more than twice the theoretical amount of sulphone, since otherwise considerable denaturation may occur. If, however, the preparations are made in the reverse order, i.e. by treatment with sulphone before iodination, it is advisable to use a higher sulphone/protein ratio when preparing derivatives of ovalbumin, but not of globulins.

When 'trace-labelling' a protein with the two isotopes the above considerations do not arise, and it is probably immaterial which of the two groups is introduced first (Table 5). We have, however, invariably introduced the sulphone groups first, since it is during the sulphone treatment that there is the greater risk of damaging the proteins, and this is less likely to occur if these proteins have been submitted to only the minimum of preliminary chemical treatment.

SUMMARY

1. Methods are described for introducing into proteins both 1:4-thiazan 1:1-dioxide groups (by treatment with di-2-chloroethyl sulphone) and 3:5-diiodotyrosine groups.

2. The number of the two groups introduced into a protein is partly dependent on the order in which the iodination and treatment with sulphone are carried out.

3. The results are discussed with reference to the positions in the protein molecule taken up by the entering groups, and the efficacy of the method for the preparation of double-labelled protein antigens.

4. A method of 'trace-labelling' proteins with both ^{131}I and ^{35}S is described.

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The Uncoupling of Oxidative Phosphorylation in Rat-Liver Mitochondria by Thyroxine, Triiodothyronine and Related Substances

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Martius & Hess (1951, 1952), Hoch & Lipmann (1953) and Maley & Lardy (1953) have shown that thyroxine, under certain conditions, can inhibit oxidative phosphorylation *in vitro*. This effect is taken to be related to the main physiological action of the thyroid hormone, as it can explain the rise in the basal metabolic rate caused by the thyroid hormone. Gross & Pitt-Rivers (1952) have suggested that triiodothyronine might be the active form of the thyroid hormone, and if this is correct this substance is expected to be at least as effective in inhibiting oxidative phosphorylation as thyroxine. The inhibitory effects of thyroxine and triiodothyronine *in vitro* were therefore compared. The procedure of Krebs, Ruffo, Johnson, Eggleston & Hems (1953), in which the rate of incorporation of radioactive phosphate into adenosine phosphates is measured, was used. Rat-liver mitochondria served as a source of the phosphorylating enzyme system and succinate or β-hydroxybutyrate as substrate.

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EXPERIMENTAL

Special chemicals. 'Phosphate medium', pH 7·4, consisting of 6·66% 0·1M-$MgCl_2$, 88·33% 0·9% (w/v) KCl and 5% 0·1M sodium phosphate buffer, pH 7·4 (by vol.), was used for suspending mitochondria. Sodium adenosine triphosphate (ATP) solution was made from barium ATP prepared according to LePage (1949); diphosphopyridine nucleotide (DPN), approx. 75% pure, was prepared by the method of LePage, modified according to Burton & Wilson (1953), and used as a neutral solution containing 15 mg./ml. Solutions of sodium L-thyroxine, D-thyroxine, L-triiodothyronine, L-diiodothyronine (all gifts from Glaxo Laboratories Ltd., Greenford, Middlesex) and sodium DL-thyroxine (British Drug Houses Ltd., Poole, Dorset: Laboratory Reagent) were prepared by dissolving 4 μmoles in 0·5 ml. 0·02N-NaOH. To this were added 0·4 ml. sheep serum in 5·5 ml. phosphate medium and then 3·1 ml. phosphate medium plus 0·5 ml. 0·02N-HCl giving a neutral 4×10^{-4}M solution. Serum served to stabilize the solution. 'Serum medium' prepared in the same way but without iodo compound was used as a control and to prepare dilutions of the test solution. L-Thyronine (Glaxo) and L-diiodotyrosine (B.D.H. Laboratory Reagent) were dissolved at a suitable concentration as above but without the addition of serum.

Mitochondria. These were prepared by a modification of the methods of Lehninger & Kennedy (1948) and of Schneider (1948), at 0°. The liver from a freshly killed male albino rat (200–250 g.) was chilled in iced 0·9% (w/v) KCl and 5 g. were disintegrated in a Potter–Elvehjem type stainless-steel homogenizer with 25 ml. 0·25M sucrose and 0·5 ml. 0·1M sodium phosphate buffer, pH 7·4. The volume was made up to 75 ml. with 0·25M sucrose and the homogenate centrifuged for 7 min. at 500 *g* to sediment red cells, nuclei and unbroken cells. The supernatant was mixed with one-ninth its volume of 1·5M-KCl and the mitochondria were sedimented by centrifuging for 15 min. at 1700 *g*. The mitochondria were twice washed with phosphate medium by resuspending and centrifuging for 7 min. at 1700 *g* and were finally suspended in 50 ml. phosphate medium. In later experiments phosphate buffer was omitted from the sucrose solution in the first step and the mitochondria were washed once only with phosphate medium.

Incubation procedure. All experiments were performed at 20° (except in Table 3) with air as the gas phase. The following components were measured into conical Warburg flasks or Dubnoff vessels of a similar shape standing on ice: 0·5 ml. 0·02M sodium ATP, 0·2 ml. 0·4M sodium DL-β-hydroxybutyrate, 0·1 ml. 0·02M DPN, 0·1 ml. $KH_2{}^{32}PO_4$ solution, 0·1 ml. 0·9% (w/v) KCl, and 1 ml. serum medium or a solution of iodo compound. When succinate was the substrate, 0·2 ml. 0·2M sodium succinate was used. Warburg flasks contained 0·2 ml. 2N-NaOH and filter paper in the centre well. The last addition was 2 ml. mitochondrial suspension (10 mg. dry wt. of tissue). To avoid the hydrolysis of ATP by mitochondria at 0°, in experiments where mitochondria were pretreated with L-thyroxine or L-triiodothyronine before incubation, 2 ml. mitochondria were added to 1 ml. iodo compound on ice and the other components added together (1 ml.) just before incubation at 20°. The concentration of iodo compound was therefore higher during the preliminary contact with the mitochondria than during incubation.

The O_2 uptake was measured after a 10 min. equilibration period in Warburg manometers shaken in a constant-temperature bath. Duplicate flasks for measuring phosphate exchange of ATP were shaken in the same bath for either 6 or 10 min. and then deproteinized by addition of 0·5 ml. of 30% (w/v) trichloroacetic acid followed by centrifuging. The supernatants were stored frozen at −20°.

When acetoacetate production alone was measured, small conical Dubnoff flasks open to air were shaken for 10 min. on a Dubnoff Shaking Incubator (Precision Instruments Ltd., Chicago).

Chemical estimations. Acetoacetate was estimated by the method of Walker (1954). Phosphate fractions were separated as bands by paper chromatography as described by Bartley (1953) with the formic acid–*iso*propyl ether solvent of Hanes & Isherwood (1949). The bands corresponding to ATP and orthophosphate were cut out and wet-ashed with the ashing fluid of Hanes & Isherwood (1949). The volume was made to 10 ml., and 2 ml. samples were analysed for phosphate by the method of Berenblum & Chain (1938).

Measurement of radioactivity. A beta-counter tube, liquid type M6 (20th Century Electronics) was used. The total ashed phosphate (10 ml.) was counted before withdrawal of samples for phosphate analysis.

Expression and calculation of results. Data from duplicate flasks did not differ by more than 5%. Average values are given. As the level of ATP in the control remained constant it was taken to represent the zero value. Labile phosphate of ATP was taken as two-thirds of the total phosphorus of ATP. Specific radioactivities are expressed as counts/μg. phosphorus/min.

The phosphorylation quotient (equivalents of organic phosphate formed/atoms of oxygen absorbed) was calculated according to Krebs *et al.* (1953) from the values for ATP and orthophosphate alone. O_2 uptake, observed between 10 and 20 min., was taken as O_2 consumed during the 10 min. incubation period. Where acetoacetate was estimated, 'moles of acetoacetate formed' during the incubation was used in place of 'atoms of O_2 absorbed'.

RESULTS

General properties of the preparation. With β-hydroxybutyrate (0·02M) as the substrate there was steady oxygen uptake for 30–40 min. with a Q_{O_2} (μl. O_2/mg. dry wt. mitochondria/hr.), of approx. −10. The blank respiration amounted to 20–30% of that with β-hydroxybutyrate as substrate. When succinate (0·01M) was the substrate the respiration was higher ($-Q_{O_2} = 20$–25).

DPN was added because it increased the oxygen uptake by 10–20% when β-hydroxybutyrate was the substrate and increased the value of the ratio μmoles acetoacetate produced/μatoms oxygen absorbed (see below). The small amount (0·04 ml.) of serum present in 4 ml. of the final reaction mixture had no effect on either the respiration, acetoacetate production or the phosphorylation quotient. For incubation periods of 5–10 min. the phosphorylation quotient approached 3 with β-hydroxybuty-

rate. The ratio specific radioactivity of P of ATP/specific radioactivity of orthophosphate rose above 0·33, indicating that both labile phosphate groups of ATP exchanged with labelled ^{32}P of orthophosphate. During a 10 min. incubation period the ratio of specific radioactivities reached about 0·15–0·2 with β-hydroxybutyrate as substrate and a rather higher value with succinate. In the later experiments reported here incubation periods shorter than 10 min. were used since it has been shown by Whittam, Bartley & Weber (1955) that the incorporation of ^{32}P into ATP may be limited by

Table 1. *Effect of* L-*thyroxine and* L-*triiodothyronine on oxygen uptake and phosphorylation*

2 ml. mitochondria (twice washed) shaken with 1 ml. of L-thyroxine or L-triiodothyronine solution and 0·5 ml. 0·02M sodium ATP, 0·2 ml. 0·4M sodium DL-β-hydroxybutyrate, 0·1 ml. 0·02M DPN, 0·1 ml. $KH_2{}^{32}PO_4$, 0·1 ml. 0·9% KCl in air at 20°. The control contained serum medium alone without iodo compound. Where succinate was the substrate, 0·2 ml. 0·2M sodium succinate was substituted for β-hydroxybutyrate and 0·1 ml. 0·9% KCl for DPN. Flasks for phosphate exchange incubated for 10 min. O_2 uptake measured after 10 min. equilibration.

Substrate	Addition	Concn. of iodo compound ($M \times 10^{-6}$)	O_2 uptake in 10 min. (μatoms)	Labile P of ATP per flask (μmoles)	Specific radioactivity of P of ATP / specific radioactivity of orthophosphate P	P/O ratio
β-Hydroxybutyrate	0	—	1·9	13·2	0·24	2·5
	L-Thyroxine	2*	1·9	12·8	0·24	2·4
		100*	1·8	12·3	0·19	2·0
β-Hydroxybutyrate	0	—	1·8	14·3	0·21	2·6
	L-Triiodothyronine	2	1·8	14·3	0·21	2·6
		100	1·4	14·1	0·18	2·9
Succinate†	0	—	3·4	8·9	0·29	1·3
	L-Thyroxine	2	3·2	8·7	0·29	1·4
		100	1·9	8·5	0·18	1·3
Succinate	0	—	4·2	12·5	0·23	1·6
	L-Triiodothyronine	2	4·3	12·1	0·22	1·4
		100	3·8	12·0	0·20	1·4

* Single flasks. † Initial ATP 80% of usual amount.

Table 2. *Effect of* L-*thyroxine and* L-*triiodothyronine on phosphorylation: pretreatment at* 0°

β-Hydroxybutyrate substrate. Conditions as in Table 1 except that mitochondria were in contact with iodo compound alone for 30 min. at 0°. The remaining components were added together (1 ml.) just before incubation at 20°. Final concentration of iodo compound 10^{-4}M.

Addition	O_2 uptake in 10 min. (μatoms)	Labile P of ATP per flask (μmoles)	Specific radioactivity of P of ATP (counts/μg. P/min.)	Specific radioactivity of P of ATP / specific radioactivity of orthophosphate P	P/O ratio
0	0·9	13·7	372	0·12	3·1
L-Triiodothyronine	0·6	11·6	37	—	~0
L-Thyroxine	0·6	11·9	12	—	~0

Table 3. *Relationship between acetoacetate production and oxygen uptake*

Mitochondria (2 ml.) pretreated with iodo compound (1 ml.) for 40 min. at 0°. Other components of the reaction mixture as in Table 1 added together (1 ml.) immediately before incubation at 30°. Flasks incubated for 10 and 25 min. and acetoacetate determined in all flasks. Oxygen uptake measured between 10 and 25 min.

Addition	Final concn. of iodo compound ($M \times 10^{-5}$)	Acetoacetate formed per flask (μmoles) 0–10 min.	0–25 min.	By difference, 10–25 min.	Oxygen uptake from 10 to 25 min. per flask (μatoms)	Ratio 10–25 min. acetoacetate / oxygen
0	—	2·8	5·5	2·7	3·4	0·79
L-Thyroxine	1	2·5	5·3	2·8	3·6	0·78
	10	1·7	3·6	1·9	2·4	0·79
0	—	2·0	4·6	2·6	3·0	0·87
L-Triiodothyronine	1	2·0	4·9	2·9	3·2	0·91
	10	1·8	3·8	2·0	2·5	0·80

the rate at which myokinase transfers the terminal P of ATP to the β position, and the error due to this limiting factor increases with the period of incubation. When mitochondria which had been washed only once to avoid depletion of myokinase were incubated with succinate as substrate for 6 min. at 20° phosphorylation quotients of 1·8–2 were obtained.

The effect of L-*thyroxine and* L-*triiodothyronine on phosphorylation.* There was no clear effect on the phosphorylation quotient when the iodo compound was added immediately before incubation as shown in Table 1. At 10^{-4}M both substances inhibited respiration. In confirmation of the findings of Martius & Hess (1951, 1952) and of Hoch & Lipmann (1953), phosphorylation was uncoupled after the mitochondria had been in contact with L-thyroxine or L-triiodothyronine for 30 min. at 0° before the incubation at 20° was carried out. Results in Table 2 show that after such pretreatment with 10^{-4}M iodo compound, specific radioactivities of ATP were not more than 10 % of the control value. Thus the inhibition was at least 90 %. When uncoupling occurred, up to 15 % of the ATP broke down and phosphorylation quotients could therefore not be calculated accurately since the method of calculation used here is based on constant levels of orthophosphate and ATP.

Comparison of uncoupling by L-*thyroxine and* L-*triiodothyronine.* When the effects of several concentrations of L-thyroxine and L-triiodothyronine were compared in the same experiment it was convenient to measure acetoacetate production and ^{32}P exchange in the same vessel. P/acetoacetate ratios were calculated instead of phosphorylation quotients. If oxygen is used solely for converting β-hydroxybutyrate into acetoacetate, the ratio μmoles acetoacetate/μatoms oxygen has a value of 1. With the method used here oxygen uptake could only be measured after a 10 min. equilibration period. For the time interval 10–25 min. the ratio μmoles acetoacetate/μatoms oxygen had a value of 0·8 in the presence or absence of L-thyroxine or L-triiodothyronine (Table 3). This shows that other oxidations were taking place, probably similar to those responsible for the blank respiration. When ^{32}P exchange and acetoacetate were measured for the same 10 min. incubation period, the P/acetoacetate ratio was therefore higher than the correct overall P/O ratio. Further, the rate of acetoacetate production in the first 10 min. was higher than subsequently and the oxygen uptake may fall in a similar manner. For this reason the phosphorylation quotients calculated in the previous experiments from the steady oxygen uptake between 10 and 30 or 40 min. may be higher by about 20 % than the correct overall P/O ratio in the first 10 min.

Table 4 gives P/acetoacetate ratios for a 10 min. incubation period with β-hydroxybutyrate as the

Table 4. *Comparison of uncoupling by* L-*thyroxine and* L-*triiodothyronine*

β-Hydroxybutyrate and succinate substrates. Conditions as in Table 2 except that pretreatment was for 40 min. With β-hydroxybutyrate as substrate, 10 min. incubation at 20° was carried out on a Dubnoff shaker and P/acetoacetate ratios were calculated from the acetoacetate production. When succinate was the substrate phosphorylation quotients were calculated from oxygen uptakes.

Substrate	Addition	Final concn. of iodo compound ($M \times 10^{-5}$)	Acetoacetate formed per flask (μmoles)	O_2 uptake in 10 min. (μatoms)	Labile P of ATP per flask (μmoles)	Specific radioactivity of P of ATP	Specific radioactivity of P of ATP / specific radioactivity of orthophosphate P	P/acetoacetate ratio	P/O ratio
β-Hydroxybutyrate	0	—	1·0	—	11·2	782	0·16	2·7	—
	L-Thyroxine	1	0·8	—	10·7	652	0·13	2·5	—
		5	1·2	—	9·0	31	—	~0	—
		10	1·2	—	9·4	32	—	~0	—
	L-Triiodothyronine	1	1·0	—	11·5	731	0·15	2·7	—
		5	1·0	—	9·8	450	0·09	1·4	—
		10	1·3	—	9·2	24	—	~0	—
Succinate	0	—	—	2·7	10·0	2326	0·23	—	1·4
	L-Thyroxine	5	—	1·7	7·5	229	—	—	~0
	L-Triiodothyronine	5	—	2·8	9·7	2270	0·21	—	1·3

substrate. At 10^{-4}M both L-thyroxine and L-triiodothyronine gave over 90% uncoupling, but neither substance uncoupled at 10^{-5}M. At the intermediate concentration of 5×10^{-5}M, L-thyroxine was more effective, causing over 90% uncoupling, whereas L-triiodothyronine only lowered the P/acetoacetate ratio to about one-half of the control value.

gave less uncoupling than the same concentration of L-thyroxine. At 2×10^{-4}M there was no uncoupling by L-thyronine or L-diiodotyrosine. Although the data are insufficient for exact quantitative comparison, they show that of the substances tested, L-thyroxine is the most effective uncoupling agent.

Uncoupling action of iodine. Whilst potassium iodide had no uncoupling effect, 2×10^{-4}M iodine in

Table 5. *Effect of substances related to* L-*thyroxine on phosphorylation*

Succinate substrate. Once-washed mitochondria (2 ml.) pretreated with substance tested (1 ml.) for 35 min. at 0°. Other components of reaction mixture as in Table 1 added together (1 ml.) and flasks shaken at 20° in air; 6 min. incubation for ^{32}P exchange. Oxygen uptake measured after 10 min. equilibration in duplicate flasks. In cases where no data for oxygen uptake are available, the ratio of specific activities gives an approximate indication of the uncoupling.

Expt. no.	Addition	Final concn. (M $\times 10^{-5}$)	O_2 uptake in 10 min. (μatoms)	Specific radioactivity of P of ATP / specific radioactivity of orthophosphate P	P/O ratio
1	0	—	3·7	0·16	1·6
	D-Thyroxine	5	3·5	0·13	1·3
	L-Thyroxine	5	3·0	0·02	0·2
2	0	—	3·9	0·18	1·9
	DL-Thyroxine*	2·5	—	0·17	—
		5	3·3	0·11	1·2
	L-Thyroxine*	2·5	—	0·16	—
		5	3·2	0·07	0·8
3	0	—	2·6	0·14	2
	L-Diiodothyronine	10	2·4	0·05	0·8
	L-Thyroxine	10	2·3	0·01	~0
4	0	—	3·4	0·16	1·9
	L-Diiodotyrosine	20	3·2	0·16	1·9
	L-Thyronine	20	3·2	0·17	2·0

* Single flasks.

Table 6. *Uncoupling of phosphorylation by iodine*

Conditions as in Table 5 except that pretreatment of mitochondria was carried out for 30 min. and flasks for ^{32}P exchange incubated for 7 min.

Expt.	Final concn. (M) KI	Final concn. (M) I_2	O_2 uptake in 10 min. (μatoms)	Labile P of ATP per flask (μmoles)	Specific radioactivity of P of ATP	Specific radioactivity of P of ATP / specific radioactivity of orthophosphate	P/O ratio
1*	0	0	2·3	12·0	3370	0·15	1·7
	2×10^{-2}	0	2·1	12·2	3040	0·14	1·8
	2×10^{-2}	2×10^{-5}	2·3	12·3	2610	0·13	1·5
2	8×10^{-3}	0	2·0	10·4	2230	0·15	1·9
	8×10^{-3}	5×10^{-5}	2·1	9·6	1490	0·1	1·0
	8×10^{-3}	2×10^{-4}	0·8†	8·7	31	—	~0

* Single flasks. † O_2 uptake fell to zero within 30 min.

This conclusion was confirmed with succinate as the substrate. At 5×10^{-5}M the phosphorylation quotient with L-triiodothyronine was only slightly lower than the control, but L-thyroxine uncoupled over 90%.

Uncoupling action of substances related to L-*thyroxine.* A comparison of L-thyroxine with related substances (Table 5) showed that D-thyroxine, DL-thyroxine and L-diiodothyronine all

potassium iodide solution uncoupled completely like L-thyroxine (Table 6). In terms of atoms of iodine, 2×10^{-4}M iodine is equivalent to 10^{-4}M L-thyroxine. However, the concentration of iodine which uncoupled also inhibited respiration to such an extent that it fell to zero in 30 min., and at this concentration iodine was adsorbed on to the mitochondria in an amount sufficient to give visible staining.

DISCUSSION

Phosphorylation quotients approaching 3 for β-hydroxybutyrate and 2 for succinate obtained here by the method of Krebs *et al.* (1953) agree with the values found previously for rat-liver mitochondria by measuring net phosphate esterified (Lehninger & Smith, 1949; Lardy & Wellman, 1952; Copenhaver & Lardy, 1952). L-Thyroxine uncoupled phosphorylation at concentrations of about 10^{-4}M only after preliminary contact with the mitochondria. This confirms the results of Martius & Hess (1951, 1952) and of Hoch & Lipmann (1953) on closely similar systems. Lardy & Feldott (1951) and Maley & Lardy (1953), using glutamate as a substrate, showed some lowering of the phosphorylation quotient by $1{\cdot}3 \times 10^{-5}$M L-thyroxine added at the beginning of incubation.

The change from no uncoupling to over 90% uncoupling by L-thyroxine occurred over the small range of concentration, 10^{-5}M–5×10^{-5}M. This differs from the wider range usually found in enzyme inhibition, for example the uncoupling by 2:4-dinitrophenol (Loomis & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949).

Since uncoupling in these experiments was only brought about by relatively high concentrations of L-thyroxine, it is of interest that Lardy & Feldott (1951), Martius & Hess (1951, 1952) and Hoch & Lipmann (1953) all found partial uncoupling in mitochondria prepared from thyroid-fed animals, and concluded that uncoupling may be a physiological effect. That uncoupling is related to the physiological action is also suggested here by the fact that, with the exception of L-triiodothyronine, the extent to which substances related to L-thyroxine uncouple phosphorylation resembles their effectiveness *in vivo*. Thus Gaddum (1930) found D- and DL-thyroxine and L-diiodothyronine less effective than L-thyroxine in raising the oxygen uptake of rats, whilst L-thyronine and L-diiodotyrosine were without activity. The greater activity *in vitro* of L-thyroxine as compared with the DL- and D- isomers differs from the earlier finding of Maley & Lardy (1953), who, under somewhat different conditions, showed that these compounds all have the same uncoupling activity. Although iodine uncoupled phosphorylation like thyroxine, it also caused visible staining of the mitochondria and soon inhibited respiration completely. It is therefore unlikely that thyroxine acted in a non-specific way on account of its iodine atoms.

In contrast to the greater *in vivo* activity of L-triiodothyronine as compared with L-thyroxine (Gross & Pitt-Rivers, 1953), L-triiodothyronine has been shown to be less effective than L-thyroxine as an uncoupling agent *in vitro*. Gross & Leblond (1951) showed that triiodothyronine is formed in the body from thyroxine, and Maclagan, Sprott & Wilkinson (1952) suggested on other evidence that triiodothyronine is the active form of the thyroid hormone formed in the body from thyroxine. However, triiodothyronine resembled thyroxine in uncoupling only after preliminary contact with the mitochondria. In the following paper a study of the uptake of L-thyroxine and L-triiodothyronine suggests an explanation for their different relative activities *in vivo* and *in vitro*.

SUMMARY

1. The action of L-thyroxine and related substances on oxidative phosphorylation by rat-liver mitochondria has been studied in a system where phosphorylation quotients were measured by the rate of incorporation of ^{32}P into ATP according to Krebs *et al.* (1953).

2. L-Thyroxine and L-triiodothyronine uncoupled phosphorylation provided there was preliminary contact for 30–40 min. with the mitochondria at 0°.

3. L-Thyroxine was a more effective uncoupling agent. About 5×10^{-5}M L-thyroxine and 10^{-4}M L-triiodothyronine were required to give over 90% uncoupling.

4. D-Thyroxine, DL-thyroxine and L-diiodothyronine all uncoupled phosphorylation less readily than the same concentration of L-thyroxine. L-Thyronine and L-diiodotyrosine were without activity at a concentration of 2×10^{-4}M.

5. Potassium iodide had no effect on phosphorylation, but 2×10^{-4}M iodine uncoupled phosphorylation and inhibited respiration.

6. These results are in agreement with the findings of other workers, and are discussed in relation to the physiological action of thyroxine and triiodothyronine.

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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