The Uncoupling of Respiratory-Chain Phosphorylation by 4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole

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1. $30\mu\text{M}$ -4,5,6,7-Tetrachloro-2-trifluoromethylbenzimidazole (TTFB) uncouples respiratory-chain phosphorylation. The respiration rate of uncoupled mitochondria is significantly greater than the state 3 rate. Neither added orthophosphate nor ATP is required to sustain this rate. The oligomycin- and octylguanidine-induced inhibitions of respiration are relieved by uncoupling concentrations of TTFB. 2. Uncoupling concentrations of TTFB elicit a high adenosine-triphosphatase activity and inhibit the ATP-dependent succinate-linked reduction of NAD catalysed by submitochondrial particles from ox-heart mitochondria. 3. The action of TTFB is similar to that of 2,4-dinitrophenol. Evidence is presented which shows that it is the anionic forms of benzimidazoles and imidazoles, having the negative charge localized on the ring nitrogen atom, which are the effective uncoupling agents.

The biological activity of substituted benzimidazoles has been studied in some detail. It has been reported that benzimidazoles inhibit the growth of many organisms, e.g. *Saccharomyces cerevisiae* (Wooley, 1944), various bacteria (Hendlin & Soars, 1951; Greer, 1958; McNair-Scott, Rogers & Rose, 1958; Bishop, Chelton & Jones, 1964), three viruses (Thompson, 1947; Brown, 1952; Tamm, Folkers, Shunk, Heyl & Horsfall, 1953), protozoans (Gillespie, Engelmann & Graff, 1954; Timmis & Epstein, 1959) and amphibian embryos (Gillespie *et al.* 1954). The mode of action has been ascribed variously to the blocking of purine utilization and interference with the metabolism of vitamin B₁₂, which contains the 5,6-dimethyl-benzimidazole moiety. Benzimidazole itself produces a variety of pharmacological responses in mammalian tissues and organs (for references see McNair-Scott *et al.* 1958).

The work in the present paper shows that TTFB* is a potent uncoupler of respiratory-chain phosphorylation. The mechanism and locus of action of TTFB are apparently identical with those of 2,4-dinitrophenol. Evidence is presented which suggests that it is the anion of TTFB which is the active uncoupling agent.

A preliminary report of this work has appeared (Büchel, Korte & Beechey, 1965).

* Abbreviation: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole.

METHODS

Preparation of mitochondria and submitochondrial particles. Rat-heart mitochondria were prepared by the method of Chance & Hagihara (1961). Ox-heart mitochondria were prepared by the method of Sanadi & Fluharty (1963). Submitochondrial particles (ETP_H) were prepared by the method of Hansen & Smith (1964).

Assay of protein. Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill & David (1949).

Estimation of respiration rates. A Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.) was used to measure oxygen concentrations in an apparatus similar to that described by Chappell (1964a). The temperature of the apparatus was maintained at 25°. Additions to the reaction vessel were made with microsyringes. The pH of all the aqueous solutions used in the reaction vessel was adjusted to 7.4. The oxygen concentration of the air-saturated reaction media was taken as $237\mu\text{M}$ (Chappell, 1964a).

Manometric assay of P/O ratios. Rat-heart mitochondria were suspended in a reaction medium containing succinate (5mM), MgCl₂ (5mM), ATP (1mM), KH₂PO₄-Na₂HPO₄ buffer, pH 7.4 (20mM), and sucrose (0.15M). A 0.9-ml. sample of this suspension (3–4 mg. of protein) was placed in the main compartment of a 5 ml. respirometer flask, with 0.1 ml. of 20% (w/v) KOH in the centre well and 0.1 ml. of 0.25 M-glucose containing 1 mg. of crystalline hexokinase (Sigma type III) in the side arm. After a 10 min. equilibration period at 25° the contents of the side arm and main compartment were mixed and the oxygen consumption was measured. The reaction was stopped by the addition of 0.1 ml. of 40% (w/v) trichloroacetic acid. The uptake of

orthophosphate was determined as the difference between the orthophosphate content of the experimental flasks and zero-time controls to which glucose-hexokinase solution was added after the addition of trichloroacetic acid. Orthophosphate was assayed by the method of Sumner (1940).

Reversed-phase thin-layer chromatography. A slurry of 13 g. of silica gel HF₂₅₄ (Merck) and 16 g. of Avicel (technical-grade microcrystalline cellulose; Avicel Sales, Marcus Hook, Pa. 19061, U.S.A.) in 83 ml. of water was spread over thin-layer plates to a thickness of 250 μ . After drying at room temperature the plates were impregnated with liquid paraffin by allowing a 10% (v/v) solution of liquid paraffin B.P. in hexane to ascend the plate and then evaporating off the hexane in a stream of air. After application of the substances to be separated the chromatogram was developed in water-acetone (4:1, v/v). The substances were located by inspection under monochromatic light (254 m μ). This method is rapid and very convenient for compounds that are not otherwise easily detectable (B. Bush, personal communication).

Fig. 1. Uncoupling action of TTFB on the coupled respiration of rat-heart mitochondria. The line represents the output from an oxygen electrode. The temperature was 25°. At A, 50 μ l. of heart mitochondria (2.1 mg. of protein), suspended in 0.25 M-sucrose-mm-EDTA, was added to a reaction mixture containing 3.5 ml. of 0.25 M-sucrose-mm-EDTA, 50 μ l. of m-potassium phosphate buffer, pH 7.4, and 50 μ l. of m-potassium glutamate. At B, 10 μ l. of 0.04 M-ADP (potassium salt) was added. At C, 5 μ l. of 10 μ M-TTFB in ethanol was added, giving a final concentration of 13.5 m μ M. At D, a further 10 μ l. of 0.04 M-ADP (potassium salt) was added. The values on the curve represent the respiration rate expressed in μ moles of oxygen/mg. of protein/hr.

RESULTS

Effects of TTFB on the coupled respiration of rat-heart mitochondria. (a) With orthophosphate and ATP present. Fig. 1 is an oxygen-electrode trace of an experiment in which mitochondria were added to an aerated reaction medium containing glutamate and orthophosphate buffer, pH 7.4. ADP (0.4 μ mole) was then added and the mitochondrial respiration rate showed a typical state 4,3,4 cycle, the ADP/O ratio being 2.8. The ADP/O ratio is calculated on the assumption that the oxygen consumption during the rapid phase of oxygen consumption after the addition of ADP, state 3, is associated with the phosphorylation of ADP to ATP (Chance & Williams, 1955). On the addition of TTFB (final concn. 11 m μ M), the respiration rate immediately changed to an increased and constant rate. A second addition of ADP again stimulated the respiration to a rate 14% greater than the state 3 respiration rate measured previously. The respiration rate eventually declined to a rate similar to that measured before the second addition of ADP. The ADP/O ratio for this state 4,3,4 cycle in the presence of TTFB was 1.1.

This type of experiment was repeated to establish the minimum concentration of TTFB that prevents the stimulation of respiration by a subsequent addition of ADP. This concentration of TTFB (30 m μ M) was designated the 'minimum uncoupling concentration'. As the concentration of TTFB was increased still further the stimulation of respiration declined and an inhibition of respiration occurred. This inhibition was not relieved by ADP.

P/O ratios were also estimated by using manometers to assay the oxygen consumption and a hexokinase-glucose trap to enable ATP synthesis to be measured by the orthophosphate-uptake method. Under these conditions 30 m μ M-TTFB completely inhibited the synthesis of ATP.

(b) With ATP absent. The stimulation of respiration by TTFB was not dependent on the prior addition of ADP and its subsequent phosphorylation to ATP. The addition of an uncoupling concentration of TTFB to mitochondria in state 4 caused a stimulation of the respiration to a rate greater than the state 3 rate measured in control experiments.

(c) With orthophosphate and ATP absent. In these experiments mitochondria were added to an aerated reaction medium similar to that described in Fig. 1, save that 2.5 mm-tris-chloride buffer, pH 7.4, was substituted for orthophosphate buffer. The addition of an uncoupling concentration of TTFB stimulated the respiration to a rate which was 90% of that of the state 3 rate measured in control experiments. A similar result was obtained with α -oxoglutarate and succinate as substrates.

Reversal of the uncoupling action of TTFB by

Fig. 2. Reversal of TTFB uncoupling by bovine plasma albumin. The line represents the output from an oxygen electrode. The temperature was 25°. At *A*, mitochondria (1.1 mg. of protein), suspended in 0.1 ml. of 0.25 M-sucrose-mm-EDTA, were added to a reaction mixture containing 4.1 ml. of 0.25 M-sucrose-mm-EDTA, 10 μ l. of M-potassium phosphate buffer, pH 7.4, and 20 μ l. of M-potassium glutamate. At *B*, 10 μ l. of 10 μ M-TTFB in ethanol was added, giving a final concentration of 24 μ M. At *C*, 2.5 mg. of crystalline bovine plasma albumin in 50 μ l. of water was added. At *D* and again at *E*, 10 μ l. of 0.04 mM-ADP (potassium salt) was added. The values on the curve represent the respiration rate expressed in μ moles of oxygen/mg. of protein/hr.

bovine plasma albumin. The oxygen-electrode trace illustrated in Fig. 2 shows rates of oxygen consumption of heart mitochondria respiring in a reaction medium containing glutamate and orthophosphate. The addition of ADP causes a state 4,3,4 cycle. The respiration rate increases on the addition of TTFB (final concn. 24 μ M) to a rate that is 18% greater than the state 3 rate. The slight increase in the electrode output is an anomaly caused by the addition of ethanol. Within 1 min. after the addition of 2.5 mg. of crystalline bovine plasma albumin the respiration rate returned to a state 4 rate. Subsequent additions of ADP stimulated typical state 4,3,4 cycles with normal P/O ratios but somewhat lower state 3 respiration rates.

Relief by TTFB of respiratory inhibition caused by oligomycin and octylguanidine. The experiment illustrated in Fig. 3(a) shows the stimulation of respiration by TTFB in an oligomycin-inhibited mitochondrial preparation. The mitochondria were respiring in a reaction medium containing orthophosphate, glutamate and malate as substrates with malonate to inhibit the oxidation of succinate. On the addition of ADP the respiration rates showed a typical state 4,3,4 cycle. The addition of 6 μ g. of oligomycin A had no effect on the state 4 respiration rate, but prevented the onset of a state 3 respiration rate when a second addition of ADP was made. However, the addition of TTFB (final concn. 140 μ M) caused an immediate stimulation

Fig. 3. Relief by TTFB of the oligomycin (a) and octylguanidine (b) inhibitions of coupled respiration. The lines represent the output from an oxygen electrode. The temperature was 25°. At *A*, mitochondria (1.6 mg. of protein), suspended in 0.1 ml. of 0.25 M-sucrose-mm-EDTA, were added to a reaction mixture containing 4.1 ml. of 0.25 M-sucrose-mm-EDTA, 50 μ l. of M-potassium phosphate buffer, pH 7.4 and 40 μ l. of a substrate solution containing potassium malonate (0.5 M), potassium malate (0.25 M) and potassium glutamate (0.25 M). At *B*, 25 μ l. of 0.04 M-ADP (potassium salt) was added. At *C*, 6 μ g. of oligomycin A in 6 μ l. of ethanol (a) or 20 μ l. of 20 mM-octylguanidine in ethanol (b) was added. At *D*, a further 25 μ l. of 0.04 M-ADP (potassium salt) was added. At *E*, 6 μ l. of 100 μ M-TTFB in ethanol was added.

of the respiration to a rate that was 90% of the previous state 3 rate.

A similar reaction medium was used in the experiment illustrated in Fig. 3(b). In this experiment octylguanidine (final concn. 125 μ M) was added

$n = 0, 1, 2, 3, 4$

Scheme 1.

while the mitochondria were in state 3. The respiration rate declined to a state 4 rate but the mitochondria did not respond to a further addition of ADP. When TTFB (final concn. 270 μM) was added the respiration rate slowly accelerated to a linear respiration rate that was 68% of the previous state 3 rate.

Effect of TTFB on mitochondrial adenosine-triphosphatase activity. The data listed in Table 1 show that, as the concentration of TTFB in the adenosine-triphosphatase assay was increased to 24 μM , the adenosine-triphosphatase activity increased to a maximum, which was 27% higher than the adenosine-triphosphatase activity stimulated by an uncoupling concentration of dinitrophenol (30 μM). The adenosine-triphosphatase activity fell slightly when the concentration of TTFB in the adenosine-triphosphatase assay was increased to 300 μM .

Effect of TTFB on the ATP-dependent succinate-linked reduction of NAD. Submitochondrial particles were prepared from ox-heart mitochondria; they catalysed the ATP-dependent succinate-

linked reduction of NAD at a rate of 50 $\mu\text{moles/mg. of protein/min.}$ at 30° when assayed under the conditions described by Sanadi & Fluharty (1963). 80 μM -TTFB caused an 86% decrease in this rate. Control experiments showed that small additions of ethanol had no effect on this reaction.

Variation of uncoupling activity with the pK_1 of the substituted benzimidazole. The uncoupling concentrations of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole, 4,5,6-trichloro-2-trifluoromethylbenzimidazole, 5,6-dichloro-2-trifluoromethylbenzimidazole, 5-chloro-2-trifluoromethylbenzimidazole, 5-nitro-2-trifluoromethylbenzimidazole and 2-trifluoromethylbenzimidazole were assayed as described above. Apparent pK_1 values for the dissociation shown in Scheme 1 were measured by titration of solutions of these compounds in 50% (v/v) ethanol with aq. potassium hydroxide solutions.

The plot of apparent pK_1 values against the log (minimum uncoupling concentration) is shown in Fig. 4. As the number of chlorine atoms substituted in the benzimidazole nucleus increases, the value of the apparent pK_1 falls and this decrease is accompanied by an approximately logarithmic increase in the uncoupling activity of the molecules. 5-Nitro-2-trifluoromethylbenzimidazole has pK_1

Table 1. *Effect of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole on the adenosine-triphosphatase activity of rat-heart mitochondria*

Mitochondria (1.1mg. of protein) suspended in 0.2ml. of 0.25M-sucrose-mM-EDTA, pH 7.4, were added at zero time to 0.8ml. of a reaction mixture containing sucrose (0.125M), tris-chloride buffer, pH 7.4 (63mM), ATP (2.5mM) and either 10 $\mu\text{l.}$ of an ethanolic solution of TTFB or an aqueous solution of dinitrophenol (3mM). The reaction was stopped after 15min. at 20° by the addition of 0.1ml. of 40% (w/v) trichloroacetic acid. The orthophosphate content of the supernatant was assayed by the method of Sumner (1944). Zero-time, enzyme and substrate controls were included. The data presented have been corrected for these control assays, which were less than 4% of the maximum activity shown here.

Adenosine-triphosphatase activity
($\mu\text{moles/hr./mg. of protein}$)

Uncoupler	Concn. (μM)	Adenosine-triphosphatase activity ($\mu\text{moles/hr./mg. of protein}$)
TTFB	3	1.3
	6	2.6
	12	4.9
	24	6.6
	30	6.5
	150	6.4
	300	6.0
Dinitrophenol	30000	5.2

Fig. 4. Relationship between the minimum uncoupling activities and the pK_1 of substituted 2-trifluoromethylbenzimidazoles. O, n -Chloro-2-trifluoromethylbenzimidazoles (n is the number of chlorine substituents); □, 5-nitro-2-trifluoromethylbenzimidazole.

6.7 and its measured minimum uncoupling concentration was $5\mu\text{M}$. Thus the data for this particular compound do not fit the relationship obtained for the chlorine-substituted 2-trifluoromethylbenzimidazoles.

4,5,6-Trichloro-*N*-methyl-2-trifluoromethylbenzimidazole at concentrations up to $75\mu\text{M}$ was completely devoid of uncoupling activity.

DISCUSSION

The results presented here show that TTFB is a potent uncoupler of respiratory-chain phosphorylation and that its mode of action appears to be identical with that of 2,4-dinitrophenol. Thus TTFB relieves the oligomycin-induced inhibition of respiration (cf. Lardy, Johnson & McMurray, 1958); TTFB also relieves the octylguanidine-induced inhibition of respiration (cf. Pressman, 1963); TTFB increases the respiration rate of mitochondria in state 4 to a rate that is significantly greater than the state 3 rate (cf. Chappell, 1964b); TTFB induces adenosine-triphosphatase activity in mitochondria (cf. Hunter, 1951); the uncoupling action of TTFB is reversed by bovine plasma albumin (cf. Garbus & Weinbach, 1963); uncoupling concentrations of TTFB also inhibit the ATP-dependent reduction of NAD by succinate (cf. Löw, Krueger & Ziegler, 1961). However, TTFB uncouples at $30\text{m}\mu\text{M}$ and hence is about 1000-fold as potent as dinitrophenol.

In the absence of orthophosphate, the stimulation of succinate oxidation by TTFB is similar to the action of dinitrophenol. However, the stimulation of α -oxoglutarate and glutamate oxidations by TTFB in absence of orthophosphate contrasts with the inertness of dinitrophenol under similar conditions (Borst & Slater, 1961).

The data illustrated in Fig. 4 show that the lower the pK_1 of the substituted benzimidazole (i.e. the greater the degree of dissociation of the compound at pH 7.4) the more efficient it is as an uncoupling agent. Thus it appears that the active uncoupler is the substituted benzimidazole anion. This conclusion is supported further by the facts that at pH 7.4 TTFB is 99.7% ionized and that a compound which cannot undergo the dissociation shown in Scheme 1, i.e. 4,5,6-trichloro-*N*-methyl-2-trifluoromethylbenzimidazole, is completely without uncoupling activity. This theory is backed by additional results obtained with substituted imidazoles. The data in Table 2 show that 2,4,5-tribromoimidazole (pK_1 6.95) is a more efficient uncoupler than 2,4-dibromoimidazole (pK_1 9.8) and also that 2,4,5-tribromo-*N*-methylimidazole is devoid of uncoupling activity.

Parker (1958) concluded that the dissociated forms of substituted nitro- and halogeno-phenols were the actual uncoupling agents. However,

Table 2. *Effect of substituted imidazoles on the coupled respiration of rat-heart sarcosomes*

The uncoupling activity was estimated by the method described in the text.

	pK_1	Minimum uncoupling concentration
2,4,5-Tribromoimidazole	6.95	$13\mu\text{M}$
4,5-Dibromoimidazole	9.8	$740\mu\text{M}$
2,4,5-Tribromo- <i>N</i> -methylimidazole	No dissociation	No uncoupling at 1.35mM
2,4-Dibromo-5-nitroimidazole	3.6	P/O ratio decreased by 20% at $700\mu\text{M}$
4,5-Dicyanoimidazole	5.05	P/O ratio decreased by 62% at $950\mu\text{M}$

Hemker (1962) and Gladtker & Liss (1959) have suggested that the uncoupling activities of 4-alkyl-2,6-dinitrophenols are directly determined by the amount of undissociated phenol dissolved in the mitochondrial lipid. The variation of the uncoupling activity shown in Fig. 4 cannot be significantly ascribed to the expected increase in lipid solubility as the number of chlorine substituents is increased, since reversed-phase thin-layer partition chromatography of these compounds showed the ratio of the maximum R_f to minimum R_f to be 4, whereas the ratio of the uncoupling activities is 104. The undissociated form of TTFB is highly lipophilic; in contrast, the anionic form is readily soluble in water at pH 7.4 and at the concentrations used in these experiments. It is envisaged that TTFB penetrates the mitochondrial membrane in the undissociated form and assumes its dissociated form when within the mitochondria. In this form it can then penetrate the polar active site of the enzyme involved. Weinbach & Garbus (1964) have proposed a similar mechanism for the penetration and mode of action of halogeno- and nitro-phenols.

Table 2 and Fig. 4 show that the benzimidazoles and imidazoles substituted with electrophilic nitro or cyano groups do not have the uncoupling activity which might be predicted from a comparison of their measured pK_1 values with those of the halogenated benzimidazoles and imidazoles. In these molecules it is highly probable that the mesomeric effect induced by the nitro and cyano groups will delocalize the negative charge produced by the dissociation of the ring $-\text{NH}-$ group. Thus, in addition to the dissociation of the benzimidazoles and imidazoles, the localization of the negative charge on the ring nitrogen atom is a prerequisite for an effective uncoupling agent. The minimum uncoupling concentration of 5-nitro-2-trifluoromethylbenzimidazole, namely $6\mu\text{M}$, is 30 times that of 4,5,6-trichloro-2-trifluoromethylbenzimidazole, a compound with a similar pK_1 . Also, 2,4-dibromo-5-nitroimidazole is at least 2000-fold less effective than would be expected from its pK_1 . (This esti-

mate is based on the data listed in Table 2.) Since the mesomeric delocalization of the negative charge would be expected to be similar in both molecules it appears that the nitro group in 2,4-dibromo-5-nitroimidazole is causing a neighbouring-group effect in addition to the delocalization of the negative charge.

Imidazole at very high concentrations (25 mM) has been shown to inhibit the ATP-dependent succinate-linked reduction of NAD (Hommes, Estabrook, Chance & Rasmussen, 1964) and to uncouple partially oxidative phosphorylation (Conover, Gonze & Estabrook, 1964) in submitochondrial particles from ox heart. Both groups of workers concluded that 'imidazole may act by competing in the energy-transfer reaction between a phosphorylated high-energy intermediate and its energy acceptor'. The present results suggest that imidazole is acting in a manner similar to dinitrophenol and hence at a site prior to the involvement of a phosphorylated intermediate (Borst & Slater, 1961; Chappell, 1964b).

With the exception of some of the benzimidazoles used by Bishop *et al.* (1964), all of the substituted benzimidazoles used in the experiments quoted in the introduction will have pK_1 values that are too high for these compounds to be active as uncoupling agents at the concentrations used. However, Bishop *et al.* (1964) state that the mode of action of 4,5-bistrifluoromethylbenzimidazole was not as an antimetabolite of vitamin B₁₂, guanine, adenine or histidine. In this case, and for other fluorine-substituted benzimidazoles quoted by these workers, the bactericidal activity of these compounds is probably due to the uncoupling of respiratory-chain phosphorylation.

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