease in H^+/O ratios in mitochondria isolated from hypothyroid rats, and that the previously reported decreased ADP/O ratio is a consequence of the breakdown of ADP to AMP under the conditions of the assay.

EXPERIMENTAL

Hormonal treatment of rats

Male Wistar rats were made hypothyroid by administration of 0.05% (w/v) PTU in their drinking water for 6 weeks (Verhoeven *et al.*, 1985) or by parathyroid–thyroidectomy under diethyl ether anaesthesia 6 weeks before use. Parathyroid–thyroidectomized rats were given a 33 μM-calcium lactate supplement in their drinking water, as described by Palacios-Romero & Mowbray (1979). Treatment was started with rats in the weight range 100–160 g. At the end of the 6 weeks the hypothyroid rats weighed 180–240 g and their littermate controls (sham-operated in the case of the parathyroid–thyroidectomized rats) approx. 400 g. PTU administration decreased circulating T_3 and T_4, assayed by a radioimmunoassay designed for human serum, from 0.89 ± 0.08 to 0.12 ± 0.04 nmol/l and from 67 ± 2.2 to 10 ± 0.47 nmol/l respectively (means $\pm$ S.E.M. for six pairs of rats). Parathyroid–thyroidectomy decreased circulating T_3 and T_4 from 1.2 ± 0.04 to 0.42 ± 0.04 nmol/l and from 86 ± 3.6 to 9.0 ± 1.2 nmol/l respectively (means $\pm$ S.E.M. for five pairs of rats).

Preparation of mitochondria

Fed hypothyroid rats and their littermate controls (sham-operated in the case of the parathyroid–thyroidectomized rats) were killed in a random order between 08:00 and 09:00 h by decapitation. Rat liver mitochondria for subsequent experiments at 37 °C were prepared as described by Palacios-Romero & Mowbray (1979) in the media defined by Holness *et al.* (1984). For experiments subsequently performed at 25 °C, rat liver mitochondria were prepared by the standard method of Chappell & Hansford (1972) in 250 mM-sucrose/5 mM-Tris/1 mM-EGTA, pH 7.4. Mitochondria from the hypothyroid and control rats were prepared simultaneously. Mitochondrial protein was assayed by the biuret method (Gornall *et al.*, 1949), with BSA as standard. The mitochondria from hypothyroid and control rats were adjusted to the same protein concentration. For the measurement of H^+/O ratios and ADP/O ratios at 2 mg of mitochondrial protein/ml, the mitochondria were resuspended at 60–80 mg of mitochondrial protein/ml. For the experiments where the mitochondrial protein concentration was varied while the ADP/O ratio was measured the mitochondria were resuspended at 40–50 mg of mitochondrial protein/ml.

Measurement of ADP/O ratios

ADP/O ratios were measured by the technique of Chance & Williams (1955) in a 3 ml sealed magnetically stirred thermostatically controlled Perspex incubation chamber. ADP/O ratios were calculated by using both the total oxygen consumed during phosphorylation of a pulse of ADP and the extra oxygen obtained by back-extrapolation of the State-4 rate. AMP was assumed to be phosphorylated twice, except in the presence of 50 μM-bisadenosyl pentaphosphate, when it was assumed not to be phosphorylated at all. The incubation medium consisted of 130 mM-KCl, 2 mM-KH_2PO_4, 2 mM-$MgCl_2$, 2 mM-EGTA, 2% (w/v) fatty-acid-free BSA, 10 mM-succinate (potassium salt) and 5 μM-rotenone. At 37 °C the medium was buffered with 5 mM-Tris, whereas at 25 °C the medium was buffered with 3 mM-Hepes. The medium was adjusted to pH 7.2 at the temperature of use. ADP/O ratios were calculated with three successive pulses each of approx. 200 nmol of ADP to the same incubation. Oxygen consumption was measured polarographically with a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). The oxygen electrode was calibrated by the method of Robinson & Cooper (1970). ADP was assayed as described by Jaworek *et al.* (1974).

Determination of ADP breakdown

Mitochondria were incubated at the lowest concentration used to determine the ADP/O ratio in Fig. 2 (0.058 mg/ml for mitochondria from control rats; 0.117 mg/ml for mitochondria from parathyroid–thyroidectomized rats) in 3 ml of 130 mM-KCl/2 mM-KH_2PO_4/2 mM-EGTA/2 mM-$MgCl_2$/5 mM-Tris/3.3 mM-succinate (potassium salt)/5 μM-rotenone, pH 7.2, at 37 °C for 20 min with 600 nmol of ADP in the presence of 5 μg of oligomycin. After incubation for 20 min, protein was precipitated with 20% (v/v) $HClO_4$, and returned to pH 7.2 with 20% (w/v) KOH in 100 mM-triethanolamine. The amounts of ADP and AMP present were then assayed by the method of Jaworek *et al.* (1974).

Measurement of H^+/O ratios

H^+/O ratios were measured by the oxygen-pulse technique of Mitchell & Moyle (1967) in the presence of NEM to inhibit rapid phosphate movement (Brand *et al.*, 1976*b*). The medium used was either (A) 130 mM-KCl/2 mM-$MgCl_2$/2 mM-EGTA/2% fatty-acid-free BSA, pH 7.2, or (B) 130 mM-KCl/2 mM-EGTA/0.8 mM-$CaCl_2$, pH 7.2 [calculated to give a free Ca^{2+} concentration of 100 nM by using the algorithm described by Fabiato & Fabiato (1979)]. Mitochondria (10 mg of protein) were incubated in 1 ml of incubation medium supplemented with 5 μM-rotenone and 10 mM-succinate (potassium salt) in a magnetically stirred thermostatically controlled Perspex chamber. A pH-sensitive combination glass electrode was inserted through the lid. Incubation medium was made anaerobic before addition of mitochondria by blowing a stream of N_2 over it. For measurement of H^+/O ratios in medium A, mitochondria were pretreated with 300 nmol of NEM at 25 °C for 2 min. After addition to the anaerobic medium, mitochondria were treated with 5 nmol of valinomycin and, in the case of medium B, 300 nmol of NEM. When the pH trace stabilized, the pH was re-adjusted to 7.2. Oxygen pulses were performed with 25 μl (medium A) or 10 μl (medium B) of 130 mM-KCl at the temperature of the incubation mixture. Air-saturated 130 mM-KCl was assumed to contain 475 and 405 nmol

of O/ml at 25 and 37 °C respectively (Reynafarje *et al.*, 1985). When the pH had returned to baseline after an oxygen pulse, the system was calibrated by injecting standard HCl. The pH was then re-adjusted to 7.2, and the oxygen pulse and calibration were repeated twice. The 10–90 % response time of the pH electrode measured in the presence of mitochondria during the acid calibration was 500 ms and 800 ms at 37 and 25 °C respectively. Extrapolation of the pH transient resulting from the oxygen pulse was performed as described by Mitchell & Moyle (1967). In brief, the logarithm of nmol of H^+ measured was plotted against time, and subjected to least-squares linear regression. The acid pulse was extrapolated to zero time. The number of protons pumped during the oxygen pulse was extrapolated back to the time it would have taken to consume half the oxygen added in the oxygen pulse at the State-3 rate. The increase relative to the peak-height H^+/O ratio produced by this extrapolation procedure was of the order of 0.3 H^+ at 25 °C in medium A, and 0.7 H^+ at 37 °C in medium B.

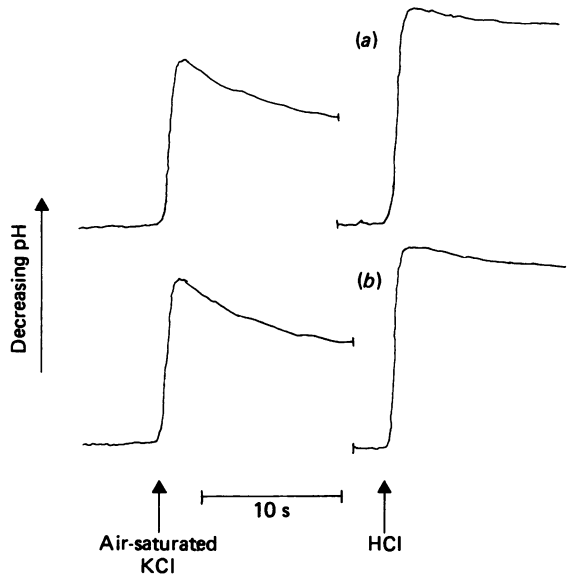

Fig. 1. Measurement of H^+/O ratios at 37 °C

Experiments were carried out with succinate as substrate in medium B as described in the Experimental section. Where indicated, 10 μl of air-saturated 130 mM-KCl or 0.3 μl of 0.1 M-HCl was injected. The traces shown are for mitochondria from control (*a*) and PTU-treated (*b*) rats. The two traces shown gave peak-height H^+/O ratios of 5.54 and 5.83 for the control and hypothyroid-rat mitochondria respectively. These values extrapolated to 6.06 and 6.42 respectively.

Statistical analysis

Several replicate measurements were made for each type of mitochondria during each experiment. These were averaged to give a single value for mitochondria from a hypothyroid rat and a single value for mitochondria from a control rat. The means of these single values were compared by Student's *t* test.

Materials

NADH (disodium salt, grade III), pyruvate kinase (type II), ADP [di(monocyclohexylammonium) salt], P^1,P^5-bis(5′-adenosyl) pentaphosphate (sodium salt), BSA (fraction V), rotenone, PTU and NEM were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Lactate dehydrogenase (type 5), myokinase and oligomycin were obtained from Boehringer Mannheim G.m.b.H. (Mannheim, W. Germany). Standard 0.1 M-HCl, catalase and phosphoenolpyruvic acid (monocyclohexylammonium salt), were obtained from BDH Chemicals (Poole, Dorset, U.K.). All other reagents were of the highest quality commercially available. Fatty-acid-free BSA was prepared by the method of Chen (1967).

RESULTS

H^+/O ratios of mitochondria from hypothyroid and control rats

H^+/O ratios were measured under three sets of conditions for rats that had been made hypothyroid by treatment with PTU, and under one of these conditions for rats made hypothyroid by parathyroid–thyroid-

Table 1. H^+/O ratios in mitochondria from hypothyroid and euthyroid rats

H^+/O ratios were measured with succinate as substrate as described in the Experimental section in a medium containing either 2 % fatty-acid-free BSA + 2 mM-$MgCl_2$ (medium A) or 0.8 mM-Ca^{2+} (medium B) at 25 or 37 °C as indicated. Hypothyroid rats were prepared by treatment with PTU or by parathyroid–thyroidectomy (PTX) as indicated. Each set of mitochondria was assayed in duplicate, with three successive pulses to each incubation. H^+/O ratios are shown as calculated by using the peak-height value, and by the extrapolation described in the Experimental section. A symbol next to a hypothyroid-rat H^+/O ratio indicates the significance of the difference from control (*$P < 0.025$); no symbol indicates no significant difference from control. Data are means ± S.E.M. for the numbers of rats indicated (*n*).

Medium	Temp. (°C)	Rats	*n*	H^+/O ratio peak	H^+/O ratio extrapolated
A	25	Control	3	4.70±0.07	4.95±0.09
		Hypothyroid (PTU)	3	4.94±0.16	5.27±0.09*
B	25	Control	2	5.70±0.31	6.13±0.31
		Hypothyroid (PTU)	2	5.65±0.26	6.11±0.33
B	37	Control	4	5.43±0.13	6.02±0.13
		Hypothyroid (PTU)	4	5.77±0.12	6.48±0.13
B	37	Control	3	5.20±0.16	5.74±0.13
		Hypothyroid (PTX)	3	5.35±0.17	6.00±0.15

ectomy. A sample trace of an experiment performed at 37 °C is shown in Fig. 1. The results are summarized in Table 1. There was no significant decrease in the H^+/O ratios in mitochondria from hypothyroid rats relative to mitochondria from control rats under any of the three conditions. In medium A (which contains 2 mM-$MgCl_2$ and 2% fatty-acid-free BSA), the H^+/O ratio for control mitochondria respiring on succinate was 4.95. This is consistent with a previous report from our laboratory, which suggested that 5 mM-Mg^{2+} lowered the H^+/O ratio from succinate to oxygen to 4.6 (Price & Brand, 1985). Subsequently Papa *et al.* (1987) have suggested that this effect of Mg^{2+} is due to modulation of the interaction of cytochrome *c* with cytochrome *c* oxidase. Under these conditions the H^+/O ratio measured for mitochondria isolated from hypothyroid rats was slightly higher than that measured for the mitochondria from control rats. In medium B (which contains 100 nM free Ca^{2+}) the measured H^+/O ratios were close to 6, the accepted value with this technique. In contrast with Crespo-Armas & Mowbray (1987) we did not observe any net acidification in the mitochondria from hypothyroid rats under any of the conditions.

We also performed oxygen-pulse experiments under the conditions of Crespo-Armas & Mowbray (1987), where the decay of the pH transient is extremely rapid, owing to small amounts of mitochondrial protein and large oxygen pulses. These workers reported a decrease in the H^+/O ratio in mitochondria isolated from hypothyroid rats. Under these conditions we observed no net acidification, in contrast with Crespo-Armas & Mowbray (1987). Attempts to fit the curve-plotting routine of Crespo-Armas & Mowbray (1987) to the experimental traces that we obtained under these conditions did not produce acceptable fits. There was no obvious difference between the traces obtained with mitochondria from hypothyroid and control rats (results not shown).

ADP/O ratios of mitochondria from hypothyroid and control rats

We have measured the ADP/O ratios for mitochondria prepared from rats made hypothyroid by parathyroid–thyroidectomy or by PTU treatment. At a concentration of 2 mg of mitochondrial protein/ml there was no significant decrease in the ADP/O ratio, calculated from either extra or total oxygen, in mitochondria prepared from hypothyroid rats (Table 2). This was true even though the medium contained 2% fatty-acid-free BSA. The ADP/O ratios measured by using total oxygen are in good agreement with those reported by Hinkle & Yu (1979). These workers reported values of 1.4 for the ADP/O ratio of mitochondria from normal rats respiring on succinate. We have measured the ADP/O ratios of mitochondria from hypothyroid and control rats at a concentration of 0.33 mg of mitochondrial protein/ml as the free Ca^{2+} concentration was titrated from 2.8 to 330 nM (calculated as described in the Experimental section). In contrast with the work of Thomas & Mowbray (1987), we observed no change in the ADP/O ratio of mitochondria isolated from hypothyroid rats under these conditions (results not shown). We have also investigated the possibility that the effect of Ca^{2+} in lowering the ADP/O ratio may be a result of it displacing another bivalent cation from EGTA. We have investigated the effects of adding micromolar (total) concentrations of Mn^{2+}, Cu^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+} and Ba^{2+}. None of these cations altered the ADP/O ratio of hypothyroid-rat or control rat mitochondria (results not shown).

Apparent dependence of the ADP/O ratio of hypothyroid-rat liver mitochondria on mitochondrial protein concentration

Having found no evidence for a change in the ADP/O ratio under standard conditions of assay, we investigated the effect of protein concentration in the assay on the ADP/O ratio. This approach was chosen because, in previous reports of a decreased ADP/O ratio in mitochondria from hypothyroid rats, ADP/O ratios were assayed at a mitochondrial protein concentration between 0.1 and 0.167 mg/ml [between 0.3 and 0.5 mg of mitochondrial protein in a 3 ml incubation chamber (Palacios-Romero & Mowbray, 1979; Crespo-Armas & Mowbray, 1987)].

Fig. 2 shows that the apparent ADP/O ratio of mitochondria from hypothyroid rats decreased as the

Table 2. ADP/O ratios in mitochondria from hypothyroid and control rats

ADP/O ratios with succinate as substrate were measured in a medium containing 2% fatty-acid-free BSA as described in the Experimental section. Mitochondrial protein concentration was 2 mg/ml in a 3 ml incubation chamber. The hypothyroid rats were prepared by treatment with PTU or by parathyroid–thyroidectomy (PTX) as indicated. ADP/O ratios were determined for each set of mitochondria in duplicate by three successive pulses of ADP to each incubation. ADP/O ratios are shown for extra or total oxygen as indicated. The symbol next to a hypothyroid-rat ADP/O ratio indicates the significance of the difference from control (*$P < 0.05$). The difference in respiration rate in mitochondria from hypothyroid rats was highly significant compared with control at all points ($P < 0.001$). Otherwise no symbol indicates no significant difference from control. Data are means ± S.E.M. for the numbers of rats indicated (*n*).

Temp. (°C)	Rats	*n*	Respiration rate (nmol of O/min per mg) State 4	Respiration rate (nmol of O/min per mg) State 3	ADP/O ratio Total	ADP/O ratio Extra
25	Control	6	32.4±1.39	146±5.78	1.40±0.01	1.81±0.02
	Hypothyroid (PTU)	6	18.9±1.45	65.3±3.91	1.37±0.03	1.93±0.04*
37	Control	5	56.9±3.54	254±18.6	1.34±0.02	1.77±0.06
	Hypothyroid (PTU)	5	31.6±1.97	141±13.3	1.38±0.04	1.83±0.08
37	Control	3	58.9±2.55	233±5.14	1.39±0.01	1.91±0.05
	Hypothyroid (PTX)	3	36.3±1.96	157±12.8	1.44±0.03	1.91±0.05

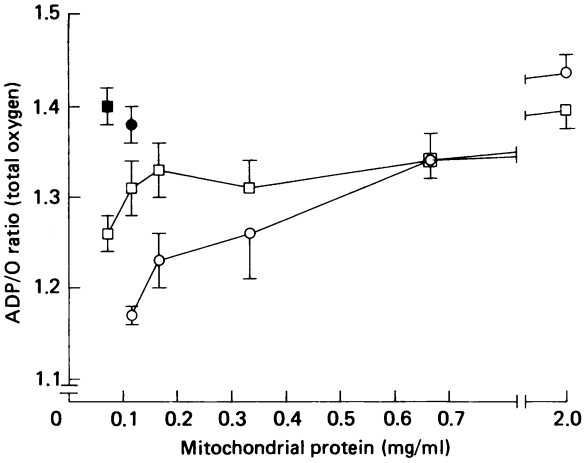

Fig. 2. Effect of mitochondrial protein concentration on the measured ADP/O ratio

Parathyroid–thyroidectomized (PTX)- and control-rat mitochondria were incubated at the concentration shown in 3 ml of 130 mM-KCl/2 mM-EGTA/2 mM-KH_2PO_4/2 mM-$MgCl_2$/5 mM-Tris/3.3 mM-succinate (potassium salt)/5 μM-rotenone/2% fatty-acid-free BSA, pH 7.2 at 37 °C. Mitochondria were given a pre-pulse of 100 nmol of ADP, and pulsed with 600 nmol of ADP to allow measurement of the ADP/O ratio. ADP/O ratios were calculated using total oxygen. Each set of mitochondria was assayed in duplicate. Error bars show S.E.M. ($n = 3$). ○, mitochondria from PTX rats; □, mitochondria from control rats; ●, mitochondria from PTX rats in the presence of 50 μM-bisadenosyl pentaphosphate; ■, mitochondria from control rats in the presence of 50 μM-bisadenosyl pentaphosphate.

amount of mitochondrial protein in the assay was decreased. The ADP/O ratio of mitochondria from hypothyroid rats decreased from 1.41 at 2 mg of protein/ml to 1.17 at 0.113 mg of protein/ml ($P < 0.01$), but there was no significant change with mitochondria from control rats over this range of protein concentration. At a concentration of 0.113 mg of mitochondrial protein/ml there was a significant decrease in the apparent ADP/O ratio measured for mitochondria from hypothyroid rats relative to that measured for mitochondria from control rats ($P < 0.05$). Even when the amount of control rat mitochondrial protein was decreased to 0.058 mg/ml to give absolute rates of oxygen consumption comparable with those for the mitochondria from hypothyroid rats assayed at 0.113 mg/ml, there was still a significant difference in the apparent ADP/O ratio of the two groups ($P < 0.02$). This indicates that the apparent fall in ADP/O ratio of mitochondria from hypothyroid rats with amount of protein assayed is not solely a consequence of absolute respiration rate. An experiment to determine the cause of the decrease in apparent ADP/O ratio is also shown in Fig. 2. The addition of 50 μM-bisadenosyl pentaphosphate completely inhibited the endogenous adenylate kinase activity, as judged by the inability of AMP to stimulate State-4 respiration under these conditions (results not shown). Such inhibition of endogenous adenylate kinase activity eliminated the difference between mitochondria from hypothyroid and control rats, and restored the ADP/O ratio to normal values. At a concentration of 0.113 mg of mitochondrial protein/ml, the apparent ADP/O ratio of mitochondria from hypothyroid rats was 1.17 in the absence of bisadenosyl pentaphosphate and 1.38 in its presence ($P < 0.01$). To determine the ADP/O ratio at the lowest concentrations reported here involved incubating the mitochondria in the oxygen-electrode chamber at 37 °C for approx. 25 minutes. It was not possible to lower the amount of mitochondrial protein assayed further, without substantial loss of respiratory control. All these experiments were performed with mitochondria with respiratory control ratios greater than 4. Under certain conditions [aging of mitochondria on ice for 6 h before measuring ADP/O ratios (where the respiratory control ratio remained above 4), or in mitochondria with low respiratory control ratios (2.5–3)] we were able to observe a much larger difference between the apparent ADP/O ratio of mitochondria from hypothyroid and control rats. On these occasions the mitochondria from hypothyroid rats showed ADP/O ratios decreased to approx. 70% of ADP/O ratios of mitochondria from control rats (results not shown). These effects showed the same characteristic concentration dependence and reversibility by bisadenosyl pentaphosphate as described above. These larger effects were, however, much harder to reproduce consistently than the effect that we have described above.

We suggest that under these conditions the apparent change in the ADP/O ratio is a consequence of the breakdown of ADP to AMP. The subsequent reconversion of this AMP into ADP by adenylate kinase leads to net phosphate-acceptor production. We therefore suggest that the apparent change in the ADP/O ratio is a result of more oxygen being consumed during State-3 respiration, because more phosphate acceptor is phosphorylated. Table 3 shows that under these conditions there is substantial breakdown of ADP to AMP for both control and hypothyroid-rat liver mitochondria. This might occur by an enzymic breakdown of ADP to AMP

Table 3. Determination of ADP breakdown by mitochondria

ADP breakdown was determined as described in the Experimental section. The blank incubation demonstrates the effect of incubating the ADP alone in the medium at 37 °C for 20 min. For the perchlorate precipitation alone, ADP was added to incubation medium at 37 °C immediately before precipitation. This experiment shows that the breakdown of ADP to AMP in the absence of mitochondria is a consequence of the perchlorate precipitation. A symbol indicates the significance of the difference of the result from the breakdown in the absence of mitochondria (*$P < 0.01$; **$P < 0.001$); no symbol indicates no significant difference. Results are expressed as a percentage of ADP added, as means ± S.E.M. for three pairs of rats.

	Percentage of ADP added recovered as:	
Rat mitochondria	ADP	AMP
Control	17.8±6.1*	72.5±4.5*
Parathyroid–thyroidectomized	10.8±6.7*	65.5±1.9**
None	71.0±7.1	31.1±3.0
Perchlorate precipitation	65.6±2.4	33.0±2.2

directly or indirectly as a consequence of the adenylate kinase reaction coupled to an ATPase.

DISCUSSION

We have measured the H^+/O ratio for succinate oxidation for mitochondria from rats that have been made hypothyroid by parathyroid–thyroidectomy or by treatment with PTU. We have used both standard protocols and protocols under which it is reported that hypothyroid-rat mitochondria show a decreased H^+/O ratio (Crespo-Armas & Mowbray, 1987). We have found no evidence for a decreased H^+/O ratio in the mitochondria from hypothyroid rats as described by those workers.

The discrepancy between our results and the results of Crespo-Armas & Mowbray (1987) may be explained by the experimental protocols and extrapolation techniques used by those workers. The experiments of Crespo-Armas & Mowbray (1987) are performed under conditions in which the extrapolated H^+/O ratio is approximately double the peak-height H^+/O ratio, so that the nature of the extrapolation is critical for the H^+/O ratio obtained. By contrast, our experiments have been carried out under conditions of high protein concentration, low buffering and small oxygen pulses, so that the size of the extrapolation required is much less, and errors in the assumptions used in extrapolation will be less important. The extrapolation procedure used by Crespo-Armas & Mowbray (1987) to measure H^+/O ratios was to fit a computer-simulated curve to the experimental points obtained during an oxygen-pulse experiment. The best-fit curve then gives values for the rate constants of proton ejection and uptake, and the H^+/O ratio. We have three objections to this extrapolation procedure. The first is theoretical, and the other two practical. The model used to generate the predicted curve has two assumptions. These are that the rate of consumption of oxygen, and thus of proton extrusion, follows a first-order exponential decay, and that the rate of re-uptake of protons also follows a first-order process. The justification used by Crespo-Armas & Mowbray (1987) for describing proton extrusion in this manner is based on a description of an exponential oxygen-consumption rate during an initial-rate method (Lehninger, 1984). This is not a valid comparison. The rate of oxygen consumption is exponential in an initial-rate method, because large amounts of oxygen are being consumed. This causes the development of a protonmotive force exerting respiratory control to inhibit further oxygen consumption. During an oxygen-pulse experiment, the size of the oxygen pulse that is used should be small, so that there is not a substantial build-up of protonmotive force. If this is the case, the rate of oxygen consumption will be linear until the oxygen concentration approaches the K_m of cytochrome *c* oxidase. If, however, because of the nature of the system or the way the experiments are performed, a substantial build-up of protonmotive force does occur, so that the first assumption of the model is correct, then the substantial protonmotive force will cause either increased proton conductance (Brown & Brand, 1986) or slip (Murphy & Brand, 1987), or both. Measurement of a mechanistic H^+/O ratio under conditions of slip is not appropriate. If, however, slip does not occur, and the non-linear relationship between respiration rate and membrane potential (Nicholls, 1974) is due to increased membrane conductance, then achieving a substantial enough protonmotive force to cause exponential oxygen consumption will cause the back-decay of the proton gradient to be multiphasic, rather than single exponential, as the permeability of the membrane changes as a function of protonmotive force. It can thus be seen that the two assumptions on which the model of Crespo-Armas & Mowbray (1987) is based cannot be true simultaneously. The peak heights in Fig. 2 of Crespo-Armas & Mowbray (1987) appear to indicate that there is a difference in H^+/O ratio of mitochondria from hypothyroid rats relative to normal ones. We suggest that this is likely to be a consequence of the slower respiration rate of mitochondria from hypothyroid rats, as well as the faster decay rate of the pH transient during an oxygen pulse for mitochondria from hypothyroid rats reported by these workers (Crespo-Armas & Mowbray, 1987).

The practical objections to the extrapolation technique used by Crespo-Armas & Mowbray (1987) are its low power of prediction and the manipulation of data that is required. For the oxygen-pulse points for mitochondria from a thyroidectomized rat that had been treated with T_3 (Fig. 2*c* of Crespo-Armas & Mowbray, 1987), the model cannot be made to fit the points unless a net acidification is subtracted. We see no justification for subtracting a net acidification when the pH transient is still decaying at the time when any remaining acidity is subtracted. At a comparable time in the oxygen pulse of mitochondria from normal rats, the pH transient has also not returned to baseline, but no net acidification is subtracted.

Concerning the low power of prediction of the model, although Crespo-Armas & Mowbray (1987) admit that with appropriate choice of rate constants an H^+/O ratio of 18 for mitochondria from normal rats can be obtained from their data by using their model, they dismiss this as 'unsupportably high' (Mowbray & Crespo-Armas, 1985; Crespo-Armas & Mowbray, 1987). The reasons for being able to exclude such numbers are not stated. Using the algorithm given, we have obtained an equally good fit for curves giving H^+/O ratios ranging from 6.35 to 10.90 for the control-rat mitochondria oxygen-pulse data in Fig. 2(*a*) of Crespo-Armas & Mowbray (1987) (results not shown). Fig. 3 shows the points and fitted curve for mitochondria from hypothyroid rats given in Fig. 2(*b*) of Crespo-Armas & Mowbray (1987), together with an example of a curve that we have generated using the same algorithm. The published curve gives an H^+/O ratio of 4.44, and provided the main evidence for a fall in H^+/O ratio in mitochondria isolated from thyroidectomized rats. Our curve is an equally good fit to the points, but gives an H^+/O ratio of 6.5. We conclude that the data and extrapolation method given by Crespo-Armas & Mowbray (1987) cannot be used as evidence for a decrease in H^+/O ratio in mitochondria from thyroidectomized rats.

We have shown that under standard conditions of assay there is no change in the measured ADP/O ratio in mitochondria from hypothyroid rats relative to control. We have also shown that, as the protein concentration in the incubation is lowered, the ADP/O ratio for mitochondria prepared from hypothyroid rats falls until, in the range of mitochondrial protein concentration where effects on ADP/O ratio are reported (0.117 mg/ml) (Palacios-Romero & Mowbray, 1979; Crespo-

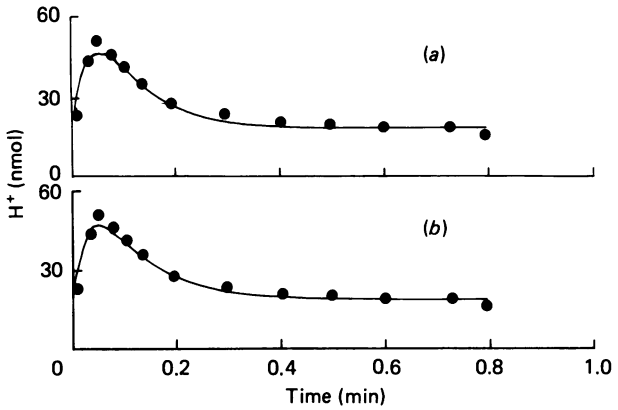

Fig. 3. H^+/O ratio in mitochondria from hypothyroid rats; data of Crespo-Armas & Mowbray (1987)

The points in Fig. 2(*b*) of Crespo-Armas & Mowbray (1987) and the fitted curve given in Table 1 of Crespo-Armas & Mowbray (1987), with the baseline shift given in that Table added, are shown (*a*). This curve [k_1 (min^{-1}) = 17; k_2 (min^{-1}) = 23; $H^+_{generated}$ (nmol) = 98] gives an H^+/O ratio of 4.44. Also shown, fitted to the same points with the same baseline shift, is the curve [k_1 (min^{-1}) = 10.8; k_2 (min^{-1}) = 31.8; $H^+_{generated}$ (nmol) = 143] (*b*). This second curve is an equally good fit to the points, but gives an H^+/O ratio of 6.5.

Armas & Mowbray, 1987), an apparent difference in the ADP/O ratio between hypothyroid-rat and control mitochondria is observed. We therefore suggest that the reason that other workers do not observe a decreased ADP/O ratio in mitochondria from hypothyroid rats relative to control is not due to the requirement for nanomolar free Ca^{2+} (Thomas & Mowbray, 1987), but is rather a consequence of the amount of mitochondrial protein in the oxygen-electrode chamber. We attribute this apparent change in the ADP/O ratio to breakdown of ADP to AMP, since it is prevented by the adenylate kinase inhibitor bisadenosyl pentaphosphate.

The partial restoration of ADP/O ratios by T_3 (Palacios-Romero & Mowbray, 1979; Crespo-Armas & Mowbray, 1987) may be caused by changes in contaminating ATPases, and hence changes in ADP breakdown during measurement of ADP/O ratios. We conclude that, when presently available techniques are used, there is no evidence for control of mitochondrial H^+/O and ADP/O ratios by thyroid hormone.

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