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Uncouplers of Rat-Liver Mitochondrial Oxidative Phosphorylation

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1. The ability of a series of compounds to uncouple oxidative phosphorylation of rat-liver mitochondria has been investigated. 2. The compounds were: 2-amino-1,1,3-tricyanopropene; carbonyl cyanide phenylhydrazone and its *m*-chloro and *p*-trifluoromethoxy derivatives; 4,5,6,7-tetrachloro-, 5-chloro-4-nitro-, 5-nitro- and 4,5,6,7-tetrachloro-1-methyl-benzotriazole; 4-hydroxy-3,5-di-iodo-, 3,5-dibromo-4-hydroxy- and 3,5-dichloro-4-hydroxy-benzonitrile; and pentafluorophenol. 3. In a medium the components and physical condition of which were, as far as possible, kept constant, each compound was tested for ability to stimulate adenosine triphosphatase, to stimulate respiration in the presence of pyruvate as substrate, to inhibit phosphate uptake and to prevent swelling by trimethyltin. 4. Each compound was also examined with respect to its ability to produce rapid rigor mortis in mice. 5. The biological properties were compared with the dissociation constant and the hexane–water partition coefficient for each compound. 6. With the exception of 4,5,6,7-tetrachloro-1-methylbenzotriazole, all the compounds behaved qualitatively as 2,4-dinitrophenol. 7. Within each class of compound there is a relation between biological activity and the physical attributes measured. 8. The most efficient uncouplers were the most acidic and the most hydrophobic.

The phenomena associated with the interaction of isolated mammalian mitochondria and 2,4-dinitrophenol occur when 2,4-dinitrophenol is replaced by some other phenols (Weinbach, 1954, 1956; Terner, 1954; Parker, 1958; Gladtke & Liss, 1958). The effects on mitochondria that these phenols have in common include uncoupling of oxidative phosphorylation, stimulation of mitochondrial respiration and adenosine-triphosphatase activity, and inhibition of the [^{32}P]phosphate–ATP exchange reaction. Certain physical properties such as acidity and lipid solubility are associated with their biological activity (Parker, 1958; Hemker, 1962, 1964).

In recent years the number of compounds that act like 2,4-dinitrophenol has been increased by classes of substances chemically unrelated to the phenols. The effect of 2-amino-1,1,3-tricyanopropene on mitochondria was described by Eberts (1961), and this was followed by descriptions of experiments with compounds of related structure, the carbonyl cyanide phenylhydrazones (Heytler & Pritchard, 1962) some of which were more efficient uncouplers of oxidative phosphorylation than 2,4-dinitrophenol itself. The possibility of other non-phenolic uncouplers was suggested in a personal communication from Dr E. F. Edson of Fison Pest Control Ltd., who had noticed that a derivative of benzotriazole induced early and intense rigor at death strikingly similar to that induced by 3,5-dinitro-*o*-cresol (Parker, Barnes & Denz, 1951). In addition to these compounds new phenolic compounds have been described as uncouplers of oxidative phosphorylation of plant tissues, e.g. 3,5-dihalogeno-4-hydroxybenzonitriles (Wain, 1963).

In the present paper these compounds have been examined and compared with respect to many of the biological effects of 2,4-dinitrophenol, as well as two physical attributes of dissociation and partition into non-aqueous solvents.

MATERIALS AND METHODS

In all the work involving mitochondria, except where the exigencies of an experiment demanded otherwise, the composition, pH and temperature of the medium were constant from one type of experiment to the other. The reasons for the composition of the medium have been fully discussed by Aldridge (1957).

Preparation of mitochondria. Rat-liver mitochondria were prepared in 0·3M-sucrose as described by Aldridge (1957), with a modified Potter–Elvehjem-type homogenizer (Webster & Smith, 1964).

Manometric measurements. Oxidative phosphorylation

and respiration in the absence of hexokinase and glucose were measured according to the method of Aldridge (1958), by using pyruvate (10mM) with fumarate (1·0mM) as substrate. Uptake of inorganic phosphate was measured over a time-interval of 12min. and compared with the oxygen uptake over 10min. by method 1 of Aldridge & Parker (1960). Inorganic phosphate was measured by the method of Fiske & Subbarow (1925). Adenosine-triphosphatase activity was measured by the method of Aldridge & Stoner (1960).

Swelling of mitochondria. Inhibition of the swelling of mitochondria was followed by changes in the extinction at 550mμ (measured with a Unicam D.G. spectrophotometer) in the adenosine-triphosphatase medium. Swelling was induced by 70μM-trimethyltin. The inhibition produced by each compound was compared with that brought about by 30μM-2,4-dinitrophenol.

Effect on intact animals. Because of the small quantities of material available in some instances, toxicity tests were not done. However, each compound in strong aqueous solution was injected intraperitoneally into two mice and the occurrence of rigor at death was compared with the same event in mice that had been injected with a lethal dose of 2,4-dinitrophenol.

Dissociation constants. These were measured for each compound either (*a*) by comparison of absorption spectra in solutions of three different but known pH values, or (*b*) by comparison of absorption spectra in acid and alkaline solution with a third solution of known pH (Rosenblatt, 1954).

Partition coefficients. Each compound in 0·1N-HCl was shaken for 30–60min. with an equal volume of hexane (spectrographically pure; British Drug Houses Ltd., Poole, Dorset). The ratio of concentration in organic and aqueous phase was determined by absorption spectroscopy by using the Unicam SP.700 recording spectrophotometer.

Calculation and comparison of results. The effect of each compound on adenosine-triphosphatase activity and on mitochondrial respiration was compared with the maximal result obtained with 30μM-2,4-dinitrophenol and expressed as a percentage. In oxidative-phosphorylation experiments, phosphate uptake was expressed as a percentage of the maximum uptake in control experiments run at the same time. The results from all three types of experiments were plotted against the negative logarithm of the concentration. Fig. 1 illustrates the type of curves obtained by this method and is typical of all the compounds that were found to be uncouplers of oxidative phosphorylation (cf. Fig. 5 in Aldridge & Street, 1964). Similar graphs were prepared for each compound and the data drawn from them for Table 1. The results for the swelling experiments did not lend themselves to this treatment. Instead, the changes in extinction were plotted and the point was estimated at which the decrease in extinction was 50% of that produced by trimethyltin alone.

Sources of materials. The benzotriazole derivatives were from Fison Pest Control Ltd. The carbonyl cyanide phenylhydrazones were from E. I. Du Pont de Nemours and Co., Wilmington, Del., U.S.A. 2-Amino-1,1,3-tricyanopropene was from Upjohn Ltd., Crawley, Sussex. The dihalogeno-4-hydroxybenzonitriles were from Professor Wain of London University, Wye College, Ashford, Kent. All other substances were from the sources mentioned in the appropriate references.

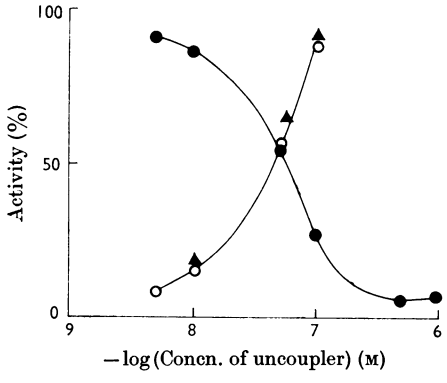


Fig. 1. Graphical method of estimating the point of 50% stimulation of adenosine-triphosphatase activity or 50% stimulation of respiration and the point of 50% inhibition of phosphate uptake: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was used in the example illustrated. ○, Adenosine-triphosphatase activity expressed as a percentage of the maximum with 30μM-2,4-dinitrophenol; ▲, respiration in the presence of pyruvate expressed as a percentage of the maximum stimulation with 30μM-2,4-dinitrophenol; ●, phosphate uptake expressed as a percentage of that in control experiments.

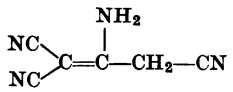
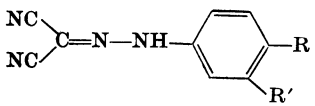
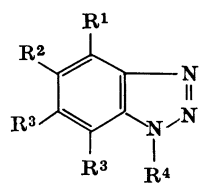
RESULTS AND DISCUSSION

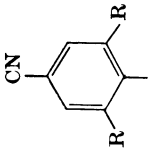
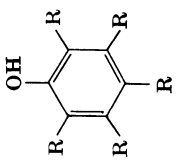
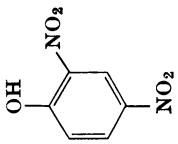
The experimental results for all the compounds together with their formulae are collected in Table 1. With the exception of 4,5,6,7-tetrachloro-1-methylbenzotriazole, all the compounds inhibited oxidative phosphorylation with little or no effect on oxygen uptake. Thus these compounds can be classed as uncouplers of oxidative phosphorylation (Aldridge & Parker, 1960; Racker, 1961). Moreover, the concentration required to inhibit oxidative phosphorylation corresponds closely to the concentration required to stimulate adenosine-triphosphatase activity or mitochondrial respiration by 50%. For most of the compounds there is a factor less than 2 between these concentrations. This effect on the three phenomena has been discussed for nitro- and halogeno-phenols and has been regarded as circumstantial evidence of a common action (Parker, 1958). These compounds therefore mimic the effect of 2,4-dinitrophenol, and in their action with respect to the three experimental procedures under discussion they are qualitatively undistinguishable from that compound.

2,4-Dinitrophenol prevents the swelling of mitochondria by trimethyltin and does so at the concentration that uncouples oxidative phosphorylation. The present series of compounds also prevent this type of swelling at concentrations close to those affecting oxidative phosphorylation. Further

Table 1. *Physical and biological properties of compounds tested for uncoupling activity*

Physical measurements and mitochondria experiments were carried out as described in the Materials and Methods section. ATPase, adenosine triphosphatase.

Name	Structure	p*K*	Hexane–water partition coefficient	Concn. causing 50% stimulation of ATPase (μM)	Concn. causing 50% stimulation of O_2 uptake (μM)	Oxidative phosphorylation: Concn. causing 50% inhibition of P_i uptake (μM)	Oxidative phosphorylation: Inhibition of O_2 uptake at 50% inhibition of P_i uptake	Concn. causing approx. 50% inhibition of swelling due to trimethyltin (μM)	Rigor
2-Amino-1,1,3-tricyano-propene		8·4 in 57% ethanol	—	500	500	800	Slight increase	230	Yes
Carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone	 R=O·CF_3, R′=H	5·8	7·5	0·04	0·04	0·06	−5%	0·03	Yes
Carbonyl cyanide *m*-chlorophenylhydrazone	R=H, R′=Cl	6·0	3·8	0·06	0·06	0·06	−8%	0·06	Yes
Carbonyl cyanide phenylhydrazone	R=R′=H	6·55	1·62	0·3	0·4	0·6	0	0·6	Yes
4,5,6,7-Tetrachloro-benzotriazole	 $R^1=R^2=R^3=Cl$, $R^4=H$	5·0	1·5	0·8	0·8	1·3	0	1·0	Yes
5-Chloro-4-nitro-benzotriazole	$R^1=NO_2$, $R^2=Cl$, $R^3=R^4=H$	6·02	0·05	6·0	5·0	10·0	−8%	80	Yes
5-Nitrobenzotriazole	$R^1=R^3=R^4=H$, $R^2=NO_2$	6·32	0·0025	20	20	5·0	0	3·0	Yes
4,5,6,7-Tetrachloro-1-methylbenzotriazole	$R^1=R^2=R^3=Cl$, $R^4=CH_3$	—	—	None	None	None	—	None	—

4-Hydroxy-3,5-di-iodo-benzonitrile		R=I	4·15	12	1·0	1·0	1·3	0	1·0	Yes
3,5-Dibromo-4-hydroxy-benzonitrile		R=Br	4·05	3·1	3·2	3·2	5·0	0	4·0	Yes
3,5-Dichloro-4-hydroxy-benzonitrile		R=Cl	4·23	0·72	9·0	12	12	0	16	Yes
Pentafluorophenol		R=F	5·2	0·5	16	16	45	−5%	25	Yes
Pentachlorophenol		R=Cl	4·5	140	—	—	9	0	—	Yes
2,4-Dinitrophenol			4·0	3·54	30	30	30	0	30*	Yes

* Concentration completely reversing swelling by trimethyltin (Aldridge & Street, 1964).

evidence of the identity of biological action is provided in the last column of Table 1: administration *in vivo* provokes a response similar to that of 2,4-dinitrophenol.

Within each class of compound the physical data conform with previous contentions that the more acidic and the more lipophilic the uncoupler the lower is the concentration necessary to affect mitochondrial systems (Parker, 1958; Hemker, 1962; Gladtke & Liss, 1958). The dissociation constant for 2-amino-1,1,3-tricyanopropene is that quoted by Eberts, Slomp & Johnson (1961), and is surprisingly high for an uncoupler, but it may be somewhat lower in a purely aqueous medium. Correspondingly, of the compounds listed in Table 1, this substance has the least effect biologically.

The order of the three carbonyl cyanide phenylhydrazones in terms of uncoupling activity is the same as that given by Heytler & Pritchard (1962), but in the present experiments under different conditions the chloro derivative is nearly as active as the trifluoromethoxy compound. These two compounds remain as the most effective uncouplers yet recorded. Both acidity and the hexane–water partition coefficient increase with increased biological activity.

The second group have not before been reported with respect to uncoupling activity. It was the observations of Dr E. F. Edson (personal communication) that 4,5,6,7-tetrachlorobenzotriazole produced immediate rigor mortis at the death of poisoned rats that led to their investigation. The dissociation constants of the three active compounds are similar to those of the carbonyl cyanide phenylhydrazones, but their partition coefficients are much lower. The 1-methyl derivative of 4,5,6,7-tetrachlorobenzotriazole illustrates by its lack of activity the need for a dissociable H^+ ion.

The third set of compounds, the 3,5-dihalogeno-4-hydroxybenzonitriles, have been reported as effective herbicides (Wain, 1963; Carpenter & Heywood, 1963). Wain (1963) also reported that these compounds were strongly active in uncoupling oxidative phosphorylation in plant tissues. With this observation and the fact that they are phenols it was expected that, provided that their dissociation constants were low, they would be uncouplers of oxidative phosphorylation in animal tissue. This was found to be the case. All three compounds have similar dissociation constants, and therefore only the increasing affinity for non-aqueous solvent reflects their increasing efficiency as uncoupling agents. Two of these compounds, the dibromo and di-iodo derivatives, were more effective than 2,4-dinitrophenol.

Finally, pentafluorophenol was compared with pentachlorophenol. Evidence that the latter compound behaves as 2,4-dinitrophenol has been

documented (Parker, 1958; Weinbach, 1954, 1956). In agreement with its greater acidity and higher hexane–water partition coefficient it is more effective than pentafluorophenol as an uncoupling agent.

For comparison, the data for 2,4-dinitrophenol are appended to Table 1. Although this compound has approximately the same dissociation constant and hexane–water partition coefficient as 3,5-dibromo-4-hydroxybenzonitrile it is tenfold less active biologically. This example illustrates a general conclusion that can be drawn from Table 1: namely, that the two physical attributes measured in the present work are insufficient to predict fully the biological activity of a particular compound. Obviously the different chemical natures of the compounds are reflected in their action.

All these compounds have five properties in common with 2,4-dinitrophenol, and for each compound there is a concentration that is equally effective in four cases (rigor mortis is not quantitatively comparable). The current view of oxidative phosphorylation postulates a 'high-energy' intermediate in the chain of events leading to the formation of ATP. It is envisaged that 2,4-dinitrophenol reacts with this intermediate in such a manner that all the phenomena associated with uncoupled oxidative phosphorylation result. On the experimental evidence, it is difficult to escape the conclusion that these compounds produce their effects by a similar mechanism. If this is so, then the differing chemical structures of these compounds present considerable difficulty in interpretation of their action on a molecular basis. On the other hand, alternative explanations of mitochondrial phenomena in terms of ion translocation, as described by Mitchell (1961), also raises difficulties in interpretation because it is unlikely that all lipid-soluble acids are uncoupling agents.

I thank Dr E. F. Edson for supplying the benzotriazole derivatives and for his information about the biological property of one of them. I am also indebted to Dr P. G. Heytler for the carbonyl cyanide phenylhydrazones, to Dr R. G. Jacomb for the 2-amino-1,1,3-tricyanopropene and to Professor R. L. Wain for the 3,5-dihalogeno-4-hydroxybenzonitriles. I am grateful to Mrs S. A. Potter for skilled technical assistance.

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