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EFFECT OF SUBSTITUTED THIOPHENS ON THE ELECTRON TRANSPORT AND ADENOSINE TRIPHOSPHATASE ACTIVITIES OF RESPIRATORY PARTICLES

T. J. FRANKLIN, C. W. JONES AND E. R. REDFEARN

Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire (Great Britain), and Department of Biochemistry, University of Leicester, Leicester (Great Britain)

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SUMMARY

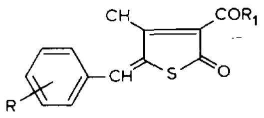
1. The cytotoxic compound 3-acetyl-5-(4-fluorobenzylidene)4-hydroxy-2-oxo 2:5 dihydrothiophen (I.C.I. 47776) inhibits both NAD- and non-NAD-linked oxidations by respiratory particles from rat liver, pig heart muscle and *Azotobacter vinelandii.*

2. Rat-liver preparations are much more sensitive than heart muscle or *A. vinelandii* particles to this compound.

3. The compound is a potent stimulator of the Mg^{2+}-dependent ATPase of rat-liver mitochondria but it strongly inhibits the 2,4-dinitrophenol-dependent ATPase activity.

INTRODUCTION

A number of substituted thiophens of the general formula



where R_1 is an alkyl or alkoxy group and R is an electron-withdrawing group suppress various immunological phenomena in the rat, including antibody synthesis[1]. These compounds are powerful inhibitors of protein and nucleic acid synthesis, and to a lesser extent of respiration, in isolated lymphocytes and a number of other cell types including rat liver *in vitro*[1]. It seemed of interest to study any possible action of the thiophen derivatives against isolated mitochondria from mammalian sources and also against electron-transport particles from *Azotobacter vinelandii.* The results described in this report show that the compounds are powerful inhibitors of the oxidative activity of rat-liver mitochondria and also have interesting effects on the adenosine triphosphatases of these particles. The thiophens also inhibit electron

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Abbreviations: I.C.I., Imperial Chemical Industries Ltd.; TMPD, *N,N,N′,N′*-tetramethylphenylenediamine hydrochloride; DNP, 2,4-dinitrophenol.

transport by preparations of pig heart muscle mitochondria and *A. vinelandii* particles although less effectively than in rat-liver mitochondria.

MATERIALS AND METHODS

Chemicals. For convenience the substituted thiophens described in this report are numbered as follows. I.C.I. 47776, R = 4-fluoro, $R_1 = CH_3$; I.C.I. 46446, R = 4-chloro, $R_1 = OC_2H_5$; I.C.I. 46452, no benzylidene group, $R_1 = CH_3$.

The synthesis and isolation of these compounds is fully described by O'MANT AND STACEY[2]. NADH and α-oxoglutaric acid were obtained from C. F. Boehringer und Soehne, Mannheim, Germany, thenoyltrifluoroacetone (4,4,4-trifluoro[2-thienyl]-1,3-butanedione) and the sodium salts of succinic, pyruvic and malonic acids from British Drug Houses, Poole, Dorset, and hexokinase from Seravac Laboratories (Pty) Ltd., Maidenhead, England.

Tissue preparations and assay procedures

Rat liver. Tissue was obtained from adult male albino rats and disrupted in 0.3 M sucrose with 15 strokes of a loosely fitting Potter–Elvehjem-type homogenizer at 1500 rev./min. Nuclei and debris were removed by spinning at 850 × *g* for 10 min at 0°. The supernatant was centrifuged at 5000 × *g* for 15 min to sediment the mitochondria which were washed once with 0.3 M sucrose and finally resuspended in 1 ml 0.3 M sucrose per g of original tissue.

The assay of Q_{O_2} and determination of P:O ratios were carried out in the Warburg apparatus at 37° for 20 min as described by ALDRIDGE[3] using approx. 5 mg of mitochondrial protein per Warburg flask. The thiophens were added to the reaction mixture dissolved in dimethyl sulphoxide so that the final concentration of the solvent was 0.5 % (v/v). This level of solvent alone had no effect on the consumption of O_2 or P_i by the mitochondria. Because of the instability of the thiophens in pure dimethyl sulphoxide it was necessary to dissolve the thiophens in the reagent immediately before adding to the aqueous reaction mixture. In aqueous solution containing 0.5 % dimethyl sulphoxide the thiophens were stable for several days.

The assay of the ATPase activity of rat-liver mitochondria was performed as described by EMMELOT AND BOS[4] at 37° for 20 min. Dimethyl sulphoxide (0.5 %, v/v) had no effect on the ATPase activity of mitochondria.

Heart-muscle preparations. These were prepared from pig heart as described by KING[5].

NADH and succinate oxidase activities were measured polarographically at 25° using a Clark electrode. The reaction mixture was as follows: 2.0 ml 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 7.4), 0.2 ml NADH (15 mg/ml) or 0.04 ml 0.3 M sodium succinate, 0.02 ml heart-muscle preparation (31.0 mg/ml). The thiophen derivatives were added in 0.02 ml dimethyl sulphoxide. This level of solvent alone was again without an inhibitory effect.

A. vinelandii preparations. Small particles were prepared as described by JONES AND REDFEARN[6]. The oxidation of succinate, DL-lactate, NADH and L-malate was measured at 30° using a Clark electrode[6]. Small particles (approx. 0.2 mg protein) were incubated for 2 min before the addition of substrate (30 μmoles, except in the case of NADH where 2.25 μmoles were used). Compound I.C.I. 47776 was dissolved in 0.05 ml ethanol and the volume of the reaction mixture was made up to 2.4 ml with glass-distilled water. Cytochrome oxidase activity was assayed using 30 μmoles sodium ascorbate *plus* 0.3 μmole *N,N,N′,N′*-tetramethylphenylenediamine hydrochloride (TMPD) with the other conditions as above.

RESULTS

Effect of thiophens on respiration of rat-liver mitochondria

The effects of compounds I.C.I. 47776, I.C.I. 46446 and I.C.I. 46452 on the consumption of oxygen by rat-liver mitochondria in the presence of either α-oxoglutarate alone, or α-oxoglutarate *plus* malonate or succinate alone were determined.

The results (Table I) indicate that thiophens possessing the benzylidene group at position 5 were potent inhibitors of mitochondrial respiration. The utilization of succinate was somewhat less sensitive than that of α-oxoglutarate.

Compound I.C.I. 46452, which lacks the benzylidene group, was very much

TABLE I

THE EFFECT OF THIOPHEN DERIVATIVES ON THE OXIDASE ACTIVITIES OF RESPIRATORY PARTICLES FROM RAT LIVER, PIG HEART MUSCLE AND *Azotobacter vinelandii*

The assay systems were as described under MATERIALS AND METHODS. O_2 uptake of rat-liver mitochondria was measured over 20 min and that of heart muscle and *A. vinelandii* particles over 5 min. The concentration of α-oxoglutarate, succinate and malonate in the rat-liver mitochondria assays was 10 mM in each case.

Compound	*Enzyme preparation*	*Substrate*	*Concentration for 50 % inhibition (μM)*	*Maximal inhibition observed (%)*	*Concentration for maximal inhibition (μM)*
I.C.I. 47 776	Rat-liver mitochondria	Succinate	6	75	200
I.C.I. 47 776	Rat-liver mitochondria	α-Oxoglutarate (+ malonate)	2	90	25
I.C.I. 47 776	Pig heart muscle mitochondria	NADH	75	83	263
I.C.I. 47 776	Pig heart muscle mitochondria	Succinate	87	88	284
I.C.I. 47 776	*A. vinelandii* particles	Ascorbate–TMPD	No significant inhibition observed at 500 μM		
I.C.I. 47 776	*A. vinelandii* particles	Succinate	375	60	500
I.C.I. 47 776	*A. vinelandii* particles	DL-Lactate	275	70	500
I.C.I. 47 776	*A. vinelandii* particles	NADH	70	95	500
I.C.I. 47 776	*A. vinelandii* particles	L-Malate	40	85	500
I.C.I. 46 446	Rat-liver mitochondria	α-Oxoglutarate	6	89	200
I.C.I. 46 452	Rat-liver mitochondria	α-Oxoglutarate	—	5	200
Thenoyltrifluoroacetone	Rat-liver mitochondria	α-Oxoglutarate (+ malonate)	27	70	200
Thenoyltrifluoroacetone	Rat-liver mitochondria	Succinate	6	90	200

less active than the other derivatives. Thenoyltrifluoroacetone also inhibited respiration but was more active against mitochondria utilizing succinate than α-oxoglutarate.

Effects of thiophens on oxidase activity of pig heart muscle preparations

I.C.I. 47776 appeared to be considerably less active against the heart muscle preparation than against rat-liver mitochondria (Table I). Compound I.C.I. 46452 had no effect on the oxidase activity of the heart muscle preparation (not shown).

Effect of I.C.I. 47776 on the oxidase activities of A. vinelandii small particles

Table I shows that I.C.I. 47776 was an effective inhibitor of NADH and L-malate oxidation, but was considerably less effective against the oxidation of succinate and DL-lactate. In these particles the oxidation of L-malate and DL-lactate is not linked to NAD$^+$ (ref. 6). The sensitivity of the bacterial oxidases to I.C.I. 47776 thus also appeared to be considerably less than that of α-oxoglutarate and succinate oxidases in rat-liver mitochondria.

Cytochrome oxidase activity (ascorbate–TMPD) was not inhibited by I.C.I. 47776 suggesting that the point of inhibition lies before the terminal oxidase.

Effects of compound I.C.I. 47776 on adenosine triphosphatase activity of rat-liver mitochondria

Compound I.C.I. 47776 was found to have interesting activity on the ATPase activity of rat-liver mitochondria. Fig. 1 shows an unusual biphasic action on the Mg^{2+}-dependent ATPase of fresh rat-liver mitochondria. Maximal stimulatory activity of I.C.I. 47776 was observed at about 4.7 μM. Above this concentration the ATPase activity fell sharply until 23.7 μM I.C.I. 47776, when the ATPase activity began to

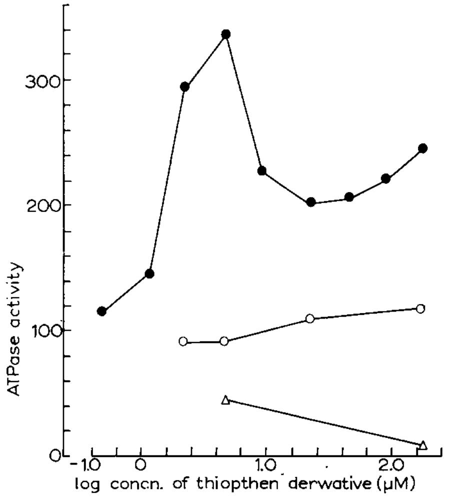

Fig. 1. Effect of I.C.I. 47776 and I.C.I. 46452 on the Mg^{2+}-dependent and -independent ATPase activity of fresh rat-liver mitochondria: ●, I.C.I. 47776 in the presence of Mg^{2+}; ○, I. C. I. 47776 in the absence of Mg^{2+}; △, I.C.I. 46452 in the presence of Mg^{2+}. The ATPase activity of fresh rat-liver mitochondria in the presence of Mg^{2+} is expressed arbitrarily as 100. Incubation time: 20 min, temperature: 37°.

rise slowly again. Compound I.C.I. 46452 was almost without activity on the Mg^{2+}-ATPase. In the absence of Mg^{2+}, I.C.I. 47776 inhibited the ATPase activity. An inhibition of DNP-stimulated ATPase activity in the presence of Mg^{2+} by I.C.I. 47776 was noted (Fig. 2) which reached a limiting value at 9.5 μM I.C.I. 47776. In the absence of Mg^{2+} (which had no effect by itself on the DNP-stimulated ATPase) the inhibition of the ATPase by I.C.I. 47776 was profound and was virtually complete at 9.5 μM I.C.I. 47776. Compound I.C.I. 46452 was without activity on the DNP-stimulated ATPase in the presence of Mg^{2+}.

The ATPase activity stimulated by ageing rat-liver mitochondria at 30° for 2 h was unaffected by I.C.I. 47776.

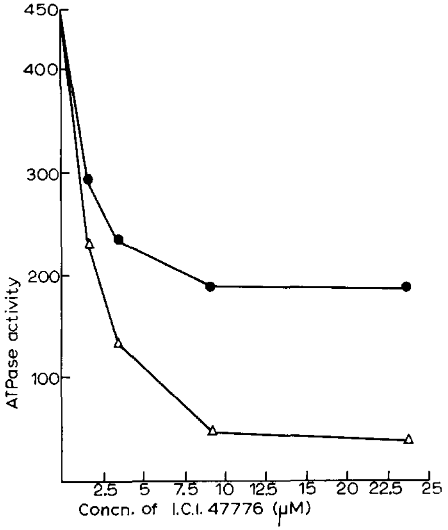

Fig. 2. Effect of compound I.C.I. 47776 on DNP-dependent ATPase activity of fresh rat-liver mitochondria: △, I.C.I. 47776 in the absence of Mg^{2+}; ○, I.C.I. 47776 in the presence of Mg^{2+}. The ATPase activity of fresh mitochondria in the absence of DNP is expressed as 100. Concentration of DNP: 10^{-5} M. Incubation time: 20 min, temperature 37°.

Effect of compound I.C.I. 47776 on the P:O ratio of rat-liver mitochondria

In view of the effects of I.C.I. 47776 on the ATPase activity of rat-liver mitochondria it was of interest to investigate any possible effect of the compound on the

TABLE II

EFFECT OF COMPOUND I.C.I. 47776 ON THE P:O RATIO IN RAT-LIVER MITOCHONDRIA

The assay system was that described by ALDRIDGE[3] utilizing α-oxoglutarate (10 mM) in the presence of malonate (10 mM). The incubation period was 20 min in air at 37°.

Additions to system	*μg atoms consumed per 5 mg mitochondrial protein*		*P:O*
	Oxygen	*Phosphorus*	
None	6.36	22.9	3.6
Dimethyl sulphoxide (0.5% v/v)	6.15	22.1	3.6
I.C.I. 47 776 (2.37 μM)	4.26	10.2	2.4
I.C.I. 47 776 (1.18 μM)	5.09	16.8	3.3

P:O ratio of these particles. The determination of P:O ratios in the presence of I.C.I. 47776 was made difficult by the greatly diminished O_2 consumption at concentrations of I.C.I. 47776 affecting ATPase activity. However, Table II indicates that the thiophen had some uncoupling activity on mitochondria utilizing α-oxoglutarate in the presence of malonate.

DISCUSSION

The experiments described in this report indicate that the thiophen derivative I.C.I. 47776 is an effective inhibitor of electron transport in respiratory particles from rat liver, pig heart muscle and *A. vinelandii*. The difference in sensitivity to I.C.I. 47776 between rat-liver preparations on the one hand and those from heart muscle and *A. vinelandii* on the other, is striking. It seems unlikely that the longer exposure of rat-liver mitochondria to the inhibitor (30 min) in the manometric procedure compared with that of heart muscle and bacterial particles in the polarographic method (7 min) can account for this difference in sensitivity since experiments with heart muscle particles in the Warburg apparatus have also indicated significantly lower sensitivity of this preparation to I.C.I. 47776 than rat-liver mitochondria. In addition, the rate of oxygen consumption by all the particles studied remained linear in the presence of I.C.I. 47776 throughout the incubation period. The sensitivity of heart muscle preparations[7] and of *A. vinelandii*[6] particles to thenoyltrifluoroacetone is also less than that found for rat-liver mitochondria. Furthermore thenoyltrifluoroacetone inhibits both NAD^+- and non-NAD^+-linked oxidations in rat-liver particles whereas in heart muscle preparations the compound inhibits succinate oxidation but has no effect on NAD^+-linked oxidations. In the case of particles from *A. vinelandii*, however, thenoyltrifluoroacetone is much more effective against NADH oxidation than succinate oxidation[6]. It seems likely therefore that sensitivity of different sites to inhibitors in respiratory particles may vary with their cellular origin. It is also conceivable that the greater instability of rat-liver mitochondria compared with heart muscle preparations might contribute to the increased sensitivity of the liver particles to I.C.I. 47776 and thenoyltrifluoroacetone.

The site of action of I.C.I. 47776 is uncertain but in view of its metal chelating properties (P. J. TAYLOR, unpublished results), it is tempting to suggest that it may interfere with the redox function of non-haem iron in the electron-transport chain. The failure of I.C.I. 47776 to inhibit cytochrome oxidase (like thenoyltrifluoroacetone[7]) places the site of action of the compound in the general region of the involvement of the non-haem iron. Clearly, however, further work is necessary in order to assign precisely the site or sites of inhibition of I.C.I. 47776.

The failure of compound I.C.I. 46452 to inhibit the oxidase activity of respiratory particles may be understandable in terms of the diminished lipophilic nature of this compound compared with I.C.I. 47776 due to the absence of the benzylidene group.

The effects of I.C.I. 47776 on the ATPase activity of rat-liver mitochondria are of considerable interest. The inhibition of DNP-dependent ATPase by I.C.I. 47776 resembles the action of sodium azide and amytal[8]. However, the inhibition of the Mg^{2+}-ATPase above 4.7 μM I.C.I. 47776 followed by further stimulation above 23.7 μM is analogous to the action of sodium deoxycholate[8] although the thiophen is very much more active. The inactivity of I.C.I. 46452 on mitochondrial ATPase may be related again to the diminished liphophilic nature of this compound.

In conclusion the thiophen derivative I.C.I. 47776 effectively inhibits the oxidase activity of various respiratory particles. This, together with the action of the compound on the ATPase activity of mitochondria would result in interference with the aerobic production of high-energy phosphate esters in intact cells. This possibility is considered elsewhere where it is shown that the action of I.C.I. 47776 on intact cells *in vitro* is typical of mitochondrial poisons*.

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