Effects of Thyroid Hormones *in vitro* on Tissue Respiration, Oxidative Phosphorylation and the Swelling of Mitochondria

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Recently (Salmony, 1956) it was shown that certain synthetic oestrogens, and some chemically closely related compounds, are able to evoke a remarkable increase in yeast-cell respiration of the type first described for 4:4′-dihydroxy-$\alpha\beta$-diethylstilbene (stilboestrol) by Shacter (1953*a*). This effect was shown (Salmony, 1956) to be associated with the ability of such compounds to bring about the uncoupling of oxidative phosphorylation in mitochondrial preparations and also with their inhibitory action on the adenosine triphosphatase activity of myosin.

Since 1951 it has been recognized that thyroxine is another hormone which is able to inhibit oxidative phosphorylations (Lardy & Feldott, 1951; Martius & Hess, 1951) and the physiological effects of this hormone have been attributed by some authors to this property. The subject has recently been extensively reviewed (Lardy & Maley, 1954; Hoch & Lipmann, 1954; Martius, 1956). An interesting difference between the action of the synthetic oestrogens and thyroxine is that whereas the depression of the P/O ratio by the former occurs without any appreciable lag period (Salmony, 1956), thyroxine, to be effective, requires preliminary contact with the mitochondria for considerable periods (usually 40 min. at 0°; Martius & Hess, 1951; Klemperer, 1955). This delayed effect has been considered as analogous to the well-known lag period in the metabolic action of thyroxine in the whole animal.

Harington & Pitt-Rivers (1952) have synthesized the analogue of DL-thyroxine in which the alanine side chain is replaced by an acetic acid residue (3:5-diiodo-4-3′:5′-diiodo-4′-hydroxyphenoxyphenylacetic acid; tetraiodothyroacetic acid or tetrac). The similar derivative corresponding to triiodothyronine (triiodothyroacetic acid or triac) has been synthesized by Pitt-Rivers (1953), who demonstrated the thyroid-hormone activity *in vivo*.

Thibault & Pitt-Rivers (1955*a*, *b*) reported that these new compounds have an immediate metabolic effect *in vivo*. Further, they describe a rapidly occurring increase in the rate of oxygen uptake of rat-kidney slices exposed to the action of these two acetic acid derivatives; large increases of up to 100% were observed, which persisted for about 30 min. and thereafter gradually diminished (Thibault & Pitt-Rivers, 1955*a*). This effect was not produced by either thyroxine or triiodothyronine, except after extraordinarily long contact (up to 3 days) with kidney slices at 4° (Thibault & Pitt-Rivers, 1955*a*, *b*).

Through the kindness of Dr Pitt-Rivers in supplying us with the thyroacetic acids, we were able to investigate their action on oxidative phosphorylations in rat-liver mitochondrial preparations. At her request, we also attempted to confirm the experiments on kidney slices of Thibault & Pitt-Rivers (1955*a*, *b*), but without success. On the other hand, we were able to observe uncoupling of mitochondrial oxidative phosphorylations in the presence of these new substances and this action, unlike that of thyroxine and triiodothyronine, was instantaneous.

Cleland (1952) showed that when sarcosomes are suspended in isotonic buffered saline, the optical density of the suspension slowly decreases, owing to the swelling of the sarcosomes, which results in less light-scattering as their refractive index decreases. This swelling can be reversed by addition of low concentrations of adenosine triphosphate (ATP) (Chappell & Perry, 1954). These authors reported that 2:4-dinitrophenol (DNP), however, completely inhibits the reversal of swelling induced by ATP. There appeared to be a correlation between the effects of DNP on the activation of the mitochondrial adenosine triphosphatase activity and its effect on the reversal of swelling. This method was used by Lehninger, Cooper & Tapley (1955) to demonstrate that thyroxine and triiodothyronine, in low concentrations at room temperature, cause a rapid swelling of rat-liver mitochondria in isotonic sucrose. Klemperer (1955) had independently described hydration of mitochondria produced by contact with L-thyroxine at 0°.

In view of the more rapid and rather greater activity of triiodo- and tetraiodo-thyroacetic acids, as compared with thyroxine, on the uncoupling of oxidative phosphorylation in mitochondria, it was of interest to compare the effect of these compounds with that of thyroxine on the swelling of mitochondria. These thyroid-hormone derivatives were also found to be highly effective inhibitors of the adenosine triphosphatase activity of myosin. On

the other hand, they had little or no action in increasing the respiration of impoverished yeast cells, such as that observed with synthetic oestrogens and related substances (Salmony, 1956).

MATERIALS AND METHODS

L-Thyroxine. This was a gift from Glaxo Laboratories Ltd.

Triiodothyroacetic and tetraiodothyroacetic acids. These were given by Dr R. Pitt-Rivers.

Hexokinase. A preparation of crude hexokinase, presented by Sigma Chemical Co., was purified by ethanol precipitation according to the method of Kunitz & McDonald (1946), and finally dissolved in 1% glucose.

Mitochondria. These were isolated essentially according to the method of Schneider (1948). The liver from a freshly killed albino rat was chilled in 0·25M sucrose at 0°, and disintegrated for 2 min. in a Potter–Elvehjem all-glass homogenizer in approx. 5 vol. of cold 0·25M sucrose (prepared in glass-distilled water). All subsequent steps were carried out at +2°. Nuclei, intact cells and cell debris were removed by centrifuging the homogenate for 10 min. at 700 *g*. The mitochondria were then sedimented at 5000 *g* for 20 min., and the opalescent supernatant was removed. The mitochondrial pellet was washed by resuspending in 0·25M sucrose and centrifuging for 10 min. at 24000 *g* and was finally suspended in 0·25M sucrose so that 1 ml. of suspension contained mitochondria from 1 g. of fresh liver.

Measurement of oxidative phosphorylation. The mitochondrial suspension (1 ml.) was added, immediately after preparation, to Warburg flasks containing 0·3 ml. of 0·2M potassium phosphate buffer, pH 7·4, 0·3 ml. of 0·01M adenosine 5′-phosphate, 0·1 ml. of 0·2M-$MgSO_4$, 0·1 ml. of 0·035M ethylenediaminetetraacetic acid (EDTA), 0·3 ml. of 0·1M sodium glutamate or succinate, 0·1 ml. of 2×10^{-4}M cytochrome *c*, 0·3 ml. of 0·26M fructose, 0·5 ml. of hexokinase (containing 50 μg.) and 0·1 ml. of 0·39M-NaF. Oxygen uptake was measured for 15 min. after 5 min. equilibration at 30°. The amount of inorganic phosphate utilized after 15 min. was determined by addition of 0·5 ml. of 70% (w/v) trichloroacetic acid from the side arm of the Warburg flasks at 0 and 15 min., and measurement of inorganic phosphate in the supernatants by the method of Fiske & Subbarow (1925).

An aqueous solution of the thyroxine derivatives was added either directly to the contents of the Warburg flasks, or together with the mitochondrial suspension after the mixture had been kept for 40 min. at 0°. Thyroxine was dissolved in the minimum quantity of dilute NaOH and a similar amount of NaOH was added to the control experiments. The final concentration of NaOH (approx. 5×10^{-4}N) did not measurably affect the pH. When malonate was used, 10 μmoles of the sodium salt was added to each flask.

Manometric measurements. The O_2 uptake of rat-kidney slices was measured by the Warburg technique. Kidney-cortex slices were incubated at 37° in an atmosphere of O_2 in 1·8 ml. of a Ringer solution containing 0·2% glucose (Ullrick & Whitehorn, 1952). A solution of the sodium salts of tri- or tetra-iodothyroacetic acid (0·2 ml.) was added from the side arm of the flask after temperature equilibration for approx. 10 min. The centre well contained 0·2 ml. of 20% (w/v) KOH. Duplicates in all experiments were in satisfactory agreement, the values in the tables being the means.

Black-hooded rats, which had been thyroidectomized at the National Institute for Medical Research, were used. Some of these were kept at 26° for 2 weeks after thyroidectomy, a procedure used in the experiments of Thibault & Pitt-Rivers (1955*a*, *b*; personal communication from Dr Pitt-Rivers).

Measurement of swelling of mitochondria. The mitochondrial suspension (0·05 ml. in 0·25M sucrose) was added at zero time to 2·85 ml. of 0·25M sucrose and either 0·1 ml. of the compound to be tested, when necessary dissolved in the minimum quantity of dilute NaOH, or 0·1 ml. of an equal concn. of NaOH, in a 1 cm. cell. The change in optical density was read in a Hilger Uvispek spectrophotometer at 520 mμ. against a blank containing 0·05 ml. of 0·25M sucrose instead of the mitochondrial suspension.

RESULTS

Effect of iodo compounds on oxidative phosphorylation

The effect of triiodothyroacetic acid and tetraiodothyroacetic acid on oxidative phosphorylation in rat-liver mitochondria was compared with that of thyroxine (Table 1). Oxygen uptake, in the presence of glutamate or succinate as substrate, and disappearance of inorganic phosphate, was measured as described under Methods. Thyroxine was able to uncouple the phosphorylation associated with the oxidation of succinate only after pretreatment of the mitochondrial suspension for 40 min. at 0°. When glutamate was used as substrate, no uncoupling was observed even after pretreatment at 0°. When malonate (10 μmoles) was added, however, to prevent the further oxidation of succinate (Lardy & Feldott, 1951), uncoupling of oxidative phosphorylation was shown, but only after pretreatment of the mitochondria with thyroxine and malonate at 0°. Both triiodothyroacetate and tetraiodothyroacetate had an immediate effect in inhibiting phosphorylation without significantly affecting the oxygen uptake in the presence of succinate when these hormones were added directly to the mitochondria in the Warburg flasks; with glutamate as substrate some inhibition of respiration occurred, especially after pretreatment at 0°. The activity of the acetic acid derivatives in uncoupling oxidative phosphorylation was not significantly increased by pretreatment of the mitochondria at 0° with these substances. Triiodothyroacetate was slightly more effective than the tetraiodo derivative in uncoupling oxidation from phosphorylation. Thibault & Pitt-Rivers (1955*a*) have reported that triiodothyroacetate was more active than the tetraiodo derivative in raising the oxygen consumption of kidney slices from thyroidectomized rats.

Effect on respiration of kidney slices

Kidney-cortex slices from thyroidectomized rats, which had been fasted up to 24 hr., were incubated in a Ringer solution in the presence of the sodium

salts of triiodothyroacetic acid and tetraiodothyroacetic acid. Respiration was measured as described under Methods. Neither of the acetic acid derivatives, at concentrations of $1{\cdot}6 \times 10^{-7}$–$1{\cdot}6 \times 10^{-5}$ M appeared to have any effect on the oxygen uptake of the tissue slices compared with that of the controls without the iodo compounds (Table 2). To reproduce exactly the conditions adopted by Thibault & Pitt-Rivers (1955*a*), some of the rats were kept at 26° for about 2 weeks, immediately after thyroidectomy. Apart from an increased dehydration of the kidneys as shown by the dry-weight determinations, which averaged 23% higher than normal, no significant effect on the respiratory rate (Q_{O_2}) was observed, in the presence of either of the acetic acid derivatives of thyroxine. The Q_{O_2} of kidney slices from intact rats, whether or not they had been maintained at 26°, was also unaffected by addition of either of the iodo compounds (Table 2).

Table 1. *Effect of* L-*thyroxine and acetic acid derivatives on oxidative phosphorylation*

The main compartment of the Warburg flasks contained 0·3 ml. of 0·2 M phosphate buffer (pH 7·4), 0·3 ml. of 0·01 M adenosine 5′-phosphate, 0·1 ml. of 0·2 M-$MgSO_4$, 0·1 ml. of 0·035 M EDTA, 0·3 ml. of 0·1 M sodium glutamate or succinate, 0·1 ml. of 2×10^{-4} M cytochrome *c*, 0·3 ml. of 0·26 M fructose, 0·5 ml. of hexokinase, 0·1 ml. of 0·39 M-NaF, ±0·1 ml. of iodo compound and 1 ml. of mitochondria suspended in 0·25 M sucrose; 0·5 ml. of 70% (w/v) trichloroacetic acid was contained in the side arm. Gas phase, air; temp. 30°; 0·2 ml. of 20% KOH in centre well; 15 min. incubation. Malonate, when present, 10 μmoles.

Substrate	Addition	Pre-treatment at 0° (min.)	Concn. of iodo compound (M)	O_2 uptake (μg. atoms)	P uptake (μg. atoms)	P/O ratio	Depression of P/O ratio (%)
Succinate	None	0	—	5·6	10·5	1·9	—
	L-Thyroxine	0	5×10^{-5}	5·8	11·0	1·9	0
	None	40	—	6·2	8·3	1·3	—
	L-Thyroxine	40	5×10^{-5}	6·5	5·3	0·8	38
	None	0	—	10·1	16·0	1·6	—
	Triiodothyroacetic acid	0	5×10^{-5}	9·2	10·4	1·1	31
	None	0	—	5·8	9·2	1·6	—
	Triiodothyroacetic acid	0	10^{-4}	5·0	3·2	0·6	62
	None	0	—	4·9	8·5	1·7	—
	Tetraiodothyroacetic acid	0	5×10^{-5}	4·8	6·1	1·3	24
	Tetraiodothyroacetic acid	0	10^{-4}	4·1	4·4	1·1	35
Glutamate	None	40	—	4·2	10·5	2·5	—
	L-Thyroxine	40	5×10^{-5}	3·8	10·6	2·8	0
	Malonate	0	—	4·8	11·8	2·5	—
	L-Thyroxine + malonate	0	5×10^{-5}	4·4	10·8	2·5	0
	Malonate	40	—	4·2	9·7	2·3	—
	L-Thyroxine + malonate	40	5×10^{-5}	4·5	5·4	1·2	47
	None	0	—	9·6	29·4	3·0	—
	Triiodothyroacetic acid	0	5×10^{-5}	6·5	13·1	2·0	33
	None	40	—	15·0	34·2	2·3	—
	Triiodothyroacetic acid	40	5×10^{-5}	6·8	9·7	1·4	39

Table 2. *Oxygen uptake of rat-kidney slices, in presence of triiodothyroacetic and tetraiodothyroacetic acids*

Rat-kidney cortex slices (approx. 9 mg. dry wt.) were incubated in 1·8 ml. of Ringer solution; 0·2 ml. of iodo compound was added from the side arm of the Warburg vessel; 0·2 ml. of 20% KOH in centre well. Gas phase, O_2; temp., 37°. Values quoted are Q_{O_2} (μl. of O_2/mg. dry wt./hr.).

	Control		$1{\cdot}6 \times 10^{-7}$ M 'Triac'		$1{\cdot}6 \times 10^{-6}$ M 'Triac'		$1{\cdot}6 \times 10^{-5}$ M 'Triac'		$6{\cdot}6 \times 10^{-6}$ M 'Tetrac'	
Conditions	0–30 min.	30–60 min.	0–30 min.	30–60 min.	0–30 min.	30–60 min.	0–30 min.	30–60 min.	0–30 min.	30–60 min.
Rat thyroidectomized, fasted 24 hr.	20·3	—	—	—	18·9	—	—	—	17·5	—
Rats thyroidectomized, fasted overnight	21·2	21·3	20·0	20·1	17·6	17·8	18·8	18·8	—	—
	17·8	17·8	—	—	18·0	18·1	—	—	—	—
Rat thyroidectomized, kept at 26° for 2 weeks, fasted overnight	17·8	18·2	—	—	18·2	18·5	—	—	20·0	20·0
Intact rat, kept at 26° for 2 weeks, fasted overnight	18·4	18·3	—	—	18·4	18·3	—	—	17·6	17·6

Swelling of mitochondria

When a freshly prepared mitochondrial suspension in isotonic sucrose is diluted with isotonic sucrose, a gradual swelling occurs, as can be shown by measuring the optical density of the suspension, as described under Methods. It has already been shown by Lehninger *et al.* (1955) that when thyroxine is added to a mitochondrial suspension in isotonic sucrose, the amount of swelling is greatly increased compared with that of the control containing only sucrose. This was confirmed, as shown in Fig. 1, and the effect of thyroxine was compared with that of tri- and tetra-iodothyroacetic acids. It was found (Figs. 1 and 2) that both these compounds, at a final concentration of $3{\cdot}3 \times 10^{-5}$M, increased the swelling of the rat-liver mitochondria to a greater extent than did the same concentration of thyroxine. When this effect was compared with that of stilboestrol, it was found that this substance, like DNP (Chappell & Perry, 1954; Lehninger *et al.* 1955), completely inhibited the swelling which occurs in isotonic sucrose (Fig. 1).

It was shown that when mitochondria were added to isotonic sucrose containing both stilboestrol and either thyroxine or one of the acetic acid derivatives, the swelling caused by the thyroid compounds was completely blocked for approximately 10 min., after which the optical density gradually decreased again in some of the preparations; Fig. 2, curve *A*, shows a typical experiment with both oestrogen and thyroid hormone. *p*-Chloromercuribenzoate had no significant effect on swelling at a concentration of 3×10^{-5}M; at higher concentrations (3×10^{-4}M), however, there was a considerable increase, similar in magnitude to that with triac and tetrac.

The addition of 0·0016M ATP to the swollen mitochondria not only completely reversed the swelling of the controls and those to which thyroxine or stilboestrol had been added, but caused a very marked shrinking of the mitochondria. ATP, however, did not have such a pronounced effect on those mitochondria which had swollen to a much greater extent due to the action of triac or tetrac. Although an immediate increase in optical density was observed, this never regained the original value in these experiments (Fig. 2).

Effect of thyroid hormones on adenosine triphosphatase activity of myosin

To complete the comparison with stilboestrol and related substances, the action of thyroxine, triac and tetrac upon the adenosine triphosphatase activity of myosin was studied under the same conditions as those adopted by Salmony (1956). The

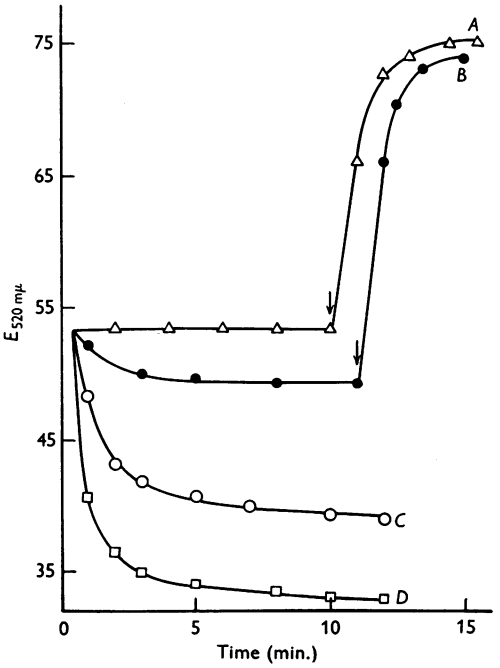

Fig. 1. Swelling of mitochondria. The system contained 2·85 ml. of 0·25M sucrose, 0·05 ml. of mitochondrial suspension and 0·1 ml. of water or compound being tested; 0·1 ml. of 0·05M ATP was added where shown (↓). *A*, $3{\cdot}3 \times 10^{-5}$M stilboestrol; *B*, control; *C*, $3{\cdot}3 \times 10^{-5}$M thyroxine; *D*, $3{\cdot}3 \times 10^{-5}$M tetrac.

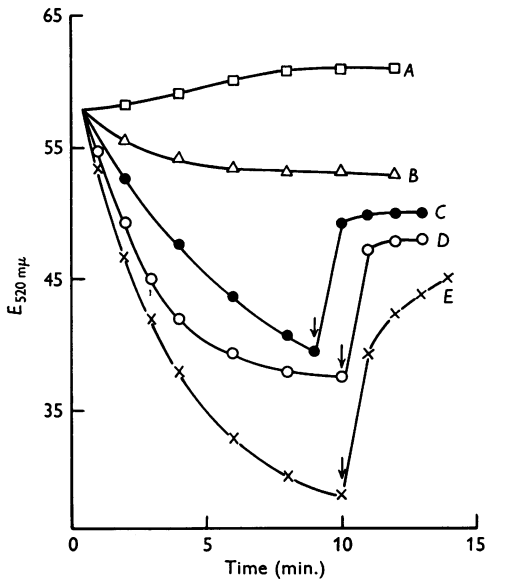

Fig. 2. Swelling of mitochondria. Conditions as in Fig. 1. *A*, $3{\cdot}3 \times 10^{-5}$M stilboestrol + $3{\cdot}3 \times 10^{-5}$M tetrac; *B*, control; *C*, $3{\cdot}3 \times 10^{-5}$M triac; *D*, $3{\cdot}3 \times 10^{-5}$M tetrac; *E*, $3{\cdot}3 \times 10^{-4}$M *p*-chloromercuribenzoate.

results (Table 3) show that although all three substances are inhibitory in low concentrations, the activity of the acetic acid derivatives exceeds that of L-thyroxine.

Effect of thyroid hormones on yeast cell respiration

No striking effects such as those previously found for stilboestrol and related compounds were observed. With thyroxine (2×10^{-4}–5×10^{-7} M) no significant increase occurred. With tetrac no increases of Q_{O_2} were seen at concentrations from 4×10^{-4} to 2×10^{-6} M; at the high level of 7×10^{-4} M the increase was slight (about 50% above the control). Under similar conditions with oestrogens, increases of 600% or more were observed (Salmony, 1956).

DISCUSSION

It has been shown previously (Salmony, 1956) that stilboestrol and some chemically related substances greatly increase the respiration of yeast cells. The present experiments were undertaken to extend these findings to other compounds, such as the thyroid hormones, whose chemical constitution resembles that of stilboestrol in possessing a 4-hydroxyphenyl group linked to another 4-substituted benzene ring.

Table 3. *Effect of thyroid hormones on adenosine triphosphatase activity of myosin*

The reaction mixture contained 1·0 ml. of 0·1 M glycylglycine buffer (pH 8·9), 0·1 ml. of 0·02 M-$CaCl_2$, 0·5 ml. of myosin, 0·3 ml. of 0·02 M ATP and 0·1 ml. of water or thyroid hormones. Incubated for 10 min. at 30°; deproteinized by addition of 0·2 ml. of 70% trichloroacetic acid. The zero time value has been subtracted in each case.

Compound added	Concn. (M)	P liberated (μg./10 min.)	Inhibition (%)
None	—	51·3	—
L-Thyroxine	2×10^{-3}	5·0	90·3
None	—	40	—
L-Thyroxine	2×10^{-4}	40	0
	5×10^{-4}	25·3	36·7
Tetrac	5×10^{-5}	40·8	0
	2×10^{-4}	18·5	53·8
None	—	41·4	—
Triac	5×10^{-5}	37·4	9·7
	2×10^{-4}	19·8	52·5

Unfortunately, we have been unable to confirm the findings of Thibault & Pitt-Rivers (1955*a*, *b*) that the oxygen uptake by kidney slices from thyroidectomized rats is strongly increased by the addition of the acetic acid analogues of thyroxine and triiodothyronine, tetrac and triac. In spite of care to reproduce as closely as possible the conditions described by these authors, we have not found any such increase of kidney-slice respiration *in vitro*.

However, corresponding with the rapid action of the acetic acid analogues *in vivo* (Pitt-Rivers, 1953) these substances have now also been shown (Table 1) to cause an immediate uncoupling of mitochondrial oxidative phosphorylation with either succinate or glutamate as substrate, whereas it is well known that thyroxine itself acts in this way only after a considerable lag period, during which the preparation is exposed at 0° to the action of the hormone. It has been confirmed that in order to demonstrate the uncoupling action of thyroxine in the presence of glutamate as substrate it is necessary to eliminate, by means of malonate, the further oxidation of succinate formed (Lardy & Feldott, 1951). When we tested triac in this system, the addition of malonate was unnecessary.

The rapid action of the acetic acid derivatives compared with that of thyroxine does not appear to be due to their more rapid penetration into the mitochondria, as it has been shown by Klemperer (1955) that thyroxine itself diffuses into the mitochondria extremely rapidly, even at 0°. One possible explanation which could account for this would be that oxidative deamination and decarboxylation of thyroxine and triiodothyronine might precede their action, and that the acetic acid derivatives might, in fact, be the active form of the hormones. Direct chromatographic evidence of this conversion occurring in the rat has now been provided by Roche, Michel, Jouan & Wolf (1955), consistent with the rapidity of action of the thyroacetic acids *in vitro* and *in vivo*.

In an attempt to study the basis of the uncoupling of oxidative phosphorylation by these substances, we have compared them with some other compounds known to be active in this respect. Table 4 summarizes the results obtained in the following systems: (1) increase of respiration of impoverished

Table 4. *Action of various inhibitors of oxidative phosphorylation on other biological systems*

Substance	Depression of P/O ratio	Increase of yeast respiration	Effect on myosin adenosine triphosphatase	Effect on swelling of mitochondria
Stilboestrol	+ + +	+ + +	Inhibits	Inhibits
2:4-Dinitrophenol	+ + +	+ +	Accelerates	Inhibits
Thyroacetic acids	+ +	±	Inhibits	Increases
Thyroxine	+*	−	Inhibits	Increases
p-Chloromercuribenzoate	+	+	Inhibits	Increases†

* After a lag-period.

† At high concentration only.

yeast cells (Shacter, 1953*a*, *b*; Salmony, 1956; and present work); (2) inhibition of oxidative phosphorylation in mitochondrial preparations [Loomis & Lipmann (1948) and others, for DNP; *p*-chloromercuribenzoate, quoted by Lehninger (1951); Salmony (1956) for stilboestrol and related compounds; Lardy & Feldott (1951) and Martius & Hess (1951) for thyroxine; present work for thyroacetic acids]; (3) effect on adenosine triphosphatase activity of myosin [Greville & Needham (1955) for DNP and *p*-chloromercuribenzoate; Salmony (1956) for stilboestrol etc.; present work for thyroid hormones]; (4) effect on the swelling of mitochondria [Chappell & Perry (1954) for DNP; Lehninger *et al.* (1955) for thyroxine; present work for stilboestrol, thyroacetic acids and *p*-chloromercuribenzoate].

There appears to be some correlation (Table 4) between the ability of this group of substances to depress the P/O ratio and to increase yeast respiration, although in this latter respect the action of the thyroacetic acids is very weak and thyroxine is inactive. Permeability differences may, of course, be involved here. Even in the comparatively simple system of adenosine triphosphatase activity of myosin, a consistent behaviour was not observed, DNP being the only one of the substances tested which was found, as previously reported by Greville & Needham (1955), to accelerate the activity, while the others were inhibitory (Table 4). The mechanism by which DNP affects this reaction remains obscure (cf. Greville & Needham, 1955).

Finally, the fact that thyroxine causes swelling of mitochondria (Klemperer, 1955) led us to test the effect of the two thyroacetic acids on this system. Both were found to be extremely powerful swelling agents towards rat-liver mitochondria in isotonic sucrose (Figs. 1 and 2), the swelling produced being reversed by subsequent addition of ATP. In this respect the thyroid hormones act similarly to *p*-chloromercuribenzoate, although the latter requires a much higher concentration to be effective (Fig. 2). Contrasting with these actions is the effect of stilboestrol, which, like DNP (Chappell & Perry, 1954), prevents the spontaneous swelling of the mitochondria (Fig. 1). This property of DNP has been suggested as being connected with its ability to uncouple oxidative phosphorylations (Raaflaub, 1953), an explanation which would agree with the similar behaviour of stilboestrol. Once more, however, the thyroid hormones form an exception, since they also inhibit oxidative phosphorylations, but nevertheless powerfully increase the swelling. Possibly some of these apparent contradictions may prove to be reconcilable by a study of the activation of the 'latent' adenosine triphosphatase of the mitochondria (Lardy & Wellman, 1953) by the group of substances studied here, but preliminary studies do not support this suggestion.

Because of the similarity of action on oxidative phosphorylation and yeast-cell respiration which we have found to exist between the thyroid hormones and the stilboestrol-like compounds, it is of interest that certain iodine-free 4-hydroxydiphenyl ethers have a definite, though weak, oestrogenic activity (Dodds & Lawson, 1938). These compounds include 4-hydroxydiphenyl ether and 4:4′-dihydroxydiphenyl ether, the structural resemblance of which to the thyroacetic acids is evident. The presence of iodine substituents in the hexoestrol molecule lowers, but does not destroy, the oestrogenic activity, since 3:3′-diiodohexoestrol is active in promoting uterine growth in immature rats (10–100 μg. dose; Hillmann-Elies & Hillmann, 1953) and produces oestrus in 100 μg. doses in adult ovariectomized rats (Lawson, personal communication).

SUMMARY

1. The action of thyroid hormones (L-thyroxine, tetraiodothyroacetic acid and triiodothyroacetic acid) has been compared with that of stilboestrol upon selected enzymic activities.

2. The increased respiration of kidney slices of thyroidectomized rats, reported for the thyroacetic acids by Thibault & Pitt-Rivers (1955*a*, *b*), has not been confirmed.

3. The inhibitory effect upon oxidative phosphorylation in rat-liver mitochondrial preparations shown by thyroxine is more pronounced with the two acetic acid analogues. Whereas preliminary contact with thyroxine is necessary, the thyroacetic acids cause immediate uncoupling of respiration and phosphorylation, similar to that previously found for stilboestrol.

4. The adenosine triphosphatase activity of myosin is powerfully inhibited by all three thyroid hormones.

5. The swelling of rat-liver mitochondria, which is increased by thyroxine, is found to be even greater with the thyroacetic acids. *p*-Chloromercuribenzoate at higher concentrations also causes the swelling of mitochondria.

6. Stilboestrol stabilizes the state of swelling of the mitochondria and neutralizes the swelling action of the thyroacetic acids.

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The Sulphatase of Ox Liver

5. SULPHATASE *C**

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The previous papers of this series have dealt with those sulphatases which can readily be extracted by water from an acetone-dried powder of ox liver (Roy, 1953*a*, *b*; 1954*a*) or which can be obtained in true solution by extraction of the fresh tissue with water (Roy, 1954*a*). These sulphatases, named sulphatases *A* and *B* (Roy, 1953*a*), are apparently predominantly localized in the mitochondria, at least in the liver of the mouse (Roy, 1953*a*) and of the rat (Roy, 1954*b*). They rapidly hydrolyse dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate), but attack simpler compounds such as *m*-tolyl, *p*-nitrophenyl and *p*-acetylphenyl sulphates relatively slowly. Previously unpublished work in this laboratory indicated the presence in mouse liver of another sulphatase which was localized in the microsomes, which rapidly hydrolysed *m*-tolyl and *p*-nitrophenyl sulphates, and which could not be obtained in true solution by the usual methods. Dodgson, Spencer & Thomas (1955) and Dodgson, Spencer & Wynn (1956) have shown the occurrence of comparable enzymes in rat and human tissues respectively.

The present paper describes the preparation and properties of this insoluble sulphatase, sulphatase *C*, of ox liver.

* Part 4: Roy (1955).

EXPERIMENTAL

Preparation of sulphatase C

Ox liver (200 g.) was cut into small pieces and treated in an Atomix blender (Measuring and Scientific Equipment Co. Ltd.) for 2 min. with 300 ml. of ice-cold water. All the following stages were carried out at 0° unless otherwise stated. The suspension was centrifuged for 5 min. at 2000 rev./min. and the residue of connective tissue, etc., discarded. The supernatant was then centrifuged for 30 min. at 20000 *g* and the loosely packed sediment collected. This sediment was washed twice by suspending it in water and recentrifuging.

The washed sediment was suspended in about 100 ml. of water and treated with 0·2 vol. of 10% Lissapol NX (I.C.I. Ltd.). After standing overnight the debris was centrifuged down at 35000 *g* and discarded, giving a clear solution of sulphatase *C*. The enzyme was precipitated by pouring its solution into 4 vol. of acetone at −20° and was centrifuged down at −15°: the sediment was washed successively with acetone and ether at −20° and finally dried rapidly *in vacuo* over P_2O_5. This yielded sulphatase *C* as a red-brown insoluble powder. Last traces of soluble enzymes were removed by suspending the enzyme in water, by the use of a loose-fitting glass homogenizer to obtain an even suspension, and sedimenting sulphatase *C* at 35000 *g*. The residue was washed twice with water, suspended in a convenient volume of water and freeze-dried, yielding approximately 1 g. of a buff-coloured powder which could readily be suspended in water.