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Properties of Substituted 2-Trifluoromethylbenzimidazoles as Uncouplers of Oxidative Phosphorylation

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1. The activity of 25 substituted 2-trifluoromethylbenzimidazoles in uncoupling oxidative phosphorylation by rat-liver mitochondria has been compared. 2. For halogen- or mixed-halogen- and alkyl-substituted analogues, uncoupling activity was proportional to the acidity of the imidazole –NH group. Tetrachloro-2-trifluoromethylbenzimidazole was the most active (50% uncoupling of oxidative phosphorylation at $7{\cdot}9 \times 10^{-8}$M, pK 5·04). Nitro-substituted analogues were less active than predicted from pK considerations or from partition-coefficient measurements. 3. Introduction of an $-NH_2$ or $-CO_2H$ substituent caused a loss of uncoupling activity, as did alkylation at position 1 of the imidazole ring. 4. Benzimidazoles active as uncouplers stimulated mitochondrial adenosine triphosphatase but not all stimulated the oxidation of succinate in the absence of a phosphate acceptor. 5. 4,5-Dichloro-2-trifluoromethylbenzimidazole inhibited the succinate-oxidase system at about the same concentration required for uncoupling (0·52 μM for 50% inhibition of both activities) and the site of this inhibition appears to lie between succinate dehydrogenase and cytochrome *b*.

The uncoupling activity of substituted 2-trifluoromethylbenzimidazoles has been described (Büchel, Korte & Beechey, 1965; Jones & Watson, 1965; Beechey, 1966). The uncoupling activity of these materials appears to be closely related to their physical properties and this relationship is described for a further series of analogues in the present paper.

It had been noted by Burton *et al.* (1965) that the 2-trifluoromethylbenzimidazoles showed interesting insecticidal and herbicidal specificity and it is therefore noteworthy that more than one site of inhibition appeared to be present in liver mitochondria. Although these compounds resemble 2,4-dinitrophenol in their uncoupling properties it is shown in the present paper that they differ in their inhibitory effects on the succinate-oxidase system. This enzyme system was most strongly inhibited by 4,5-dichloro-2-trifluoromethylbenzimidazole at concentrations that had little effect on the oxidation of other substrates. The localization of the site of inhibition between succinate dehydrogenase and cytochrome *b* is described.

MATERIALS AND METHODS

Preparation of mitochondria. Finely chopped rat liver was homogenized manually with 0·25M-sucrose at 0° in a Potter–Elvehjem-type homogenizer and a loose-fitting Teflon pestle. All subsequent steps in the isolation of the mitochondria were as described by Hogeboom (1955).

Manometric measurement of oxidative phosphorylation. Mitochondria, suspended in 0·5ml. of 0·25M-sucrose, were added to the main body of respirometer flasks containing 2·15ml. of incubation mixture made up as follows: glycylglycine, pH 6·8 (50 μmoles), KCl (375 μmoles), $MgCl_2$ (42 μmoles), AMP (6 μmoles), ATP (6 μmoles), cytochrome *c* (0·036 μmole), potassium phosphate, pH 7·4, (46·2 μmoles) and substrate (30 μmoles). With pyruvate as substrate 3 μmoles of fumarate were included. Benzimidazoles or other uncouplers were added in 0·3ml. of water or aqueous ethanol. The centre well contained 0·15ml. of 20% (w/v) KOH. The side arm contained, in 0·35ml., glucose (180 μmoles) and yeast hexokinase (2mg.; 50units/mg.; Darrow & Colowick, 1962). The gas phase was air and the temperature 30°. After 5min. equilibration the side-arm contents were tipped and uptake of O_2 was measured. The final pH was 6·8–6·9. The reaction was stopped by adding 0·5ml. of 50% (v/v) $HClO_4$. Uptake of inorganic phosphate was measured by the method of Fiske & Subbarow (1925).

Mitochondrial protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Zero-time controls were included in all experiments. Flask concentrations of ethanol were 1% (v/v) or less; controls containing no uncoupler all received ethanol at the same concentration as the experimental flasks.

Effect of uncouplers on mitochondrial respiration. Respiration in the absence of phosphate acceptors was measured in a reaction mixture similar to that described above, except that AMP was omitted. Uncouplers, or glucose and hexokinase (0·35ml.), were added from the side arm

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after the basic respiration rate had been determined. Water or aqueous ethanol was tipped from the side arm of control flasks.

Determination of respiration rates with an oxygen electrode. The incubation mixture described for oxidative phosphorylation incubations was used. An oxygen electrode and stirrer assembly (Rank Brothers, Bottisham, Cambridge) was used to measure concentration of O_2. The value for the solubility of O_2 in this medium was assumed to be 0·474 μg.atom of O/ml. (Chappell, 1964). Incubations were carried out at 25°.

Mitochondrial adenosine triphosphatase. The assay was based on that described by Aldridge & Stoner (1960). Addition of uncouplers [in 0·3 ml. of 10% (v/v) ethanol] and mitochondria (0·3 ml.) were added to 2·4 ml. of a stock solution containing (per ml.) KCl (100 μmoles), $MgCl_2$ (14·0 μmoles), EDTA (1·0 μmole), glycylglycine, pH 6·8 (10·67 μmoles), ATP (3·32 μmoles) and sucrose (25·0 μmoles). The pH was adjusted to 6·7–6·8 before addition of the mitochondria. Incubation was at 30° for 30 min. The reaction was stopped by the addition of 0·3 ml. of 50% (v/v) $HClO_4$ and inorganic phosphate measured by the method of Fiske & Subbarow (1925).

Catalase. Ox-liver catalase (stock no. C–10) was purchased from Sigma Chemical Co. (London) and assayed as described in the Sigma catalogue.

Peroxidase. Crude horseradish peroxidase was purchased from Sigma Chemical Co. (London) and assayed as described by Chance & Maehly (1955).

Reduced-cytochrome c oxidase. Cytochrome c was reduced with hydrogen in the presence of palladized asbestos (Smith, 1954) and the reduced-cytochrome c-oxidase activity of mitochondria determined as described by Smith (1954), with a recording spectrophotometer set at 551 mμ.

Succinate–cytochrome c reductase. The succinate-linked cytochrome c-reductase activity of mitochondria was determined at 30° in cuvettes containing (final volume 1·1 ml.) potassium phosphate, pH 7·4 (30 μmoles), sodium succinate (21 μmoles), KCN, pH 7·7 (1·4 μmoles) and oxidized cytochrome c (14 mμmoles). The reaction was started by the addition of mitochondria (about 25 μl.) and the reduction of cytochrome c followed at 551 mμ with a recording spectrophotometer.

Succinate dehydrogenase. Succinate dehydrogenase of mitochondria was determined by the phenazine methosulphate method of Bernath & Singer (1962).

Thin-layer chromatography. 2-Trifluoromethylbenzimidazoles were chromatographed on thin layers of silica gel HF_{254} (E. Merck A.-G., Darmstadt, Germany) developed with benzene-acetic acid (9:1, v/v). Their position was located by their suppression of the u.v.-induced fluorescence of the added dye.

Partition coefficients. Each compound in 0·1 N-HCl was shaken for 30–60 min. with an equal volume of cyclohexane (spectrographically pure; British Drug Houses Ltd., Poole, Dorset). The ratio of the concentrations in the organic and aqueous phases was determined spectroscopically.

Dissociation constants. These were determined in aqueous solution (D. Hughes, unpublished work), by using a spectroscopic method based on that of Biggs (1954).

Reagents. Hexokinase (Grade I) was purchased from Seravac Laboratories (Pty) Ltd., Maidenhead, Berks. Cytochrome c and ADP were purchased from British Drug Houses Ltd. AMP, ATP and phenazine methosulphate were purchased from Sigma Chemical Co., London.

RESULTS

Uncoupling of succinate-oxidase-linked phosphorylation in rat-liver mitochondria. The rapid onset of rigor mortis at the death of animals treated with 2-trifluoromethylbenzimidazoles was very similar to that noted when animals were dosed with tetrachlorobenztriazole, a compound known to be an uncoupler of oxidative phosphorylation that resembles 2,4-dinitrophenol in its effects (Parker, 1965), and that has been included in many of our experiments for purposes of comparison. In Fig. 1 the effects of some 2-trifluoromethylbenzimidazoles on P/O ratios of rat-liver mitochondria are shown. The uncoupling effect found resembles that of 2,4-dinitrophenol in the rate of increase of activity with increase of concentration; also, there is a wide variation between analogues in the concentration required for 50% uncoupling.

In Fig. 2 (A and B) are shown the results of experiments where 2-trifluoromethylbenzimidazoles of a wide range of pK values were examined as uncouplers of oxidative phosphorylation. For 12 compounds with increasing halogen substitutions, or mixed halogen and alkyl substitution, ranging from the unsubstituted to the tetrahalogen 2-trifluoromethylbenzimidazoles, it was possible to draw a smooth curve between pK and uncoupling activity (Fig. 2A) with the compound of lowest pK being the most active uncoupler. However, when nitro substituents were introduced to obtain derivatives of even lower pK, the activities did not

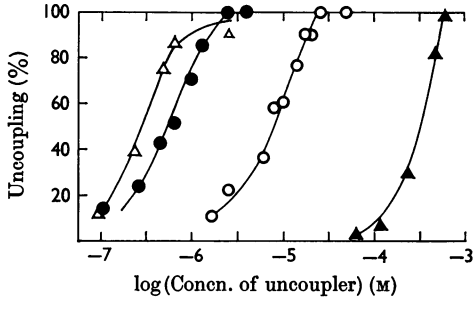


Fig. 1. Comparison of effect of 2-trifluoromethylbenzimidazoles and 2,4-dinitrophenol as uncouplers of oxidative phosphorylation of rat-liver mitochondria. Experimental conditions were as described in the Materials and Methods section. The substrate was succinate. ○, 2,4-Dinitrophenol; △, 4,5,6-trichloro-2-trifluoromethylbenzimidazole; ●, 6-chloro-4-nitro-2-trifluoromethylbenzimidazole; ▲, 2-trifluoromethylbenzimidazole. The average P/O ratio for the untreated mitochondria was 1·4.

lie on this curve (Fig. 2B) and the compounds, although very active as uncouplers, were less active than had been predicted. The results of these determinations of 50%-uncoupling concentrations are shown in Table 1. The introduction of nitro substituents frequently causes the partition of a compound between organic and aqueous phase to favour the latter and it appeared possible that the failure of the nitro-substituted 2-trifluoromethylbenzimidazoles to show the high uncoupling activity predicted from their low pK might be due to their failure to penetrate the lipids of the mitochondrial membrane. Some measurements of partition between cyclohexane and 0·1N-hydrochloric acid were carried out to see if this value could help to explain the anomalous activities (Table 1). A wide range of partition coefficients was found, and although of some assistance in interpreting uncoupling results, did not fully correlate with biological activity. The increase in activity with increased halogen substitution was greater than could be explained simply by increased lipid solubility.

Our experiments had shown that when an ionizing substituent such as $-CO_2H$ or $-NH_2$ was introduced there was an almost complete loss of uncoupling activity (Table 1). Thus 0·64μM-5-nitro-2-trifluoromethylbenzimidazole caused 50% uncoupling of oxidative phosphorylation whereas the 5-amino analogue was inactive at 1mM. It appeared possible that, if the mitochondria contained an active aromatic nitro reductase capable of reducing nitrophenyl groups to aminophenyl groups, there would be an apparent low uncoupling activity of the nitrophenyl compound. Such reducing activity has been shown in chloroplasts where 2,4-dinitrophenol was reduced to 2-amino-4-nitrophenol (Wessels, 1965). Evidence for such

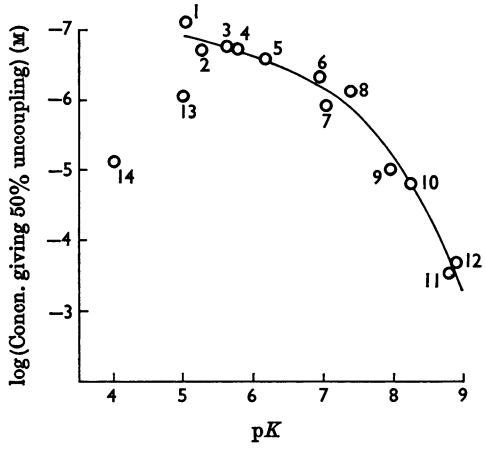


Fig. 2 (A). Relationship between pK and uncoupling activity of halogen- and mixed-halogen alkyl-substituted 2-trifluoromethylbenzimidazoles. Experimental points represent concentrations required to give 50% uncoupling of oxidative phosphorylation of rat-liver mitochondria, with succinate as substrate, and are derived from concn./activity curves as shown in Fig. 1. Experimental conditions were as described in the Materials and Methods section. The compounds studied were: 1, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; 2, 4-bromo-5,6,7-trichloro-2-trifluoromethylbenzimidazole; 3, 4,6,7-trichloro-2-trifluoromethylbenzimidazole; 4, 4,5,6,7-tetrabromo-2-trifluoromethylbenzimidazole; 5, 4,5,6-trichloro-2-trifluoromethylbenzimidazole; 6, 4,5-dichloro-2-trifluoromethylbenzimidazole; 7, 4,6-dichloro-5-methyl-2-trifluoromethylbenzimidazole; 8, 5,6-dichloro-2-trifluoromethylbenzimidazole; 9, 5-chloro-2-trifluoromethylbenzimidazole; 10, 6-chloro-5-methyl-2-trifluoromethylbenzimidazole; 11, 2-trifluoromethylbenzimidazole; 12, 5-methyl-2-trifluoromethylbenzimidazole; 13, 4,5,6,7-tetrachlorobenzotriazole; 14, 2,4-dinitrophenol.

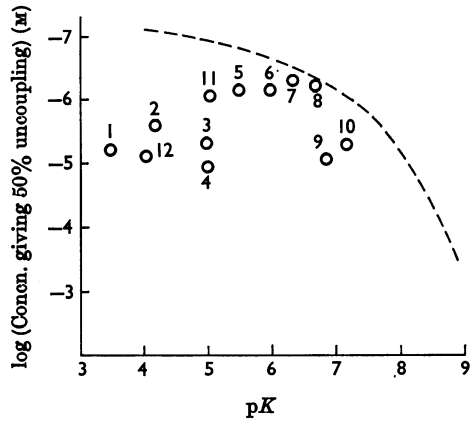
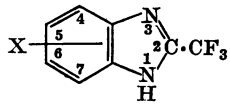


Fig. 2 (B). Relationship between pK and uncoupling activity of nitro-, mixed nitro-halogen-substituted and other 2-trifluoromethylbenzimidazoles. Experimental points represent concentrations required to give 50% uncoupling of oxidative phosphorylation of rat-liver mitochondria, with succinate as substrate, and are derived from concentration/activity curves as shown in Fig. 1. Experimental conditions were as described in the Materials and Methods section. The curve obtained with halogenated benzimidazoles is shown by a broken line. The compounds studied were: 1, 4,6-dichloro-5,7-dinitro-2-trifluoromethylbenzimidazole; 2, 4,6,7-trichloro-5-nitro-2-trifluoromethylbenzimidazole; 3, 4,6-dinitro-2-trifluoromethylbenzimidazole; 4, 5,6-dinitro-2-trifluoromethylbenzimidazole; 5, 4-chloro-6-nitro-2-trifluoromethylbenzimidazole; 6, 6-chloro-5-nitro-2-trifluoromethylbenzimidazole; 7, 6-chloro, 4-nitro-2-trifluoromethylbenzimidazole; 8, 5-nitro-2-trifluoromethylbenzimidazole; 9, 4-nitro-2-trifluoromethylbenzimidazole; 10, 5-cyano-2-trifluoromethylbenzimidazole; 11, 4,5,6,7-tetrachlorobenzotriazole; 12, 2,4-dinitrophenol.

Table 1. *Physical properties and uncoupling activities of a series of 2-trifluoromethylbenzimidazoles*

Physical measurements and determinations of uncoupling were carried out as described in the Materials and Methods section. The 50%-uncoupling concentrations were determined for each compound by plotting activity against concentration of uncoupler as shown in Fig. 1. n.d., Not determined.

Compound	p*K*	Cyclohexane–0·1 N-HCl partition coefficient	Concn. required for 50% uncoupling with succinate as substrate (μM)
2,4-Dinitrophenol	4·0	n.d.	7·88
4,5,6,7-Tetrachlorobenzotriazole	5·0	n.d.	0·91
2-Trifluoromethylbenzimidazoles:			
			
Substituents (X)			
(i) Halogen and halogen-alkyl derivatives			
Nil	8·8	0·11	305
5-Methyl-	8·9	0·33	220
6-Chloro-5-methyl-	8·3	0·86	16·2
4,6-Dichloro-5-methyl-	7·0	15·0	1·2
5-Chloro-	8·0	0·49	10·4
4,5-Dichloro-	7·0	2·0	0·52
5,6-Dichloro-	7·4	*	0·80
4,6,7-Trichloro-	5·6	2·6	0·18
4,5,6-Trichloro-	6·2	3·7	0·29
4,5,6,7-Tetrachloro-	5·0	8·7	0·079
4-Bromo-5,6,7-trichloro-	5·3	*	0·21
4,5,6,7-Tetrabromo-	5·8	*	0·20
(ii) Nitro, nitro-halogen and other derivatives			
4-Nitro-	6·8	0·80	9·2
5-Nitro-	6·7	0·02	0·64
4,6-Dinitro-	5·0	0·15	5·0
5,6-Dinitro-	5·0	0·08	11·7
6-Chloro-4-nitro-	6·3	2·37	0·53
4-Chloro-6-nitro-	5·5	0·18	0·74
6-Chloro-5-nitro-	6·0	0·86	0·75
4,6-Dichloro-5,7-dinitro-	3·4	0·55	6·4
4,6,7-Trichloro-5-nitro-	4·1	1·70	2·6
5-Cyano-	7·2	0·07	5·4
5-Amino-	4·5	*	Inactive at 1000
5-Carboxy-	8·4	*	Inactive at 1000
4,5,6-Trichloro-1-ethyl-	—	n.d.	Inactive at 1

* Too low for accurate determination.

a reductase, capable of acting on nitro-substituted benzimidazoles, was sought in mitochondria.

When preparations of mitochondria were incubated with 2-trifluoromethylbenzimidazoles it was found possible by repeated extraction with methylene chloride at pH 5·0 followed by thin-layer chromatography (Materials and Methods section) to recover over 70% of the benzimidazole at the end of the incubation. No differences were found between nitro- and halogen-substituted benzimidazoles in the extent of recovery. It was certain that insufficient nitro-substituted benzimidazole could have been reduced to account for a decrease in uncoupling activity of several hundredfold from that predicted from p*K* considerations.

Effect of 2-trifluoromethylbenzimidazoles on mitochondrial adenosine triphosphatase. As would be expected for compounds resembling 2,4-dinitrophenol in activity, the benzimidazoles stimulated adenosine triphosphatase of mitochondria (Table 2). In all cases it proved possible to determine the concentration giving a maximum stimulation and

Table 2. *Stimulation of rat-liver mitochondrial adenosine triphosphatase by 2-trifluoromethylbenzimidazoles*

Conditions for determining adenosine triphosphatase were as described in the Materials and Methods section. The concentration of additive giving maximum stimulation was determined by plotting a graph of concentration of additive against inorganic phosphate released and the values quoted were obtained from a large number of experiments. Since adenosine-triphosphatase activity varied in different preparations of mitochondria a standard material (2,4-dinitrophenol) was included in each experiment and the activities were adjusted to allow for variation in the stimulation produced.

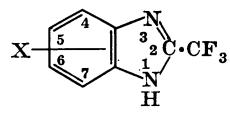
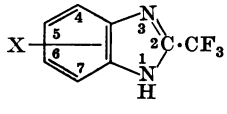
Addition	Concn. required for maximum stimulation of adenosine triphosphatase (μM)	P_i liberated (μmoles/mg. of protein/30min.)
2-Trifluoromethylbenzimidazoles:		
		
Substituents (X)		
Nil	—	0·65
4,5,6,7-Tetrachloro-	0·096	5·23
4,5,6-Trichloro-	0·20	6·37
6-Chloro-4-nitro	1·25	6·41
4,5-Dichloro-	2·87	6·33
4,6-Dichloro-5,7-dinitro-	5·44	5·77
5-Chloro-	21·3	4·30
2,4-Dinitrophenol	14·4	5·17
4,5,6,7-Tetrachlorobenzotriazole	1·72	6·33

Table 3. *Effect of 2-trifluoromethylbenzimidazoles on the mitochondrial succinate-oxidase system, measured in intact mitochondria, in the presence of a phosphate acceptor*

The determinations were carried out, as described in the Materials and Methods section, with concentrations of benzimidazole previously found by the determination of uncoupling activity (shown in Fig. 2 and Table 1) to permit significant phosphorylation.

Addition	Concn. (μM)	Inhibition of oxidative phosphorylation (%)	Inhibition of uptake of P_i (%)	Inhibition of uptake of O_2 (%)
2 4 Dinitrophenol	8·0	58	59·3	3
2-Trifluoromethylbenzimidazoles:				
				
Substituents (X)				
Nil	341	58	66·1	19·6
4,5,6,7-Tetrabromo-	0·33	71	72	3·8
4,5,6,7-Tetrachloro-	0·079	50	48·9	8
5-Chloro-	10·4	50	63·7	33·2
4,5-Dichloro-	0·52	50	70·3	51·7
5,6-Dichloro-	1·25	65·6	64·9	0
6-Chloro-4-nitro-	0·71	57·1	61·3	11·5
4,6-Dichloro-5,7-dinitro-	5·0	51	43·9	10·4
5-Methyl-	10·3	36·6	48·8	19·2
4-Nitro-	10·0	60·6	77·4	44·3
5-Nitro-	0·625	45	55·1	19·3
6-Chloro-5-nitro-	0·7	40	54·3	23·8

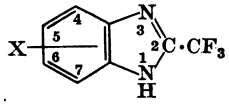
increases beyond this concentration were less effective. The experimental conditions differ from those previously used (Jones & Watson, 1965) and the values obtained are not strictly comparable; the reaction was carried out at pH 6·7–6·8 instead of 7·4 and the incubation mixture contained Mg^{2+} ions.

Effects of 2-trifluoromethylbenzimidazoles on mitochondrial respiration. During experiments to determine the uncoupling activity of the substituted benzimidazoles it became apparent that they differed in the degree to which they inhibited succinate oxidation. The most powerful uncoupler, tetrachloro-2-trifluoromethylbenzimidazole, was almost without inhibitory effect on the succinate-oxidase system over the uncoupling concentration range whereas the 4,5-dichloro, the 5-chloro and the 4-nitro compounds caused significant inhibition of respiration (Table 3). These effects are shown in Fig. 3 (A and B) in more detail for two of these compounds. Tetrachloro-2-trifluoromethylbenzimidazole, although much more active as an uncoupler than 2,4-dinitrophenol, resembled the latter in its relative effects on oxygen uptake and phosphate uptake, but 4,5-dichloro-2-trifluoromethylbenzimidazole inhibited respiration severely although some 10% of the succinate-oxidase system was highly resistant to the inhibitor.

In the absence of a phosphate acceptor the benzimidazoles differed in their effects on succinate-linked respiration (Table 4), ranging from complete lack of stimulation to the usual 2,4-dinitrophenol-type stimulation, but these effects were not related to uncoupling activity of the materials nor in any obvious way to their physical properties. The oxidation of substrates other than succinate was stimulated in the absence of a phosphate acceptor by all the active uncouplers (Table 5). The specificity of this effect is further illustrated for 4,5-dichloro-2-trifluoromethylbenzimidazole in Table 6, where 51·7% inhibition of succinate-linked respiration was found at 0·52 μM under phosphorylating conditions whereas only 18·5% inhibition of α-oxoglutarate oxidation was found at 1·7 μM

Table 4. *Effect of 2-trifluoromethylbenzimidazoles on succinate-linked respiration of rat-liver mitochondria in the absence of a phosphate acceptor*

Assay conditions were as described in the Materials and Methods section. Additions (in 0·35ml.) were made from the side arm of the Warburg flasks. Pre-tipping respiration rates have been represented as equivalent to 100%. Concentrations of additive were selected to give near-maximum stimulation of respiration (or lowest inhibition). The final volume of incubation mixture was 3ml.

Addition	Concn. (μM)	Effect on respiration (%)
Water	—	+ 15
2,4-Dinitrophenol	22	+ 80
4,5,6,7-Tetrachlorobenzotriazole	5·0	+124
	1·0	+ 48
2-Trifluoromethylbenzimidazoles:		
		
Substituents (X)		
Nil	100	+ 28
5-Chloro-	10	+ 28
5,6-Dichloro-	0·8	+ 83
4,5-Dichloro-	1·0	+ 20
	2·5	− 17
4,5-Dichloro-+ATP (6mM)	2·5	+ 7
4,5-Dichloro-+2,4-dinitrophenol (22 μM)	3·0	− 13
4,5,6-Trichloro-	0·3	+ 72
4,5,6,7-Tetrachloro-	0·4	+ 87
	0·08	+ 54
6-Chloro-5-nitro-	0·5	+ 43
5-Nitro-	0·6	+ 47
4,6-Dinitro-	7·5	+ 61
	1·0	+ 20
6-Bromo-4-nitro-	0·9	+ 68

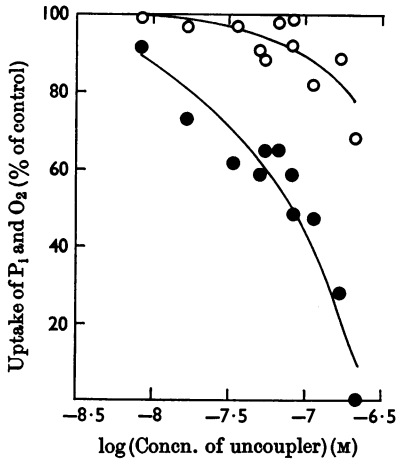


Fig. 3 (A). Effect of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole on the uptake of oxygen and inorganic phosphate by rat-liver mitochondria. The substrate was succinate. Experimental conditions were as described in the Materials and Methods section. ○, Uptake of oxygen; ●, uptake of phosphate. Similar curves for 2,4-dinitrophenol are shown in Fig. 3 (B). The average P/O ratio for the untreated mitochondria was 1·4.

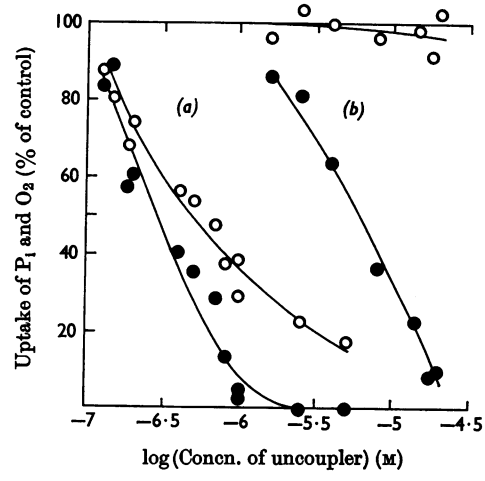
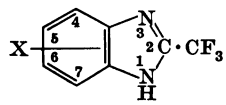


Fig. 3 (B). Effect of (*a*) 4,5-dichloro-2-trifluoromethylbenzimidazole and (*b*) 2,4-dinitrophenol on the uptake of oxygen and inorganic phosphate by rat-liver mitochondria. The substrate was succinate. Experimental conditions were as described in the Materials and Methods section. ○, Uptake of oxygen; ●, uptake of phosphate. The average P/O ratio for the untreated mitochondria was 1·4.

Table 5. *Effect of 2-trifluoromethylbenzimidazoles on the rate of oxidation of various substrates by rat-liver mitochondria*

Rates of respiration were measured, in the absence of a phosphate acceptor, before and after tipping in the additive (0·35ml.) from the side arm of the Warburg flask (see the Materials and Methods section). Each 2-trifluoromethylbenzimidazole was added at a concentration that had been found in preliminary experiments to give the maximum stimulation (or lowest inhibition) for that particular additive–substrate combination. *b*/*a* represents rate of respiration in the presence of additive/rate of respiration in the absence of additive. Hexokinase was used at 250K.M. units/flask. Average protein content was 4·15mg. of mitochondrial protein/flask. Tipping water alone (0·35ml.) from the side arm caused a stimulation of respiration giving a mean *b*/*a* 1·14. n.d., Not determined.

	Substrate							
	Glutamate		Pyruvate		α-Oxoglutarate		Succinate	
Additive	Concn. of additive (μM)	*b*/*a*	Concn. of additive (μM)	*b*/*a*	Concn. of additive (μM)	*b*/*a*	Concn. of additive (μM)	*b*/*a*
								
Substituents (X)								
4,5,6,7-Tetrachloro-	0·3	2·4	0·2	2·24	0·2	2·24	0·25	1·85
4,5,6-Trichloro-	0·28	2·26	n.d.	n.d.	n.d.	n.d.	0·25	1·72
4,5-Dichloro-	1·5	3·52	2·9	3·07	2·5	2·06	0·74	1·18
5-Chloro-	25	2·43	n.d.	n.d.	n.d.	n.d.	5	1·42
Hexokinase–glucose	—	2·99	—	2·87	—	1·9	—	1·7
2,4-Dinitrophenol	22·3	2·95	22·3	3·45	22·3	1·81	22·3	1·88

and even less effect on the oxidation of other substrates.

The effect of 4,5-dichloro-2-trifluoromethylbenzimidazole on various components of the succinate-oxidase complex and the model haem enzymes, catalase and peroxidase, was studied (Table 7). The succinate-oxidase system was severely inhibited at 1 μM, reduced-cytochrome *c* oxidase was unaffected but succinate-cytochrome *c* reductase was inhibited. Succinate dehydrogenase (phenazine methosulphate-linked) was unaffected at concentrations up to 20 μM, although this enzyme was inhibited in the usual way by malonate (Table 7). No effects were noted on catalase and peroxidase.

A similar inhibition of succinate-oxidase activity was reported by Heytler (1963) with the uncoupler *m*-chlorocarbonylcyanide phenylhydrazone and also by other workers with various uncouplers, including 2,4-dinitrophenol and arsenate (cf. Azzone & Ernster, 1960). The effects have been attributed to the inhibition of succinate hydrogenase by accumulated oxaloacetate.

Various methods of overcoming such an oxaloacetate inhibition were therefore examined. The failure to stimulate succinate oxidation shown by 4,5-dichloro-2-trifluoromethylbenzimidazole was not prevented by high (6mM) concentrations of ATP (Table 4), nor was any inhibition reversed by the addition of Ca^{2+} (2mM). The added Ca^{2+} stimulated the succinate-oxidase system by 69% but the addition of benzimidazole at 1 μM produced

Table 6. *Comparison of the effect of 4,5-dichloro-2-trifluoromethylbenzimidazole on the oxidation of a number of substrates by intact rat-liver mitochondria*

Substrates were present at a concentration of 10mM. Pyruvate oxidation was measured in the presence of 1mM-fumarate. Determinations were carried out as described in the Materials and Methods section. Concentrations of 4,5-dichloro-2-trifluoromethylbenzimidazole giving 50% uncoupling of oxidative phosphorylation were determined as described for Fig. 1.

Substrate	Concn. required for 50% uncoupling (μM)	Inhibition of respiration at 50%-uncoupling concn. (%)
Succinate	0·52	51·7
Glutamate	1·76	13·5
α-Oxoglutarate	1·7	18·5
β-Hydroxybutyrate	1·35	6·8
Pyruvate	1·9	2·3

Table 7. *Effect of 4,5-dichloro-2-trifluoromethylbenzimidazole on some respiratory enzymes of rat-liver mitochondria and on other haem enzymes*

The enzymes were assayed by standard procedures (see the Materials and Methods section).

Enzyme activity	Inhibition (%)	Concn. of inhibitor (μM)
Succinate-oxidase system	62·7	1
Reduced-cytochrome *c* oxidase	5	10
Succinate–cytochrome *c* reductase	50	1
Succinate dehydrogenase, linked to phenazine methosulphate	10*	20
Peroxidase (horseradish)	0	10
Catalase (ox liver)	0	10

* 64·5% with sodium malonate as inhibitor at 1·7mM.

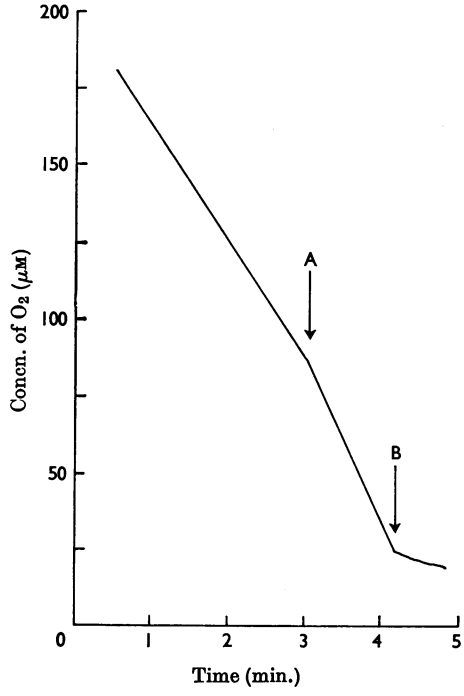


Fig. 4. Oxygen-electrode trace of the effect of halogen-substituted 2-trifluoromethylbenzimidazoles on succinate-oxidase activity of rat-liver mitochondria. The part of the trace showing the initial rate of respiration on addition of succinate (20 μmoles in 200 μl.), and the stimulation on addition of ADP (0·2 μmole in 10 μl.), has been omitted. The composition of the basic incubation mixture was that used in mitochondrial respiration experiments except that ATP was omitted. The temperature was 25°. The reaction volume was 3·0ml. At A, 1·0 μl. of 5mM-4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole in ethanol was added, followed, at B, by 1·0 μl. of 5mM-4,5-dichloro-2-trifluoromethylbenzimidazole in ethanol giving a final concentration 1·67 μM for each compound.

a 70% inhibition of respiration in both Ca^{2+}-treated and control mitochondria. When 2,4-dinitrophenol (22 μM) was added to inhibited succinate-oxidase preparations no relief of inhibition was found (Table 4). The inhibition of the succinate-oxidase system still occurred in the presence of sodium Amytal (2mM).

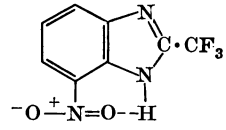
As shown in Fig. 4, the inhibition of succinate respiration takes place very rapidly without any lag phase such as had been reported by Heytler (1963) with *m*-chlorocarbonylcyanide phenylhydrazone, which argues against the inhibitions being due to the accumulation of oxaloacetate.

DISCUSSION

This investigation of the effects of a larger number of substituted 2-trifluoromethylbenzimidazoles on oxidative phosphorylation confirms many of the properties that were reported for tetrachloro-2-trifluoromethylbenzimidazole (Beechey, 1966) and a few related compounds (Büchel *et al.* 1965; Jones & Watson, 1965; Parker, 1965). Of the 27 compounds described in Table 1, all, with the exception of $-NH_2$- and $-CO_2H$-substituted derivatives, and the 4,5,6-trichloro-1-ethyl analogue, possessed some activity in uncoupling oxidative phosphorylation; this activity was highest for tetrachloro-2-trifluoromethylbenzimidazole, which gave detectable uncoupling at 8×10^{-9}M.

Introduction of an ionizing group, such as $-NH_2$ or $-CO_2H$, into the benzene ring (Table 1) markedly decreased the activity, no uncoupling being detected at 1mM. The effect was possibly due to the low lipid solubility of these molecules, which would be highly dissociated at the pH of the incubation medium. Uncoupling activity varied with the acidity of the benzimidazole –NH group but this relationship was clearcut only in the absence of nitro substituents. For the halogen-substituted compounds there was a regular relationship between pK_a and uncoupling activity; the more acidic the compound the more potent the uncoupler (Fig. 2A). This suggests that the active uncoupler is the anion, a hypothesis that is supported by the inactivity of *N*-methyl or *N*-ethyl derivatives of benzimidazoles (Büchel *et al.* 1965; Jones & Watson, 1965) and by the observation (O. T. G. Jones & W. A. Watson, unpublished work) that if the substituent on the imidazole nitrogen is $-CO_2R$, where R is CH_3 or C_2H_5, then uncoupling activity is found to parallel the formation of the parent benzimidazole, after enzymic hydrolysis of the ester and spontaneous loss of carbon dioxide. The requirement for the anion for activity conflicts with the generally accepted requirement of lipid solubility for penetration through the mitochondrial membrane to the site of activity and may explain the finding of an optimum p*K* for uncoupling activity at 5·04; compounds of lower p*K* would be almost completely ionized and of consequently low lipid solubility. Such an interpretation is complicated by the fact that all compounds of low p*K* that were available to us were nitro-substituted and this substitution was found in general to cause a lowering of activity below that predicted from consideration of p*K* alone (Fig. 2B). Nitro groups tend to increase the partition of aromatic molecules from oil into water, as shown in Table 1, and so might be expected to decrease their penetration of lipid membranes. For a group of compounds of about equal p*K*, the 4,5,6,7-tetrachloro-, the 4,6-dinitro- and the 5,6-dinitro-2-trifluoromethylbenzimidazoles, the order of activity in uncoupling was the same as their order of partition from acid into cyclohexane but the differences in the magnitude of uncoupling effects were much greater than differences in partition coefficient and do not explain the nitro-substituent effect (Table 1). The partition coefficients in Table 1 illustrate the large differences between compounds with a 4- or 7-nitro substituent and those with a 5- or 6-nitro substituent. The value for the 4-nitro compound was 0·8, for the 5-nitro compound 0·022, for the 6-chloro-4-nitro compound 2·37, and for the 4-chloro-6-nitro compound 0·175. Similarly the 5,6-dichloro-4,7-dinitro compound gave 1·1, and the 4,7-dichloro-5,6-dinitro compound 0·12. These large differences are possibly due to the formation of internal hydrogen bonds between a nitro substituent at position 4 or 7 and the –NH group of the imidazole ring (I). Such hydrogen-bonded species would be expected to be of greater lipid solubility, but their effects on uncoupling, as shown in Table 1 and Fig. 2(B), are not consistently increased.

Beechey (1966) found that 5-nitro-2-trifluoromethylbenzimidazole was a less active uncoupler than would be expected from its p*K*. Although we have failed to confirm his observation with this particular compound (Table 2B), we have frequently found nitro derivatives to be of lower activity than predicted. From his work with nitro- and nitrile-substituted imidazoles Beechey (1966) has suggested that the lower activity can be explained by the effects of these substituents on the mesomeric delocalization of the negative



(I)

charge produced by the dissociation of the ring –NH group. It is apparent that the uncoupling activity of the nitro-substituted benzimidazoles may be affected by a variety of physical factors that are not so important in the halogen-substituted series.

Although the NH-substituted acidic 2-trifluoromethylbenzimidazoles behaved like 2,4-dinitrophenol in stimulating the adenosine triphosphatase of mitochondria, in the absence of added inorganic phosphate, with activity following the order of activity found in uncoupling experiments (Table 2), they differed from it in their effect on respiration. Many of the 2-trifluoromethylbenzimidazoles, like 2,4-dinitrophenol, did not significantly inhibit succinate oxidation at concentrations causing 50% uncoupling of oxidative phosphorylation. The inhibition of succinate oxidation by some 2-trifluoromethylbenzimidazoles, particularly the 4,5-dichloro analogue (Tables 3, 4, 5 and 6) was very striking and did not appear to result from an inhibition of succinate dehydrogenase by accumulated oxaloacetate, since the effect was very rapid (Fig. 4), not reversed by high concentrations of ATP (Table 4), Amytal or added Ca^{2+}. The decreased rate of succinate oxidation reported by Azzone & Ernster (1960) followed preincubation of mitochondria with uncouplers before the addition of succinate (conditions that did not obtain in our experiments) and was attributed to the hydrolysis of 'high-energy' phosphate required for oxidizing succinate at the maximum rate. Such an inhibition was partly overcome by added ATP or Amytal (Azzone & Ernster, 1960). 4,5-Dichloro-2-trifluoromethylbenzimidazole affected succinate oxidation severely and pyruvate, glutamate and α-oxoglutarate oxidation less so. The slight effect upon glutamate and α-oxoglutarate may be due to the fact that succinate oxidation contributed to the total oxygen uptake with these substrates. It might be expected that the site of inhibition would be found near the succinate-dehydrogenase site and not at sites in the electron-transport pathway that are common to the oxidation of all substrates. It was found (Table 7) that, although succinate-cytochrome *c* reductase was inhibited, succinate dehydrogenase linked to phenazine methosulphate was unaffected. This suggests that this inhibitor may act at the same point as thenoyltrifluoroacetone [4,4,4-trifluoro-1-(thien-2-yl)-1,3-butadione], between succinate and ubiquinone or cytochrome *b*, possibly at the non-haem iron (Ziegler, 1961). We have not found any evidence for the formation of iron complexes of the benzimidazoles, which suggests that their mode of action is not concerned with complex-formation with non-haem iron, as has been suggested for thenoyltrifluoroacetone (Tappel, 1960), and indeed studies with that compound have shown that its inhibitory effects may not be associated with complex-formation with iron (Piper & Thorn, 1965). Like the 2-trifluoromethylbenzimidazoles, thenoyltrifluoroacetone has a hydrogen atom that may be activated by its proximity to a trifluoromethyl group, but the reason for the selectivity of the inhibitory effect of some of the benzimidazoles on succinate dehydrogenase is not apparent. The activity does not follow uncoupling activity or any obvious physical property.

Although imidazoles are known to form co-ordination complexes with haem pigments (cf. Falk, 1964), it was found that 4,5-dichloro-2-trifluoromethylbenzimidazole had no effect on the haem enzymes catalase, peroxidase or reduced cytochrome *c* oxidase, nor did a number of other benzimidazoles that were examined.

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