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MODE OF ACTION OF 5,5′-DIPHENYLTHIOHYDANTOIN—SITES OF ACTION OF 5,5′-DIPHENYLTHIOHYDANTOIN IN MITOCHONDRIA IN RELATIONSHIP TO THYROXINE-STIMULATED RESPONSES*

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Abstract—The effects of 5,5-diphenylthiohydantoin (DPTH) on mitochondria were examined *in vitro* and *in vivo* on well-delineated sites known to be profoundly affected by thyroid hormones, in order to test the hypothesis that thyroid hormones regulate metabolic responses through mitochondrial and cytosolic processes. The effect of methimidazole, another antithyroid drug, was studied *in vitro* in parallel systems. Addition of DPTH greatly decreased the capacity of mitochondria to synthesize precursors for gluconeogenesis as a result of the inhibition of pyruvate carboxylation. DPTH was found to inhibit α-glycerophosphate dehydrogenase, the enzyme involved in the regulation of hydrogen transfer to cytochromes through the cytosolic and mitochondrial α-glycerophosphate cycle. DPTH also inhibited β-hydroxybutyrate dehydrogenase. The state 3 respiration with pyruvate, α-ketoglutarate, succinate and α-glycerophosphate was inhibited by DPTH. Methimidazole had no effect in blocking the peripheral action of thyroxine and was non-inhibitory in the above systems studied. *In vivo*, in experiments where DPTH was added to the diets, a large inhibition of liver gluconeogenic enzyme activities, α-glycerophosphate dehydrogenase and oxygen consumption by rat liver tissue slices was observed.

IT HAS been established that thryroid hormone affects carbohydrate metabolism[1–5] and the metabolic rate in liver, kidney, heart, skeletal muscle and other tissues.[6–10] The oxygen consumption in these tissues has been reported to be markedly increased. Thyroxine also had a highly specific[10–13] effect on mitochondrial α-glycerophosphate dehydrogenase; a large increase in its activity was observed in thyroxine-treated animals. The physiological importance of this stimulation of α-glycerophosphate dehydrogenase activity by thyroxine may be in its ability to act as a carrier of cytoplasmic reducing equivalents to mitochondrial cytochrome carriers.[14–17] The effect of this hormone is to lower the extramitochondrial NADH/NAD^+ ratio which can then stimulate carbohydrate and glycogen catabolism in cytosol as suggested by Lee and Lardy.[10]

Ruegamer *et al.*[11] have screened over 60 compounds for possible antithyroid activity in the rat. One of these compounds, 5,5′-diphenylthiohydantoin (DPTH) was

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found to be a very potent inhibitor of thyroxine-stimulated metabolic responses. DPTH at 0·06 per cent of the diet totally inhibited metabolic responses to thyroid hormone (20 mg/kg). The present study was undertaken to examine both *in vitro* and *in vivo* the effects of DPTH on the sites known to be affected by thyroid hormone.

EXPERIMENTAL

Methods

Experiments in vitro. All experiments were performed with intact rat liver mitochondria isolated according to the method of Schneider,[18] except that the mitochondria were centrifuged at 10,000 *g* for 10 min. The incubation, processing of samples, analyses for pyruvate, malate, α-glycerophosphate, dihydroxyacetone phosphate, citrate, $^{14}CO_2$ incorporated, acetoacetate (AcAc), β-hydroxybutyrate (β-OH) and mitochondrial nitrogen were performed according to methods previously cited.[19,20] Oxygen utilization, ADP/oxygen ratios and respiratory control indices were determined by the method of Chance and Williams[21] using an Oxygraph (Gilson Medical Electronics).

Experiments in vivo. The rats were fed powdered laboratory Purina chow diets to which DPTH was added in amounts described in the legends to the tables. There were no differences in body weight gain or food consumption between control and DPTH-fed rats.

Enzyme assays. Rats were killed by decapitation and the liver was removed rapidly and weighed in prepared beakers containing cold media (0·25 M sucrose, 0·02 M tris, pH 7·3, 0·001 M EDTA and 0·001 M reduced glutathione). Homogenates were adjusted to 10% (w/v) of liver.

Pyruvate carboxylase (EC 6.5.1.1), phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose 1,6-diphosphatase (EC 3.1.3.11) and glucose 6-phosphatase (EC 3.1.3.9) were assayed by previously cited methods.[22]

Measurement of oxygen consumption by tissue slices. The liver was quickly removed and cooled in Krebs–Ringer phosphate medium on ice. Slices of liver were prepared freehand, blotted on moist filter paper to remove excess solution, and weighed in a torsion balance. The slices were then placed directly in Warburg flasks containing 3 ml of Krebs–Ringer phosphate solution with 10 mM glucose as substrate. Flasks were placed in a 37° water bath, shaken and gassed with 100% oxygen for a 5-min period. Side-arms were then closed and measurements of oxygen consumption were commenced at 15 min after placing the flasks in the water bath. Standard manometric techniques and calculations were utilized to measure oxygen consumption of the slices and results are expressed as micromoles of oxygen per gram of dry tissue per minute.

Materials

The $NaH^{14}CO_3$ was purchased from Tracer Laboratories and was diluted with unlabeled $KHCO_3$ to give a final specific activity of 0·22 μc/μmole. The specific radioactivity of the solution used was determined as previously described by Mehlman *et al.*[19] The enzymes were purchased from Boehringer Mannheim Corp. All other reagents were of the highest purity commercially available. L-Octanoylcarnitine was a generous gift of Dr. Yuzo Kawashima of Otsuka Pharmaceutical Factory, Naruto, Japan.

RESULTS

Experiments in vitro

Influence of DPTH and methimidazole (MMI) on α-glycerophosphate and L-*octanoylcarnitine metabolism in rat liver mitochondria.* It has been shown that administration of thyroxine to rats causes an increase in mitochondrial α-glycerophosphate dehydrogenase.[10,17] When mitochondria from normal rat livers were incubated with DPTH, the conversion of α-glycerophosphate to dihydroxyacetone phosphate was inhibited (Table 1). DPTH, 0·6 mM, inhibited α-glycerophosphate conversion by 81 per cent. However, methimidazole had no inhibitory effect.

TABLE 1. EFFECT OF 5,5-DIPHENYL-2-THIOHYDANTOIN (DPTH) AND METHIMIDAZOLE (MMI) ON α-GLYCEROPHOSPHATE METABOLISM IN RAT LIVER MITOCHONDRIA*

Addition to system (mM)	Metabolite changes (nmoles/mg N/min)	
	α-Glycerophosphate used	Dihydroxyacetonephosphate found
None	75·0	63·3
DPTH (0·3)	31·3	21·0
DPTH (0·6)	14·6	10·2
MMI (1·46)	77·1	61·5

* The reaction mixture contained 3·3 mM ATP, 10 mM $MgSO_4$, 13·3 mM $KHCO_3$, 13·3 mM potassium phosphate and 13·3 mM potassium triethanolamine buffers, pH 7·3, 5 mg of fatty acid-poor albumin and 20 mM α-glycerophosphate. Mitochondria from 0·5 g liver were suspended in 0·5 ml of 0·25 M sucrose and contained 2·4 mg nitrogen. The final volume was 3·0 ml adjusted with 0·7–1·2 ml of 0·154 M KCl. The incubation time was 12 min.

Results in Table 2 show the effect of varied DPTH concentrations on L-octanoylcarnitine metabolism to ketone bodies in the absence of any additional substrate. It can be seen that DPTH strongly inhibits formation of total ketone bodies and also inhibits conversion of AcAc to β-OH butyric acid. The degree of inhibition of fatty acid oxidation is dependent on the concentration of DPTH in the incubation media.

Inhibition by DPTH of L-*octanoylcarnitine oxidation in disrupted rat liver mitochondria.* Since fatty acid oxidizing enzyme systems are located in the mitochondrial membrane,[23] it was possible that DPTH blocked membrane sites that were involved in oxidation of fatty acids. Results in Table 3 show the effect of 1·2 mM DPTH on mitochondrial oxidation of fatty acids in intact, sonicated, frozen-thawed and digitonin-treated mitochondria. It is clear that total ketone body production was markedly decreased in the presence of DPTH in intact mitochondria as well as in fragments formed by sonication, freeze-thawing or digitonin treatment.

At this high concentration of DPTH (1·2 mM), even acetoacetate formation was greatly decreased, from 20·96 to 7·81 nmoles/mg N/min. The ratio of β-hydroxybutyrate to acetoacetate in intact mitochondria in the presence of DPTH was also decreased. Sonication or treatment of mitochondria with digitonin greatly altered the β-hydroxybutyrate to acetoacetate ratios. In the presence of 1·2 mM DPTH, the total amount of ketones formed was so small that the ratio of β-OH to AcAc which was

TABLE 2. METABOLISM OF L-OCTANOYLCARNITINE BY LIVER MITOCHONDRIA IN THE PRESENCE OF VARIED CONCENTRATIONS OF 5,5-DIPHENYL-2-THIOHYDANTOIN*

DPTH added to system (mM)	Metabolite changes (nmoles/mg N/min)			
	Acetoacetate found	β-Hydroxybutyrate found	Total ketones	Ratio of β-hydroxybutyrate acetoacetate
None	41·14	104·68	145·82	2·54
	40·10	94·27	134·37	2·35
0·15	35·93	84·63	120·56	2·35
	36·71	82·03	118·74	2·23
0·30	45·57	8·85	54·42	0·19
	46·35	8·07	54·42	0·17
0·60	36·45	4·94	41·30	0·13
	36·71	5·20	41·91	0·14
0·90	28·38	3·90	32·28	0·13
	23·43	4·68	28·11	0·20

* The reaction mixture contained 3·3 mM ATP, 10 mM $MgSO_4$, 13·3 mM $KHCO_3$, 13·3 mM potassium phosphate and 13·3 mM potassium triethanolamine buffers, pH 7·3, 5 mg of fatty acid-poor albumin and 1·0 mM L-octanoylcarnitine. Mitochondria from 0·5 g liver were suspended in 0·5 ml of 0·25 M sucrose and contained 3·2 mg nitrogen. The final volume was 3·0 ml adjusted with 0·7–1·2 ml of 0·154 M KCl. The incubation time was 12 min.

reported is probably unreliable under these circumstances. In frozen-thawed mitochondria, the ratio of β-OH to AcAc was also decreased. This ratio obtained with frozen-thawed mitochondria in the presence of DPTH is probably reliable, since total amounts of ketones formed are several times larger than those with sonicated or digitonin-treated mitochondria. These experiments indicate that DPTH inhibition of fatty acid oxidation is not a membrane-dependent phenomenon.

Effect of DPTH on synthesis of precursors for gluconeogenesis by rat liver mitochondria. The utilization of pyruvate, the formation of malate and citrate, and the incorporation of $^{14}CO_2$ into organic acids by intact liver mitochondria in the presence of DPTH, MMI and L-octanoylcarnitine (OC) are shown in Table 4.

The major organic acids accumulated by isolated rat liver mitochondria are malate, citrate and fumarate. L-Octanoylcarnitine (0·7 mM) added to the incubation media decreased pyruvate utilization and decreased the ratio of pyruvate used to $^{14}CO_2$ incorporated. This decrease in pyruvate utilization in the presence of L-octanoylcarnitine was a result of competition of pyruvate oxidase and the fatty acid oxidase system for CoA.[19,24] Addition of 0·6 mM DPTH decreased pyruvate utilization from 545·8 to 379·1 nmoles, $^{14}CO_2$ incorporation from 206·2 to 89·5 nmoles, and increased the ratio of pyruvate used to $^{14}CO_2$ incorporated from 2·6 to 4·3.

Addition of 0·6 mM DPTH and 0·7 mM OC further decreased pyruvate utilization from 395·8 nmoles (in the presence of OC alone) to 291·2 nmoles. The $^{14}CO_2$ incorporation was 89·5 nmoles, the same as with DPTH alone. The effect of MMI with and without OC on pyruvate metabolism was minimal.

TABLE 3. METABOLISM OF L-OCTANOYLCARNITINE IN INTACT, SONICATED, FROZEN-THAWED AND DIGITONIN-TREATED RAT LIVER MITOCHONDRIA IN THE PRESENCE OF 5,5-DIPHENYL-2-THIOHYDANTOIN (DPTH)*

Addition to system and treatment (mM)	Metabolite changes (nmoles/mg N/min)			
	Acetoacetate found	β-hydroxybutyrate found	Total ketones	Ratio β-hydroxybutyrate/ acetoacetate
Intact, none	20·96	38·75	59·71	1·84
	24·06	45·62	69·68	1·89
DPTH (1·2)	7·81	4·18	11·99	0·53
	5·62	3·18	8·80	0·56
Sonicated,† none	21·34	5·18	26·52	0·24
	21·50	5·50	27·00	0·26
DPTH (1·2)	0·96	1·56	2·52	1·61
	0·68	1·56	2·24	2·30
Frozen-thawed,‡ none	23·62	22·90	46·52	0·97
	24·53	20·40	44·93	0·83
DPTH (1·2)	6·40	3·09	9·49	0·49
	4·84	3·93	8·77	0·88
Digitonin,§ none	20·68	2·81	22·49	0·13
	16·84	3·68	20·52	0·21
DPTH (1·2)	1·96	0·81	2·77	0·41
	1·31	0·50	1·81	0·38

* The reaction mixture was the same as that described in Table 2, except that 0·67 mM L-octanoylcarnitine was used as substrate.
† Sonicated for 90 sec.
‡ Frozen for 20 min and thawed under running cold water.
§ Digitonin was added at 0·05% (w/v) to mitochondria for 5 min before incubation.

The estimation of pyruvate carboxylation based on $^{14}CO_2$ incorporation is minimal because labeled $^{14}CO_2$ may be lost by oxidative decarboxylation during forward metabolism in the tricarboxylic acid (TCA) cycle. In addition, unlabeled CO_2 produced from oxidation of pyruvate[20] may be fixed into organic acids. Thus, the observed changes in $^{14}CO_2$ incorporation may be influenced by the rates of pyruvate oxidation and by the rates of TCA cycle decarboxylation reactions. A more accurate way to estimate the amount of pyruvate carboxylated is by summing all products synthesized (malate, citrate, fumarate and succinate) as previously described.[23] When pyruvate carboxylase activity was estimated by summing up total products formed, DPTH inhibited carboxylation by 43 per cent in the absence of OC and by 47 per cent in the presence of OC.

Effect of succinate and 2,4-*dinitrophenol* (*DNP*) *on DPTH inhibition of* L-*octanoylcarnitine oxidation by rat liver mitochondria.* Table 5 shows the effects of succinate and DNP on ketone body accumulation from fatty acid oxidation, in the presence of pyruvate. DPTH significantly inhibited ketone accumulation (187·38–52·41 nmoles; $P < 0·01$) and decreased β-OH/AcAc ratios from 1·47 to 0·60 ($P < 0·001$). Succinate,

6·6 mM, in the absence of DPTH did alter significantly ketone body accumulation. However, the redox ratio was significantly ($P < 0.001$) increased (1·47–2·65). In the presence of DNP there was a large increase in the amount of acetoacetate found ($P < 0.001$) and a decrease in the redox ratio from 1·47 to 0·36. However, the total

TABLE 4. EFFECT OF 5,5-DIPHENYL-2-THIOHYDANTOIN (DPTH) AND METHIMIDAZOLE (MMI) ON PYRUVATE METABOLISM BY RAT LIVER MITOCHONDRIA*

Addition to system (mM)	Metabolite changes (nmoles/mg N/min)				
	Pyruvate used	$^{14}CO_2$ incorporated	Malate found	Citrate found	Ratio pyruvate used/ $^{14}CO_2$ incorporated
None	545·8	206·2	131·2	85·4	2·6
OC (0·7)	395·8	172·9	137·5	58·3	2·2
DPTH (0·6)	379·1	89·5	81·2	43·7	4·3
DPTH (0·6), OC (0·7)	291·2	89·5	66·6	33·3	3·1
MMI (1·43)	525·0	175·0	116·6	91·6	3·0
MMI (1·43), OC (0·7)	420·8	185·4	135·4	64·5	2·2

* The reaction mixture contained 10 mM pyruvate, 3·3 mM ATP, 10 mM $MgSO_4$, 13·3 mM $KH^{14}CO_3$, 13·3 mM potassium phosphate and 13·3 mM potassium triethanolamine buffers, pH 7·3, 5 mg of fatty acid-poor albumin and 1·0 mM L-octanoylcarnitine (OC). Mitochondria from 0·5 g liver were suspended in 0·5 ml of 0·25 M sucrose and contained 3·2 mg nitrogen. The final volume was 3·0 ml adjusted with 0·7–1·2 ml of 0·154 M KCl. The incubation time was 15 min.

TABLE 5. EFFECT OF 5,5-DIPHENYL-2-THIOHYDANTOIN, SUCCINATE AND 2,4-DINITROPHENOL (DNP) ON FATTY ACID METABOLISM IN RAT LIVER MITOCHONDRIA*

Addition to system (mM)	Metabolite changes (nmoles/mg N/min)			
	Acetoacetate found	β-Hydroxybutyrate found	Total ketones found	Ratio β-hydroxybutyrate/acetoacetate
None	78·47 ± 10·74	108·91 ± 0·58	187·38 ± 11·08	1·47 ± 0·20
DPTH (0·6)	32·83 ± 1·37	19·58 ± 1·16	52·41 ± 0·10	0·60 ± 0·06
Succinate (6·6)	46·10 ± 0·46	122·22 ± 2·31	168·32 ± 2·37	2·65 ± 0·06
DNP (0·1)	129·44 ± 3·33	46·10 ± 2·30	175·54 ± 5·31†	0·36 ± 0·01
DPTH (0·6), Succinate (6·6)	13·24 ± 1·09	19·01 ± 0·58	32·25 ± 1·08	1·21 ± 0·23†
DPTH (0·6), DNP (0·1)	67·98 ± 1·82†	28·83 ± 2·74	96·81 ± 1·24	0·43 ± 0·05
DPTH (0·1), DNP (0·1), Succinate (6·6)	10·94 ± 0·74	19·58 ± 0·67	30·52 ± 1·37	1·56 ± 0·31†

* The reaction mixture contained 8·4 mM pyruvate, 3·3 mM ATP, 10 mM $MgSO_4$, 13·3 mM $KHCO_3$, 13·3 mM potassium phosphate and 13·3 mM potassium triethanolamine buffers, pH 7·3, 1 mM L-octanoylcarnitine and 5 mg fatty acid-poor albumin. Mitochondria from 0·5 g liver were suspended in 0·25 M sucrose and contained 2·4 mg nitrogen. The final volume was 3·0 ml adjusted with 0·7–1·2 ml of 0·15 M KCl. Mitochondria were from four different animals. All values are means ± S.E.M. The significance was determined by Student's *t*-test. All the means are significantly different (P 0·025), except those indicated.

† Not significant.

TABLE 6. RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF SUBSTRATES BY RAT LIVER MITOCHONDRIA IN THE PRESENCE OF 5,5-DIPHENYL-2-THIOHYDANTOIN (DPTH)*

Substrate (mM)	Qo_2 (mμmoles O_2/mg P/min) State 3 Without DPTH	State 3 With DPTH	State 4 Without DPTH	State 4 With DPTH	Ratio state 3/state 4 Without DPTH	Ratio state 3/state 4 With DPTH	Ratio ADP/O Without DPTH	Ratio ADP/O With DPTH	Qo_2 (mμmoles O_2/mg P/min) (DNP 0·1 mM) Without DPTH	Qo_2 (mμmoles O_2/mg P/min) (DNP 0·1 mM) With DPTH
Pyruvate (10)	34·7 ± 2·6	20·3 ± 1·8 ($P<0·005$)	8·7 ± 0·7	8·3 ± 0·2 (NS)	4·10 ± 0·15	2·36 ± 0·25 ($P<0·001$)	2·30 ± 0·02	2·04 ± 0·05 ($P<0·05$)	40·3 ± 3·3	22·8 ± 2·7 ($P<0·005$)
α-Ketoglutarate (10)	25·0 ± 0·3	8·7 ± 0·6 ($P<0·001$)	4·7 ± 0·2	3·2 ± 0·2 ($P<0·005$)	5·51 ± 0·18	2·95 ± 3·5 ($P<0·001$)	2·81 ± 0·03	2·08 ± 0·07 ($P<0·001$)	25·5 ± 0·3	9·7 ± 0·5 ($P<0·005$)
Succinate (60)	82·0 ± 3·8	52·5 ± 2·5 ($P<0·001$)	29·3 ± 1·7	24·2 ± 2·2 (NS)	2·85 ± 0·24	2·23 ± 0·16 ($P<0·001$)	1·57 ± 0·03	1·35 ± 0·02 ($P<0·005$)	78·5 ± 6·6	62·4 ± 3·7 ($P<0·05$)
α-Glycerophosphate (15)	26·8 ± 2·1	21·5 ± 1·0 ($P<0·05$)	9·5 ± 0·5	10·5 ± 0·5 (NS)	2·81 ± 0·19	2·08 ± 0·09 ($P<0·005$)	2·24 ± 0·04	1·96 ± 0·07 ($P<0·025$)	23·4 ± 1·1	19·5 ± 0·5 ($P<0·05$)

* The reaction mixture contained pyruvate (10 mM) plus malate (1 mM) or succinate (60 mM) or α-ketoglutarate (10 mM) or α-glycerophosphate (15 mM), Tris buffer (20 mM), EDTA (1 mM), KCl (15 mM), $MgCl_2$ (5 mM), potassium phosphate (30 mM) and ADP (0·36 mM). The final volume of reaction mixture in the polarographic chamber was 2·0 ml at pH 7·3. Values are means ± S.E.M. NS = not significant; n = 8.

ketone body accumulation did not differ from that of controls. Addition of succinate with DPTH did not reverse the DPTH inhibition of OC oxidation to ketones. In the presence of both DPTH and DNP, the total ketones found were higher than with DPTH alone, but not so high as those of control group A. The redox state ratio remained low. The combination of succinate, DPTH and DNP suppressed the total ketones formed by 84 per cent.

TABLE 7. RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF PYRUVATE AND SUCCINATE BY RAT LIVER MITOCHONDRIA IN THE PRESENCE OF 5,5-DIPHENYL-2-THIOHYDANTOIN (DPTH) AND PENTABARBITOL*

	State 3					
	Oo_2 (mmoles O_2/mg P/min)			Ratio ADP/O		
Substrate (mM)	Without DPTH	With DPTH†		Without DPTH		With DPTH†
Pyruvate (10)	34·6 ± 2·6	20·3 ± 1·8	(P < 0·001)	2·30 ± 0·02	(P < 0·05)	2·04 ± 0·05
Pyruvate (10) + pentabarbitol	17·3 ± 0·4‡	18·3 ± 0·6	(NS)	2·22 ± 0·03	(P < 0·005)	1·90 ± 0·02
Succinate (60)	82·0 ± 3·8	52·5 ± 2·5	(P < 0·001)	1·57 ± 0·03	(NS)	1·50 ± 0·02
Succinate (60) + pentabarbitol	84·0 ± 1·0	50·3 ± 0·8	(P < 0·001)	1·50 ± 0·04	(P < 0·05)	1·36 ± 0·02

* Same reaction mixture as that described in Table 6.
† DPTH (0·6 mM).
‡ P < 0·001, significance level between pyruvate and pyruvate plus pentabarbitol.

Effect of DPTH on respiration and oxidative phosphorylation of pyruvate, α-ketoglutarate, succinate and α-glycerophosphate by rat liver mitochondria. Table 6 illustrates the effect of DPTH on respiration of rat liver mitochondria in the presence of various substrates. The state 3 respiration rates were significantly decreased with all four substrates studied. The respiratory rate in state 4* was significantly decreased with α-ketoglutarate as substrate (P < 0·005) in the presence of DPTH, whereas with pyruvate, succinate and α-glycerophosphate there was no significant difference. Highly significant decreases were observed in the respiratory control ratios with all substrates (Table 6). The ratio of ADP/oxygen was slightly, but significantly, decreased with all substrates in the presence of DPTH (Table 6).

Results in Table 7 show the state 3* respiration rates and ratio of ADP/oxygen* in the presence of DPTH with and without added pentabarbitol. With pyruvate as

* State 3 is the condition in which all required components are present and the respiratory chain itself is the rate-limiting factor. State 4 is the condition in which only ADP is lacking. The ratio of state 3 to state 4 is defined as the respiratory control index. This ratio is a more sensitive criterion of the intactness of mitochondrial structure than a high phosphorus/oxygen ratio.[25] The ADP/O ratio is equivalent to the phosphorus/oxygen ratio.

substrate, pentabarbitol caused no further decrease in state 3 oxygen consumption than did DPTH. Pentabarbitol did not inhibit succinate oxidation as DPTH did. With or without pentabarbitol in the system, the ratio of ADP/oxygen was significantly lower in the presence of DPTH with pyruvate as substrate.

Experiments in vivo

Effect of DPTH on α-glycerophosphate metabolism by rat liver mitochondria. Table 8 shows that mitochondria isolated from rats fed dietary DPTH for a total of 39 days almost completely inhibited the conversion of α-glycerophosphate to dihydroxyacetone phosphate. This inhibitory effect by DPTH *in vivo* is in good agreement with results obtained *in vitro* in Table 1.

TABLE 8. EFFECT OF DIETARY 5,5-DIPHENYL-2-THIOHYDANTOIN (DPTH) ON α-GLYCEROPHOSPHATE METABOLISM IN RAT LIVER MITOCHONDRIA

Treatment	Dihydroxyacetone phosphate found (nmoles/mg/min)
Control (5)	46·04 ± 1·45
DPTH-treated (5)*	0·021 ± 0·062

* Animals were kept on a 0·06% DPTH diet for 24 days and for 15 additional days on a 0·12% DPTH diet prior to sacrificing. Same reaction mixture as that described in Table 4, except that α-glycerophosphate (10 mM) instead of pyruvate was used as substrate.

TABLE 9. EFFECT OF DIETARY 5,5-DIPHENYL-2-THIOHYDANTOIN (DPTH) ON OXYGEN CONSUMPTION BY RAT TISSUE SLICES WITH GLUCOSE AS SUBSTRATE

Treatment	Oxygen consumed (μmoles O_2/g dry wt./min)
Control (7)	4·21 ± 0·29 ($P < 0·001$)
DPTH-treated (9)*	2·51 ± 0·15

* Animals were kept on a 0·10% DPTH diet for 2 weeks. Slices weighing 60–80 mg were then placed in Warburg flasks containing 3 ml of Krebs–Ringer phosphate solution and 10 mM glucose as substrate. The pH of the solutions was adjusted with phosphate buffer to 7·3. Flasks were placed in a 37° water bath, shaken and gassed with 100% oxygen for 5 min. Side-arms were then closed and measurement of oxygen consumption was commenced 10 min after the flasks were placed in the water bath. The oxygen consumption was resumed for 1 hr.

Inhibition of oxygen consumption by rat liver tissue slices from DPTH-fed rats. Results in Table 9 show that there is a large decrease in oxygen consumption from 4·21 ± 0·29 to 2·51 ± 0·15 μmoles O_2/g dry wt./min by liver tissue slices of DPTH-treated rats. This decrease in oxygen consumption by tissues from DPTH-treated animals may be due in part to decrease in respiration, as seen in Table 6 where DPTH was added to rat liver mitochondria.

DISCUSSION

The metabolic changes that are evoked by thyroid hormones are associated with the electron transport system, reactions in the tricarboxylic acid cycle and enzyme systems associated with generation and the transport of reducing equivalents between mitochondria and cytosol.

In the present study we have examined the effects of DPTH and MMI on sites in mitochondria that are known to be affected by thyroid hormones. MMI had no effect *in vivo* in blocking the peripheral action of thyroxine,* and was used in our studies to demonstrate that the compound was also inactive in the systems *in vitro* in comparison with DPTH, which has strong inhibiting effects.

Menahan and Wieland[26] have shown that in hypothyroid rats there was a large decrease in the activities of two key rate limiting gluconeogenic enzymes, pyruvate carboxylase and phosphoenolpyruvate carboxykinase.[27] Treatment of hypothyroid rats with thyroid hormones greatly increased the activities of pyruvate carboxylase and phosphoenolpyruvate carboxykinase.[3,28–30] In our studies (Table 10) we have also noted that feeding animals 0·10% DPTH diets for 2 weeks resulted in a large, significant decrease ($P < 0.001$) in pyruvate carboxylase and phosphoenolpyruvate carboxykinase activities, in addition to total inhibition of mitochondrial α-glycerophosphate dehydrogenase activity (Table 8) after 39 days of feeding of DPTH. The inhibition of these enzymes by DPTH is consistent with its peripheral antithyroid activity.

TABLE 10. EFFECT OF DIETARY 5,5-DIPHENYL-2-THIOHYDANTOIN (DPTH) ON LIVER GLUCONEOGENIC ENZYME ACTIVITY

Enzyme activity	Control (μmoles/g liver/min)	DPTH-treated* (μmoles/g liver/min)	P value
Pyruvate carboxylase (μmoles $^{14}CO_2$ fixed)	7·64 ± 0·60	2·83 ± 0·21	< 0·001
Phosphoenolpyruvate carboxykinase (μmoles $^{14}CO_2$ fixed)	1·57 ± 0·12	0·73 ± 0·11	< 0·001
Glucose 6-phosphatase (μmoles Pi liberated)	16·1 ± 0·51	11·80 ± 0·17	< 0·001
Fructose 1,6-diphosphatase (μmoles NADP reduced)	6·43 ± 0·89	4·66 ± 0·16	< 0·05

* Animals were fed a 0·1% DPTH diet for 2 weeks; n = 4.

Addition of DPTH *in vitro* greatly decreased mitochondrial synthesis of gluconeogenic precursors, as shown in Table 4 where carboxylation of pyruvate to dicarboxylic acids was markedly inhibited by DPTH.

Thyroid hormone may regulate hydrogen transfer to the cytochromes by acting on the cytosolic and mitochondrial α-glycerophosphate cycle. This was proposed by Bücher and Klingenberg[16] and by Estabrook and Sacktor.[13] In our studies, α-glycerophosphate dehydrogenase was strongly inhibited by DPTH *in vitro* (Table 1) and *in vivo* (Table 8).

* W. R. Ruegamer, unpublished observations.

The inhibition of fatty acid oxidation by DPTH, possibly at the β-hydroxybutyrate dehydrogenase step, appears to be an effect not related to its antithyroid properties since the rate of β-hydroxybutyrate oxidation decreased during thyroid hormone treatment while the dehydrogenase activity remained unchanged.[10] This aspect requires further investigation. Perhaps these observations indicate a new site for thyroxine action.

The stimulation of oxidative metabolism as represented by increased oxygen consumption is a characteristic of thyroid hormone administration.[6–10,15] It has been suggested that stimulation of mitochondrial respiration and basal metabolic rate by thyroid hormone is not the result of uncoupling of oxidative phosphorylation or of mitochondrial structural changes, but is possibly related to synthesis of respiratory chain components.[30–35] Thus, inhibition of state 3 respiration with pyruvate, α-ketoglutarate, succinate and α-glycerophosphate by DPTH as shown in Table 6 is consistent with the antithyroid effects of this compound, in accord with observations of Bronk[36] and Volfin *et al.*[35] which showed that both state 3 and state 4 respirations were greatly decreased in thyroidectomized animals.

The uncoupling of oxidative phosphorylation by DNP was significantly lower in the presence of DPTH. This suggests that DPTH may exert its inhibitory effect within the respiratory chain. We have shown that DPTH is a potent inhibitor (Table 2) of NAD-linked β-hydroxybutyrate dehydrogenase reaction and that it also inhibits respiration with both NAD^+ and FAD^+ linked substrates. It can be seen in Table 7 that, with pyruvate as substrate in the presence of pentabarbitol, DPTH did not further inhibit state 3 respiration, but the ADP/O ratio (Table 7) was decreased to a larger degree with both pentabarbitol and DPTH in the system. In the presence of both pentabarbitol and DPTH, state 3 respiration of succinate was inhibited to the same extent as with DPTH alone, while the ADP/oxygen ratio was decreased in the presence of DPTH but not pentabarbitol. From these studies it can be concluded that DPTH may interact at more than one phosphorylation site in the respiratory chain.

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