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The Uptake of Thyroxine and Triiodothyronine by Rat-Liver Mitochondria

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In the previous paper (Klemperer, 1955) it was shown that the uncoupling of oxidative phosphorylation by L-thyroxine and L-triiodothyronine (Martius & Hess, 1951; Lardy & Feldott, 1951; Hoch & Lipmann, 1953) occurred only at relatively high concentrations, and after a latent period during which the mitochondria were preincubated with the iodo compound. L-Thyroxine was a more active uncoupling agent than L-triiodothyronine in contrast to the relative activities in the intact animal found by Gross & Pitt-Rivers (1953*b*). This paper describes quantitative studies of the uptake of L-thyroxine and L-triiodothyronine at concentrations considerably higher than those obtaining *in vivo*. The results are of interest in relation to the delayed action and relative activities of these two substances *in vivo* and *in vitro*, and in relation to the mechanism of their uptake by mitochondria.

EXPERIMENTAL

The general techniques and reagents were as described by Klemperer (1955) except that phosphate buffer was omitted from the sucrose homogenizing medium for preparing mitochondria. The solutions of iodo compound were made up as before in a 'serum medium' containing sheep serum with a total dry weight (serum proteins plus non-protein solutes) of 93 mg./ml. Trichloroacetic acid (TCA; 30% w/v) was freshly prepared for each experiment. *n*-Butanol was once distilled and saturated with aqueous 0·01 N-NaOH.

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To measure the uptake of thyroxine and triiodothyronine by mitochondria, the iodo compound, in serum medium, was incubated at 0° with mitochondria. Usually 4×10^{-4} M iodo compound in 2 ml. serum medium (containing 0·08 ml. serum) was mixed with 2 ml. mitochondrial suspension (approx. 10 mg. dry wt. mitochondria) giving 2×10^{-4} M iodo compound and 2% (v/v) serum in 4 ml. final volume. Elsewhere the quantities were altered to suit the needs of individual experiments. After the required period of incubation, the mitochondria were separated from the medium by centrifuging in the high-speed head of a refrigerated centrifuge. The centrifuge was accelerated to a speed corresponding to 13 000 *g* in 10–13 sec. and maintained at this speed for 1 min. Sedimentation was complete within 30 sec. of starting the centrifuge, but the longer time was allowed to pack the mitochondria more firmly. When the uptake over short intervals was measured, the iodo compound and the mitochondria were mixed in tubes already set in the centrifuge head. On other occasions larger amounts were mixed in conical flasks and suitable quantities withdrawn at intervals. Where the centrifuge was started immediately after mixing ('zero time' point) the duration of contact between mitochondria and iodo compound was taken as *ca.* 30 sec.

Estimation of iodo compounds in mitochondria and medium. After centrifuging, the supernatant was decanted into a test tube and 2 ml. were transferred to a 10 ml. stoppered tube containing 0·25 ml. 30% (w/v) TCA. The centrifuge tube was drained and dried inside with filter paper. The mitochondria were suspended in 1 ml. phosphate medium (Klemperer, 1955) and were quantitatively transferred to a 10 ml. stoppered tube containing 0·25 ml. 30% (w/v) TCA by washing with a further 1 ml. phosphate medium.

Thyroxine or triiodothyronine was extracted from the supernatant and mitochondria into 2 ml. *n*-butanol by

shaking for 1 min. In preliminary work *n*-butanol saturated with 0·01 N-NaOH was used; this was continued unchanged in later experiments even though TCA was greatly in excess during the extraction of the iodo compound. Centrifuging for 10 min. at 400 *g* separated the emulsion into an upper butanol and a lower aqueous layer. From the butanol layer 1 ml. was transferred to a test tube and thyroxine or triiodothyronine estimated colorimetrically by the nitrous acid reaction, as used by Gross & Pitt-Rivers (1953*a*). The optical density was read at 465 mμ. in a Unicam spectrophotometer and corrected for the blank due to mitochondria or serum medium alone. The optical density × 1·41 corresponded to μmoles thyroxine, and optical density × 1·44 to μmoles triiodothyronine extracted into the whole original 2 ml. *n*-butanol. This relationship was established by estimations of standard thyroxine and triiodothyronine solutions in serum medium.

Accuracy of the method. Up to 1 μmole iodo compound was estimated by this method. Provided that the amount of iodo compound in each determination exceeded about 0·05 μmole, duplicate estimations agreed to within 5%, and the recovery of thyroxine and triiodothyronine added to mitochondria and medium was 95–100%. Smaller quantities gave rise to errors, especially when blank values were raised by the use of large amounts of serum or mitochondria.

Small quantities of sodium L-thyroxine dissolved in 2 ml. 0·005 N-NaOH were completely extracted into 2 ml. *n*-butanol when shaken in the presence of 0·25 ml. 30% (w/v) TCA. The amount of thyroxine detectable in this way was of the order of 2 μg.

Weight determinations. For wet-weight determination, mitochondria were centrifuged from 4 ml. suspending medium as described above. The supernatant was decanted and the tube drained for 5–10 min. and dried inside with filter paper before weighing. For dry-weight determination, the mitochondria separated from the suspending medium in the same way were dried by heating in an oven at 110° for 12 hr. The dry weight of serum was derived from the volume added and the weight of a known volume when evaporated to a constant weight at 110°.

Presentation of results. Results given are averages of single estimations on duplicate samples and are expressed as total μmoles in medium and in mitochondria, taking 1 μmole as equal to 651 μg. triiodothyronine and 799 μg. sodium L-thyroxine. Elsewhere (except in Tables 2–4) μmoles/g. dry wt. of mitochondria or serum are given. In this way a comparison on a similar basis was possible between the amount of iodo compound remaining in the serum and that bound to the mitochondria.

The term '% dry weight' means $\frac{\text{dry weight}}{\text{wet weight}} \times 100$.

RESULTS

Uptake of L-*thyroxine and* L-*triiodothyronine by mitochondria at* 0°

When mitochondria were incubated with thyroxine or triiodothyronine at 0° there was a progressive uptake of the iodo compound by the mitochondria. This was measured by separating mitochondria at successive intervals from the suspending medium. Table 1 shows that the very rapid initial uptake was followed by a slower increase. After 40 min. no further rise took place, and Expt. 1, in which approximately equimolar amounts of thyroxine and triiodothyronine were added, shows a higher molar uptake of triiodothyronine at this time. After 40 min. suspension in thyroxine, the dry weight of the mitochondria was approximately 10% of their wet weight (see below, Table 3) so that the final concentration of iodo compound (Table 1) in unit volume was over 50 times greater in the mitochondria than in the medium.

Table 1. *Uptake of* L-*thyroxine and* L-*triiodothyronine by rat-liver mitochondria at* 0°

Mitochondria were separated by centrifuging from a mixture of 2 ml. mitochondrial suspension and 2 ml. iodo compound solution in serum medium. Times refer to the intervals between mixing mitochondria with iodo compound and starting the centrifuge. In Expt. 1, 4 ml. mixture contained 8 mg. dry wt. mitochondria and 0·85 μmole L-thyroxine or 0·88 μmole L-triiodothyronine; in Expt. 2 there were 10 mg. dry weight mitochondria and 0·82 μmole L-triiodothyronine.

	Expt. 1						Expt. 2		
	L-Thyroxine found (μmoles)			L-Triiodothyronine found (μmoles)			L-Triiodothyronine found (μmoles)		
Time (min.)	In 8 mg. mitochondria	In 4 ml. medium	Total	In 8 mg. mitochondria	In 4 ml. medium	Total	In 10 mg. mitochondria	In 4 ml. medium	Total
0	—	—	—	—	—	—	0·34	0·44	0·78
2·25	0·33	0·54	0·87	—	—	—	—	—	—
5	—	—	—	—	—	—	0·42	—	—
10	0·41	—	—	—	—	—	0·49	—	—
20	0·44	—	—	—	—	—	0·50	—	—
40	0·46	0·40	0·86	0·54	0·36	0·90	0·55	0·28	0·83
60	0·45	—	—	—	—	—	0·54	0·27	0·81
80	0·46	0·42	0·88	0·54	0·32	0·86	0·54	—	—

Distribution of L-*thyroxine between mitochondria and medium*

The high concentrations of iodo compound used here (2×10^{-4} M) were made possible by the addition of serum. It is also known that thyroxine present naturally in plasma is combined with plasma proteins (Taurog & Chaikoff, 1948). The greater solubility of the iodo compounds in the presence of serum may be attributed to the formation of complexes between serum protein and iodo compound. When a thyroxine solution was prepared in the usual way except that serum was omitted, or if serum proteins were precipitated with TCA, the amount of thyroxine remaining in solution in 2 ml. was detectable and estimated as approximately 10 μg. This is about half the amount expected from the solubility in water (0·001 g./100 ml.). Thus it seems likely that a thyroxine solution stabilized by serum contained less than 10 μg. free thyroxine/ml., and that in a 2×10^{-4} M thyroxine solution less than 6 % of the thyroxine occurs in the free form.

Variation of the serum concentration and the L-thyroxine concentration showed that the uptake of thyroxine at 0° was due to a passive distribution between mitochondria and serum, and that the amount taken up depended on the relative amounts of serum and mitochondria and the affinity of the iodo compound for each protein. In Table 2 approximately similar final concentrations of thyroxine/mg. dry weight mitochondria attained after 40 or 60 min. are arranged together. The ratio

$$\frac{\mu\text{g. thyroxine/mg. dry weight mitochondria}}{\mu\text{g. thyroxine/mg. dry weight serum}} \quad (1)$$

expresses the distribution between mitochondria and serum, and this is shown to be approximately constant. On the other hand, the ratio

$$\frac{\mu\text{g. thyroxine/g. wet weight mitochondria}}{\mu\text{g. thyroxine/ml. suspending medium}} \quad (2)$$

which expresses the distribution between mitochondria and medium is not constant but decreases when the proportion of serum in the medium rises. Ratio (2) is calculated with the help of the percentage dry weight of mitochondria. Table 3 shows that mitochondria suspended for 40 min. at 0° in thyroxine underwent swelling as compared with mitochondria suspended in serum medium alone. The amount of swelling appeared to depend on the amount of thyroxine taken up by the mitochondria. An average value of 10 % dry weight was adopted for the calculation in ratio (2) (Table 2) since the range of percentage dry weight was small compared with the fluctuation of this ratio, and since in any case the comparison is between mitochondria with approximately the same thyroxine concentration.

Table 2. *Distribution of* L-*thyroxine between supernatant and rat-liver mitochondria*

Mitochondria were separated by centrifuging after 40–60 min. contact at 0° with L-thyroxine solution. The amounts of mitochondria and L-thyroxine and the % (v/v) of serum in the final medium differed in individual cases. Results of ten experiments are arranged in three groups, so that approximately similar final concns. of L-thyroxine/mg. dry weight of mitochondria fall in the same group. Corresponding final concns. of L-thyroxine remaining in solution in the medium are given. In both columns the amounts are expressed as μg. sodium L-thyroxine. Dry weight of serum was 93 mg./ml. Dry weight of mitochondria was assumed to be 10 % of the wet weight.

Sodium L-thyroxine found (μg.)				
Per mg. dry weight mitochondria (a)	Per ml. medium (b)	Serum in medium (%, v/v) (c)	Ratio: thyroxine/mg. mitochondria / thyroxine/mg. serum $\left(\frac{a}{b} \times \frac{c}{100} \times 93\right)$	Ratio: thyroxine/g. wet wt. mitochondria / thyroxine/ml. medium $\left(\frac{a \times 100}{b}\right)$
10	10	1	0·9	100
11	12	1	0·9	92
10	16	2	1·2	63
13	24	2	1	54
12	45	4	1	27
7	28	4	0·9	25
18	18	1	0·9	100
22	25	1	0·8	88
21	38	2	1	55
21	32	2	1·2	66
18	60	4	1·1	30
25	85	4	1·1	29
26	75	4	1·3	35
44	80	2	1	55
47	65	2	1·3	72
44	115	4	1·4	38
48	130	4	1·4	37

Table 3. *Percentage dry weight of rat-liver mitochondria and* L-*thyroxine uptake*

Wet weight and L-thyroxine content determined for mitochondria separated from 4 ml. mixture containing 11 mg. dry weight mitochondria and L-thyroxine (expressed as μg. sodium L-thyroxine) as shown. The control contained 2 ml. serum medium instead of L-thyroxine. Other details as in Table 1.

Time (min.)	Sodium L-thyroxine (μg.) Present in 4 ml. mixture	Sodium L-thyroxine (μg.) Found per mg. dry wt. mitochondria	Wet weight mitochondria (mg.)	Dry weight (% of wet wt.)
0 } control	—	—	64	17·2
40 } control	—	—	70	15·7
40	170	9	101	10·9
40	340	19	112	9·8
40	680	35	122	9

Table 4. L-*Thyroxine uptake and swelling of rat-liver mitochondria*

Details as in Table 3. Each 4 ml. of mixture contained 12 mg. dry weight mitochondria and 660 μg. sodium L-thyroxine.

Time (min.)	Sodium L-thyroxine found (μg.) in 12 mg. mitochondria	Wet weight mitochondria (mg.)	Increased wet weight over control (mg.)	Dry weight (% of wet wt.)
0	190	72	3	16·7
1	220	75	6	16·0
3	250	86	17	14·0
10	290	107	38	11·2
20	320	118	49	10·2
40	380	123	54	9·8
60	390	124	55	9·7
0 } control	—	69	—	17·4
40 } control	—	71	2	16·9

Time course of swelling and of thyroxine uptake

Both the wet weight and the thyroxine content of mitochondria were determined at intervals during incubation at 0°. The increased wet weight as compared with the initial wet weight of mitochondria suspended in serum medium alone was taken as a measure of the amount of swelling. Table 4 shows that most of the swelling occurred after the greater part of the thyroxine had been taken up. Over half of the final level of thyroxine in the mitochondria was reached after about 1 min. but little swelling had taken place at this time, and 50 % of the final amount of swelling was reached only between 3 and 10 min. These results suggest that swelling was a consequence of the uptake of thyroxine.

Comparison of the uptake of L-*thyroxine and* L-*triiodothyronine*

The distribution of L-thyroxine and L-triiodothyronine between mitochondria and serum is compared in Table 5. The concentrations of iodo compound are expressed in μmoles/g. dry weight to facilitate comparison of the data. The ratio, concn. in mitochondria/concn. in serum, shows that about 25 % more triiodothyronine was taken up than thyroxine, and at low concentrations this difference may have been greater, although in this region errors may be considerable owing to the small amount of iodo compound estimated and the indirect derivation of the amount remaining in solution.

The rates at which L-thyroxine and L-triiodothyronine were taken up at 0° were compared over the early rapid phase where differences in uptake would be greatest. Duplicate experiments gave results which differed by less than 5 %. Table 6 shows that in both cases over 50 % of the final level of iodo compound was reached in the mitochondria within 1 min., but that triiodothyronine approached the final level more rapidly than did thyroxine.

Exchange of L-*thyroxine and* L-*triiodothyronine between mitochondria and serum*

When mitochondria which had taken up L-thyroxine or L-triiodothyronine from a solution were suspended in a medium containing serum, the iodo compound moved from the mitochondria into the medium. As in the case of transfer from serum

Table 5. *Distribution of* L-*thyroxine and* L-*triiodothyronine between rat-liver mitochondria and serum*

Details as in Table 2 except that in all cases 4 ml. of the final mixture contained 2 ml. serum medium (or 2%, v/v, serum). Results of seven experiments. Figures in italics are derived by subtraction of the amount of iodo compound recovered in the mitochondria from the amount originally added.

L-Thyroxine found (μmoles)			L-Triiodothyronine found (μmoles)		
Per g. dry wt. mitochondria (*a*)	Per g. dry wt. serum (*b*)	Ratio: *a*/*b*	Per g. dry wt. mitochondria (*x*)	Per g. dry wt. serum (*y*)	Ratio: *x*/*y*
7	*10*	0·7	9	*7*	1·3
10	12	0·8	13	7	1·9
12	*13*	0·9	15	*8*	1·9
13	11	1·2	15	10	1·5
17	*16*	1·1	20	*10*	2·0
26	25	1·0	30	17	1·8
27	21	1·3	32	18	1·8
48	53	0·9	53	41	1·3
50	49	1·0	60	35	1·7
52	45	1·2	61	37	1·7
54	41	1·3	67	48	1·4
58	54	1·1	68	41	1·7
59	43	1·4	73	42	1·7

Table 6. *Rate of uptake of* L-*thyroxine and* L-*triiodothyronine by rat-liver mitochondria*

Details as in Table 1. Each 4 ml. of mixture contained 9 mg. dry weight mitochondria and 0·81 μmole L-thyroxine or 0·91 μmole L-triiodothyronine. Rate of uptake expressed as percentage of final amount of iodo compound in the mitochondria at 50 min. Times refer to intervals between mixing mitochondria with iodo compound and starting the centrifuge.

	L-Thyroxine found		L-Triiodothyronine found	
Time (min.)	In 9 mg. mitochondria (μmoles)	% of 50 min. value	In 9 mg. mitochondria (μmoles)	% of 50 min. value
0·25	0·3	62·5	0·43	69·5
1	0·33	69	0·47	76
2·5	0·38	79	0·51	82
50	0·48	100	0·62	100

Table 7. *Loss of* L-*thyroxine and* L-*triiodothyronine from rat-liver mitochondria at* 0°

Mitochondria suspended in L-thyroxine or L-triiodothyronine solution (initial concn. 2×10^{-4} M) at 0° were separated by centrifuging after 10 min. or 50 min. and resuspended in a similar medium at 0° containing serum but no iodo compound. After 50 min. L-thyroxine or L-triiodothyronine was determined in mitochondria and medium. In all cases 10·8 mg. dry weight mitochondria and 2 ml. serum medium were used in 4 ml. reaction mixture.

	L-Thyroxine found (μmoles)			L-Triiodothyronine found (μmoles)		
Time suspended (min.)	Per g. dry wt. mitochondria (*a*)	Per g. dry wt. serum (*b*)	Ratio: *a*/*b*	Per g. dry wt. mitochondria (*x*)	Per g. dry wt. serum (*y*)	Ratio: *x*/*y*
10	23	22	1·0	29	19	1·5
50	27	23	1·2	36	21	1·7

to mitochondria, transfer in the reverse direction was completed in less than 50 min., and the final ratio (Table 7), concentration in mitochondria/concentration in serum, had approximately the same value for each compound as in Table 5. This ratio of concentrations may therefore reflect an equilibrium in an exchange of iodo compound between mitochondria and serum. However, mitochondria which had previously been suspended in thyroxine or triiodothyronine solution for 50 min. gave up less iodo compound to serum than mitochondria in the same experiment suspended for only 10 min., suggesting that the uptake after 10 min. is less readily reversible than in the initial phase.

Mechanism of uptake of iodo compound

The following experiments provide further evidence for the passive nature of the mechanism by which mitochondria take up L-thyroxine and L-triiodothyronine.

The uptake of L-thyroxine was not affected by raising the temperature to 20° and so appreciably increasing the rate of metabolism (Table 8). There was no increased uptake even though adenosine triphosphate, diphosphopyridine nucleotide and β-hydroxybutyrate were added to maintain respiration. The experiment was of short duration since in the presence of thyroxine at 20° adenosine triphosphate decomposed and there were progressive changes in the mitochondria leading to disintegration.

Mitochondria denatured by heating for 5 min. on a water bath at 100° and then suspended in L-thyroxine or L-triiodothyronine solution at 0° took up over twice as much iodo compound as did native mitochondria (Table 9).

Table 10 shows that the uptake of thyroxine at the concentrations studied did not depend on the optical configuration since both isomers reached the same final concentration in the mitochondria.

Table 8. *Uptake of* L-*thyroxine at* 0° *and at* 20°

For incubation at 0°, 2 ml. mitochondria (10 mg. dry weight) were added to 1 ml. L-thyroxine solution in serum medium and 1 ml. phosphate medium. Incubation at 20° was carried out in conical Dubnoff flasks shaken in air on a Dubnoff shaker. The mixture was the same as that incubated at 0° except that 1 ml. phosphate medium was replaced by 0·5 ml. 0·02M adenosine triphosphate, 0·2 ml. 0·4M sodium DL-β-hydroxybutyrate, 0·1 ml. diphosphopyridine nucleotide (containing 1·5 mg. 75% pure) and 0·2 ml. phosphate medium. Initial thyroxine concentration approximately 10^{-4}M in all cases. Other details as in Table 1.

Time (min.)	L-Thyroxine found per 10 mg. mitochondria (μmoles) At 0°	At 20°
3	0·16	0·14
8	0·19	—
10	—	0·19
15	0·21	—
16·5	—	0·21

DISCUSSION

The observations reported in this paper show that the distribution of L-thyroxine and L-triiodothyronine between rat-liver mitochondria and serum is a passive process and is not connected with metabolism. The uptake of these iodo compounds by mitochondria has features in common with the binding of dyes by proteins. Thus the greater uptake of iodo compound by heat-treated mitochondria recalls the increased binding of dyes by heat-denatured proteins (Oster & Grimsson, 1949) and the similar uptake of D- and L-thyroxine resembles the adsorption onto serum albumin of stereoisomers of dyes at high concentration (Karush, 1952). Thyroxine in serum, whether present naturally or added *in vitro*, is bound to serum protein (Taurog & Chaikoff, 1948), and it is suggested that the uptake

Table 9. *Uptake of* L-*thyroxine and* L-*triiodothyronine by native and denatured rat-liver mitochondria*

Mitochondria denatured by heating suspension for 5 min. on water bath at 100°. Mitochondrial suspension (2 ml., dry weight 8·5 mg. native, 9 mg. denatured) incubated for 50 min. at 0° with 2 ml. solution of iodo compound in serum medium. Initial concn. iodo compound approximately 2×10^{-4}M in all cases.

Mitochondria	L-Thyroxine found (μmoles) Per g. dry wt. mitochondria (*a*)	Per g. dry wt. serum (*b*)	Ratio: *a*/*b*	L-Triiodothyronine found (μmoles) Per g. dry wt. mitochondria (*x*)	Per g. dry wt. serum (*y*)	Ratio: *x*/*y*
Native	54	41	1·3	62	34	1·8
Denatured	74	24	3·1	77	20	3·9

Table 10. *Uptake of* D- *and* L-*thyroxine by rat-liver mitochondria at* 0°

Mitochondrial suspension (2 ml., dry weight 12 mg.) incubated for 50 min. at 0° with 1 ml. solution iodo compound in serum medium and 1 ml. phosphate medium.

Approx. concn. iodo compound ($M \times 10^{-5}$)	L-Thyroxine found (μmoles) Per g. dry wt. mitochondria (*a*)	Per g. dry wt. serum (*b*)	Ratio: *a*/*b*	D-Thyroxine found (μmoles) Per g. dry wt. mitochondria (*x*)	Per g. dry wt. serum (*y*)	Ratio: *x*/*y*
5	10	—	—	11	—	—
10	23	18	1·3	24	18	1·3

of iodo compound studied in this paper is a distribution between serum and mitochondrial protein, the greater uptake of L-triiodothyronine than of L-thyroxine by mitochondria reflecting the different relative affinities of these iodo compounds for serum and mitochondrial proteins. In agreement with this is the finding that serum α-globulin binds L-thyroxine more firmly than L-triiodothyronine (Deiss, Albright & Larson, 1953).

The concentrations at which the uptake of thyroxine was studied in this paper were much higher than those occurring *in vivo*. Carr & Riggs (1953) reported tissue iodine levels on a wet-weight basis and showed that the concentration of iodine in liver mitochondria was the same as for whole liver. Their data for normal dogs correspond approximately to $2{\cdot}5 \times 10^{-3}$ μg. atoms iodine/g. dry weight of liver mitochondria and $1{\cdot}25 \times 10^{-3}$ μg. atoms iodine/g. dry weight of serum. Assuming that all tissue iodine is in the form of thyroxine the levels of thyroxine in mitochondria and serum given in Table 10 (initial thyroxine concentration 10^{-4} M) are 4×10^4 times higher than *in vivo*. It is of interest, however, that the distribution of thyroxine given by the ratio (concentration in mitochondria/concentration in serum) is of the same order in both cases. The uptake of thyroxine by the tissues *in vivo* may therefore also depend on a passive distribution similar to that reported here.

Since concentrations of L-thyroxine below 5×10^{-5} M give partial uncoupling of oxidative phosphorylation (Klemperer, 1955) and iodine levels in thyroid-fed dogs are about 5–9 times higher than normal, the mitochondrial level of thyroxine when phosphorylation is uncoupled is also much higher than *in vivo* even after thyroid feeding. However, this need not necessarily represent the concentration of thyroxine at some specific centre sensitive to the action of the iodo compound. Moreover, the swelling of mitochondria in presence of concentrations of L-thyroxine which uncouple phosphorylation resembles the swelling of mitochondria in presence of the uncoupling agent dinitrophenol (Chappell & Perry, 1954; Price & Davies, 1954) and corresponds to what is found at much lower concentrations *in vivo*, namely the increased tendency to swell of liver mitochondria prepared from thyroid-fed rats (Aebi & Abelin, 1953).

Mitochondria took up L-thyroxine and L-triiodothyronine at a very rapid rate. It is therefore unlikely that the time required for uptake contributes significantly to the latent period observed before the iodo compounds uncouple phosphorylation (Martius & Hess, 1951; Hoch & Lipmann, 1953; Klemperer, 1955). Since L-triiodothyronine was taken up by mitochondria to a greater extent than L-thyroxine, the lower uncoupling activity of L-triiodothyronine cannot be explained in terms of the uptake. The latent period might therefore correspond to the formation of some active form of the hormone different from both thyroxine and triiodothyronine. On the other hand, the faster uptake of L-triiodothyronine by mitochondria, which corresponds to its more rapid removal from the circulation by the tissues (Rawson *et al.* 1953; Blackburn, McConahey, Keating & Albert, 1954; Roche, Michel, Michel & Tata, 1954; J. Gross, personal communication in 1954), may explain its greater activity *in vivo*, assuming that uncoupling is the physiological action of thyroxine and triiodothyronine. Apart from combining with the tissues where they exert their effect, the iodo compounds are subject in the whole animal to degradation and excretion. By combining more rapidly than thyroxine with the tissues a greater proportion of a dose of triiodothyronine may have the opportunity of exerting its physiological action.

SUMMARY

1. Mitochondria rapidly took up L-thyroxine and L-triiodothyronine at 0° from solution in a medium containing serum. After 40 min. there was no further rise in concentration in the mitochondria.

2. When equilibrium was reached, the ratio, concentration of L-thyroxine in mitochondria/concentration in serum, had a constant value for a variety of concentrations.

3. There was swelling of the mitochondria in the presence of L-thyroxine at 0° but this mostly occurred after thyroxine uptake was nearly complete.

4. Mitochondria took up about 25% more L-triiodothyronine than L-thyroxine on a molar basis. L-Triiodothyronine was taken up more rapidly than L-thyroxine. Within the first minute both iodo compounds reached over 50% of the final level in the mitochondria.

5. The uptake of both substances was largely, but not entirely reversible.

6. The uptake of L-thyroxine at 20° was the same as at 0°. Heat-denatured mitochondria took up over twice as much L-thyroxine and L-triiodothyronine as native mitochondria. D-Thyroxine was taken up to the same extent as L-thyroxine.

7. The findings indicate that the distribution of iodo compounds between serum and mitochondria is 'passive', being controlled by the physical or chemical binding power of serum and mitochondria, and independent of metabolic activity.

8. The level of thyroxine which uncoupled oxidative phosphorylation was much higher than that found *in vivo*, even after thyroxine feeding. The available evidence suggests that probably neither L-thyroxine nor L-triiodothyronine is the form of the thyroid hormone active in the tissues.

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The Mechanism of Itaconic Acid Formation by *Aspergillus terreus*

1. THE EFFECT OF ACIDITY

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It has been reported that in cultures of *Aspergillus terreus* a substantial accumulation of itaconic acid can only be obtained under acid condition; a pH of about 2 has been found to be the optimum (Lockwood & Reeves, 1945; Moyer & Coghill, 1945; Lockwood & Nelson, 1946). Discussing their results, Lockwood & Reeves (1945) advanced two possible explanations to account for this phenomenon: (1) itaconic acid is produced by the mould only at low pH; (2) itaconic acid is produced over a wide pH range, but is further metabolized at higher pH. In their discussion these authors report the results of an experiment in which they investigated the rate of utilization of itaconic acid by *A. terreus* at different pH values. They found that the higher the starting pH of the culture, the faster the disappearance of itaconic acid. These results do not, however, throw much light upon the problem in question, since it may well be that itaconic acid is preferentially metabolized at higher pH even if it is formed only at low pH.

This paper presents the results of an investigation on the influence of pH on the formation of itaconic acid by *A. terreus*. The results indicate that a low pH is necessary for the formation of the enzyme system producing itaconic acid.

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EXPERIMENTAL

Fermentation experiments. The experiments were carried out with *A. terreus*, strain NRRL 1960. Cultures were grown in 500 ml. flat-bottom flasks containing 100 ml. medium of the following composition: 5 g. glucose monohydrate; 0·3 g. $(NH_4)_2SO_4$; 0·3 g. anhydrous $CaSO_4$; 0·05 g. $MgSO_4,7H_2O$; 0·01 g. KH_2PO_4; made up to 100 ml. with tap water. The medium was autoclaved at 10 lb./sq.in. for 10 min., each flask inoculated with 1–2 sq.cm. of a spore mat prepared according to Moyer & Coghill (1945) and incubated on a reciprocating shaker (4 cm. amplitude, 180 oscillations/min.) at 30°. The above medium has a pH of 5·5 at the time of inoculation. During growth of the mycelium the pH decreases to about 2·1, and remains constant at this value during itaconic acid production (Larsen & Hovden, in preparation). For fermentation experiments at pH 6·0 the same procedure as described above was used, but with 1% (w/v) dry sterilized $CaCO_3$ added aseptically to the autoclaved medium.

Reagents. The glucose monohydrate and anhydrous $CaSO_4$ used were laboratory reagents from British Drug Houses Ltd. The other chemicals were of analytical grade.

Analytical methods. CO_2 produced during fermentation was determined by absorption in Ascarite, a sodium hydrate–asbestos absorbent (Eimer and Amend, New York). For this purpose a slow stream of CO_2-free air was drawn through the cultures and absorption tubes. In cultures where $CaCO_3$ was a constituent of the medium, total CO_2 was determined at the end of the experiment by adding an excess of H_2SO_4.