Thyroid Hormone Action on Mitochondria

IV.* Redox States of Intrinsic Pyridine Nucleotides in Hypothyroidism, and Influence of L-Thyroxine†

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Abstract

Measurements of fluorescence at >420 nm and extracted NADPH in mitochondria obtained from the livers of hypothyroid rats show that the addition of Pi, ADP and glutamate rapidly reduces over 90% of the total reducible intrinsic pyridine nucleotides in State 3, compared with 20% in normals. The total fluorescence intensity change and reducible $NADP^+$ is about twice normal in hypothyroid mitochondria. Adding 6-30 μM L-thyroxine to hypothyroid mitochondria *in vitro* decreases and delays the substrate-induced reduction of pyridine nucleotides, and excludes both $NADP^+$ from such reduction and NADPH from oxidation by added ADP + Pi, without changing the high NADP(H) content. The correcting actions of the hormone are rapidly reversed by albumin, probably by binding free hormone. Changes in respiration do not appear to account for these observations. There is indirect evidence for decreased phosphorylation of added ADP in hypothyroid mitochondria, and a correction by added hormone. The hormonal actions on NADP(H) redox reactions are not reproduced by 1 to 6 μM dinitrophenol *in vitro*. L-Thyroxine appears to specifically block the participation of NADP(H) in redox reactions in mitochondria from hypothyroid rats, perhaps by effecting a sequestration of the nucleotide, by inhibiting the pyridine nucleotide transhydrogenase, or by activating an energy-linked process that competes with transhydrogenation.

Introduction

On the basis of simultaneous measurements of respiration and endogenous pyridine nucleotides (PN),* Klingenberg and Slenczka [1] have correlated the metabolic states [2, 3] of mitochondria with the PN redox states. Rat liver mitochondria contained 1.23 n moles of $NAD^+ + NADH$ and 2.24 n moles of $NADP^+ + NADPH$ per mg of protein. Addition of ADP + Pi (State 2) produced 100% oxidation of the NAD^+ and 60% oxidation of the $NADP^+$. The subsequent addition of glutamate or β-hydroxybutyrate produced first a state of rapid respiration (State 3) in which the PN remained partly reduced, with $[NADH] = 0.04 \, ([NAD^+] + [NADH])$ and $NADPH = 0.72 \, ([NADP^+] + [NADPH])$. When the ADP was phosphorylated respiration slowed (State 4) and the PN became more reduced, with $[NADH] = 0.42$ $([NAD^+] + [NADH])$ and $[NADPH] = 0.95 \, ([NADP^+] + [NADPH])$. Measurements of extracted PN(H) showed redox ratios similar to those calculated from changes in the intensity of fluorescence excited at $366 \, m\mu$.

In liver mitochondria obtained from hypothyroid rats the partial reduction of endogenous PN in State 3, as measured fluorimetrically, is transient or absent [4] and the change of fluorescence signal is about twice normal [4, 5]. Additional features of the PN redox reactions, and the corrective actions of LT_4 *in vitro,* are herein reported. Preliminary reports have been made† [6].

Materials and Methods

Male Sprague Dawley rats weighing 100-200 g and thyroidectomized rats weighing 50-75 g were obtained from the Hormone Assay Company, Chicago, Illinois. The normal rats were maintained on Rockland Rat/Mouse food *ad lib.* The thyroidectomized rats were injected intraperitoneally with 0.25 mCi of ^{131}I as NaI within a few days after surgery, and maintained for at least 3 weeks on a low-iodine diet (Nutritional Biochemicals, Inc., Cleveland, Ohio). For previous studies it was found necessary to preselect hypothyroid rats for an abnormally low calorigenic response to DNP [7] ; in the experiments to be reported here, all of the early groups of hypothyroid rats responded subnormally to DNP, so the preselection procedure was discontinued. Rats were fasted overnight before use.

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* Abbreviations used: PN, PNH: oxidized and reduced pyridine nucleotide coenzymes; LT_4: L-thyroxine; DNP: 2,4-dinitrophenol.
† Hoch, F. L. (1972), presented before the American Thyroid Association, 48th meeting, Chicago, Illinois, September 20-23.

Mitochondria were prepared from the livers of decapitated rats, in 0.25 M sucrose [7]. To obtain equivalent amounts of liver homogenate, two hypothyroid animals were used or one normal, because of the differences in organ size. The final suspension of mitochondria, in 0.25 M sucrose, was adjusted to contain 15 mg of protein per ml after estimation of protein by a rapid biuret method [8]. The use of a protein baseline for comparison between normals and hypothyroids is supported by the similarity between the ratios cytochrome aa_3 : cytochrome b : protein (unpublished).

Reagents of analytical or comparable grade were obtained commercially. Water was double-distilled. Reaction mixtures for fluorimetric and polarographic measurements were at pH 7.4 and 25°, and contained 0.25 M sucrose, 21.2 μM ethylenediamine tetraacetate, 10.9 mM KCl, 10.9 mM Tris buffer, and 4.23 mM potassium mono- and di-hydrogen phosphate. Mg^{++} ions were not added because they modify the actions or effects of LT_4 [9]. For the fluorimetric assays, the volume was 2.0 ml, which included 0.1 ml of mitochondrial suspension. For polarographic assays of respiration, using a Clark electrode and a Gilson Electronics Oxygraph, the volume was 3.0 ml, with 0.15 ml of mitochondria; PN reduction and respiration were measured separately. Small volumes (0.01-0.05 ml) of reactants were added during the course of the reactions and no correction for dilutions was considered necessary. L-Thyroxine pentahydrate (Sigma Chemical Co., St. Louis, Missouri) was dissolved in 0.005 N KOH immediately before addition in *in vitro* assays; additions of equivalent amounts of KOH had no effects.

Changes in the reduction level of intrinsic pyridine nucleotides in suspensions of mitochondria were measured fluorimetrically [10]. Amplification was adjusted to give a signal that was half of full scale when 10 nmoles of NADH were added to a reaction mixture. The noise level was less than 1% of full scale. Tracings of the original recorder charts are shown here. PN redox states in mitochondria were originally studies spectrophotometrically at 340-380 nm [1]; our absorption measurements at 340-374 nm were similar to the fluorimetric data presented here, when the mitochondria were obtained from either normal or hypothyroid rats.

The terminology of Chance [2, 3] for the various metabolic states of mitochondria is used. In one set of experiments State 2 was produced by adding ADP to mitochondria suspended in the reaction mixture, and allowing full oxidation of the PN. Then State 3 was initiated by adding glutamate; the time of transition to State 4 was determined from separate measurements of respiration under the same conditions. State 5 was produced by adding rotenone. In another set of experiments, the PN were first reduced with glutamate (State 4), then partly oxidized by adding ADP (State 3), and allowed to become reduced again when phosphorylation ended (State 4). Analogous but less striking differences

between normals and hypothyroids were observed with β-hydroxy-butyrate as substrate [4].

The NADPH content of mitochondria was estimated enzymatically, using an NADPH-glutathione reductase (Sigma Chemical Co.) assay [11] and measuring NADPH oxidation fluorimetrically. The PN redox state of a 2 ml reaction mixture was monitored fluorimetrically. State 2 was taken to exist at near minimal fluorescence, as in [1], to avoid leakage of endogenous oxidized PN [12]. State 4 was taken at maximal fluorescence. Alcoholic KOH was added to stop the reaction in State 2 or State 4. The protein precipitate was spun off, and the NADPH content of the neutralized extract was determined. NADPH content is given as n moles per 1.5 mg of mitochondrial protein, the amount of mitochondria in the 2.0 ml reaction mixtures used in the fluorimetric measurements. The amount of ADP and ATP in the reaction mixtures was measured enzymatically in neutralized perchlorate extracts obtained in State 2 or State 4 as above. ATP was determined with hexokinase and glucose-6-phosphate dehydrogenase (Sigma Chemical Co.), measuring NADPH fluorimetrically [13]. The ADP assay used phospho-enolpyruvate, pyruvate kinase and lactic dehydrogenase (Sigma Chemical Co.), and measured NADPH oxidation spectrophotometrically [14].

Results

Action of LT$_4$ in vitro: In mitochondria obtained from normal rats, glutamate increases the fluorescence signal first to a distinct intermediate steady level (State 3, according to ref. [1]), then to a maximum (State 4) about 4 times greater (Fig. 1A). In mitochondria from hypothyroid rats the increase in fluorescence proceeds rapidly and without any distinct inflection, and the increase from State 2 to State 4 is almost 3 times greater than normal. The fluorescence at the end of State 3, as judged from parallel measurements of the respiration of hypothyroid mitochondria, is about 90% of maximal. Hypothyroid mitochondria contain 75% to 100% more NADPH than normals in State 2 and State 4.

In hypothyroid mitochondria plus 12.8 μM LT$_4$, the addition of glutamate intensified fluorescence first to a transient level, then to a 1.5-times greater maximum in State 4, and the total State 2–State 4 signal is about that in normals. L-Thyroxine increases the NADPH content in State 2 and decreases it in State 4; the amount of NADPH$^+$ reduced after glutamate addition (State 4 minus State 2) is 3.3 n moles in normal mitochondria, 7.8 n moles in hypothyroid mitochondria, and 3.5 n moles in the latter plus 12.8 μM LT$_4$. As the concentration of LT$_4$ is raised from 6.4 to 25.6 μM, the NADPH content in State 2 rises and that in State 4 falls (Fig. 1B). In the presence of 25.6 μM LT$_4$, glutamate

addition produces almost no $NADP^+$ reduction but increases fluorescence considerably (probably due to NAD^+ reduction). Less than 1 nmole of mitochondrial $NADP^+$ is further reduced by anaerobiosis (State 5, after rotenone addition in Fig. 1B). The hormone does not make the intramitochondrial NADP(H) unavailable for redox reactions by releasing these coenzymes from the mitochondria, as shown by their absence from centrifugal supernatants after incubation with as much as $65 \mu M$ LT_4 plus substrate. It appears that the hormone decreases both the glutamate-induced (and (β-hydroxybutyrate-induced*) reduction of $NADP^+$ and the ADP + Pi induced oxidation of NADPH in hypothyroid mitochondria.

The transitions between States 4–3–4 are also abnormal in mitochondria from hypothyroid rats (Fig. 2). ADP + Pi decreases PNH fluorescence almost twice more than in normal mitochondria. After minimal fluorescence (State 3) the return to the reduced level (State 4) is more rapid. Hypothyroid mitochondria in State 4 contain 50% more NADPH than normal, and almost twice as much NADPH is oxidized after addition of ADP as in normal mitochondria. The addition of $25.6 \mu M$ LT_4 to hypothyroid mitochondria in State 4 partly and slowly depresses the PNH fluorescence to a lower level, and oxidizes 18% of the NADPH. The new steady state of lowered fluorescence is now maximal for a subsequent State 4–3–4 cycle. The addition of ADP oxidizes less NADPH (1.0 nmole as compared to 6.0 nmoles in the absence of LT_4), and the return of fluorescence to the reduced State 4 condition is delayed and reaches only the partly oxidized level at which the ADP was added. The hormone *in vitro* thus limits the accessibility of the intramitochondrial NADP(H) to oxidation after addition of ADP + Pi and to reduction after glutamate addition, although the total NADP(H) content remains high. The transitions in the State 4–3–4 cycle are slowed to resemble those in normal mitochondria.

Concentration curves for the *in vitro* actions of LT_4 on fluorescence intensity and NADPH content in the various stages of the cycle State 4 – + LT_4 – State 3 – State 4 – State 5 in hypothyroid mitochondria are shown in Fig. 3. Adding more LT_4 to the mitochondria in State 4 progressively lowers the glutamate-induced State 4 intensity of fluorescence (–PNH) and $NADP^+$ reduction (–NADPH), decreases the further drop in fluorescence after the subsequent addition of ADP + Pi (+ PNH), and prolongs the half-time for restoration of fluorescence to the new LT_4-controlled level (t 1/2). AT $25.6 \mu M$ LT_4, rotenone-induced anaerobiosis reduces only an additional 0.6 n moles of $NADP^+$ but

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* β-Hydroxybutyrate or succinate also reduce more PN in hypothyroid mitochondria than in normals. With β-hydroxybutyrate the State 3 reduction of PN in hypothyroids resembles that with glutamate, but succinate reduces PN very rapidly in State 3 in normal mitochondria and no increase can be detected in hypothyroid mitochondria [4].
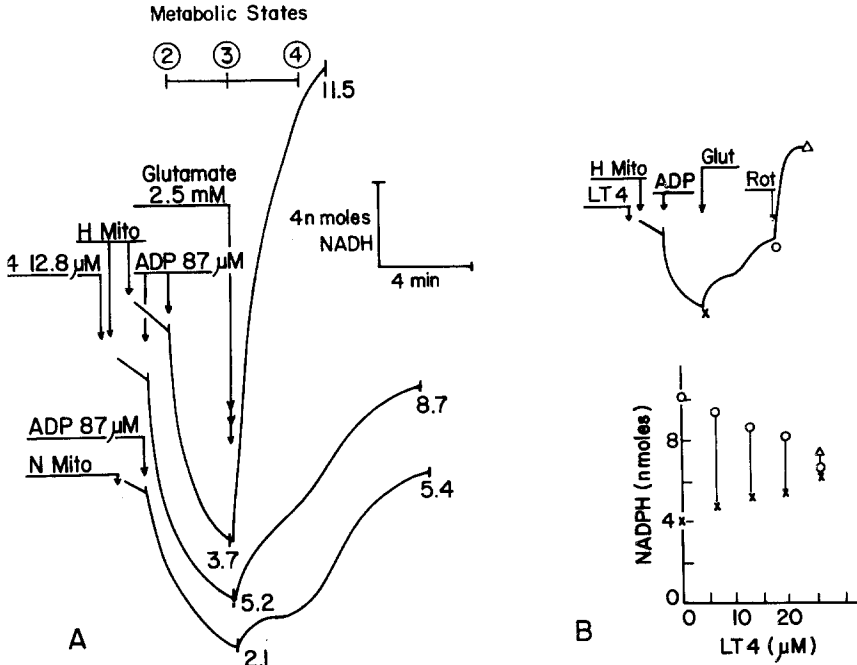
Figure 1. The kinetics of the States 2–3–4 redox transitions of pyridine nucleotides, and changes in the NADPH content, in liver mitochondria from hypothyroid rats (H) compared to those from normal rats (N); and the action of added LT$_4$ on the H mitochondria. Medium: 0.25 M sucrose; 21.1 μM EDTA; 10.0 mM KCl; 10.9 mM Tris buffer; 4.13 mM Pi; mitochondria in 0.25 M sucrose, 1.5 mg protein; volume 2.0 ml; pH 7.4. Fluorescence is measured as a function of time, and the scale is calibrated by adding NADH. (A) To N or H mitochondria are added first ADP, then glutamate; in the middle tracing, 12.8 μM LT$_4$ is added to the reaction mixture before the H mitochondria. Each curve was obtained on mitochondria from one normal or two hypothyroid rats, and is chosen as average for the groups. The beginning of the metabolic States 2, 3, and 4, obtained from parallel measurements of respiration of H mitochondria (top curve), are indicated above the tracings. In other parallel experiments, the reaction was stopped with alcoholic KOH at State 2 or State 4. The NADPH content of the extract was measured enzymatically; total nmoles NADPH per 1.5 mg protein is shown. (B) The diagram shows the sequence of additions and the points at which NADPH is measured. The concentration of LT$_4$ is varied and NADPH content is determined at State 2 ($\times$), State 4 ($\bigcirc$), and State 5 ($\triangle$, after addition of 22.5 n M rotenone); the total n moles of NADPH (per 1.5 mg mitochondrial protein) are plotted against [LT$_4$].

increases fluorescence considerably. At higher concentrations of LT$_4$, addition of ADP + Pi induces very little change in fluorescence intensity.

The decrease in fluorescence that occurs when LT$_4$ is added to mitochondria in State 4 is reversed by bovine serum albumin (Fig. 4A), in direct proportion to the amount of albumin and in inverse proportion
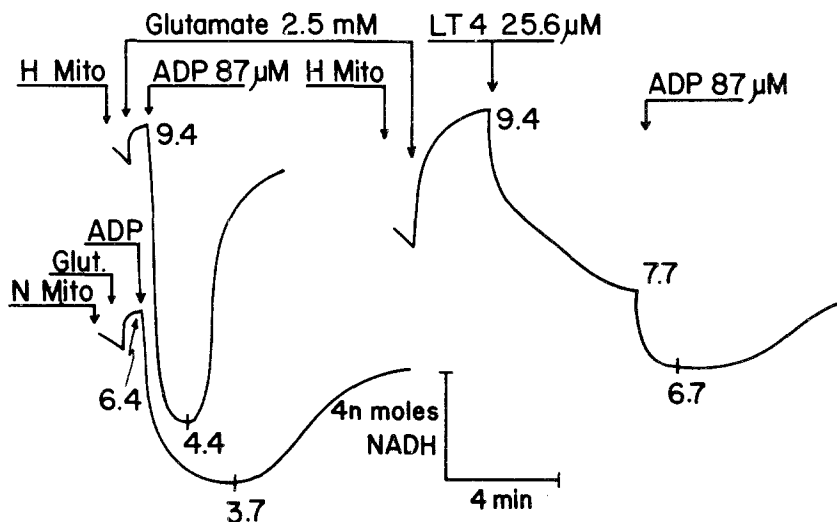
Figure 2. The States 4–3–4 cycle of the redox changes of pyridine nucleotides, and the NADPH content, in liver mitochondria from hypothyroid rats (H) compared to those from normal rats (N); and the action of added LT$_4$ on the H mitochondria. Conditions are as in Fig. 1. LT$_4$ is added to the H mitochondria after the glutamate (State 4), and when the new steady state of PN redox is reached, ADP is added. The nmoles NADPH for each group of mitochondria is shown at State 4 and State 3, and after addition of LT$_4$.

to the concentration of LT$_4$. It takes 17.5 nmoles of albumin to reverse exactly the action of 12.8 nmoles of LT$_4$, and 32.1 nmoles of albumin to reverse that of 38.4 nmoles of LT$_4$; the mean albumin : LT$_4$ ratio is 1.1. Higher ratios of albumin : LT$_4$ produce a fluorescence intensity greater than that seen in the absence of LT$_4$. NADPH contents were not measured in these experiments because albumin interfered with the assays.

As noted above, the hormone decreases the amplitude of the change in the PNH fluorescence that occurs after added ADP is phosphorylated (State 4 minus State 3 in Fig. 2, right side). Albumin added at the end of the State 4–3–4 cycle rapidly reverses this hormonal action (Fig. 4B), and an albumin : LT$_4$ ratio of 1.8 increases the fluorescence to the level seen before the LT$_4$ was added.

The involvement of electron-transport in the changes in PN reduction was studied polarographically. We have previously reported that the rates of State 3 respiration with glutamate or β-hydroxybutyrate as substrate are not significantly different in mitochondria from normal or hypothyroid rats [15]. Therefore differences in the rate of electron-transport do not account for the excessive fluorescence signal in State 3 in hypothyroidism. The addition of increasing concentrations of LT$_4$ to
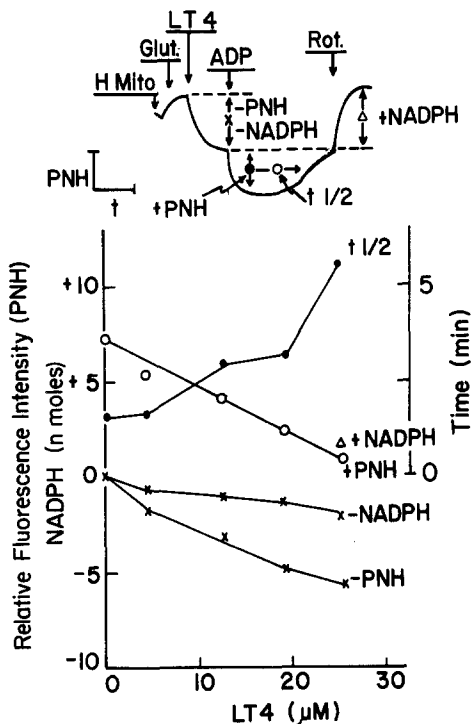
Figure 3. The actions of varying concentrations of L-thyroxine (LT$_4$) on the NADPH content, the level of reduction of the intrinsic mitochondrial pyridine nucleotides, and the extent and duration of the subsequent States 4–3–4 oxidation-reduction cycle, as measured fluorimetrically. The diagram outlines the sequence of additions in the fluorimetric assay and the points at which NADPH is assayed: to a reaction mixture containing the hypothyroid mitochondria (as in Fig. 2), 2.5 mM glutamate (GLUT) is added, then varying amounts of LT$_4$, then 87 μM ADP, and finally 22.5 nM rotenone; the parameters are defined in the diagram, and are plotted versus [LT$_4$] as relative fluorescence intensity (–PNH and + PNH), or n moles of extracted NADPH, or minutes for the half-time of the State 3–4 transition (t 1/2).

hypothyroid mitochondria, in the range that is effective in decreasing and slowing the fluorescence changes in the transitions from State 2 to State 4 (Fig. 1), progressively inhibits respiration (glutamate) in State 3, by up to –40% at 32 μM LT$_4$ (Fig. 5). State 2 respiration is inhibited less, and State 4 not at all under these conditions. The duration of State 3 is unchanged by up to 20 μM LT$_4$, and approximately doubled at higher concentrations.

The amount of ADP remaining in the reaction mixture in State 2 is directly related to the duration of the partly reduced PN condition in
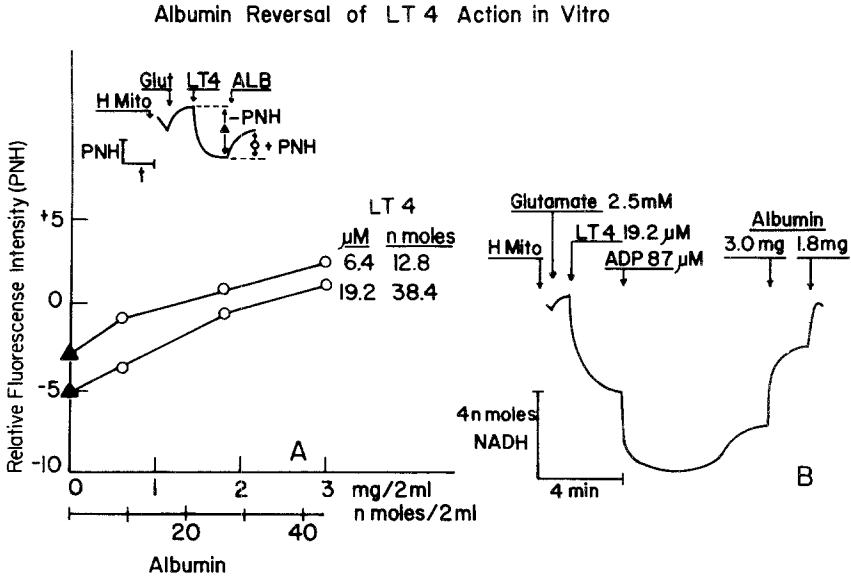
Albumin Reversal of LT 4 Action in Vitro

Figure 4. Reversing action of bovine serum albumin (ALB) on the LT_4-induced depression of (A) the steady State 4 (glutamate) condition of intrinsic PN, and (B) the subsequent ADP-induced States 4–3–4 oxidation-reduction cycle. (A) The diagram outlines the sequence of additions in the fluorimetric measurements under conditions as in Fig. 2: the reaction mixture containing hypothyroid mitochondria receives glutamate (GLUT), then LT_4, then albumin. The LT_4-induced decrease in relative PNH fluorescence intensity is indicated on the graph at albumin = 0; the subsequent increase in fluorescence when albumin is added (+ PNH, n moles) is plotted against the concentration of albumin, in mg per 2 ml reaction mixture or in n moles per 2 ml. The concentrations and amounts of LT_4 added are indicated. (B) The fluorimetric tracing and the sequence of additions are shown.

State 3 [4]. Phosphorylation of more ADP in State 2 in hypothyroid mitochondria, perhaps through the presence of more endogenous substrates than in normals, might account for the transience of State 3, and a specific inhibition of State 2 phosphorylation by LT_4 might prolong State 3. Accordingly, the amounts of ADP and ATP were measured in State 2 (Table 1). The reaction mixtures contained normal or hypothyroid mitochondria (1.5 mg of protein), which contributed 13 and 20 nmoles of endogenous ATP + ATP, making the total ADP + ATP equal about 190 n moles. The percent recovery in the perchlorate extracts was 80% + 2.6 SEM. Mitochondria obtained from hypothyroid rats phosphorylate slightly less added ADP in State 2 than do normals, leaving more ADP to be phosphorylated in State 3. The addition of 12.8 μM LT_4 to hypothyroid mitochondria does not change phos-
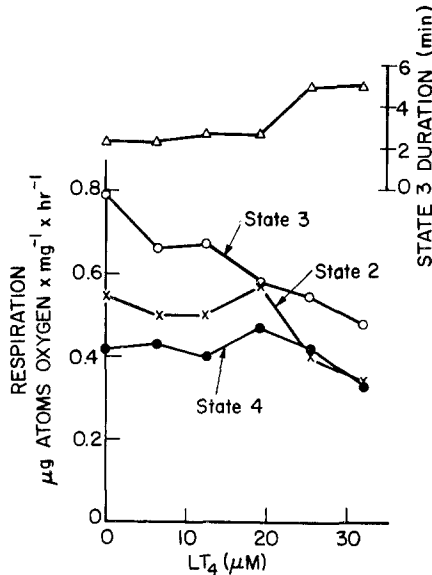
Figure 5. The actions of varying concentrations of L-thyroxine (LT$_4$) on the respiration of mitochondria obtained from hypothyroid rats. The conditions and sequence of additions were as in Fig. 1A. The rates of respiration in States 2, 3 and 4 are shown below, and the duration of State 3 respiration, above.

phorylation in State 2. Changes in State 2 phosphorylation do not seem to account for the observations in the current studies. However, added LT$_4$ up to 19.2 μM concentrations depresses State 3 respiration but does not prolong State 3 (Fig. 5), suggesting that the hormone does promote phosphorylation in State 3.

Dinitrophenol: The actions of DNP on mitochondria obtained from hypothyroid rats were compared with those of LT$_4$. Figure 6A shows that 2 μM DNP *in vitro* produces the following: a distinct delay in the development of maximal fluorescence; a slight decrease in the amplitude of the signal in the transition from State 2 to State 4; only a slight decrease in the NADPH content in States 2 and 4; and no change in the amount of NADP$^+$ that is reduced upon glutamate addition. *In vivo* the injection of DNP, 10 μg/g, into a hypothyroid rat 2 min before sacrifice alters the kinetics and the NADPH content similarly to DNP *in vitro*, but does not diminish the fluorimetric signal between States 2 and 4. Increasing concentrations of DNP added *in vitro* produce little if any change in the NADPH in States 2 and 4, or in the total amount of NADP$^+$ reduced after adding glutamate (Fig. 6B). The hormone acts differently on both NADPH content and NADP$^+$ reduction (cf. Fig. 1).
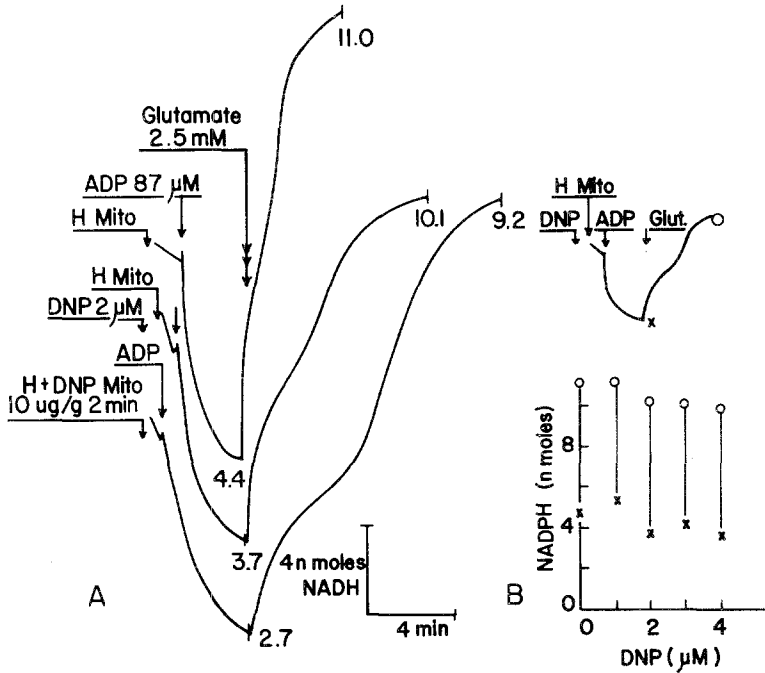
Figure 6. The *in vitro* and *in vivo* actions of dinitrophenol (DNP) on the States 2–3–4 cycles of PN redox kinetics in mitochondria from the livers of hypothyroid rats. Experimental conditions are as in Fig. 1. (A) The fluorescence of mitochondria from hypothyroid rats is shown in the top two tracings, the lower in the presence of 2 μM DNP. The third tracing is of mitochondria from two other hypothyroid rats killed 2 min after intraperitoneal injection of DNP, 10 μg/g. The NADPH content in States 2 and 4 is indicated, in nmoles. (B) The concentration of DNP, added as in (A), is plotted against the NADPH content, in nmoles per 1.5 mg protein, in State 2 (x) and State 4 (○).

Figure 7 shows that adding DNP to hypothyroid mitochondria in State 4 (glutamate) decreases fluorescence and NADPH content, and limits the extent of further depression of fluorescence when ADP is added to produce a subsequent State 4–3–4 cycle (Fig. 7). Rotenone does not reduce $NADP^+$ further at the end of such a cycle, although it increases PNH fluorescence. Concentrations between 1 and 6 μM DNP are increasingly effective. The low concentrations of DNP accelerate the State 3–4 transition (decrease t 1/2), and higher concentrations increase t 1/2 only slightly; in contrast, LT_4 increases t 1/2 markedly (Fig. 4). The hormone and DNP act similarly on the other parameters of this cycle.
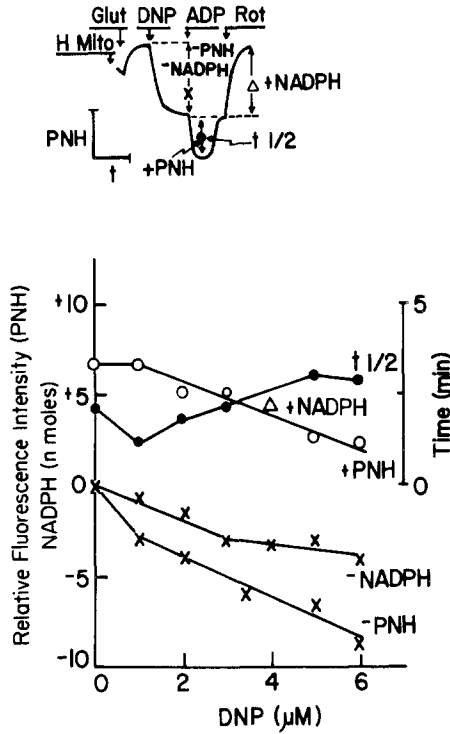
Figure 7. The actions of varying concentrations of dinitrophenol (DNP) on the NADPH content and on the States 4–3–4 cycle in hypothyroid mitochondria, measured as in Fig. 3.

Discussion

L-Thyroxine added *in vitro* appears to correct some aspects of the abnormal redox behavior of liver mitochondrial pyridine nucleotides in hypothyroidism. This is a physiological action in the sense that it restores the normal condition. However, the large amounts of LT_4 that are necessary raise the question of a pharmacological action. A concentration of 10 μM LT_4 *in vitro* must be compared with the 0.4 pM concentration of free LT_4 in the plasma [16] that maintains normal function in the mitochondria.

The rapid reversal of these actions of LT_4 by approximately stoichiometric amounts of albumin suggests that albumin binds free LT_4—as it is known to do [17]—and thereby competes with a

mitochondrial site of direct hormonal reaction. However, high albumin : LT_4 ratios increase the PNH fluorescence above that seen in the absence of LT_4 (Fig. 4). Albumin is said to reduce intrinsic PN in normal mitochondria by binding uncoupling agents of endogenous origin, probably fatty acids [18]. The variety of substances bound by albumin suggests caution in the interpretation of the mechanism of reversal of LT_4 action [19, 20].

The present studies do not define the biochemical basis for the abnormalities in mitochondrial PN redox reactions in hypothyroidism. The reduction of mitochondrial PN competes with oxidative phosphorylation [21], perhaps for a common source of energy. Either an abnormally slow or a rapid rate of phorphorylation might account for the excessive and early reduction of PN in State 3 in hypothyroidism. For instance, normal mitochondria oxidize succinate in State 3 more rapidly than NAD^+-dependent substrates, and they phosphorylate added ADP and then reduce internal PN very rapidly and completely. However, State 3 oxidation of glutamate or β-hydroxybutyrate is not faster than normal in intact mitochondria from hypothyroid rats [15]. A depressed phosphorylation rate, such as is specifically effected by oligomycin [4, 22, 23], aurovertin, or atractyloside [22] in normal mitochondria, produces a rapid and extensive PN reduction in State 3 that resembles the pattern in hypothyroid mitochondria [4]. Initial rates of phosphorylation of added ADP are lower than normal in mitochondria from hypothyroid rats (Chen, Neymark, Dimino and Hoch, unpublished data). Adenine nucleotide translocation is slower than normal in liver mitochondria from hypothyroid rats when measured at $0°$ [24, 25] but is normal or higher at temperatures above $25°$ [25], and so does not seem to be a cause for decreased phosphorylation of added ADP in our measurements. This defect in phosphorylation, taken together with the observation that hypothyroid mitochondria phosphorylate less than the normal amount of ADP in State 2 and thereby leave more ADP to be phosphorylated in State 3 (Table I), suggests one basis for the early PN reduction.

The corrective actions of LT_4 *in vitro* can also not be ascribed to one mitochondrial process at this time. The hormone, in the concentration range that corrects PN reduction, inhibits State 3 respiration (Fig. 5). In spite of this, LT_4 appears to stimulate phosphorylation of added ADP (Fig. 5). A competition with phosphorylation would account for the hormone-induced delay and limitation in PN reduction, that resembles the pattern in normal mitochondria. However, it does not necessarily follow that the LT_4 present in normal mitochondria, which is 5 times that in hypothyroids [26], actually stimulates phosphorylation. Equally feasible alternatives are that the hormone added to hypothyroid mitochondria or present in normals inhibits an energy-linked process that competes with phosphorylation, or that a true correction of the defects

TABLE I.[a] Phosphorylation of added ADP by mitochondria in metabolic
State 2, and the influence of hypothyroidism and added 12.8 μM LT$_4$

	nmoles	
	ADP	ATP
Normal (3)	92.3	49.1
Hypothyroid (3)	109.4	41.9
Hypothyroid + 12.8 μM LT$_4$ (3)	106.7	39.5

[a] To a 2-ml reaction mixture containing mitochondria from normal or hypothyroid
rats was added 87 μM ADP, and the reduction level of the intrinsic PN was
monitored fluorimetrically as in Fig. 1. The mitochondria from the hypothyroids
were also examined in the presence of 12.8 μM LT$_4$, added to the reaction mixture
as in Fig. 1. Similar reaction mixtures were set up in parallel, and the reaction was
stopped with 1 N perchloric acid at Stat 2. The extracts were freed of protein by
centrifugation, neutralized and freed of perchlorate, and assayed enzymatically for
ADP (14) and ATP (13). The means of the ADP and ATP contents aae shown.

in hypothyroidism involves protein synthesis (since the hormone *in vitro*
does not correct the abnormally high mitochondrial PN contents).

The actions of added LT$_4$ on the degree of substrate-induced
reduction of intramitochondrial NADP$^+$ may be of some help in deciding
among these alternatives. The hormonal actions appear to be specific, in
that DNP does not significantly affect the degree of NADP$^+$ reduction
(Fig. 6); others have also concluded that thyroactive compounds and
DNP act on different mitochondrial energy-linked reactions [27]. The
redox reactions of NADP(H) in normal rat liver mitochondria are
relatively few in number, as will be discussed below. In contrast, a large
number of factors is known to affect the redox reactions of NAD(H),
which includes [ADP], [Pi] and [O$_2$], substrate concentrations and
availability, reverse electron-flow, and the specific activities, amounts
and availabilities of NAD$^+$-dependent dehydrogenases and electron-
transport components. The present data on intact mitochondria do not
seem adequate for assigning for assigning a role of LT$_4$ among these
factors in NAD(H) metabolism.

The hormone added *in vitro* appears to exclude the large amounts of
NADP(H) in hypothyroid mitochondria from participation in redox
reactions. Increasing concentrations of LT$_4$ progressively decrease the
substrate-induced reduction of NADP$^+$ and prevent significant further
reduction even when electron-transport is blocked (Fig. 1, 3); LT$_4$ also
progressively decreases the ADP + Pi-induced oxidation of NADPH.
These observations might be explained if intramitochondrial NADP(H)
were made physically unavailable by being bound to a membrane or a
soluble component, for example, the properties of which were changed
by the hormone [28]. Or LT$_4$ might alter the enzyme reactions that

metabolize NADP(H). Either the glutamate dehydrogenase plus gluta-
mate [29, 30] or the pyridine nucleotide transhydrogenase plus NADH
[31, 32] reduce mitochondrial $NADP^+$, in reactions that require an
energy source [33–35]. The glutamate dehydrogenase does not seem to
be a site of LT_4 action because the oxidation of β-hydroxybutyrate gives
results similar to those seen with glutamate [4], and the mitochondrial
β-hydroxybutyrate dehydrogenase reacts specifically with NAD^+ [36].
The oxidation of NADPH involves the non-energy-linked PN trans-
hydrogenase. Thyroxine is known to inhibit both the energy-linked [37,
38] and the non-energy-linked [33, 39, 40] PN transhydrogenase, which
appear to be the same enzyme [37]. A de-inhibited energy-linked
transhydrogenation reaction in hypothyroidism would compete more
successfully for high-energy intermediates to drive the reduction of
$NADP^+$, which would account for our observations.

There is evidence for both alternatives. In hypothyroidism the inner
membrane of liver mitochondria has an altered composition and physical
properties [41, 25], and the activity of the energy-linked PN
transhydrogenase is higher than normal [42].

Acknowledgment

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