Influence of Mitochondrial Inhibitors on the Respiration and Energy-Dependent Uptake of Iodide by Thyroid Slices

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The influence of mitochondrial inhibitors, including oligomycin, antimycin and rotenone, on the iodide and oxygen uptake and the nucleotide content of incubated sheep thyroid slices was investigated. Each inhibitor strongly suppressed both iodide and oxygen uptake, and decreased the nucleoside triphosphate content of the slices. In most cases the addition of glucose or mitochondrial substrates restored iodide uptake in inhibitor-treated slices. Inhibitor concentrations sufficient to inhibit iodide uptake strongly had only slight effects on the thyroidal $Na^+ + K^+$-activated adenosine triphosphatase. It is concluded that the inhibitors produce their effects by the inhibition *in vivo* of mitochondrial oxidative phosphorylation. ATP synthesis appears to be essential for iodide uptake to occur, and the high-energy intermediates (or energized state) of oxidative phosphorylation cannot be used to energize the uptake process. To a limited extent glycolytic ATP synthesis can support iodide uptake, which is therefore not exclusively dependent on aerobic metabolism. The mechanism of energy-linked iodide uptake is discussed.

Surviving thyroid slices are able to concentrate inorganic iodide from the incubation medium by a process that is independent of thyroid-hormone biosynthesis (Franklin, Chaikoff & Lerner, 1944; Schachner, Franklin & Chaikoff, 1944). The concentration factor, termed the tissue/medium ratio (Freinkel & Ingbar, 1955*a,b*), is lowered to values close to 1 by treatments that inhibit or uncouple mitochondrial oxidative phosphorylation, e.g. the addition of 2,4-dinitrophenol or cyanide or incubation under nitrogen. These studies suggested that thyroidal iodide uptake requires energy from oxidative phosphorylation (Freinkel & Ingbar, 1955*a,b*). At that time little was known about energy metabolism in the thyroid. More recently, phosphorylating mitochondria have been isolated from thyroid homogenates (Turkington & Nordwind, 1962). These preparations contain the phosphorylating respiratory-chain system present in other types of mammalian mitochondria, and oligomycin, antimycin and rotenone have been found to exert their usual effects on the electron- and energy-transfer reactions (Lamy, Rodesch & Dumont, 1964; Gonze & Tyler, 1965; Degroot, Dunn & Jaksina, 1966).

As pointed out by Wolff (1964), it is not known whether the abolition of iodide uptake by 2,4-

dinitrophenol or anaerobic conditions is due to an effect on the synthesis of ATP or of high-energy intermediates of oxidative phosphorylation. Huijing & Slater (1961) suggested that oligomycin should be a useful reagent to test whether the high-energy intermediates can be directly utilized for energy-requiring reactions without having first to be converted into ATP. With isolated mitochondria, oligomycin prevents the use of high-energy intermediates for ATP synthesis, but has no effect on their use in other energy-linked reactions (Ernster & Lee, 1964). In the present study the influence of oligomycin and other mitochondrial inhibitors on the iodide and oxygen uptake of sheep thyroid slices was investigated to determine whether high-energy intermediates (or the energized state; Mitchell, 1966) of oxidative phosphorylation could be used directly to energize iodide uptake.

When thyroid slices are incubated with exogenous glucose, mitochondrial respiration accounts for about 80% and glycolysis for about 20% of the synthesis of 'high-energy' phosphate bonds (Dumont, 1965). The influence of glycolytic ATP synthesis on iodide uptake was therefore also evaluated.

There is strong evidence that the activity of the thyroidal $Na^+ + K^+$-activated ouabain-sensitive ATPase† ('transport ATPase') is a prerequisite for

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† Abbreviation: ATPase, adenosine triphosphatase.

iodide uptake (Wolff, 1964). The effect of mitochondrial inhibitors on the thyroid transport-ATPase activity was measured, and attempts were made to restore iodide uptake in inhibitor-treated slices by the addition of suitable substrates. The results of these studies prompted the measurement of the nucleotide contents of thyroid slices incubated in various metabolic states.

A preliminary account of this work has been presented (Tyler, Gonze & Dumont, 1966).

MATERIALS AND METHODS

Oligomycin and antimycin were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.); rotenone, dicyclohexylcarbodi - imide, ouabain, butyl 4 - hydroxy - 3,5 - diiodobenzoate, $K^{131}I$ and methimazole were from S. B. Penick and Co. (New York, N.Y., U.S.A.), British Drug Houses Ltd. (Poole, Dorset), California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.), Laboratoires Pharmaceutiques Corbière (Paris, France), C.E.N. (Mol, Belgium) and Christiaens S.A. (Brussels, Belgium) respectively. Atractyloside was a generous gift from Dr B. C. Pressman.

The methods used in the preparation and for the incubation of sheep thyroid slices were as described by Dumont (1964). The slices were incubated in a Warburg apparatus or in a Dubnoff metabolic incubator under an atmosphere of air or oxygen at 37°. Similar results were obtained with either gas phase. Each flask contained about 100 mg. wet wt. of slices and 2 ml. of incubation medium. The medium used was usually Krebs–Ringer phosphate buffer, pH 7·4, (Dumont, 1965), containing methimazole (2 mM) and $K^{131}I$ (0·8 μM; specific activity 100 c/mole). No glucose or other substrate was added except when explicitly stated. This medium is referred to as 'standard incubation medium'. In some experiments, Na^+ ions were replaced completely by K^+ ions ('Na^+-free medium'). Control flasks contained the same quantity of solvent (usually 10 μl. of ethanol) as the inhibitor-containing flasks. At the end of the incubation, slices were removed rapidly from the flasks, blotted on filter paper, weighed and placed in counting tubes. The radioactivity of the slices and of samples of the incubation medium was measured in a well-type scintillation counter (PW 4003; Philips) with a statistical error of less than 1%. The tissue/medium ratios were calculated from the formula (Freinkel & Ingbar, 1955a,b):

$$\text{Tissue/medium ratio} = \frac{\text{Radioactivity/100 mg. of slices}}{\text{Radioactivity/0·1 ml. of medium}}$$

In several experiments it was established that none of the radioactivity of the thyroid slices was precipitated by trichloroacetic acid, i.e. that methimazole inhibited completely the conversion of inorganic iodide into iodinated compounds. When slices were incubated with $KClO_4$ (2 mM) the tissue/medium ratio was always close to 1. The logarithmic mean of the tissue/medium ratio obtained under these conditions in 40 experiments was 1·0 (0·92–1·08), where 0·92 and 1·08 are the antilogarithms of the mean ± S.D.

In short-term experiments the kinetics of respiratory inhibition after the addition of mitochondrial inhibitors to thyroid slices was measured by the oxygen-electrode technique (Chappell, 1964). Respiration rates are expressed as μl. of O_2 consumed/100 mg. wet wt. of slices/hr.

The ATPase preparation was obtained from 10% (w/v) homogenates of sheep thyroids in ice-cold glass-distilled water. The particulate material sedimented after centrifugation of the homogenate at 100000g for 30 min. in a Spinco model L ultracentrifuge was washed twice with water and finally resuspended. The preparation was either used fresh or, usually, was stored at −20° in the form of a freeze-dried powder (Wolff & Halmi, 1963). Between 10 and 15 mg. of powder was usually obtained for each 100 mg. wet wt. of fresh thyroid homogenized. The powder was weighed out and resuspended by homogenization in water just before use. ATPase activity was assayed in a 2 ml. reaction mixture containing tris–HCl buffer, pH 7·4 (50 mM), $MgCl_2$ (2 mM), ATP (3 mM), NaCl (100 mM), KCl (10 mM) (Van Groningen & Slater, 1963) and enzyme. The amount of enzyme preparation added was equivalent to between 60 and 80 mg. wet wt. of fresh thyroid. The samples were incubated in a Dubnoff shaker for 30 min. at 37°. The reaction mixture was stopped by the addition of 0·1 ml. of 70% (w/v) $HClO_4$, and the precipitated protein was removed by centrifugation. Then P_i was measured in the supernatant fluid by the method of Fiske & Subbarow (1925). The transport-ATPase activity was assumed to be that fraction of the total ATPase activity (usually 15–25%) inhibited by 0·1 mM-ouabain. The ouabain-sensitive ATPase activity of the freshly isolated preparation and of the freeze-dried rehydrated preparation showed similar responses towards the inhibitors tested.

Nucleotides were extracted from the slices and measured by the method of Adam (1963). The cytochrome a content of sheep thyroid mitochondria was assumed to be similar to that of ox thyroid mitochondria. The value used was 0·2 nmole/mg. of protein (Tyler & Gonze, 1967). The mitochondrial content of sheep thyroids, based on attempts at the quantitative recovery of the mitochondrial fraction of five sheep thyroid homogenates, was found to be 0·6–1·3 mg. of protein/100 mg. wet wt. (mean 1·05 mg. of protein).

Results are expressed as the mean values of several duplicates ± maximum ranges of values.

RESULTS

Inhibition of iodide uptake and respiration. The influence of oligomycin, antimycin and rotenone on the iodide uptake and the endogenous respiration rate of sheep thyroid slices during a 3 hr. incubation period is shown in Fig. 1. Each inhibitor lowered the tissue/medium ratio to 1–3 and also strongly inhibited the rate of oxygen uptake. When the inhibitor concentration used was 1–5 μg./2 ml./ 100 mg. of slices, antimycin and rotenone inhibited oxygen uptake by about 95%. Similar concentrations of oligomycin usually inhibited oxygen uptake by 60–75%, but with two out of 12 preparations the inhibition was only about 30%. In the latter preparations the tissue/medium ratio was also lowered to below 3, despite the relative insensitivity of the respiration to the inhibitor.

The extent of inhibition induced by a fixed inhibitor concentration depended on the weight of
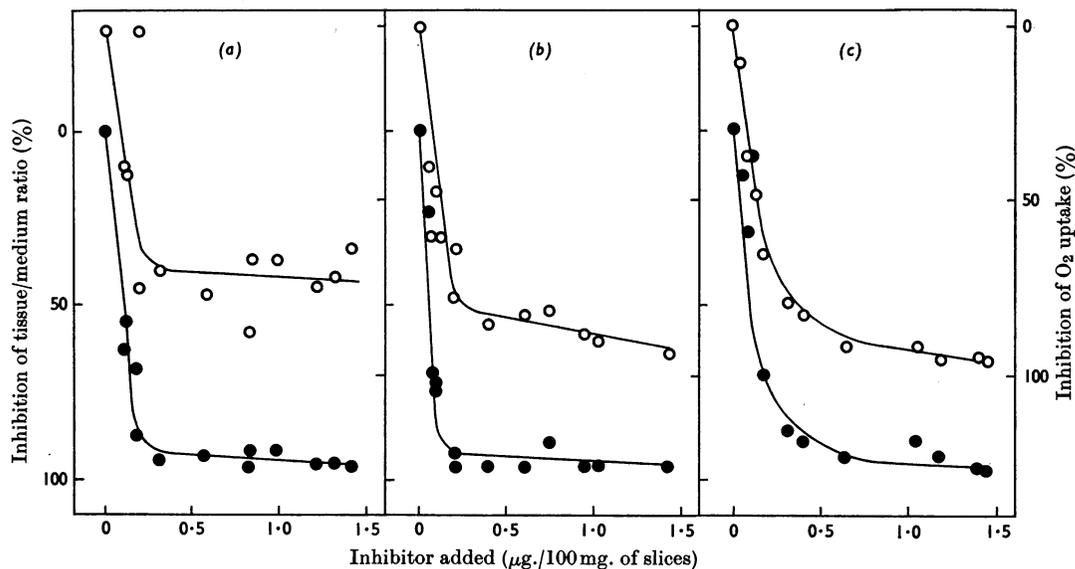
Fig. 1. Effect of oligomycin (a), antimycin (b) and rotenone (c) on the tissue/medium ratio (●) and respiration rate (○) of thyroid slices. The slices were added to manometer flasks containing standard incubation medium and various amounts of inhibitor and were incubated at 37° for 3 hr. before assay of the tissue/medium ratio. The mean values of the tissue/medium ratio and respiration rate of control samples were 43·7 and 37·2 μl. of O_2/100 mg./hr. respectively.

thyroid slice added to the incubation medium. The percentage inhibitions of iodide and oxygen uptake were therefore plotted against the amount of inhibitor added/100 mg. of slices, as in Fig. 1, and not against inhibitor concentration/ml. A similar phenomenon was observed by Ernster, Dallner & Azzone (1963) during experiments with isolated liver mitochondria. They found that the degree of inhibition of reactions concerned in oxidative phosphorylation by the same inhibitors was dependent on the quantity of mitochondria present in the assay system. With isolated mitochondria, about 1 mole of inhibitor/mole of cytochrome a was required to inhibit ATP synthesis strongly. The possibility that a similar relationship existed between the amount of inhibitor added and the quantity of mitochondria in the thyroid slices was therefore investigated. A value for the cytochrome a content of 100 mg. of thyroid slices was derived as indicated in the Materials and Methods section and the data of Fig. 1 were replotted to show the relationship between the inhibition of the tissue/medium ratio and the molar ratio of inhibitor to cytochrome, giving the result shown in Fig. 2. A 50% inhibition of iodide uptake was obtained with about 0·5 mole of antimycin/mole of cytochrome a or about 1·5 moles of oligomycin or rotenone/mole of cytochrome a. At higher inhibitor/cytochrome
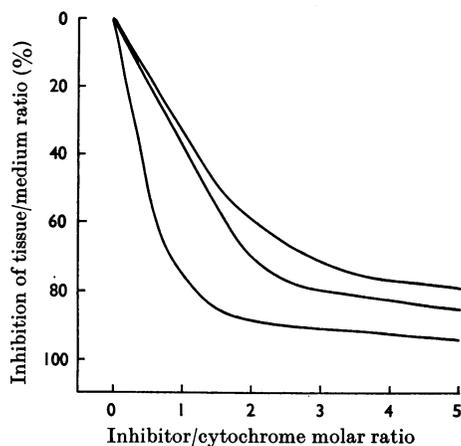
Fig. 2. Relationship between the tissue/medium ratio and the molar ratio of inhibitor to mitochondrial cytochrome used in the experiments of Fig. 1. Curves A, B and C refer to the results obtained with oligomycin, antimycin and rotenone respectively.

ratios, increments in the amount of inhibitor added produced progressively decreasing increments in the inhibition of iodide uptake, particularly when
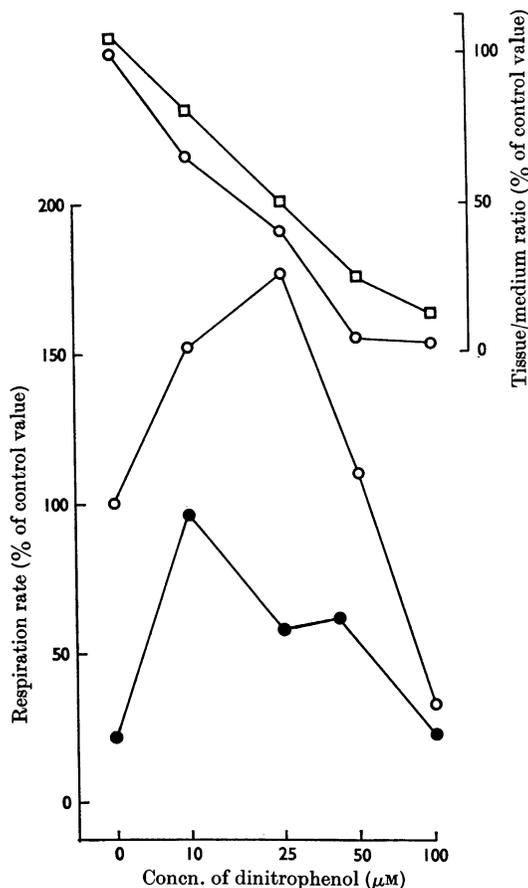
Fig. 3. Influence of 2,4-dinitrophenol concentration on the respiration rate and tissue/medium ratio. Thyroid slices were incubated in standard medium at 37° for 90min. before assay of the tissue/medium ratio. The mean values of the tissue/medium ratio and respiration rate of control samples were 24·7 and 43·7 μl. of O₂/100mg./hr. respectively. ○, No further addition; ●, oligomycin (1μg./ml.) present; □, glucose (14mM) present.

oligomycin or rotenone was used. These results suggest that the inhibitors were bound primarily by the mitochondria in the cells of the tissue slices, and that they were also bound by other sites after most of the mitochondrial sites had been occupied.

Studies of the kinetics of respiratory inhibition, recorded with the oxygen electrode, showed that no more than 3–4 min. incubation with the inhibitor was required to establish a maximal inhibitory effect. The inhibition of iodide uptake was less rapidly established, since the tissue/medium ratio in the presence of inhibitor declined gradually during a 3hr. incubation. In a typical experiment with 2 μg. of antimycin/100mg. of slices/2ml., mean values (four duplicates) of the tissue/medium ratios were 4·1, 2·7 and 1·3 after 20min., 1 hr. and 3hr. of incubation respectively. A similar phenomenon was observed when either oligomycin or rotenone was the inhibitor used.

The oligomycin-inhibited respiration of isolated mitochondria is restored by the uncoupling agent 2,4-dinitrophenol (Lardy, Johnson & McMurray, 1958). When 2,4-dinitrophenol was added to oligomycin-treated slices a marked stimulation of respiration was observed, but the rate of oxygen uptake remained lower than that obtained with 2,4-dinitrophenol-treated slices incubated in the absence of oligomycin (Fig. 3). Tobin & Slater (1965) observed a similar inhibition of the 2,4-dinitrophenol-stimulated respiration of rat kidney slices by oligomycin. A possible explanation is suggested by the work of Chappell & Greville (1961), who showed that in 2,4-dinitrophenol-treated mitochondria oxidations that were dependent on the conversion of α-oxoglutarate into succinate remained rate-limited by the availability of ADP if oligomycin was also present. In thyroid-slice preparations the respiration is due to citric acid-cycle activity, including this oxidation step (Dumont, 1962).

Iodide uptake was inhibited by 50% by 18μM-

Table 1. *Effect of added glucose on the tissue/medium ratio of inhibitor-treated slices*

The slices were added to cold Dubnoff flasks containing standard incubation medium and the concentrations of inhibitor and glucose indicated. The flasks were transferred rapidly to a bath at 37° and incubated with shaking for 90min. before assay of the tissue/medium ratio.

Expt. no.	Inhibitor	Concn. (μg./ml.)	Tissue/medium ratio		
			No added glucose	With 8mM-glucose	With 24mM-glucose
1	None	—	38·1±1·3	31·1±0·1	—
2	None	—	45·5±3·5	—	34·6±3·6
1	Oligomycin	0·5	3·2±0·8	14·0±4·9	—
2	Oligomycin	1·0	2·8±0·7	—	14·2±4·8
1	Oligomycin	5·0	2·8±0·8	8·8±2·9	—
1	Antimycin	0·5	1·6±0·0	8·6±1·5	—
2	Antimycin	1·0	2·2±0·5	—	9·4±3·0
2	Rotenone	1·0	1·5±0·2	—	9·0±0·8

2,4-dinitrophenol and was almost eliminated by 50 μM-2,4-dinitrophenol, in good agreement with the data of Freinkel & Ingbar (1955a,b). 2,4-Dinitrophenol was slightly less effective when glucose was added to the incubation mixture (Fig. 3). The finding that uncoupling agents were effective inhibitors of iodide uptake was confirmed by studies with the uncoupling agent butyl 4-hydroxy-3,5-di-iodobenzoate, which has been found to suppress iodide uptake by the thyroid *in vivo* (Lawson & Searle, 1952). Butyl 4-hydroxy-3,5-di-iodobenzoate inhibited iodide uptake by 50% at 10 μM concentration and fully inhibited the system at 50 μM. The effect of oligomycin was similarly confirmed by studies with dicyclohexylcarbodi-imide, which acts like oligomycin as an inhibitor of energy-transfer reactions in mitochondria (Beechey, Holloway, Knight & Roberton, 1966). Iodide uptake was inhibited by 50% by 6 μg. of dicyclo-hexylcarbodi-imide/100mg. of slices and fully inhibited by 20 μg./100mg. of slices. The influence of atractyloside, which inhibits the exchange of adenine nucleotides across the membranes of isolated mitochondria (Chappell & Crofts, 1965; Heldt, Jacobs & Klingenberg, 1965), was also studied. With two slice preparations atractyloside increased the tissue/medium ratio at the concentrations tested (1–10 μg./100mg. of slice) by up to 30% during a 3hr. incubation period.

Influence of added glucose. Thyroid slices contain very little glycogen-like material (Freinkel, 1964) and lactate formation is dependent on added glucose (Dumont & Tondeur-Montenez, 1965). These observations suggested that very little endogenous glycolytic ATP synthesis was taking place under the conditions of the experiments presented in Fig. 1. For this reason the influence of added glucose on the iodide uptake of inhibitor-treated slices was measured. As shown above, glucose addition produced a small increase in the tissue/medium ratio of 2,4-dinitrophenol-treated slices (Fig. 3). A more marked increase in the tissue/medium ratio was observed when glucose was added to slices that had been treated with oligomycin, antimycin or rotenone (Table 1). The restoring effect of glucose was more striking in 1 hr. than in 3hr. incubations. Glucose did not modify the tissue/medium ratio of slices incubated with perchlorate or in the absence of inhibitors.

Influence of mitochondrial substrates. Attempts were made to restore iodide and oxygen uptake in inhibitor-treated slices, including Amytal-treated slices, by the addition of suitable mitochondrial substrates. Amytal was found to be a relatively ineffective inhibitor. For example, 2mM-Amytal inhibited respiration by less than 50% and 5mM-Amytal gave only 66% inhibition (Table 2). With isolated mitochondria, including sheep thyroid

Table 2. *Effect of succinate on the tissue/medium ratio and respiration rate of inhibitor-treated slices*

The slices were added to cold manometer flasks containing all other components. Standard incubation medium was used in Expts. 1–4 and Krebs–Ringer bicarbonate medium, pH7·4 (Dumont, 1965), was used in Expt. 5. The flasks were equilibrated at 37° and the respiration rate was recorded for 3hr. before assay of the tissue/medium ratio. Oligomycin (1 μg./ml.), rotenone (1 μg./ml.) and succinate (15 mM) were added as indicated. Column (a): tissue/medium ratio; column (b): respiration rate (μl. of O_2/100mg./hr.).

Addition	Expt. 1 (a)	(b)	Expt. 2 (a)	(b)	Expt. 3 (a)	(b)	Expt. 4 (a)	(b)	Expt. 5 (a)
None	33·5±1·0	22·7±1·3	56·8±13·3	34·3±2·9	60·1±2·6	34·8±4·8	48·6±10·4	27·0±3·9	101·5±18·5
Succinate	24·2±3·7	53·5±4·2	66·6±15·4	76·8±20·8	48·9±7·9	64·2±8·1	38·4±6·6	50·0±6·3	141·0±3·0
Rotenone	1·5±0·5	2·7±2·1							1·7±0·3
Rotenone+succinate	2·2±1·4	39·3±12·4							1·5±0·0
Oligomycin			2·0±0·2						
Oligomycin+succinate			1·6±0·4						
Amytal (2mM)					36·4±1·5	21·7±0·4			
Amytal (2mM)+succinate					40·0±6·4	58·7±7·3			
Amytal (5mM)							15·5±9·3	11·3±2·6	
Amytal (5mM)+succinate							9·7±2·1	56·2±18·5	

Table 3. *Effect of ascorbate and* NNN'N'-*tetramethyl*-p-*phenylenediamine on the tissue/medium ratio and respiration rate of inhibitor-treated slices*

Standard incubation medium was used. Antimycin (1 μg./ml.), rotenone (1 μg./ml.), succinate (15 mM), ascorbate (10 mM) and NNN'N'-tetramethyl-p-phenylenediamine (TMPD) (0·2 mM) were added as indicated. Other conditions were similar to those specified in Table 2. Column (a): tissue/medium ratio; column (b): respiration rate (μl. of O_2/100 mg./hr.).

| | Expt. 1 | | Expt. 2 | Expt. 3 | Expt. 4 | Expt. 5 |
Addition	(a)	(b)	(a)	(a)	(a)	(b)
None	44·3 ± 5·4	55·5 ± 5·0	21·6 ± 7·5	61·8 ± 5·1	42·5 ± 4·3	35·1 ± 2·1
TMPD	15·6 ± 0·0	39·3 ± 0·0	13·2 ± 0·0	—	—	19·8 ± 6·2
Ascorbate + TMPD	14·3 ± 0·0	112·0 ± 0·0	5·4 ± 0·0	—	—	61·0 ± 0·0
Antimycin	1·4 ± 0·3	3·3 ± 0·1	2·8 ± 0·2	2·5 ± 0·6	7·4 ± 0·9	4·8 ± 2·0
Antimycin + TMPD	—	—	5·1 ± 1·6	—	14·3 ± 0·7	3·9 ± 2·0
Antimycin + TMPD + ascorbate	9·9 ± 1·1	107·7 ± 20·2	8·5 ± 0·8	33·5 ± 2·0	22·6 ± 0·9	73·8 ± 6·3
Antimycin + TMPD + ascorbate + rotenone	—	—	—	3·6 ± 1·0	—	—
Antimycin + TMPD + ascorbate + succinate	—	—	—	30·4 ± 0·0	—	—

mitochondria, 2 mM-Amytal induces an almost total inhibition of NAD-linked oxidations. These results are therefore difficult to understand, since experiments *in vivo* indicate that Amytal can rapidly enter other mammalian tissues (Chance, Williamson, Jamieson & Schoener, 1965). Oxygen uptake was restored by the addition of succinate to rotenone-treated slices in the absence of antimycin (Table 2) or by the addition of ascorbate and NNN'N'-tetramethyl-p-phenylenediamine to antimycin-treated slices (Table 3). Oxygen uptake supported by ascorbate and NNN'N'-tetramethyl-p-phenylenediamine in the presence of antimycin was accompanied by a significant restoration of iodide uptake. In contrast, succinate oxidation (tested at substrate concentrations varying between 0·2 and 15 mM), or ascorbate and NNN'N'-tetramethyl-p-phenylenediamine oxidation, in rotenone-treated slices failed to support any significant iodide uptake. The tissue/medium ratio remained close to 1 when rotenone-treated slices were incubated with succinate in bicarbonate buffer instead of the Krebs–Ringer phosphate buffer usually employed (Table 2). The results obtained with rotenone-treated slices were unexpected since in isolated rotenone-treated mitochondria ATP synthesis is supported either by succinate oxidation or by ascorbate and NNN'N'-tetramethyl-p-phenylenediamine oxidation (Ernster & Lee, 1964), and these results have been confirmed with isolated sheep thyroid mitochondria (J. Gonze & D. D. Tyler, unpublished work). The failure of succinate oxidation to energize iodide uptake in rotenone-treated slices might have been due to the fact that succinate was oxidized only by uncoupled mitochondrial particles released into the medium from

damaged cells. To test this hypothesis the oxygen uptake of thyroid slices was measured for 3 hr. in the presence of succinate and rotenone. The slices were then removed from the manometer flasks and the measurement was continued with the cloudy incubation medium. Removal of the slices was found to decrease the rate of oxygen uptake by about 80%. The material remaining in the flasks, which was responsible for the other 20% of the oxygen uptake, could be sedimented by centrifugation at less than 100 g for 10 min. It is concluded from this experiment that after a 3 hr. incubation period virtually all the oxygen uptake recorded is due to cellular respiration and that a significant proportion of whole cells becomes detached from the tissue slice during the incubation period. In the absence of inhibitors succinate had only slight effects on the tissue/medium ratio and did not alter the iodide uptake supported by ascorbate and NNN'N'-tetramethyl-p-phenylenediamine in antimycin-treated slices. Thus the failure of succinate oxidation to support iodide uptake in rotenone-treated slices is attributed to an effect of rotenone and not to an effect of succinate.

Inhibition of transport ATPase. Whittam, Wheeler & Blake (1964) found that a high concentration (10 μg./ml.) of oligomycin and ouabain (a specific inhibitor of transport-ATPase activity) both inhibited the respiration rate of rabbit kidney slices to the same extent. They proposed that the inhibitory action of oligomycin on respiration was due to an inhibition of transport-ATPase activity, which thus prevented the formation of ADP, the rate-controlling compound of mitochondrial respiration. Van Groningen & Slater (1963) showed that in brain preparations the mitochondrial ATPase is far

Table 4. *Effect of inhibitors on the thyroidal transport-ATPase activity*

For experimental conditions see the Materials and Methods section. The specific activity of control tubes was 1·6–2·1 μmoles of P_i/100 mg. wet wt./hr.

Inhibitor	Concn.	Inhibition (%)
Oligomycin	2·5 μg./ml.	28 ± 3
Oligomycin	5·0 μg./ml.	41 ± 3
Oligomycin	7·5 μg./ml.	50 ± 4
Oligomycin	40·0 μg./ml.	78 ± 6
Rotenone	7·5 μg./ml.	0 ± 6
Antimycin	5·0 μg./ml.	15 ± 4
Atractyloside	5·0 μg./ml.	4 ± 3
Dicyclohexylcarbodi-imide	100·0 μg./ml.	34 ± 6
Mersalyl	10·0 μM	82 ± 4
Mersalyl	50·0 μM	97 ± 0
Fluoride	12·5 mM	77 ± 10
Fluoride	50·0 mM	90 ± 3

more sensitive than is the transport ATPase to low concentrations of oligomycin, and they concluded that it is possible to distinguish between these two effects by carefully controlling the oligomycin concentration. Since ouabain has been found to abolish iodide uptake (Wolff & Maurey, 1958) and to inhibit the respiration of sheep thyroid slices (Dumont & Burelle, 1964), it seemed necessary to test the effect of oligomycin on the thyroidal transport-ATPase activity. For this purpose the action of oligomycin on iodide uptake by a known weight of tissue slices was compared with the action on the transport-ATPase activity of a sample of thyroid homogenate containing an equivalent weight of tissue. The concentrations of oligomycin required for 50% inhibition were 0·1 μg./100 mg./ 2 ml. and 15 μg./100 mg./2 ml. for the tissue/medium ratio and the transport-ATPase activity respectively (Table 4). Iodide uptake was thus inhibited by much lower oligomycin concentrations than were required to inhibit the ATPase. Rotenone, antimycin and atractyloside had little or no effect on the transport ATPase. The enzyme was partially inhibited by dicyclohexylcarbodi-imide and strongly inhibited by either fluoride or the organic mercurial mersalyl (Table 4). The latter result suggests that the thyroid enzyme contains functional thiol groups, and in this respect shows similar properties to the kidney enzyme (Skou, 1963).

Influence of oligomycin in Na+-free medium. The omission of Na+ ions from the incubation medium abolished iodide uptake and decreased the respiration rate by about 40%, in agreement with the results of Iff & Wilbrandt (1963) and Dumont & Van Sande (1965) (Fig. 4). These effects are presumably due to the suppression of the Na+ + K+-
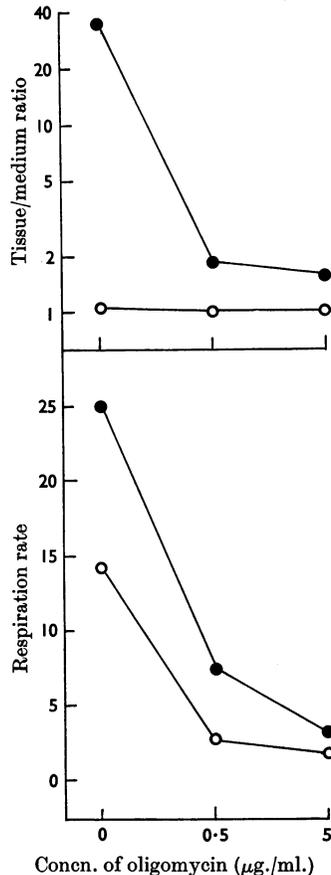
Fig. 4. Influence of oligomycin concentration on the tissue/medium ratio and the respiration rate (μl. of O_2/100 mg./hr.). Thyroid slices were incubated for 3 hr. at 37° in either standard incubation medium (●) or in Na+-free incubation medium (○) before assay of the tissue/medium ratio.

activated transport ATPase, which is thus unable to regenerate ADP. Oligomycin was found to inhibit the respiration rate of slices incubated in Na+-free medium, thus confirming that the influence of oligomycin was not confined to an inhibition of transport ATPase (Fig. 4).

Influence of inhibitors on the nucleotide content. The results presented above suggested that the inhibition of iodide uptake by mitochondrial inhibitors was due to an inhibition of mitochondrial ATP synthesis *in vivo*. This hypothesis was tested by measuring the effect of incubation with inhibitor on the nucleotide content of thyroid slices. The inhibitors were found to have two main effects. First, the total amount of nucleotide was decreased in inhibitor-treated slices. Secondly, the inhibitors strongly decreased the nucleoside

Table 5. *Effect of inhibitors on the nucleotide content of thyroid slices*

The slices were incubated in standard medium at 37° for 1 hr. before the nucleotide assay. Oligomycin (1 μg./ml.), antimycin (1 μg./ml.), rotenone (1 μg./ml.), succinate (15 mM) and dicyclohexylcarbodi-imide (0·8 mM) were added as indicated.

| | | Total nucleotide content (μmole/g. wet wt.) | | % of total | | | | | |
| | | | | Nucleoside triphosphate | | Nucleoside diphosphate | | AMP | |
Inhibitor	Expt. no. ...	1	2	1	2	1	2	1	2
None		—	0·293	86	91	14	8	0	1
Oligomycin		0·202	0·183	10	28·5	25	14	65	57·5
Antimycin		0·201	0·140	10·5	23	13·5	8	76	69
Rotenone		0·159	0·152	4·5	28	13	14	82·5	58
Rotenone + succinate		—	0·158	—	21·5	—	18	—	60·5
Dicyclohexylcarbodi-imide		0·154	—	17·5	—	20	—	62·5	—

triphosphate/nucleoside diphosphate ratio and induced a large increase in the AMP content (Table 5). These results show that the inhibitors strongly decrease the phosphorylating activity of the slices, and, since the nucleoside triphosphates other than ATP require ATP for their formation, the effects of the inhibitors must be due to an inhibition of ATP synthesis. The addition of succinate to rotenone-treated slices had no significant effect on the nucleotide content, indicating that in this system, which cannot support iodide uptake, no ATP synthesis is occurring. Exogenous ATP failed to restore iodide uptake in antimycin-treated slices.

DISCUSSION

Inhibition of iodide uptake. The addition at low concentration of the mitochondrial inhibitor oligomycin, antimycin and rotenone to sheep thyroid slices strongly inhibited iodide and oxygen uptake and caused a marked decrease in both the nucleoside triphosphate content and the nucleoside triphosphate/nucleoside diphosphate ratio. There is evidence supporting the conclusion that the inhibitors produce their effects through an action on mitochondrial oxidative phosphorylation *in vivo* and that these effects are similar in most respects to those on isolated mitochondria. First, the inhibitors produced marked effects when added in amounts approximately equimolar with the mitochondrial cytochrome content of the tissue. With isolated rat liver mitochondria a similar inhibitor/cytochrome ratio results in a powerful inhibition of ATP synthesis (Ernster *et al.* 1963), and experiments with isolated sheep thyroid mitochondria have yielded similar results (J. Gonze & D. D. Tyler, unpublished work). Secondly, the stimulating

effect of 2,4-dinitrophenol on the oligomycin-inhibited respiration of thyroid slices was similar to the influence of these reagents on isolated mitochondria (Lardy *et al.* 1958). Thirdly, the finding that glucose addition, and hence glycolytic ATP synthesis, could be used to energize iodide uptake in inhibitor-treated slices suggests that the inhibitors do not markedly influence the cell-membrane transport system for iodide uptake. This conclusion is supported by the finding that rotenone and antimycin had only slight effects on the thyroidal transport-ATPase activity, which is an essential part of the iodide-uptake mechanism (Wolff, 1964). With inhibition by oligomycin it is important to distinguish between two possible sites of action, the mitochondrial energy-transfer reactions and the transport ATPase. Ouabain, which abolished iodide uptake through an inhibition of the thyroidal transport ATPase (Wolff, 1964), inhibits sheep thyroid-slice respiration by about 40% (Dumont & Burelle, 1964). The influence of oligomycin differed from that of ouabain in important respects, showing that the influence of oligomycin was not restricted to an action on the transport ATPase. For example, oligomycin induced a more pronounced inhibition of respiration than did ouabain, and it also inhibited respiration occurring in the absence of added Na⁺ ions. Under the latter conditions, the transport-ATPase activity is strongly depressed and presumably has little influence on cellular metabolism. The concentration of oligomycin required for 50% inhibition of iodide uptake was very much lower than that required for 50% inhibition of the transport ATPase, and even at very high oligomycin concentrations the inhibition of the ATPase was incomplete. Dicyclohexylcarbodi-imide was also found to be a more

effective inhibitor of iodide uptake than of the transport ATPase. The similarity in the effects of oligomycin and dicyclohexylcarbodi-imide, both on mitochondrial oxidative phosphorylation and on the transport ATPase, suggests a basic similarity in their mechanisms of enzyme inhibition.

Further evidence about the site of action of the inhibitors is provided by studies of their effects on the nucleotide content of the slices. The decrease in the amount of nucleoside triphosphate induced by the inhibitors indicates that their mode of action is to inhibit ATP synthesis and not ATP utilization. This result therefore also suggests the mitochondria as the site of inhibition, and is inconsistent with an action on the membrane transport system for iodide uptake, which is linked to ATP utilization.

In general, the effects of added substrates on iodide and oxygen uptake in inhibitor-treated slices were compatible with the known effects of the compounds on subcellular preparations. For example, the restoration of iodide uptake by glucose was presumably supported by glycolytic ATP synthesis, a process that is not known to be affected by either oligomycin, antimycin, rotenone or 2,4-dinitrophenol. Thyroid slices contain very little glycogen-like material, and no lactate formation is observed during incubations *in vitro* unless glucose is added. In the absence of glucose ATP synthesis therefore depends almost entirely on aerobic mitochondrial metabolism; this factor explains the nearly total inhibition of iodide uptake by uncouplers and inhibitors of mitochondrial oxidative phosphorylation.

The restoration of iodide and oxygen uptake by the addition of ascorbate and *NNN'N'*-tetramethyl-*p*-phenylenediamine to antimycin-treated slices is to be expected, since under these conditions ATP synthesis can occur through the terminal phosphorylation site of the mitochondria (Jacobs, 1960). It was surprising that no significant iodide uptake was promoted by the addition of succinate to rotenone-treated slices, despite the restoration of a high rate of oxygen uptake. In isolated mitochondria, two phosphorylation sites are coupled to succinate oxidation in the presence of rotenone (Ernster *et al.* 1963). Since glucose restored iodide uptake in rotenone-treated slices, the site of action of rotenone *in vivo* appears to be restricted to the mitochondria, because a second site of inhibition, e.g. on the iodide-transport mechanism, would prevent the use of glycolytic ATP synthesis to support iodide uptake. The failure of succinate to alter the nucleotide pattern in rotenone-treated slices suggests that, in contrast with results obtained with mitochondria *in vitro*, no oxidative phosphorylation occurs with succinate and rotenone-treated thyroid mitochondria *in vivo*. It appears therefore that rotenone not only inhibits the

respiration of thyroid slices through the well-characterized inhibition of electron transport in the NADH–flavine region of the respiratory chain, but also uncouples the second and third phosphorylation sites. In agreement with this conclusion rotenone was found to prevent iodide uptake supported by ascorbate and *NNN'N'*-tetramethyl-*p*-phenylenediamine oxidation in the presence of antimycin. The discrepancy between the failure of rotenone to uncouple oxidative phosphorylation *in vitro* and the postulated uncoupling effect *in vivo* may be due to the conversion of rotenone *in vivo* into a compound with uncoupling activity.

Atractyloside inhibits the translocation of adenine nucleotides across the mitochondrial membrane, and the inhibition is competitive with ADP (Bruni, Contessa & Luciani, 1962; Chappell & Crofts, 1965). Unlike the other mitochondrial inhibitors tested atractyloside failed to lower the tissue/medium ratio. This failure may be due either to the inability of atractyloside to penetrate the thyroid cell membrane or to the fact that the intracellular ADP concentration was maintained sufficiently high to compete successfully with the inhibitor.

Respiratory control in vivo. Tobin & Slater (1965), in discussing their own work and that of other investigators, emphasized the relative insensitivity of the respiration of muscle, brain and kidney slices to oligomycin. They concluded that in the presence of oligomycin the energy of high-energy intermediates of oxidative phosphorylation is either largely dissipated or directly utilized. This conclusion is valid provided that the oxygen uptake observed is due to respiratory-chain activity, but often no evidence is presented in support of this assumption. In thyroid-slice metabolism the powerful inhibition of respiration by low concentrations of rotenone and antimycin indicates that virtually all the observed oxygen uptake is due to respiratory-chain activity. With isolated mitochondria oligomycin abolishes the 'active', ADP-stimulated, state of respiration (state 3), but has no effect on the 'resting' state of respiration (state 4) observed in the absence of ADP (Lardy *et al.* 1958). 2,4-Dinitrophenol, on the contrary, activates mitochondrial respiration to a rate similar to the state 3 respiration rate. The rates of respiration observed in thyroid slices treated with oligomycin or 2,4-dinitrophenol therefore provide an estimate of the state 4 and state 3 respiration rates respectively *in vivo*. The respiratory control ratio of mitochondria in thyroid slices, calculated as the ratio of these two rates, is about 7. This value compares well with the ratios (namely 3–5) that are commonly observed with isolated thyroid mitochondria. The strong inhibition of thyroid-slice respiration by oligomycin suggests that in thyroid

cells nearly all the energy conserved in oxido-reductions in the respiratory chain must be incorporated into ATP before it can be used.

Nature of the iodide-transport system. It may be concluded that the iodide uptake by thyroid slices is energized by ATP, which can be supplied by either aerobic mitochondrial ATP synthesis or by glycolytic ATP synthesis. The lowering of the tissue/medium ratio by oligomycin indicates that high-energy intermediates of oxidative phosphorylation cannot be used directly instead of ATP to energize iodide uptake. Exogenous ATP appears to be unable to replace endogenous ATP as the energy source, suggesting that the mechanism for iodide uptake in the thyroid cell membrane is arranged in a vectorial manner and can accept ATP to energize the system only from the inner side of the membrane. The nature of the iodide-transport system remains unknown. Since low concentrations of ouabain depress both the tissue/medium ratio and the thyroidal transport-ATPase activity (Wolff, 1964), the ATP requirement for iodide uptake may be necessary solely to energize opposing Na^+ and K^+ movements across the cell membrane. An important feature of the effects of mitochondrial inhibitors is that, whereas inhibition of respiration, and hence of ATP synthesis, was established within 5 min. of incubation, the full effect on the tissue/medium ratio was observed only after about a 3 hr. incubation. This result suggests that, although ATP synthesis is required to maintain iodide uptake, some other property of the system is able to support temporarily an iodide gradient between the tissue and the medium. There is other evidence that iodide uptake is not a simple consequence of the transport-ATPase activity (Iff & Wilbrandt, 1963; Scranton & Halmi, 1965; Brunberg & Halmi, 1966). Many of these observations, and those of the present work, can be explained by assuming that the mechanism of iodide uptake by the thyroid cell is similar to that proposed by Crane (1965) to explain the uptake of sugar by intestinal cells. By analogy with his hypothesis, the thyroid cell membrane is postulated to contain a specific carrier, which requires Na^+ to bind iodide. The iodide-binding site may be provided by the thyroidal phospholipid described by Vilkki (1962). The operation of the carrier alone would merely equilibrate the distribution of Na^+ and iodide across the thyroid cell membrane, but the asymmetric distribution of Na^+ maintained by transport-ATPase activity drives the iodide carrier in favour of net iodide entry into the cells.

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