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UNCOUPLING OF OXIDATIVE PHOSPHORYLATION BY TRIBROMOIMIDAZOLE DERIVATIVES AND FATTY ACIDS

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Abstract—A number of 2,4,5-tribromoimidazole derivatives and long chain fatty acids have been examined with respect to their action upon oxidative phosphorylation by rat liver mitochondria. Uncoupling of oxidative phosphorylation in the absence of mitochondrial swelling occurred only with those tribromoimidazole derivatives which were capable of being degraded to 2,4,5-tribromoimidazole. A stable derivative, 1-vinyl tribromoimidazole only caused uncoupling at concentrations which produced swelling but its saturated analogue, 1-ethyl tribromoimidazole was without effect. The rate of breakdown of the unstable derivatives was enhanced by increasing the alkalinity or temperature of the medium and also by the addition of subcellular fractions of rat liver. The uncoupling effects of unsaturated fatty acids could be correlated with mitochondrial swelling. Saturated fatty acids caused little or no effect.

IT HAS been reported[1] that 1-vinyl 2,4,5-tribromoimidazole administered to rats induced symptoms characteristic of uncouplers of oxidative phosphorylation[2,3] and that survivors of the LD_{50} dose developed ataxia of the hind limbs. 1-Vinyl tribromoimidazole was one of a number of tribromoimidazole derivatives, the toxicity of which were investigated in these laboratories on behalf of the World Health Organization. Subsequent experiments (unpublished) indicated that other tribromoimidazole derivatives produced effects identical to those of 1-vinyl tribromoimidazole. It was therefore relevant to examine the affects of these compounds upon oxidative phosphorylation using rat liver mitochondrial preparations for this purpose. As a metabolite of these compounds could be 2,4,5-tribromoimidazole and as this compound has been reported to be an uncoupling agent,[4] the *in vitro* stability of the compounds was also examined.

The general formula of the derivatives is given in Fig. 1. In the present work it has been found that many of the compounds behave at a certain concentration, like the classical uncoupling agent, 2-4-Dinitrophenol (DNP) but only under conditions which favour their metabolic breakdown to the free 2,4,5-tribromoimidazole.

At higher concentrations some of these unstable compounds and in addition, the vinyl derivative, induce mitochondrial swelling in contrast to the commonly used uncouplers such as DNP[5] or carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) (unpublished observation). Such swelling may be more closely related to the effects of some long chain fatty acids upon mitochondria.[6] It was relevant, therefore, to compare this action of tribromoimidazole derivatives with the effects of fatty acids under similar conditions.

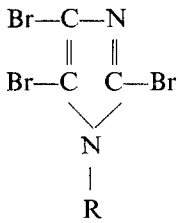


FIG. 1. General formula of 2,4,5-tribromoimidazole derivatives.

Three compounds were stable under the conditions of these experiments and were entirely without effect with respect to either oxidative phosphorylation or to mitochondrial swelling.

MATERIALS AND METHODS

Special chemicals. 2,4,5-Tribomoimidazole and its derivatives, except 1-ethyl tribromoimidazole, were gifts from Messrs. Boots Ltd., Nottingham. 1-Ethyl tribromoimidazole was prepared by bromination of imidazole followed by ethylation. Sodium lauryl sulphate was supplied by E. I. Du Pont de Nemours & Co., Wilmington, U.S.A. Oleic, linoleic, stearic and palmitic acids were purchased from British Drug Houses Chemicals Ltd., Poole, Dorset. Butyric, hexanoic, decanoic and lauric acids were purchased from Sigma Chemical Co., London. Other reagents and biochemicals were from the same sources as reported previously by Aldridge and Street.[7]

Preparation of subcellular fractions of rat liver. The mitochondrial fraction was obtained as described by Aldridge and Street.[7] Microsomes were isolated from the postmitochondrial supernatant by centrifugation at 103,000 g_{av} × 1 hr. The pellet was resuspended in 1·15% KCl and recentrifuged at 103,000 g_{av} × 1 hr. The final pellet was resuspended in 12 ml of 0·1 M phosphate buffer, pH 7·4. The suspension was equivalent to 8 g liver. The supernatant derived from the first centrifugation at 103,000 *g* was used as cell sap.

Determination of ATP synthesis. ATP synthesis was measured as inorganic phosphate uptake with ADP as acceptor in the presence of hexokinase (EC 27.1.1.) and glucose. The mitochondrial suspension (0·3 ml equivalent to 3–4 mg protein) was added to the medium which contained (final concentrations) KCl (0·1 M), $MgCl_2$ (14 mM), EDTA (1 mM), glycylglycine (16·7 mM), Na_2 ATP (3 mM) potassium phosphate (16·6 mM), sucrose (30 mM), glucose (60 mM), hexokinase (Sigma Type VI, 40 units per flask), Na pyruvate (10 mM) and Na fumarate (1 mM) in a final volume of 3·0 ml, pH 6·8. The incubation was carried out with mechanical shaking for 20 min at 37° in air. The reaction was stopped by the addition of 7 ml of ice-cold perchloric acid (0·725 M). Agents under test were added mainly as alcoholic solutions in quantities in most cases of not more than 30 μl.

Determination of ATP hydrolysis. ATP hydrolysis was measured as inorganic phosphate released from a medium which contained (final concentrations) KCl, (0·1 M), $MgCl_2$ (14 mM), EDTA (1 mM) glycylglycine (16·7 mM), Na_2 ATP (3·25 mM), 0·3 ml mitochondrial suspension (3–4 mg protein) the final volume being 3·0 ml at pH 6·8. The reaction was stopped by the addition of 7 ml ice-cold perchloric acid

(0·725 M). Determination of mitochondrial protein and inorganic phosphate was as described previously.[7]

Determination of oxygen uptake. This was measured polarographically (Beckmann Oxygen Sensor 39076) with Model 777 Oxygen Analyser from Beckmann Instruments Ltd., with 3 ml of medium in the cell at 37°. The medium was as described for the measurement of ATP synthesis except for the omission of glucose, hexokinase and ATP.

Mitochondrial enzyme activities of control experiments and treatment of results. Unstimulated rate of ATP-hydrolysis = 0·53 ± 0·05 (S.E.) (24) μmole P released/milligram protein/hour. Stimulated rate in presence of 30 μM DNP = 16·48 ± 0·32 (S.E.) (26) μmole P released/milligram protein/hour. ATP-synthesis, 25·87 ± 0·67 (S.E.) (17) μmole P taken up/milligram protein/hour. Stimulation of respiration (polarographic technique) by 30 μM DNP = 3·9 ± 0·2 (S.E.) (16). S.E. = Standard error of the mean. Figures in parentheses = number of experiments. Stimulation by DNP of respiration and ATP-hydrolysis to the extent shown were regarded as criteria of satisfactory preparations of mitochondria. Concentrations of each test compound producing 50 per cent inhibition of ATP-synthesis or 50 per cent stimulation of ATP-hydrolysis was determined graphically as described previously.[5] All experiments upon the test compounds were preceded by exploratory tests to establish the range of effective concentrations. The results quoted in the Tables are of single experiments. Some experiments were repeated but the results did not differ from one another to any greater extent than did the reference compound, DNP, from the control results quoted above.

Determination of mitochondrial swelling. Mitochondrial swelling was equated with the decrease in absorbance of a mitochondrial suspension measured at 550 nm and 37°. The medium used for ATP-hydrolysis (2·95 ml) previously heated to 37° was placed in a standard 10 mm cuvette in the temperature controlled unit of a Unicam SP 800 spectrophotometer. After 1 min, 50 μl mitochondrial suspension (approximately 1 mg protein) was added and the mixture rapidly stirred. Automatic readings at 20 sec intervals were recorded until constant values were observed. The test compound was added, the mixture stirred and the absorbance recorded until again constant. Recordings of the test cuvette was balanced against one containing the ATP-hydrolysis medium only.

Rate of breakdown of tribromoimidazole compounds (a) in the presence of mitochondrial preparations. To avoid error due to concomitant swelling, the mitochondrial membranes were disrupted by freezing and thawing (twice). The mitochondrial suspension was then sonicated for 30 sec at 2 A in a Soniprobe, Type 7530A. The amount of this final suspension added to the ATP-hydrolysis medium was balanced between the amount necessary to give a measurable rate of breakdown of the TB1 compound and the amount producing an inconveniently large 'blank' adsorbance. The recording spectrophotometer (Unicam SP 800) was set at the wavelength of maximum absorbance for the particular compound being studied. The final volume was 3·0 ml. Temperature and pH were adjusted according to the experiment. Recordings were made at fixed time intervals.

(*b*) *Measurement of breakdown in absence of animal tissue.* This was measured as described above with the omission of the mitochondrial preparation and with the substitution of phosphate buffer for the ATP-hydrolysis medium. In both cases, the

readings were corrected for the absorbance when complete breakdown had occurred and the corrected readings were used for log plots in order to obtain first order rate constants and half-lives of the compounds.

Lipid solubility of tribromoimidazole. 100 μM tribromoimidazole was portioned between equal volumes of 0·1 M HCl and cyclohexane. After shaking for 1 hr the tribromoimidazole in each solvent was measured spectrophotometrically and the amount calculated by use of standard curves.

Thin-layer chromatography. Identification of tribromoimidazole was achieved by using commercially prepared thin layer chromatographic plates Silica gel 25 u.v. 254. After incubation at 37° under conditions promoting metabolism of the TB1 compound the products were extracted from the solution with diethyl ether 5-times. The combined extracts were dried over anhydrous sodium sulphate, filtered and evaporated to dryness. The extract was dissolved in 0·2 ml ethanol 20 μl of which was spotted on to the plates. The plates were developed with cyclohexane–ethyl acetate (1 : 1 v/v) or with toluene. Positions of the components were identified by u.v. irradiation of the plate.

Measurement of carbon dioxide evolved from breakdown of TB1 methyl carboxylate. The rate of evolution of carbon dioxide from TB1 methyl carboxylate was followed manometrically. The Warburg flasks contained 2·7 ml 0·1 phosphate buffer, pH 8·0 in the main compartment, and 0·3 ml 0·1 M HCl in the side arm. After an intial period of temperature equilibration at 37° the manometers with attached flasks were momentarily removed from the bath. An alcoholic solution (50 μl) of the compound was quickly injected into the main compartment and the flasks and manometers rejoined. The final concentration of TB1 methyl carboxylate was 2·2 mM. After a brief incubation of approximately 30 sec the taps on the manometers were closed and readings of manometric fluid levels taken. The acid was tipped from the sidearm of different flasks into the main compartment at different times. Observations of the manometric levels were continued until readings were constant. The manometric readings were converted to μl CO_2. A correction factor (100/75) for the retention of carbon dioxide in phosphate buffer was obtained by measuring the recovery of carbon dioxide from known amounts of sodium bicarbonate. The first order rate constant and hence the half-life of the compound were derived graphically.

RESULTS

Tribromoimidazole (TB1) and derivatives

Effect upon mitochondrial enzyme systems. The tribromoimidazole compounds are listed in Table 1. Initial experiments using the polarographic technique revealed that many of these compounds behaved towards isolated rat liver mitochondria in a manner similar to that of DNP. With pyruvate and fumarate as substrates and at concentrations of between 5 to 10 μM they increased mitochondrial respiration by an extent comparable to that of 30 μM DNP. The compounds effective in this manner were tribromoimidazole, all of the carboxylate derivatives and the cyano tribromoimidazole. Vinyl tribromoimidazole behaved similarly but was required at a higher concentration (25–30 μM). 1-Methyl and 1-ethyl and dimethyl carbamoyl tribromoimidazole had no effect.

Stimulation of mitochondrial respiration suggested that these compounds were acting as uncouplers of oxidative phosphorylation. Confirmation of this point was

TABLE 1. CORRELATION OF EFFECTS BY TRIBROMOIMIDAZOLE AND ITS DERIVATIVES ON MITOCHONDRIAL FUNCTION AND THE STABILITY OF THESE COMPOUNDS IN THE PRESENCE AND ABSENCE OF DISRUPTED MITOCHONDRIA

Compounds	ATP-synthesis (A) Concn inhibiting P-uptake 50%* (μM)	ATP-hydrolysis (B) Concn stimulating hydrolysis 50% (30 μM DNP = 100%) (μM)	Stability in mitochondrial preparation (C) half-life (min)	Stability in 0·066 M Phosphate buffer (D). Half-life (min)
Tribromoimidazole N-derivatives TBI-R				
R = —Methyl	No effect	No effect	Compound unchanged	Compound unchanged
= —Ethyl	No effect	No effect	Compound unchanged	Compound unchanged
= —Dimethyl carbamoyl	No effect	No effect	Compound unchanged	Compound unchanged
= —Vinyl	30	33	Compound unchanged	Compound unchanged
= —Cyano	5	5	29	47
= —H	9	5	Compound unchanged	Compound unchanged
Tribromoimidazole *N'*-carboxylates (R = —CO_2R')				
R' = —Methyl	9	5	0·73	90
= —*n*-Butyl	9	5	Not done	190
= —*iso*-Butyl	10	3	1·66	330
= —Phenyl	9	5	0·46	7
= —*p*-Tolyl	5	4	0·40	47
= —2·6-Dimethyl	7	4	2·16	12

* Separate polarographic experiments using the ATP-synthesis medium showed that at these concentrations there was no inhibitory effect upon oxygen uptake.

Effect of tribromoimidazole and its derivatives upon mitochondrial ATP-synthesis (A) and ATP-hydrolysis (B) compared with stability of these compounds in mitochondrial preparations (C) and 0·066 M phosphate buffer (D), ATP-synthesis and ATP-hydrolysis as described in Methods section. Stability of each compound in presence of sonicated, frozen and thawed mitochondria pH 7·0 and in phosphate buffer pH 7·4 was measured as decrease of absorbance at appropriate wavelengths at 37°. Half-lives calculated from rate constants obtained from first order log plots. Other details in Methods section.

sought by examining the effect of these compounds upon mitochondrial ATP-synthesis and ATP-hydrolysis.

Table 1 shows that those compounds previously found capable of stimulating mitochondrial respiration inhibited phosphate uptake (ATP-synthesis) and stimulated ATP-hydrolysis. The inhibition of phosphate uptake was not accompanied by inhibition of oxygen uptake. The effective concentrations of the compounds for both types of mitochondrial enzyme response were essentially similar except in the case of the vinyl compound which was not effective below 25 μM. In agreement with their lack of effect upon mitochondrial respiration, the methyl, ethyl tribromoimidazoles and dimethyl-carbamoyl derivative had no effect upon either ATP-synthesis or ATP-hydrolysis.

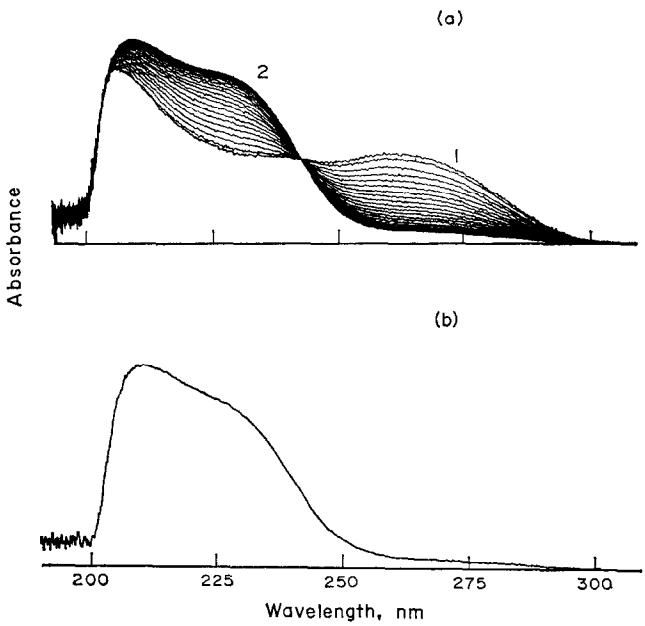


FIG. 2. (a) Initial (1) and final (2) spectra of 100 μM Methyl-tribromoimidazole carboxylate in 0·1 M phosphate buffer, pH 8·0, 37°. (b) Spectrum of 100 μM tribromoimidazole in 0·1 M phosphate buffer, pH 8·0, 37°. In (a) spectral change was followed by continuous automatic scanning on Unicam SP 800 spectrophotometer.

Stability of tribromoimidazole derivatives in aqueous media. Since the concentration of tribromoimidazole required to inhibit ATP-synthesis or to stimulate ATP-hydrolysis is virtually the same as the corresponding concentrations of the carboxylate derivatives (Table 1), it was possible that the compounds were being degraded rapidly to a common form. Tribromoimidazole possesses an ionisable hydrogen atom and has been reported to be an uncoupler of oxidative phosphorylation. Therefore this compound was a possible candidate for the uncoupling effect of the carboxylate derivatives.

In order that the above hypothesis should be feasible it was necessary to show that the derivatives of tribromoimidazole were unstable under the conditions of the oxida-

tive phosphorylation experiments. As a preliminary step the stability of these compounds in buffered aqueous medium was examined spectrophotometrically. Figure 2 illustrates a typical example of many of the tribromoimidazole carboxylates and the cyano derivative. The initial spectrum (1) shows a maximum at approximately 275 nm. After a variable time, dependent upon the compound, the final curve closely resembled that of tribromoimidazole. (2) The disappearance of the maximum at 274 nm paralled the rate of appearance of the tribromoimidazole curve. Where the possible leaving group was aromatic, as in the case of the phenyl carboxylate, the final curve was a summation of the curves due to equimolar concentrations of the phenol and tribromoimidazole. Under the same conditions the vinyl, methyl, ethyl and dimethyl-carbamoyl tribromoimidazole showed no spectral change. By recording, at fixed time intervals,

TABLE 2. EFFECT OF pH AND TEMPERATURE ON RATE OF BREAKDOWN OF TBI-DERIVATIVES IN PHOSPHATE BUFFER (0·1 M)

Compound	pH	Half-life (min) Temp. 20°	30°	37°
Methyl carboxylate	7	90	51	36
	8	45	23	9
	9	18	6	3
n-Butyl carboxylate	7	186	168	109
	8	64	46	25
	9	33	14	8·3
iso-Butyl carboxylate	7	330	224	126
	8	86	62	28
	9	40	17	8·3
Phenyl carboxylate	7	7·4	3·6	2·6
	8	5·1	1·9	0·8
	9	2·0	0·8	0·4
Cyano-TBI	7	47	18	4·5
	8	13	5	2·7
	9	10	4	1·0

Rate breakdown measured as in Methods section. Half-lives calculated from first order rate constants.

the absorption at a constant wavelength corresponding to the maximum for each compound it was possible to calculate the rate of breakdown. The calculated half-lives of each compound are seen in Table 1. There is considerable variation, ranging from 7 min for the phenyl carboxylate to more than 5 hr for the isobutyl compound. It is seen that the compounds which were inaffective towards the mitochondrial systems were stable in buffered solutions. The vinyl derivative was stable but was effective against the mitochondrial enzyme systems.

Factors affecting these rates were investigated in selected compounds. Table 2 illustrates that the rate is increased at high pH and therefore the reaction is base catalysed. The rate is also increased with respect to increases in temperature by an amount normally expected of a chemical reaction.

Rate of breakdown by cell fractions. The rates of breakdown in aqueous medium are inadequate to account for the rapid onset of uncoupling observed in the polarographic experiments. It was therefore probable that the mitochondrial preparations may have contained an enzyme or enzymes capable of catalysing the breakdown. By measuring the rate of decrease in absorbance, as before, it was found that suspensions of disrupted mitochondrial fractions considerably accelerated the degradation of the carboxylate derivatives and the degradation of the cyano derivative (Table 1). Some of the rates were slow enough to lead one to expect that a time lag should have been evidence in the polarographic records of respiration stimulation. This was sometimes observable but not consistently. Short time lags of a few seconds would have been missed by the initial jump in the tracing due to dissolved oxygen in the alcohol solvent.[8] Those compounds previously found to be stable in aqueous medium showed no evidence of breakdown by mitochondrial fractions.

Further experiments with two derivatives (Table 3) showed that the greatest catalytic activity was associated with the microsomal fraction. The activity associated

TABLE 3. BREAKDOWN OF TRIBROMOIMIDAZOLE CARBOXYLATE DERIVATIVES BY SUBCELLULAR FRACTIONS OF RAT LIVER

Fraction	TB1-*n*-butyl carboxylate (first order rate constant min^{-1}/g liver)	TB1-methyl carboxylate (first order rate constant min^{-1}/g liver)
Mitochondria once washed	19·3	18·3
Four-times washed		3·5
Denatured mitochondria		2·0
Microsomes	32·5	115·0
Cell sap	13·5	13·5

First order rate constants of each compound were measured as described in Methods section after correction for corresponding rates in phosphate buffer at 37°, pH 7. Results for the cell sap fractions were calculated from the rate constants obtained in the first few minutes. Denatured mitochondria were obtained by boiling the standard preparation of mitochondria for 5 min. The denatured mitochondria were then resuspended in 0·3 M sucrose.

with the mitochondrial preparations could be reduced by repeated washing with 0·3 M sucrose and by heat denaturation. Unlike all previous experiments those dealing with the cell sap did not produce simple first order log plots. Instead two straight lines were obtained, the rate over the first few minutes being approximately 3-times faster than the subsequent rate. The half life given in Table 3 was derived from the first of these rates.

To provide more evidence that the TB1 derivatives were being degraded to a common species, phenyl tribromoimidazole carboxylate was incubated at 37° in phosphate buffer, pH 8·0 for 15 min and then the solution extracted with ethyl ether and finally into alcohol (Materials and Methods). The alcoholic extract was chromatographed on thin layer plates. Under u.v. light one spot was revealed with R_f value of 0·40, identical to that of tribromoimidazole.

Mechanism of the breakdown. Preliminary manometric experiments indicated that incubation under alkaline conditions of the carboxylate derivatives resulted in the

evolution of carbon dioxide as well as the production of tribromoimidazole. A direct comparison between the rate of CO_2 production and the rate of absorbance change was possible in the case of only one derivative, the methyl carboxylate. The experiment with the methyl carboxylate (2·2 mM) was performed in 0·1 M phosphate buffer, pH 8·0 at 37°. Parallel observations of the absorbance change were conducted upon solutions incubated under the same conditions, but diluted in phosphate buffer, pH 6·0 immediately before recording the absorbance. At pH 6·0 and at room temperature the rate of further degradation was negligible. The rate of breakdown of the compound as calculated from the CO_2 production and the rate of absorbance change were of first order. Half-life for the absorbance change was 9·0 min and that of CO_2 production was 60 min.

In the case of the cyano derivative similar manometric experiments showed that after tipping acid from the Warburg flask side-arm CO_2 was evolved. The other possible product, ammonia, was detected either by direct nesselerization or indirectly by using dilute acid in the centre well to absorb any ammonia. Qualitative tests by the copper sulphate pyridine-reaction were indicative of the presence of cyanate.[9]

Vinyl tribromoimidazole. The previous results are strongly indicative of a degradation of the carboxylate derivatives and of the cyano derivative to tribromoimidazole which is the compound involved in uncoupling oxidative phosphorylation. Where no evidence of instability was observed as in the case of the methyl, ethyl TB1 or dimenthyl carbamoyl derivatives then likewise no uncoupling was manifest. An anomaly in the general thesis that degradation precedes uncoupling is the behaviour of the vinyl compound. No evidence was obtained that this compound was anything but stable. The absorption curve was similar to that of tribromoimidazole and therefore possible degradation by change in absorbance was not detectable. Infrared spectra of this compound compared with that of tribromoimidazole indicated that although the presence of the latter compound in the vinyl preparation could not be excluded, nevertheless the major component was a compound other than tribromoimidazole. Two different elution systems were devised for thin layer chromatography by which tribromoimidazole could be differentiated from its derivatives. Chromatography of the vinyl derivative indicated the presence of a minor amount of tribromoimidazole but previous incubation of the vinyl compound in either phosphate buffer pH 7·0, in 0·1 M HCl, in 0·1 M NaOH at room temperature for 24 hr or in mitochondrial suspensions for 30 min at 37° failed to alter the pattern of the thin layer chromatograms or to alter the relative density of the spots.

Structurally, the compound does not contain a dissociable hydrogen atom and therefore was not expected to behave as DNP or tribromoimidazole. It is well known that certain long chain fatty acids such as oleic acid[6] or detergents produce some of the phenomena of uncouplers of oxidative phosphorylation. Consequently the possibility that vinyl tribromoimidazole was acting in a similar fashion was considered. Suspensions of intact mitochondria in ATP-hydrolysis media were examined on an automatic recording spectrophotometer at 550 nm and the change of turbidity recorded. A range of concentrations were examined of which two are recorded in Table 4. It is seen that the vinyl derivative produced a marked swelling at 100 μM but little or none at 10 μM. This would account for the fact that 30–33 μM was required to affect ATP-synthesis and ATP-hydrolysis. Following this observation, the other compounds were examined and Table 4 illustrates that many of the compounds shared this property of causing

mitochondria to swell. As in the case of the vinyl compound, little effect was obtained at 10 μM, a concentration which for any of the other compounds would have had a marked uncoupling effect. Those compounds previously found to be stable and to have no effect upon mitochondrial enzymes, the methyl TBI ethyl TBI and dimethyl carbamoyl derivatives, were also inert in respect to mitochondrial swelling. It is of interest that ethyl TBI, which differs from the vinyl derivative only in the absence of the double bond, was completely inert in all respects. In addition the parent compound,

TABLE 4. EFFECT OF TRIBROMOIMIDAZOLE AND DERIVATIVES ON MITOCHONDRIAL SWELLING

Carboxylate derivatives	Concn (μM)	Change in absorbance 1 min after addition (%)
—Methyl	100	6·5
	10	3·0
—*n*-Butyl	100	68·0
	10	4·0
—*iso*-Butyl	100	75·0
	10	3·5
—Phenyl	100	78·0
	10	6·0
—*p*-Tolyl	100	77·0
	10	5·5
2,6 Dimethyl phenyl	100	59·0
	10	10·0
Tribromoimidazole derivatives		
—H	100	4·5
	10	Not done
—Cyano	100	26·0
	10	12·0
—Vinyl	100	41·0
	10	3·0
Dimethyl carbamoyl	100	3·0
	10	Not done
Methyl	100	3·0
	10	1·0
Ethyl	100	2·0

Mitochondrial swelling was measured as decrease in absorbance at 550 nm in the ATP-hydrolysis medium, 37°, pH 6·8. Spontaneous decrease in absorbance = 3–4 per cent. Details in Methods section.

tribromoimidazole, was ineffective as was the methyl carboxylate. It is possible that the small amount of swelling induced by 10 μM 2,6 dimethyl phenyl carboxylate and the cyano derivative may be indicative of some contribution of this aspect towards the uncoupling of these compounds. As certain types of mitochondrial swelling are prevented by DNP[10,5] where swelling did occur the experiments were repeated in the presence of 30 μM DNP. In every case DNP did not change the extent or time course of the swelling (results not shown).

As a test of the specificity of the effect of these compounds upon biological membranes their action upon red blood cells was examined. Table 5 demonstrates that in

general those tribromoimidazole compounds which at 100 μM caused mitochondria to swell also caused haemolysis of horse red blood cells. One exception was the cyano derivative. This compound had some effect upon mitochondria but none on blood cell membranes.

TABLE 5. EFFECT OF TRIBROMOIMIDAZOLE AND ITS DERIVATIVES ON HORSE RED BLOOD CELLS

Tribromoimidazole carboxylate derivatives	Absorbance* (407 nm)
—Methyl	0·38
—*n*-Butyl	1·19
—*iso*-Butyl	1·21
—Phenyl	0·68
—*p*-Tolyl	1·21
—1·6 Dimethyl phenyl	1·20
Tribromoimidazole derivatives	
—H	0·0
—Cyano	0·0
—Vinyl	1·05
—Dimethyl carbamoyl	0·0
—Methyl	0·0

* Less spontaneous haemolysis amounting to an increase of absorbance of 0·21 for 7 min.

Saline washed r.b.c. diluted 1:500 with normal saline. 5 ml r.b.c. plus 50 μl TBI compound, final concentration 100 μM. Incubated in shaking metabolic waterbath at 37° for 7 min. Centrifuged at 1000 rev/min for 10 min. Decanted supernatant read at 407 nm.

Effect of long chain fatty acids upon rat liver mitochondria. Tables 1 and 4 demonstrate that tribromoimidazole carboxylate derivatives and the cyano derivative uncouple oxidative phosphorylation at lower concentrations than those required to induce mitochondrial swelling. The vinyl derivative promotes swelling at the concentration which also uncouples, and three compounds which did not induce swelling had no effect upon oxidative phosphorylation. The behaviour of the vinyl compound resembled that of some long chain fatty acids[6] and it was decided, therefore, to compare under the same experimental conditions, the effects of the vinyl derivative with these of fatty acids along with the "classical" uncoupler, DNP and a detergent, lauryl sulphate.

The acids studied were two unsaturated fatty acids, oleic and linoleic, and two saturated fatty acids, stearic and palmitic. Other fatty acids, namely butyric, hexanoic, decanoic, and lauric, were investigated from the point of view of their effects upon ATP-hydrolysis and mitochondrial swelling only. In addition to the experiments previously described, the affect of each compound upon ATP-hydrolysis partially stimulated by DNP was examined.

Table 6 summarizes the experimental results. The unsaturated fatty acids and lauryl sulphate produced effects upon ATP-hydrolysis and ATP-synthesis similar to those produced by DNP. Unlike the latter compound, however, at the same concentrations as used previously, the fatty acids and the detergent caused rapid large scale changes

TABLE 6. SUMMARY OF EFFECT OF DNP, TRIBROMOIMIDAZOLE, 1-VINYL TRIBROMOIMIDAZOLE AND FATTY ACIDS UPON RAT LIVER MITOCHONDRIA

Compound	Concn	ATP-hydrolysis	ATP-synthesis	Swelling	ATP-hydrolysis in presence of 5 μM DNP*
DNP	5	Stimulation	Inhibition	No effect	+ve
Tribromoimidazole	5	Stimulation	Inhibition	No effect	+ve
1-Vinyl tribromoimidazole	30	Stimulation	Inhibition	Swelling	+ve
Oleic acid	60–80	Stimulation	Inhibition	Rapid swelling	−ve
Linoleic acid	50–90	Stimulation	Inhibition	Rapid swelling	−ve
Stearic acid	100	Partial stimulation	Partial inhibition	Slow swelling	−ve
Palmitic acid†	100	No effect	No effect	No effect	−ve
Lauryl sulphate	200–250	Stimulation	Inhibition	Rapid swelling	+ve

* +ve = Additive stimulation, −ve = no additive effect.
† Butyric, hexanoic, decanoic and lauric acid produced results as for palmitic acid.
Stimulation = stimulation to 50 per cent of the rate induced by 30 μM DNP.
Inhibition = inhibition by 50 per cent of control rate of P uptake.
Partial stimulation = stimulation by less than 10 per cent of that induced by 30 μM DNP.
Partial inhibition = inhibition by less than 30 per cent of control rate of P uptake.
Rapid swelling = decrease in absorbance by 60–70% in 1 min.
Slow swelling = decrease in absorbance by 30 per cent in 22 min.
Experiments involving ATP-hydrolysis, ATP-synthesis and mitochondrial swelling described in Methods section. ATP-hydrolysis in presence of 5 μM DNP (see text).

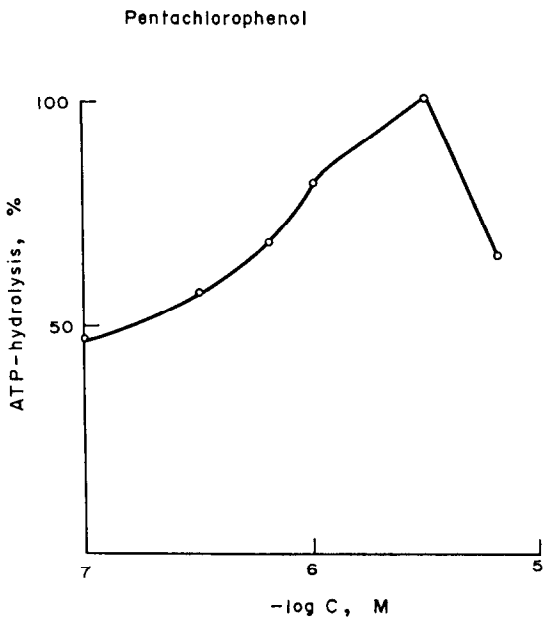


FIG. 3. Effect of pentachlorophenol on ATP-hydrolysis of rat liver mitochondria in presence of 5 μM DNP. For medium and method see Methods section. 100 per cent hydrolysis $\equiv$ 30 μM DNP. 5 μM DNP = 48 per cent.

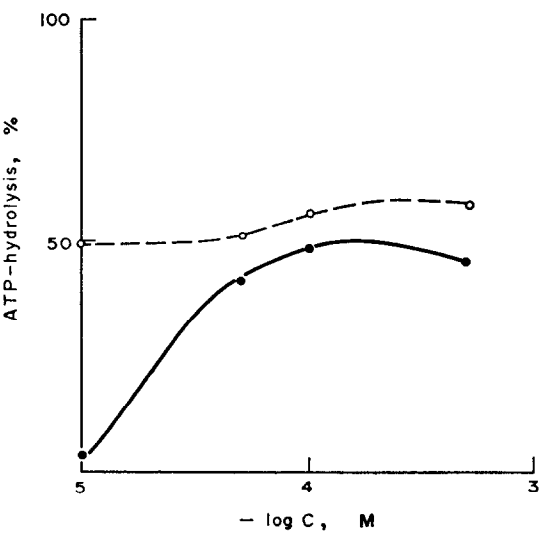


FIG. 4. Effect of oleic acid on ATP-hydrolysis of rat liver mitochondria in presence of 5 μM DNP. For medium and method see Method section. 100 per cent hydrolysis $\equiv$ 30 μM DNP. 5 μM DNP = 42 per cent. ○- - -○, oleic acid + 5 μM DNP; ●——●, Oleic acid.

in absorbance of mitochondrial suspensions indicative of mitochondrial swelling. Stearic acid caused a slow progressive decrease in absorbance during the course of the experiment but the extent of the change was much less than that produced by oleic or linoleic acids. Correspondingly, there was a relatively small effect upon ATP-hydrolysis. Palmitic acid did not produce mitochondrial swelling and had no effect upon ATP-synthesis or hydrolysis. As with swelling brought about by tribromoimidazole compounds the addition of DNP had no effect upon either the time course or degree of swelling produced by fatty acids or by lauryl sulphate.

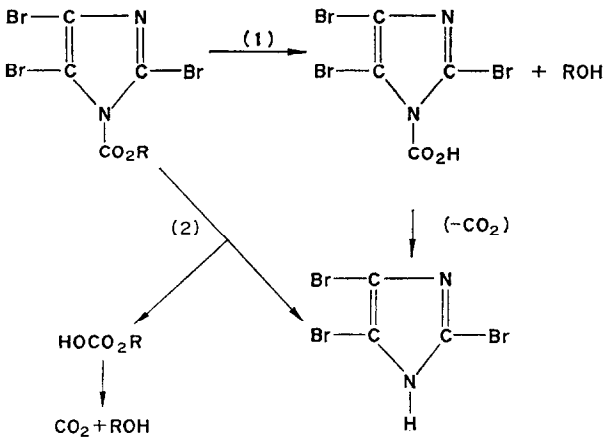
DNP (5 μM) stimulates ATP hydrolysis by approximately 50 per cent of the maximum attainable (30 μM DNP). The addition of increasing concentrations of a second uncoupler, e.g. pentachlorophenol, causes an increase in ATP-hydrolysis until the expected maximum is reached (Fig. 3). The last column in Table 6 and Fig. 4 indicates that in the case of the fatty acids such additive effects were not obtained. It is also seen that in this respect vinyl tribromoimidazole resembled tribromoimidazole and DNP. However, taking all the experiments into account vinyl tribromoimidazole behaved similar to lauryl sulphate.

DISCUSSION

There are now several examples of different classes of chemical compounds which it is claimed behave towards mitochondria in the same manner as the classical uncoupler (DNP).[16] Although diverse in chemical structure most of these compounds have in common the property of being lipophilic weak acids. Their effectiveness towards mitochondrial enzyme systems, is within limits, correlated with their degree of acidity and their lipid solubility.[5,11,12] Structurally the tribromoimidazole derivatives do not exhibit the characteristics previously associated with uncoupling compounds in that they are not acids. It was expected therefore, and circumstantial evidence conforms to the expectation, that those of them which uncouple oxidative phosphorylation do so after degradation to tribromoimidazole or to some other common breakdown product. The one exception was vinyl tribromoimidazole which whilst inducing the overt manifestations of uncoupling does so without any evidence of degradation to another compound.

The spectral changes and the chromatographic evidence as well as the coincidence of concentrations required to uncouple oxidative phosphorylation indicate that the effective agent is tribromoimidazole. This compound has a pK of 6·95[4] and by partition between cyclohexane and 0·1 M HCl found to be poorly lipid soluble (unpublished). As compared with other uncouplers[5,15] this compound would not have been expected to be very effective. However, present studies demonstrate that tribromoimidazole is as effective as DNP.

The derivation of tribromoimidazole from the carboxylic compounds could conceivably arise by two different mechanisms. These substances can be regarded as N^1 disubstituted derivatives of carbamate esters. The mechanism proposed for the degradation of these esters differ in details,[13,14] but all involve an initial de-esterification with the production of an alcohol or phenol followed by the simultaneous evolution of carbon dioxide and production of the amine (Route 1). Alternatively, (2) the initial hydrolysis could occur between the nitrogen atom in the (1) position on the tribromoimidazole and the carbon on the carboxylate group. The substituted carbonate would undergo decarboxylation to form CO_2 and an alcohol or phenol



(Route 2). If Route 1 operated it would be expected that the rate of production of CO_2 should be the same as the rate of production of tribromoimidazole. Moreover, tribromoimidazole carboxylate would be the common intermediate and thus the rate of production of CO_2 from all of the carboxylate derivatives would be the same. In contrast, by Route 2 it would not necessarily be the case that the tribromoimidazole production would proceed at the same rate as the evolution of CO_2. As the different compounds would give rise to different carbonate esters it is likely that the rate of CO_2 production from these esters would vary. The experiment with tribromoimidazole methyl carboxylate showed that the rate of degradation as measured by the change in absorbance was much more rapid than the rate of evolution of CO_2 measured under the same conditions. For solubility reasons only semi-quantitative experiments could be performed with the other compounds. Such experiments (unpublished) however, indicated that in all cases the rate of CO_2 evolution was very slow as compared with the absorbancy changes and also the rate of CO_2 production varied between the different derivatives. The evidence therefore favours the second of these two schemes. The results with the cyano derivative were consistent with the hypothesis that the initial step in the degradation of this compound involves hydrolysis to tribromoimidazole and cyanate. The latter could then undergo further hydrolysis to CO_2 and ammonia.

The greatly enhanced rates of breakdown in the presence of subcellular liver fractions and the fact that such rates can be considerably decreased by heat denaturation indicates that the process is enzymic. The activity appears to be associated mainly with the microsomal fraction although repeated washing of the mitochondrial fraction did not altogether reduce the rate in this subcellular fraction to that in phosphate buffer alone.

Although the uncoupling activity of the carboxylate and cyano compounds is explicable by virtue of the breakdown of these compounds to tribromoimidazole, the present experiments do not provide an explanation of the effect of these compounds in relation to mitochondrial swelling. Stability does not appear to be a necessary requirement for swelling activity. The three stable compounds not reactive to mitochondria do not induce swelling whereas the vinyl derivative which proved to be stable under various experimental conditions caused swelling. The unstable compounds also do not

show a good correlation between stability and swelling for the *p*-tolyl derivative which has the most rapid rate of breakdown is also a very good swelling agent. On the contrary, the methyl carboxylate exhibits no swelling and yet is more stable than the *p*-tolyl carboxylate.

In the case of the vinyl derivative there may be an association between chemical structure and swelling activity. This compound possesses a carbon–carbon double bond. It is significant that the saturated analogue, 1-ethyl tribromoimidazole, is entirely ineffective both as regards swelling and uncoupling activity. In this respect the behaviour of the vinyl derivative resembles that of the unsaturated fatty acids which are most effective in promoting swelling, whereas the saturated fatty acids have little or no activity. Unlike the fatty acids, however, 1-vinyl tribromoimidazole has an additive effect towards partially stimulated ATP-hydrolysis. Again it has long been known that the fatty acids exert a detergent-like activity towards mitochondria[6] but there is no indication from the structure of the tribromoimidazole compounds that these substances would be capable of detergency. The mechanisms and factors involved in causing mitochondrial swelling are complex and imperfectly understood but that there are different types of swelling is well known[10]. In the present instance, the swelling induced by the tribromoimidazole compounds is of a different nature than that caused in other ways. It takes place in a medium containing ATP, EDTA and relatively high concentration of $MgCl_2$, all substances which prevent many types of swelling.[10] Moreover, the swelling is not inhibited by DNP.

In conclusion, the present study demonstrates that although tribromoimidazole derivatives, fatty acids, and DNP provoke the manifestation of uncoupled oxidative phosphorylation, distinct differences can be observed in their behaviour towards mitochondria. The DNP-type compounds such as DNP itself and tribromoimidazole do not cause mitochondria to swell and show an additive effect upon ATP-hydrolysis partially stimulated by DNP. The unsaturated fatty acids do cause mitochondria to swell but do not have an additive effect upon partially stimulated ATP-hydrolysis. 1-vinyl tribromoimidazole exemplifies a third type of behaviour where this compound does cause swelling, but like the DNP type uncoupler has an additive effect upon partially stimulated mitochondrial ATP-hydrolysis. Finally, as far as the tribromoimidazole carboxylate derivatives are concerned, these *in vitro* studies may explain the DNP-type symptoms, in the acute phase of poisoning, after *in vivo* administration to rats. The relevance of the *in vitro* to the *in vivo* effects of the 1-vinyl derivative and of the stable compounds remains unknown. This and the ataxic effect of these compounds is subject to further investigation.

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REFERENCES

1. J. M. BARNES, *The Scientific Basis of Medicine Annual Reviews* p. 183. British Postgraduate Medical Federation, University of London, Athlone Press (1969).
2. W. DIECHMANN, W. MACHLE, K. V. KITZMILLER and G. THOMAS, *J. Pharmac. exp. Ther.* **76**, 104 (1942).
3. V. H. PARKER, J. M. BARNES and F. A. DENZ, *Br. J. Indust. Med.* **8**, 226 (1951).
4. B. B. BEECHEY, *Biochem. J.* **98**, 284 (1966).
5. V. H. PARKER, *Biochem. J.* **97**, 658 (1965).

6. B. C. Pressman and H. A. Lardy, *Biochim. biophys. Acta.* **21,** 458 (1956).
7. W. N. Aldridge and B. W. Street, *Biochem. J.* **124,** 221 (1971).
8. J. B. Chappell and A. R. Crofts, *Biochem. J.* **95,** 707 (1965).
9. A. I. Vogel, *Qualitative Chemical Analysis,* p. 256. Longmans, London, (1945).
10. A. L. Lehninger, *Physiol. Rev.* **42,** 467 (1962).
11. H. C. Hemker and W. C. Hulsmann, *Biochim. biophys. Acta* **48,** 221 (1961).
12. E. Gladtke and E. Liss, *Biochem. Z.* **331,** 65 (1959).
13. P. Adams and F. A. Baron, *Chem. Rev.* **65,** 567 (1965).
14. M. L. Bender and R. B. Homer, *J. Org. Chem.* **30,** 3975 (1965).
15. O. T. G. Jones and W. A. Watson, *Biochem. J.* **102,** 564 (1967).
16. E. C. Weinbach and D. M. Garbus, *Nature* **221,** 1016 (1969).