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THE INFLUENCE OF THYROXINE ON OXIDATIVE PHOSPHORYLATION AND RELATED REACTIONS

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SUMMARY

Studies on the influence of thyroxine on oxidative phosphorylation and associated reactions in submitochondrial particles have revealed several new thyroxine effects. The rate of the ATP–^{32}P exchange reaction was reduced by thyroxine and this inhibition was doubled by adding the thyroxine to the incubation medium after the particles rather than before. The increased inhibition of the exchange reaction was associated with a reduction in P/O ratios and a loss of the stimulatory effect of thyroxine on substrate oxidation.

The improvement in P/O ratios in the presence of thyroxine reported earlier has now been shown to be largely due to an inhibition of the ATPase activity of the particles.

The previously reported stimulation of substrate oxidation by thyroxine has been shown to be more pronounced at lower particle concentrations. At 12° this stimulatory effect was masked by the slow rate of the terminal phosphate-transferring reactions unless phosphorylation was uncoupled by 2,4-dinitrophenol.

Thyroxine also increased the absorbancy of the submitochondrial particle suspensions, but this effect appeared to be unrelated to any of the other thyroxine effects.

These data suggest that inhibition of the terminal phosphate-transferring reactions by thyroxine and some of its analogues may be responsible for the reduction in phosphorylation efficiency by these compounds. Depending upon the type of preparation used and on the incubation conditions these inhibitory effects may mask the stimulation of substrate oxidation.

INTRODUCTION

Many studies have suggested that there is an association between the effects of the thyroid hormones on oxidative phosphorylation *in vitro* and the action of these hormones *in vivo*. However, the results obtained with isolated mitochondria have not been consistent[1]. Some investigators have reported uncoupling by thyroxine[2,3], some have found no effects[4] and others have reported a stimulation of oxidation[5,6]. In this laboratory the influence of thyroxine on oxidative phosphorylation has been studied with submitochondrial particles[4,7,8]. This work established that thyroxine stimulated the rate of oxidation of succinate or DPNH and improved the P/O ratios

obtained. These stimulatory effects were always found when thyroxine was added to the incubation medium prior to the submitochondrial particles. However, when the order of additions was reversed so that the thyroxine was added to the medium after the particles, its effects on oxidative phosphorylation were markedly altered. Under such conditions thyroxine reduced P/O ratios and did not increase the oxidation rate. The data in this paper provide an explanation for the change in thyroxine effects which result from reversing the order of additions. Thyroxine has been found to inhibit the exchange of inorganic phosphate with the terminal phosphate of ATP. The extent of the inhibition was doubled by adding the thyroxine to the incubation mixture after the submitochondrial particles. Thus, the inhibition of phosphorylation which occurs under similar conditions may be due to a slowing of the terminal phosphate-transferring reactions.

As well as establishing an inhibitory effect of thyroxine on the ATP–^{32}P exchange, the present study has emphasized the distinction between the effects of thyroxine on oxidative phosphorylation and those of 2,4-dinitrophenol. Although both compounds under appropriate conditions can reduce P/O ratios and inhibit the ATP–^{32}P exchange process, DNP accelerates ATPase activity while thyroxine has been found to inhibit ATPase activity. This inhibition of ATPase activity by thyroxine is probably largely responsible for the increases in P/O ratio in the presence of thyroxine which were previously reported[4].

The data in this paper also indicate that thyroxine cannot stimulate oxidation at low temperatures unless 2,4-dinitrophenol is present to uncouple phosphorylation. These findings suggest that no stimulation of oxidation by thyroxine can be observed under conditions in which the terminal phosphate-transferring reactions are rate-limiting for oxidative phosphorylation[9].

Studies by PACKER AND TAPPEL[10] indicated, for suspensions of another type of mitochondrial fragments, that a relationship existed between absorbancy changes and the degree of coupling of oxidative phosphorylation. Thyroxine was found to increase the absorbancy of suspensions of the particles used in this laboratory, but the changes did not coincide with any of the other thyroxine effects.

Thus, the results presented below indicate that thyroxine influences the component reactions of the oxidative phosphorylation system in at least four distinct ways. These findings make it possible to explain some of the apparently conflicting reports in the literature.

METHODS AND MATERIALS

Submitochondrial particles were prepared by sonic disintegration of rat-liver mitochondria according to the method of KIELLEY AND BRONK[11]. The particles were resuspended in 0.03 M phosphate (pH 7.0) to give a stock suspension of 2 to 4 mg N/ml from which an aliquot was taken with a calibrated micropipette for each incubation.

For measurement of oxygen consumption the Clark oxygen electrode was used and the output was recorded linearly, as previously described[11]. With submitochondrial particles, phosphorylation was measured by estimating the incorporation of ^{32}P into ATP as before[11].

The rate of the ATP–^{32}P exchange process was measured according to the method

of BRONK AND KIELLEY[9]. ATPase activity was measured as the appearance of inorganic phosphate.

The measurements of absorbancy were made at 410 mμ with either a Beckman DU spectrophotometer or with a Zeiss spectrophotometer in 1-cm cuvettes.

All biochemicals were obtained commercially. L-Thyroxine was used as the sodium salt. L-Isomers of all thyroxine analogues were used with the exception of D,L-thyronine.

RESULTS

The influence of thyroxine on the rate of substrate oxidation

Fig. 1 shows the relationship between the stimulation of oxidation by thyroxine and the concentration of submitochondrial particles. For both substrates the percentage increase with thyroxine was larger at the lower levels of particle nitrogen. This increased effectiveness of thyroxine coincided with a decline in the oxidative activity/mg N in the controls. In fact, Part A indicates that in the presence of

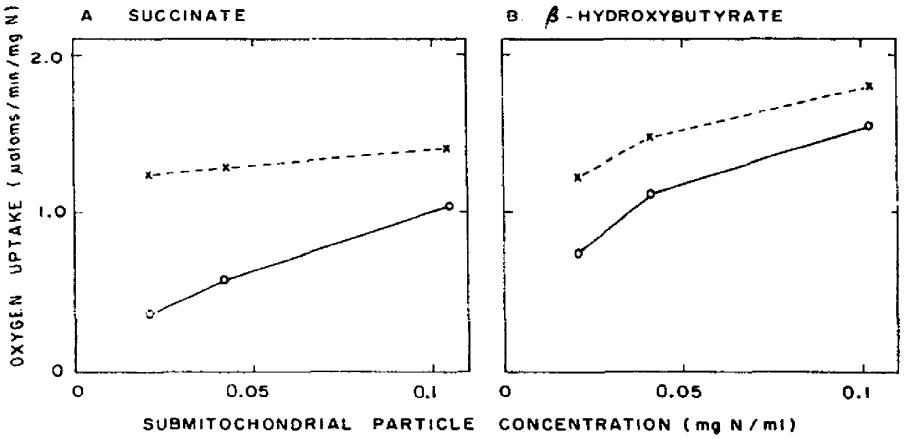

Fig. 1. The influence of thyroxine on the oxidation of succinate and β-hydroxybutyrate by submitochondrial particles. O—O, Control incubations without thyroxine; ×—×, incubations in the presence of $2.5 \cdot 10^{-5}$ M thyroxine. Conditions: 10 μmoles ADP, 10 μmoles AMP, 10 μmoles $MgCl_2$, 20 μmoles phosphate (pH 7.0) 10^6 counts/min ^{32}P, 5 μmoles succinate or 20 μmoles β-hydroxybutyrate and 2 μmoles DPN^+ in 1.9 ml total volume at 38°.

thyroxine the oxidative activity with succinate was almost proportional to particle nitrogen. Part B shows that the thyroxine effect was smaller with β-hydroxybutyrate, and that, even with thyroxine, the oxidative activity declined disproportionately at low nitrogen levels.

The data in Fig. 1 were obtained at 38°. Table I shows that the influence of thyroxine on succinate oxidation was less pronounced at 28°, while at 12° thyroxine produced no change in the oxidation rate. Table I also indicates that in the presence of DNP thyroxine did stimulate succinate oxidation markedly at the lower temperatures. Thus, the stimulation of succinate oxidation by thyroxine did not depend on coupled phosphorylation. Furthermore, when oxidative phosphorylation was uncoupled by 2,4-dinitrophenol, the thyroxine effect on succinate oxidation was less temperature dependent.

TABLE I

THE INFLUENCE OF THYROXINE AND 2,4-DINITROPHENOL ON THE RATE OF SUCCINATE OXIDATION AT VARIOUS TEMPERATURES

Conditions: 10 μmoles ADP, 10 μmoles AMP, 10 μmoles $MgCl_2$, 20 μmoles phosphate (pH 7.0) 10^6 counts/min ^{32}P, 5 μmoles succinate, approx. 0.21 mg particle nitrogen added per incubation vessel. Incubation period 2–8 min; 1.9 ml total volume.

Temperature	Final concentration of dinitrophenol present	Final concentration of thyroxine present	Oxygen uptake	
			(μatoms/min)	Control (%)
12°	None	None	0.018	—
12°	None	$2.5 \cdot 10^{-5}$ M	0.018	100
12°	$2 \cdot 10^{-4}$ M	None	0.022	122
12°	$2 \cdot 10^{-4}$ M	$2.5 \cdot 10^{-5}$ M	0.026	145
28°	None	None	0.068	—
28°	None	$2.5 \cdot 10^{-5}$ M	0.086	126
28°	$2 \cdot 10^{-4}$ M	None	0.080	118
28°	$2 \cdot 10^{-4}$ M	$2.5 \cdot 10^{-5}$ M	0.106	156
38°	None	None	0.122	—
38°	None	$2.5 \cdot 10^{-5}$ M	0.192	157
38°	$2 \cdot 10^{-4}$ M	None	0.128	105
38°	$2 \cdot 10^{-4}$ M	$2.5 \cdot 10^{-5}$ M	0.202	165

TABLE II

THE INFLUENCE OF THYROXINE ON ATPase, AND PHOSPHORYLATION AS A FUNCTION OF TEMPERATURE

Conditions: For ATPase: 5 μmoles ATP, 5 μmoles ADP, 6 μmoles phosphate (pH 7.0). For phosphorylation see the legend of Table I. Thyroxine added where indicated to give a final concentration of $2.5 \cdot 10^{-5}$ M. Incubation time 1–3 min, 1.9 ml total volume. The reaction was initiated by the addition of $MgCl_2$ for ATPase and of succinate for phosphorylation. In all cases the particles were equilibrated with the incubation medium for 30 sec before the reaction was started. Each activity was measured with a separate particle preparation.

mg N/ml	Measurement	At 28°			At 38°		
		Control	Incubation in the presence of thyroxine		Control	Incubation in the presence of thyroxine	
				Control (%)			Control (%)
0.106	ATPase activity (μmoles ATP split/min)	0.32	0.32	100	(0.80)	0.71	89
0.042	ATPase activity	0.21	0.16	76	(0.50)	0.32	64
0.102	P/O ratio	0.56	0.62	111	(0.66)	0.73	122
0.041	P/O ratio	0.48	0.58	121	(0.45)	0.62	138

The influence of thyroxine on ATPase and phosphorylation efficiency

Fig. 2 shows that at 38° thyroxine inhibited the ATPase activity of the submitochondrial particles. However, it is also clear that, in the controls, as the level of particles was reduced the ATPase activity/mg N increased. The presence of $2.5 \cdot 10^{-5}$ M thyroxine prevented this increase. These findings suggested that the increases in P/O ratio caused by thyroxine at low particle levels could be largely due to the suppression of ATPase activity by thyroxine. Table II shows a comparison

of the effects of thyroxine on ATPase activity and P/O ratio. At both 28° and 38°, the ATPase activity increased disproportionately as the particle concentration was reduced, while the P/O ratio decreased. At 38° thyroxine inhibited the ATPase activity at both particle levels, but at 28° the ATPase activity was inhibited only at the lower particle level. On the other hand, thyroxine increased the P/O ratio in all four sets of conditions. Thus, the data in Table II indicate that the inhibition of ATPase activity was probably not entirely responsible for the improvement in P/O ratio in the presence of thyroxine.

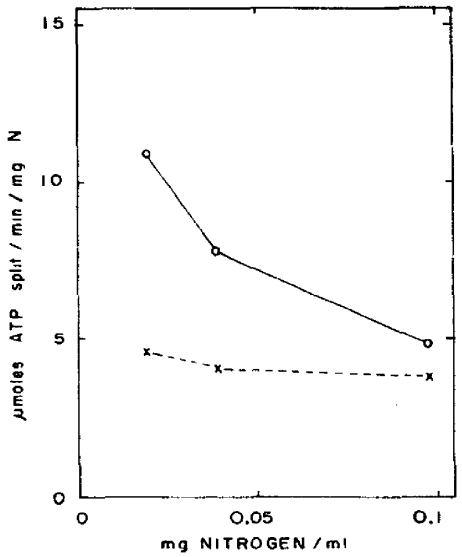

Fig. 2. The influence of thyroxine on the ATPase activity of submitochondrial particles. O—O, Control incubations without thyroxine; ×—×, incubations in the presence of $2.5 \cdot 10^{-5}$ M thyroxine. Conditions: 5 μmoles ATP, 5 μmoles ADP, 6 μmoles phosphate (pH 7.0) in 1.9 ml total volume at 38°.

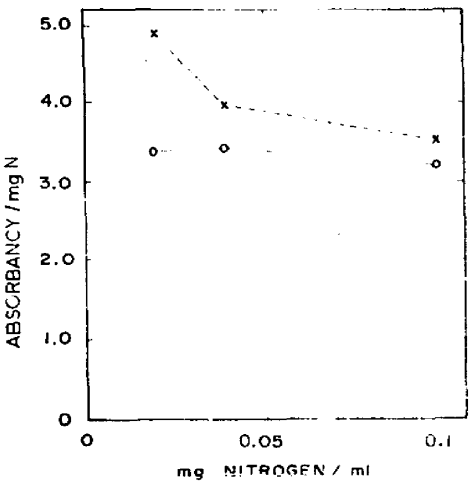

Fig. 3. The absorbancy of the submitochondrial particle suspension in the presence and absence of thyroxine. O—O, The absorbancies of control suspensions at 410 mμ in the absence of thyroxine; ×—×, absorbancies in the presence of $2.5 \cdot 10^{-5}$ M thyroxine. Conditions: same as described in the legend for Fig. 1. The absorbancy increased for several minutes after addition of the particles, and in each case the maximum value was reported. The absorbancies in the presence of thyroxine were corrected for the changes in absorbancy caused by the addition of thyroxine alone.

The influence of thyroxine on the ATP–^{32}P exchange reaction

The data in Table III indicate that thyroxine inhibited the incorporation of ^{32}P into ATP. Approximately the same percentage inhibition was obtained over a three-fold range of particle concentrations. Column B shows that, if the order of additions was reversed so that the thyroxine was added to the incubation mixture after the submitochondrial particles, the percentage inhibition was approximately doubled. The reason for the increased inhibitory effect of thyroxine when it was added to the incubation medium after the particles is not known. However, one possibility is suggested by the work of LISSITZKY, ROQUES AND BENEVENT[12] which demonstrated that a large proportion of the thyroxine molecules in an aqueous solution at neutral pH may become bound to the glass container. In the present ex-

TABLE III

THE INFLUENCE OF THE ORDER OF ADDITIONS ON THE INHIBITION OF THE ATP–^{32}P EXCHANGE BY THYROXINE

Conditions: 5 μmoles ATP, 5 μmoles ADP, 20 μmoles phosphate (pH 7.0) 10^6 counts/min ^{32}P. Column A: reaction started with $MgCl_2$ added to incubation medium 30 sec after particles: Column B: thyroxine added 90 sec after particles, reaction started with $MgCl_2$ added after an additional 30 sec. 0.036, 0.077 and 0.189 mg particle nitrogen added per incubation with preparations 1, 2 and 3 respectively; incubation time 2.5 min with preparations 1 and 2; and 1 min with preparation 3.

Preparation	Final concentration of thyroxine present	$MgCl_2$ (μmoles/ml)	A		B	
			Thyroxine added before particles		Thyroxine added after particles	
			μmoles phosphate exchanged/min	Control (%)	μmoles phosphate exchanged/min	Control (%)
1	0	5	0.060	—	0.056	—
	$5 \cdot 10^{-7}$ M	5	0.060	100	0.059	105
	$2.5 \cdot 10^{-6}$ M	5	0.058	97	0.054	96
	$1 \cdot 10^{-5}$ M	5	0.053	88	0.028	50
	$2.5 \cdot 10^{-5}$ M	5	0.036	60	0.018	32
	$1 \cdot 10^{-4}$ M	5	0.029	48	0.004	7
2	0	5	0.085	—	0.082	—
	$2.5 \cdot 10^{-5}$ M	5	0.058	68	0.035	43
	0	10	0.078	—	0.069	—
	$2.5 \cdot 10^{-5}$ M	10	0.054	69	0.022	32
3	0	5	0.194	—	0.174	—
	$2.5 \cdot 10^{-6}$ M	5	0.139	72	0.095	55
	$5 \cdot 10^{-5}$ M	5	0.119	61	0.058	33

periments the thyroxine which was added before the particles may have been largely bound to the glass, whereas that added after the particles may have become bound to the particles.

Table III also shows that increasing the $MgCl_2$ concentration did not reduce the inhibitory effect of the thyroxine.

The influence of thyroxine on the absorbancy of the particle suspension

Fig. 3 shows that the absorbancy/mg N was unaffected by dilution of the submitochondrial particle suspension over a five-fold range. The addition of $2.5 \cdot 10^{-5}$ M thyroxine altered this picture considerably, however. Thyroxine increased the absorbancy slightly at a particle level of 0.1 mg N/ml, but at 0.02 mg N/ml the increase amounted to about 50% of the control. The significance of the effect of thyroxine on absorbancy is not known, but it does not appear to be related to any of the other thyroxine effects in either direction or extent.

The relationship between thyroxine concentration and its effects on submitochondrial particles

In Fig. 4 the relative changes in the various measurements made with submitochondrial particles are plotted against the log of the molar concentration of thyroxine. The data indicate that for all of the measurements except absorbancy, the lowest effective concentration of thyroxine was $5 \cdot 10^{-6}$ M and that the thyroxine

effects were only slightly increased by raising the concentration from $2.5 \cdot 10^{-5}$ M to $5 \cdot 10^{-5}$ M. On the other hand, thyroxine had little effect on absorbancy below $2.5 \cdot 10^{-5}$ M but raising the concentration from $2.5 \cdot 10^{-5}$ M to $5 \cdot 10^{-5}$ M more than tripled the increase in absorbancy.

If the percent change at $1 \cdot 10^{-5}$ M thyroxine is compared with the percent change at $5 \cdot 10^{-5}$ M thyroxine, one can calculate that, for oxidative rate, P/O ratio,

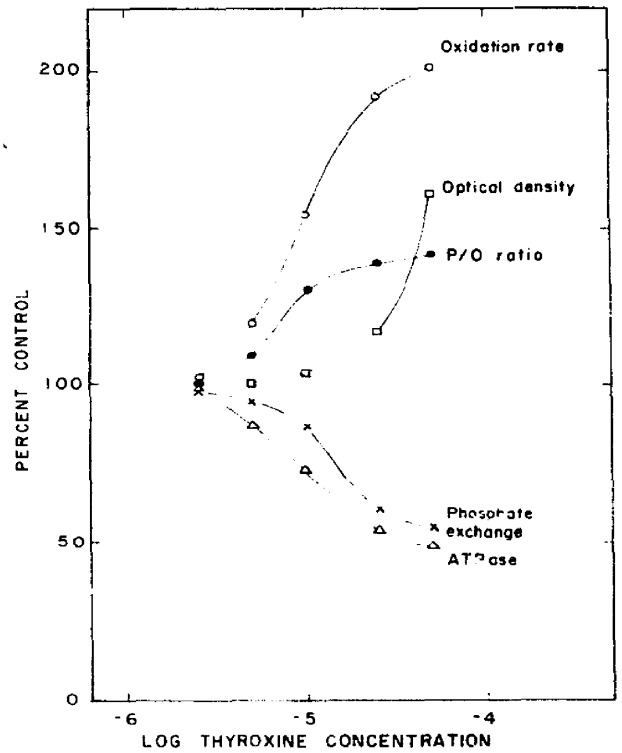

Fig. 4. The relationship between the molar concentration of thyroxine and the various measurements made with submitochondrial particles. The points at each thyroxine concentration were obtained by calculating the ratio of the value obtained in the presence of thyroxine to that of the control and multiplying by 100. Each point represents the average of such ratios obtained with two separate particle preparations. Except for the measurements of oxidative rate and P/O ratio, which were made in the same experiments, each of the activities was measured using separate particle preparations. ○—○, Rate of succinate oxidation; ●—●, P/O ratio with succinate; □—□, absorbancy at 410 mμ; ×—×, rate of ATP–^{32}P exchange; △—△, ATPase activity. For each incubation approximately 0.08 mg of submitochondrial particle nitrogen was used in a final volume of 1.9 ml at 38°; for other conditions see the legends of Figs. 1 to 3, and Tables II and III.

and ATPase activity, the percent change at the lower thyroxine concentration was between $^1/_2$ and $^2/_3$ of that at the higher concentration. For ATP–^{32}P exchange on the other hand, the percent change at $1 \cdot 10^{-5}$ M was less than $^1/_3$ of the value at $5 \cdot 10^{-5}$ M. This fact suggests that the inhibition of exchange was distinct from the other thyroxine effects. Also it is clear that at the higher thyroxine concentrations the increase in oxidative rate and P/O ratio may be limited by a suppression of the terminal phosphate-transferring reactions.

TABLE IV

THE INFLUENCE OF THYROXINE ANALOGUES ON OXIDATION, PHOSPHORYLATION, ATP ^{32}P EXCHANGE AND ATPASE ACTIVITY

Conditions: Same as described in the legends of Tables I, II and III. 0.054 mg of particle nitrogen per incubation vessel. Thyroxine and each of the analogues were used at a final concentration of $5 \cdot 10^{-5}$ M at 38° in 1.9 ml total volume.

Addition	*Oxidation rate (μatoms/min)*		*P/O ratio*		*ATP ^{32}P exchange (μmoles phosphate exchanged/min)*		*ATPase (μmoles ATP split/min)*	
		Control (%)		*Control (%)*		*Control (%)*		*Control (%)*
None	0.032	—	0.38	—	0.070	—	0.68	—
Thyroxine	0.063	200	0.68	184	0.041	54	0.38	55
3,5,3′-Triiodothyronine	0.051	162	0.42	114	0.026	34	0.38	55
3,5-Diiodothyronine	0.038	121	0.37	100	0.033	44	0.47	68
Thyronine	0.032	102	0.36	97	0.074	98	0.60	87
Tetraiodothyroacetic acid	0.031	98	0.00	0	0.000	0	0.23	33
3,5,3′-Triiodothyroacetic acid	0.027	86	0.00	0	0.002	3	0.30	43
None	0.031	—	0.36	—	0.075	—	0.70	—

TABLE V

THE INFLUENCE OF THE ORDER OF ADDITIONS ON THE THYROXINE EFFECTS

Conditions: Same as indicated in the legends of Tables I, II and III for oxidation, phosphorylation, ATP-^{32}P exchange and ATPase; thyroxine added as indicated to give a final concentration of $2.5 \cdot 10^{-5}$ M. Absorbancy measurements made at 410 mμ. 0.030 and 0.055 mg particle nitrogen/ml for preparations 1 and 2 respectively. In column A the thyroxine was added immediately before the particles, and the reaction was started by the addition of substrate or $MgCl_2$ 30 sec later. In column B the thyroxine was added 90 sec after the particles and the reaction was started by $MgCl_2$ addition after an additional 30 sec.

Preparation	*Temperature*	*Measurement*	*A*			*B*		
			Control	*Thyroxine added before particles*		*Preincubated control*	*Thyroxine added after particles*	
					Control (%)			*Control (%)*
1	38°	Oxydative rate (μatoms/min)	0.052	0.081	156	0.050	0.052	104
	38°	Phosphorylation rate (μmoles ATP formed/min)	0.027	0.055	204	0.025	0.017	68
	38°	P/O Ratio	0.53	0.69	130	0.52	0.33	64
	38°	ATP-^{32}P exchange (μmoles phosphate exchanged/min)	0.085	0.058	68	0.082	0.035	43
	38°	ATPase (μmoles ATP split/min)	0.55	0.32	58	0.53	0.29	55
2	28°	ATP-^{32}P exchange (μmoles phosphate exchanged/min)	0.043	0.021	49	0.052	0.014	27
	28°	ATPase (μmoles ATP split/min)	0.19	0.15	79	0.18	0.14	78
	28°	Absorbancy	0.270	0.327	121	0.269	0.317	118

The influence of thyroxine analogues

The data in Table IV show that the presence of iodine atoms on the thyronine structure was essential in order to observe any of the effects described above on oxidative phosphorylation, ATP–^{32}P exchange or ATPase activity. Except for a slight inhibition of ATPase activity, thyronine itself had no effect. 3,5-Diiodothyronine was about 25% as effective as thyroxine for stimulating oxidation and it had no effect on the P/O ratio, in spite of its inhibition of ATPase activity. Its lack of effectiveness in stimulation of oxidative phosphorylation may be due to its striking inhibition of the ATP–^{32}P exchange reaction. Similar comments can be made about 3,5,3'-triiodothyronine which stimulated oxidation but also profoundly inhibited the ATP–^{32}P exchange process.

The acetic acid analogues of both thyroxine and 3,5,3'-triiodothyronine were reported earlier to inhibit phosphorylation markedly[7,8]. These results are confirmed by the data in Table IV, and it is clear that the inhibition of phosphorylation coincided with the complete inhibition of the ATP–^{32}P exchange. The fact that the acetic acid analogues inhibit ATPase activity as well as the phosphate exchange reaction and phosphorylation, indicates that they are acting as true inhibitors and not as uncoupling agents of the 2,4-dinitrophenol type.

A comparison of the various effects of thyroxine as a function of the sequence of additions

Table V shows the results of two representative experiments in which a number of thyroxine effects were studied with the same preparation. In each case incubations were carried out in which thyroxine was added to the incubation medium before and after the submitochondrial particles. These data show that $2.5 \cdot 10^{-5}$ M thyroxine added after the particles abolished the stimulation of oxidation rate and phosphorylation rate, and markedly inhibited the ATP–^{32}P exchange. However, altering the sequence of additions did not change the inhibition of ATPase activity or the increase in absorbancy caused by thyroxine. These findings confirm the lack of correlation between the changes in absorbancy and the thyroxine effects on oxidative phosphorylation and ATP–^{32}P exchange, and make it unlikely that the same inhibitory action of thyroxine was involved in suppressing the rate of ATP–^{32}P exchange and ATPase activity.

On the other hand, the results do establish that there was a clear association between the increased inhibition of the ATP–^{32}P exchange process that accompanied the addition of thyroxine after the particles, and the inhibition of phosphorylation. It is also apparent that the inhibition of phosphorylation was not a true uncoupling since it was not accompanied by an increase in oxidation rate or in ATPase activity.

Finally, it should be noted that Table V shows the inhibition of ATPase and ATP–^{32}P exchange by thyroxine under conditions in which the submitochondrial particles were added to an incubation medium which was complete except for $MgCl_2$. The $MgCl_2$ was added to start the reaction. If the order of additions was changed so that the particles were added to a medium containing phosphate and $MgCl_2$, and the reaction was started by the addition of nucleotides, the same inhibitory effects of thyroxine were observed. Thus, only the time of addition of the thyroxine altered its effects on the ATP–^{32}P exchange.

DISCUSSION

The various thyroxine effects described above can be considered together by reference to a proposed mechanism of oxidative phosphorylation[8,9]:

$$A_{(reduced)} + B_{(oxidized)} + X \longrightarrow A_{(oxidized)} + B_{(reduced)} + X^* \quad (1)$$

$$X^* \xrightarrow{(DNP)} X + \text{energy} \quad (2)$$

$$X^* + P \rightleftharpoons X \sim P \quad (3)$$

$$X \sim P + ADP \overset{Mg^{2+}}{\rightleftharpoons} ATP + X \quad (4)$$

In this formulation A and B are consecutive members of the electron transport chain; X is a hypothetical intermediate which can exist in a high energy form X*; and P represents inorganic phosphate. Eqn. 2 is responsible for at least some of the ATPase activity and for the low P/O ratios observed with the particles. Previous studies[9] indicated that Eqn. 2 was the site of 2,4-dinitrophenol action. In tightly coupled intact mitochondria Eqn. 2 is almost completely absent. The ATP–^{32}P exchange process is represented by the sum of Eqns. 3 and 4, while Eqn. 4 is presumably catalysed by the ADP exchange enzyme of WADKINS AND LEHNINGER[13] and requires Mg^{2+} (see ref. 9). In LEHNINGER's formulation[14] Eqn. 4 involves an additional step in which phosphate is transferred from X ~ P to the enzyme.

In terms of this mechanism the results presented above and those published earlier[4,8] indicate that the stimulation of oxidation occurs by an acceleration of Eqn. 1. This reaction is necessarily vague since the manner in which phosphorylation is coupled to electron transport is unknown. The data are equally consistent with a thyroxine effect on the coupling process, the dehydrogenases, or whatever other step may be rate-limiting for electron transport. However, Table I shows that thyroxine did not stimulate succinate oxidation when Eqn. 3 was rate-limiting unless 2,4-dinitrophenol was added to accelerate Eqn. 2. The data in Fig. 1 also suggest that some component essential for the oxidative system may be lost on dilution of the particles. Thyroxine produced a partial restoration of the lost activity.

The inhibition of ATPase activity probably occurred through a slowing of Eqn. 2. This inhibitory effect of thyroxine is directly opposite to that of 2,4-dinitrophenol and results in increased P/O ratios by raising the proportion of X* entering Eqn. 3.

The inhibition of ATP–^{32}P exchange was due to a slowing of the overall rate of Eqns. 3 plus 4. This inhibition was doubled by adding the thyroxine to the incubation medium after the particles rather than before. In terms of the above mechanism the inhibition of ATP–^{32}P exchange should cause lower P/O ratios under any conditions in which the rate of Eqn. 3 was slowed substantially more than the rate of Eqn. 2. Table III and Fig. 4 show that this condition was met at a thyroxine concentration of $1 \cdot 10^{-5}$ M, or more, added after the particles. Earlier data[4] indicated that at least $2.5 \cdot 10^{-5}$ M thyroxine added after the particles was required to lower the P/O ratios. These findings strongly suggest that the reductions in P/O ratio reported previously[4] and in Table V (column B) as the result of thyroxine addition were due to an inhibition of Eqns. 3 and 4. Table IV shows that the inhibitory effects of tetraiodothyroacetic acid and triiodothyroacetic acid[7] also resulted from a blocking of the ATP–^{32}P exchange rather than from a true uncoupling.

The observed increases in the absorbancy of the particle suspensions in the presence of thyroxine failed to coincide with any of the other thyroxine effects. Thus, it appears unlikely that the thyroxine effects on oxidative phosphorylation resulted from any gross physical changes in the particles.

Although some investigators using submitochondrial particles have failed to find thyroxine effects on oxidative phosphorylation[15], PENEFSKY, PULLMAN, DATTA AND RACKER[16,17] showed that 10^{-5} M 3,5,3'-triiodothyronine inhibited the soluble ATPase they found to be a component of the phosphorylation system of heart mitochondria. Their experiments also indicated that at a higher concentration of 3,5,3'-triiodothyronine P/O ratios and oxidative rate were reduced. The more highly fragmented particles used by PENEFSKY *et al.*[17] and this author may be physically more accessible to thyroxine than those prepared by TAPLEY *et al.*[15] using digitonin. Furthermore, it is clear that any variation in the relative activities of the component reactions of the oxidative phosphorylation system could alter the direction or extent of the resultant effect of thyroxine on the process.

Incubation conditions are also important in determining the overall thyroxine effect. This author missed both the inhibition of ATPase and of the ATP–^{32}P exchange in an earlier series of experiments[4] in which the reactions were initiated by the addition of the particles and no equilibration period was allowed.

The effect of thyroxine on the ATP–^{32}P exchange in submitochondrial particles strongly suggests that it is an inhibition of the terminal phosphate-transferring reactions which is responsible for the "uncoupling" effect of thyroxine in whole mitochondria. Evidence supporting this view will be published shortly.

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