Biochemical Pharmacology, Vol. 18, pp. 1389–1401. Pergamon Press. 1969. Printed in Great Britain

UNCOUPLING ACTION OF 2,4-DINITROPHENOLS, 2-TRIFLUOROMETHYLBENZIMIDAZOLES AND CERTAIN OTHER PESTICIDE CHEMICALS UPON MITOCHONDRIA FROM DIFFERENT SOURCES AND ITS RELATION TO TOXICITY*

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(*Received* 11 *August* 1968; *accepted* 29 *November* 1968)

Abstract—In order to elucidate the mode of action of representative pesticide chemicals and related substituted 2,4-dinitrophenols, 2-trifluoromethylbenzimidazoles, salicylanilides, carbonyl cyanide phenylhydrazones and certain other compounds, studies were made on their selectivity as uncouplers of respiratory-chain phosphorylation under conditions *in vitro*, their effects *in vivo* on mitochondrial enzymes and the relationship between their uncoupling potency and toxicity, using various insects and mammals. Generally, mitochondria from mouse liver are less sensitive to uncouplers than mitochondria from mouse brain or from insect tissues. Some of the uncouplers are nonselective while others are active at a much lower concentration with a particular mitochondrial source. Partial correlations are evident between the potency of the compounds for uncoupling *in vitro* of mitochondria from housefly thoraces, honey bee heads and thoraces, and mouse brain and liver and the toxicity to these species. Brain mitochondria and, in a few cases, liver mitochondria isolated from mice treated with the above-mentioned substances and with certain inhibitors of the electron transport chain generally are completely uncoupled or inhibited only when the dose used results in severe symptoms of poisoning. Thus, effects on mitochondrial function probably are most important in the mammalian brain from a toxicological standpoint. Five chemicals of high pesticidal activity but of widely varying chemical type did not uncouple or inhibit brain or liver mitochondria in mice with severe symptoms of poisoning and so their mode of action involves other mechanisms.

A NUMBER of pesticide chemicals are uncouplers or inhibitors of oxidative phosphorylation, an action which may be related to their biological activity. The types of such compounds which act or potentially act in this manner include the following: substituted 2,4-dinitrophenols (DN),[1–3] substituted 2-trifluoromethylbenzimidazoles (TFB),[2–8] salicylanilide (SA) derivatives,[3, 9] carbonyl cyanide phenylhydrazones (CCP),[10] carbazoles (C),[11] rotenone,[12] piericidin[13] and organotin compounds.[14] The existing data on these compounds pertain mainly to investigations in which they are added, under conditions *in vitro*, to mitochondrial preparations and which involve enzymes from only a few species and tissues. Only a little information exists in regard

* Supported in part by grants from the United States Public Health Service, National Institutes of Health (Research Grant No. ESGM 00049; Training Fellowship No. 3 FOSTW-1001-02 for one of us), and Fisons Pest Control Ltd., Chesterford Park, Saffron Walden, England.

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to possible correlations between toxicity, uncoupling activity and physical properties.[8] There is a scarcity of data on the selectivity of these chemicals under conditions *in vitro* and on their effects *in vivo* on the enzymes involved. Therefore, the current speculations on their mode of action in living animals are largely extrapolations from findings *in vitro* and definitive information is lacking on the possible relationship between their ability to uncouple or inhibit oxidative phosphorylation in the living animal and their toxic effects.

Earlier difficulties in studies on uncouplers of oxidative phosphorylation have been overcome by the use of an improved isolation medium for mitochondria from the housefly thorax and mouse brain which results in preparations that are very sensitive to these chemicals.[2] With this medium, the mitochondrial enzymes function normally without the addition of bovine serum albumin (BSA) to minimize the effects of endogenous inhibitors released on homogenization or aging of the preparations. This is important because mitochondria are insensitive to uncouplers in the presence of BSA.[2, 15] These improved assay conditions allow a comparative biochemical and toxicological study using mitochondria from various species (houseflies, honey bees and mice) and various tissues or body regions (head, thorax, liver and brain); they also permit consistent assays for respiration and oxidative phosphorylation on mitochondria prepared from the brain and liver of mice poisoned with various compounds.

This study considers the potency of uncouplers under conditions *in vitro*, using mitochondria from both insects and mammals. Also, it concerns the relationship of potency as uncouplers to toxicity and the activity of mitochondria prepared from mice poisoned with respiratory uncouplers, inhibitors and certain other pesticide chemicals.

MATERIALS AND METHODS

Chemicals and test animals. Sources for the chemicals tested as potential uncouplers or inhibitors were those given in Tables 1 and 2. Adenosine diphosphate (ADP, A grade) and BSA (Fraction V, B grade) were obtained from Calbiochem, Los Angeles, Calif. Male albino mice (each weighing 18–20 g) were obtained from the Berkeley Pacific Laboratories, Berkeley, Calif. Housefly (*Musca domestica* L.) adults (each weighing 15–17 mg) from laboratory cultures of the SCR strain were used between 3 and 7 days after emergence.[2] Honey bee (*Apis mellifera* L.) workers (each weighing 120–140 mg) were provided by Professor N. Gary and Dr. J. Marston of the University of California, Davis, Calif.

Oxidative phosphorylation measurements in vitro. After anesthesia of the test animals with carbon dioxide, mouse brain and liver tissues, honey bee heads and thoraces and housefly thoraces were quickly removed, chilled and mitochondria were prepared from them in a medium consisting of 250 mM sucrose, 5 mM ethylenediaminetetraacetic acid (EDTA) and 6 mM each of citrate, α-ketoglutarate and succinate, adjusted to pH 7·4, by the procedure of Ilivicky *et al.*.[2] The mouth parts were cut off from the honey bee heads prior to use in order to increase the brain protein content of the preparation. In the case of the mouse preparations only, the mitochondria were washed once by resuspension and resedimentation from the same medium. Finally, the particles were resuspended in the same medium as used for isolation, an aliquot of about 0·2 ml was added to the standard reaction mixture and respiration at 25° was measured polarographically using the Clark oxygen electrode covered by a Teflon membrane.[16] The standard reaction mixture, having a volume of

TABLE 1. POTENCY OF VARIOUS 2,4-DINITROPHENOLS, 2-TRIFLUOROMETHYLBENZIMIDAZOLES AND CERTAIN OTHER COMPOUNDS AS UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION AND THEIR TOXICITY TO MICE, HOUSEFLIES AND HONEY BEES

Compound*		Minimum uncoupling concn, μ[M], with mitochondrial preparations from					LD_{50}, injected (μmoles/kg)		
		Mouse		Housefly	Honey bee		Mouse	Housefly	Honey bee
Code	Substituent	Liver	Brain	Thorax	Head	Thorax			
		2,4-Dinitrophenols (DN)							
DN-1	Unsubstituted (DNP)	50	1·0	50	30	50	141	1630	108
DN-2	6-Cyclohexyl- (DNOCHP)	1·5	1·0	0·2	0·8	1·0	95	131	3·5
DN-3	6-Methyl- (DNOC)	20	20	20	20	30	94	732	18
DN-4	6-*sec*-Butyl- (DNBP)	1·0	0·5	0·8	0·5	0·8	42	146	11
DN-5	6-*sec*-Butyl-1-isopropyl carbonate (Dessin)	80	80	100	100	200	383		
		2-Trifluoromethylbenzimidazoles (TFB)							
TFB-1	Unsubstituted	>500	>500	150	200	400			
TFB-2	5-Chloro-	20	10	10	10	15	206	295	181
TFB-3	4,5-Dichloro-†	2·0	2·0	10	4·0	5·0	57	126	39
TFB-4	4,6-Dichloro-	4·0	0·8	0·2	2·0	3·0	96	70	20
TFB-5	4,7-Dichloro-	20	10	5·0	3·0	7·0	100	57	20
TFB-6	5,6-Dichloro-†	7·5	1·0	1·0	1·0	1·0	139	94	52
TFB-7	4-Nitro-6-chloro-	3·0	0·7	0·2	0·6	2·0	84	19	8·5
TFB-8	4,5,6-Trichloro-	0·5	0·5	0·6	0·2	1·0	57	46	7·7
TFB-9	4,6,7-Trichloro-	4·0	7·0	5·0	1·0	5·0	64	34	23
TFB-10	4,5,6,7-Tetrachloro-	1·0	0·2	5·0	1·0	3·0	67	52	15
TFB-11	5,6-Dichloro-1-phenoxycarbonyl-	10	5	100	20	70	118		
		Salicylanilides (SA)							
SA-1	3-*tert*-Butyl-2',5-dichloro-4'-nitro-	0·5	0·05	0·05					
SA-2	5-Chloro-3-(4-chlorophenyl)-4'-fluoro-2'-methyl-	1·0	0·8	0·2	0·2	0·4	385		
SA-3	3-(4-Chlorophenyl)-4',5-dichloro-	1·0	0·8	0·4	0·5	0·8	185		
		Carbonyl cyanide phenylhydrazones (CCP)							
CCP-1	3-Chloro-	0·4	0·2	0·5	0·5	0·6	40		
CCP-2	4-Trifluoromethoxy-	0·3	0·08	0·07	0·08	0·2	32		
		Carbazoles (C)							
C-1	1,3,6,8-Tetranitro-†	0·7	0·4	0·4	0·5	0·7			

* Sources: DN-1, Matheson, Coleman & Bell, Ohio, N. J.; DN-2 and DN-3, Chemical Insecticide Corp., Metuchen, N. J.; DN-4 and DN-5, D. L. Heywood, Union Carbide Corp., South Charleston, West Va., purified by H. Matsuo; TFB-1 through TFB-11 and other related compounds, E. F. Edson, Fisons Pest Control Ltd., Nr. Saffron Walden, England; SA-1, SA-2 and SA-3 from P. C. Hamm, Monsanto Chemical Co., St. Louis, Mo.; CCP-1 and CCP-2, P. G. Heytler, E. I. DuPont de Nemours and Co., Wilmington, Del.; C-1 and carbazole, Aldrich Chemical Co., Milwaukee, Wisc.

† Compounds inactive at 100 μ[M] are TFB derivatives with the following substituents: 5,6-dichloro-4-hydroxy-, 4,5-dichloro-6-hydroxy-, and 4,5-dichloro-7-hydroxy- with each of the enzyme sources; 5,6-dichloro-1-(β-D-glucopyranose)-2-trifluoromethylbenzimidazole with each of the honey bee and housefly preparations; carbazole with each of the mouse and housefly preparations.

TABLE 2. UNCOUPLING AND INHIBITION OF BRAIN AND LIVER MITOCHONDRIA AFTER INJECTION OF MICE WITH VARIOUS DINITROPHENOLS, 2-TRIFLUOROMETHYLBENZIMIDAZOLES AND CERTAIN OTHER PESTICIDE CHEMICALS

Compound code or name*	Dose (μmoles/kg)	Holding time (min)	Severity of symptoms†	Magnitude of uncoupling or inhibition‡	
				Brain	Liver
2,4-Dinitrophenol (DN) uncouplers					
DN-1	269	20	2	0	0
DN-2	38	20	0	0	0
	94	20	2	2	1
DN-4	63	20	0	0	0
	100	20	1	1	1
	150	20	2	2	2
DN-5	181	30	1	1	0
	181	60	2	2	0
Dinocap	275	30	1	1	0
	550	30	2	2	2
2-Trifluoromethylbenzimidazole (TFB) uncouplers					
TFB-6	49	20	0	0	0
	98	20	2	2	1
TFB-10	23	20	0	0	0
	46	20	1	1	0
	115	20	2	2	2
TFB-11	125	20	0	0	0
	250	20	1	1	0
Salicylanilide (SA), carbonyl cyanide phenylhydrazone (CCP) and carbazole (C) uncouplers					
SA-2	33	20	0	1	0
	117	20	2	2	1
CCP-2	16	20	0	0	0
	32	20	1	1	0
	80	20	2	2	2
C-1	63	20	0	0	0
	126	20	1	0	0
Inhibitors					
4-*sec*-Butyl-2,6-dinitrophenol	50	20	1	1	0
	100	15	2	2	1
Rotenone	2·5	20	0	0	0
	6·2	20	2	2	2
Piericidin A	6	20	1	0	0
	18	20	2	2	2
Triethyltin chloride	10	20	1	1	0
	30	20	2	2	1
Tetraethyltin	333	30	0	0	0
	1000	30	2	1	0
Other modes of action					
Callicarpone	11	20	2	0	0
Carzol	2000	20	2	0	0
DDT	423	20	2	0	0
Justicidin B	34	20	2	0	0
Morestan	1060	20	1	0	0

* Sources: Compounds indicated by code numbers, see Table 1; dinocap [2,4-dinitro-6-(1-methylheptyl)phenyl crotonate], a fungicide and acaricide, Rohm and Haas, Philadelphia, Pa.; 4-*sec*-butyl-2,6-dinitrophenol, H. Matsuo, Division of Entomology, University of California, Berkeley, Calif.; DDT and rotenone, two insecticide chemicals, Aldrich Chemical Co.; piericidin A (an insecticide produced by the fungus, *Streptomyces mobaraensis*[17]), N. Takahashi, Department of Agricultural Chemistry, The University of Tokyo; triethyltin chloride and tetraethyltin, A. Ross, M & T Chemicals, Inc., Rahway, N. J.; callicarpone (a fish poison isolated from the shrub, *Callicarpa candicans*[18]), K. Kawazu, Department of Agricultural Chemistry, Kyoto University; Carzol [*N,N*-dimethyl-*N'*-(2-methyl-4-chlorophenyl)-formamidine], an acaricide, Morton Chemical Co., Woodstock, Ill.; justicidin B (a synthetic sample[19] identical with one component of the fish poisons in the plant, *Justicia hayatai* var. *decumbens*[20]), K. Munakata, Department of Agricultural Chemistry, Nagoya University; Morestan (6-methyl-2,3-quinoxalinedithiol cyclic carbonate), Chemagro Corp., Kansas City, Mo.

† The severity of the symptoms are graded as follows: 0, not any, same as control; 1, mild, survival of animal is probable; 2, severe manifestation, death of animal is expected.

‡ Effects on mitochondria are graded as follows: 0, not any, same as control; 1, partial uncoupling or inhibition; 2, complete uncoupling or inhibition.

2 ml, contained sucrose (250 mM), EDTA (5 mM), sodium phosphate (20 mM), magnesium chloride (0·5 mM) and pyruvate (4 mM); it was adjusted to pH 7·4 with sodium hydroxide. Additions to the reaction mixture, besides the mitochondria, were made as aqueous solutions, at pH 7·4, with a 20-μl volume for the 480 mμmoles of ADP added and 100-μl volumes for each of BSA (60 mμmoles) and the uncouplers, if any. In the case of the SA and CCP compounds, 5 μl of dimethyl sulfoxide (DMSO) was used instead of 100 μl of aqueous solution for the additions. (DMSO, in itself, does not alter the normal activity of the preparations.) Respiratory control (R.C., the ratio of the rate of oxygen uptake in the presence of ADP to the rate of respiration after complete phosphorylation of the added ADP) and ADP/O ratios were calculated as previously reported.[2] The mitochondrial protein levels were determined by the procedure of Ilivicky *et al.*,[2] the levels ranging from 1·8 to 2·2 mg for each reaction mixture.

The potency of the uncouplers used in the studies *in vitro* was reported in terms of the minimum concentration necessary for complete uncoupling, i.e. the amount necessary to add to the incubation mixture to produce both stimulation of respiration and no further response after addition of ADP. On a routine basis, BSA was added, after the initial uncoupler and ADP additions, to ascertain if this protein resulted in a reversal of uncoupling so that subsequent addition of ADP elicited the normal response of the coupled mitochondrial system. Each experiment was repeated two or more times to establish the consistency of the results obtained.

Uncoupling in vivo *and inhibition experiments.* The test compound in 50 μl of DMSO solution was administered intraperitoneally to each of two mice; after appropriate holding times, usually 20 min, the nature and intensity of the symptoms were recorded, the animals were killed with carbon dioxide and the liver and brain tissues were removed. Mitochondrial preparations were made and tested as described in the previous section. The preparation was considered to be normal if the respiratory rate, ADP/O and R.C. corresponded to those of untreated mice, and if subsequent addition *in vitro* of the same compound, if an uncoupler, at its minimum uncoupling level elicited the same response as with preparations from normal mice. The injection vehicle, DMSO, for the test compounds had no effect whatsoever on the normal activity of the mitochondria and it gave no symptoms of poisoning. Results are reported in terms of partial inhibition or partial uncoupling as well as complete effects of these types on the preparation. Preparations from treated mice were considered to be uncoupled, as a result of injection of the test material, if the respiration rate was unusually rapid and R.C. was absent; in each case, an additional criterion was that BSA restored the ADP/O and R.C. to values corresponding to those of untreated mice. Partial uncoupling was taken as less than half of the normal values for ADP/O and R.C. Inhibition was considered to be the action resulting from injection of the test compound when the respiration rate, initially, became very low and respiration stopped completely within a period of 2–4 min. Partial inhibition was taken as a reduced initial respiration rate which continued for at least 8 min before no further respiration was evident.

Toxicity experiments. The test compounds were administered by i.p. injection, in 15 μl of DMSO solution, to the mice and by intrathoracic injections, in 1·0 μl of 5% sodium bicarbonate solution, to the houseflies and honey bees. The bicarbonate solution was used in the insect studies to convert the acidic uncouplers to their more

soluble sodium salts. In some cases, definitive toxicity data were not obtained because the compounds involved were not sufficiently soluble in the medium used; these cases resulted in the missing values in the tabulation of results. At least forty houseflies, forty honey bees and six mice were used for each test dose. LD_{50} values, on a μmole per kg basis, were determined from observations made 24 hr after injection of the test compounds by visual inspection of the log dosage-probit mortality curves.

RESULTS

General characteristics of the preparations. The ADP/O ratio of each normal mitochondrial preparation is consistently 3·0 and the average R.C. values are as follows: 4·6, 3·8, 4·4, 4·0 and 3·8, for mouse liver and brain, housefly thoraces and honey bee thoraces and heads respectively. With each mitochondrial source, tests with oligomycin and atractylate, by the general procedure described by Ilivicky *et al.*,[2] established that the mechanism of phosphorylation concomitant with the oxidation of

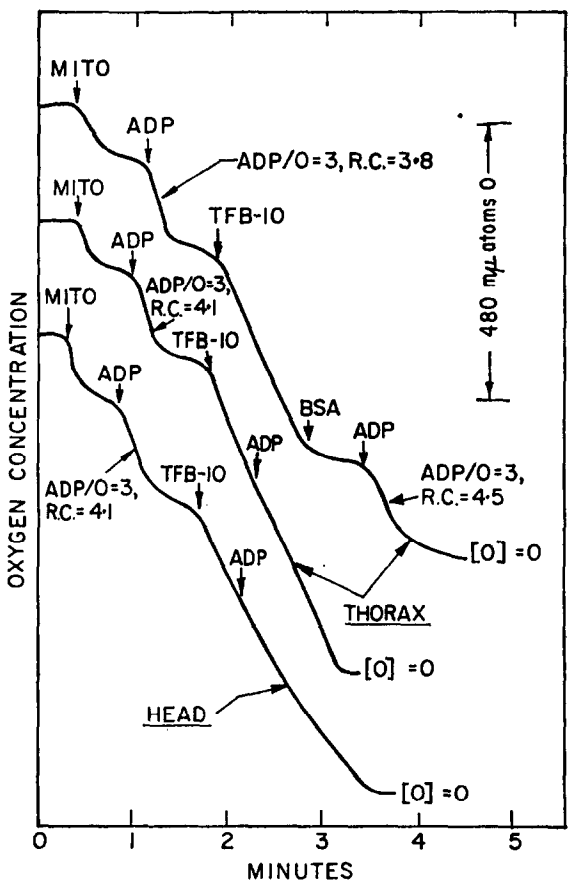

FIG. 1. Respiratory control during pyruvate oxidation by mitochondria prepared from the thoraces and heads of honey bees, as affected by TFB-10 with or without subsequent addition of BSA, in typical experiments. Additions to the 2-ml reaction volume were as follows: ADP = 480 mμmoles; BSA = 60 mμmoles; TFB-10 = 2 mμmoles for head mitochondria and 6 mμmoles for thorax mitochondria. Ratios for ADP/O and R.C. are indicated after each addition of ADP.

substrate has properties appropriate for electron transport phosphorylation. In each of the preparations, ADP- or uncoupler-stimulated respiration is inhibited by oligomycin; on the other hand, atractylate completely abolishes the stimulation of respiration induced by ADP but does not inhibit the release of respiration induced by uncouplers, when ADP is absent. The response to uncouplers is illustrated in Fig. 1 with the honey bee head and thorax preparations, and in a previous report[2] with mitochondria from housefly thoraces. BSA, at 60 mμmoles, produces a rapid and complete reversal of the uncoupling, as illustrated in Fig. 1 and in the earlier report;[2] this effect of BSA is common to each of the uncouplers in each preparation studied.

Selectivity of uncouplers with mitochondria from different tissues and organisms. Some specificity is evident in the action of various uncouplers on the mitochondrial preparations studied (Table 1). Although not of a great magnitude, it is of interest that sensitivity differences exist with species and tissues and that the substituents on the uncoupler molecule govern not only the potency but the selectivity.

In general, the mouse-brain mitochondria are the most sensitive to uncoupling and the mouse-liver mitochondria are the least, the insect preparations being intermediate in sensitivity. Without exception, the brain mitochondria are uncoupled at the same or at up to a 50-fold lower level of compound than the liver mitochondria. With the 4,6-disubstituted-2-trifluoromethylbenzimidazoles (TFB-4 and TFB-7), the fly thorax shows a sensitivity ten times that for the mouse-liver preparation. With the tetrachloro-2-trifluoromethylbenzimidazole (TFB-10), the fly-thorax preparation is unusually resistant and the mouse brain is, by far, the most sensitive. Little selectivity is evident between honey bee head and thorax preparations. Compounds which generally provide complete uncoupling, with all preparations, at levels of 1 μ[M] or less, are as follows: the 2,4-dinitrophenols, DN-2 and DN-4; the 2-trifluoromethylbenzimidazole, TFB-8; and each of the salicylanilides, carbonyl cyanide phenylhydrazones and tetranitrocarbazole. Certain of the compounds that are metabolites of TFB-3 and TFB-6* are not active as uncouplers (see footnote to Table 1).

Dessin (DN-5) and TFB-11 are esters of potent uncouplers (DN-4 and TFB-6 respectively). The difference in potency of the phenol or benzimidazole and their corresponding esters may be related to the ease of ester cleavage, by the particular mitochondrial preparation involved; if this is so, then TFB-11 is readily hydrolyzed by mouse-liver mitochondria and less readily by mouse-brain or insect mitochondria. On the same basis, Dessin is not readily hydrolyzed to DNBP by any of the preparations.

Relation of potency as uncouplers to toxicity. Two approaches are taken in considering the relation between potency as uncouplers and toxicity: first, comparison of the levels for uncoupling *in vitro* to the toxic levels, as LD_{50} values, for the test compounds and the various species used (Table 1, Fig. 2); second, evaluation of the degree of uncoupling *in vivo* of mouse-brain and -liver mitochondria in intoxicated animals in relation to the symptoms (Table 2, Fig. 3).

Plotting the minimum uncoupling concentrations, with a series of compounds, for a particular mitochondrial preparation against the LD_{50} values for the species involved, shows that toxicity tends to increase with increasing uncoupler potency (Table 1, Fig. 2). However, in any case, the correlation is not a particularly good one, indicating that factors other than uncoupler potency, as evaluated under conditions *in vitro*, are

* D. M. Bower, S. K. Bandal and J. E. Casida, unpublished results.

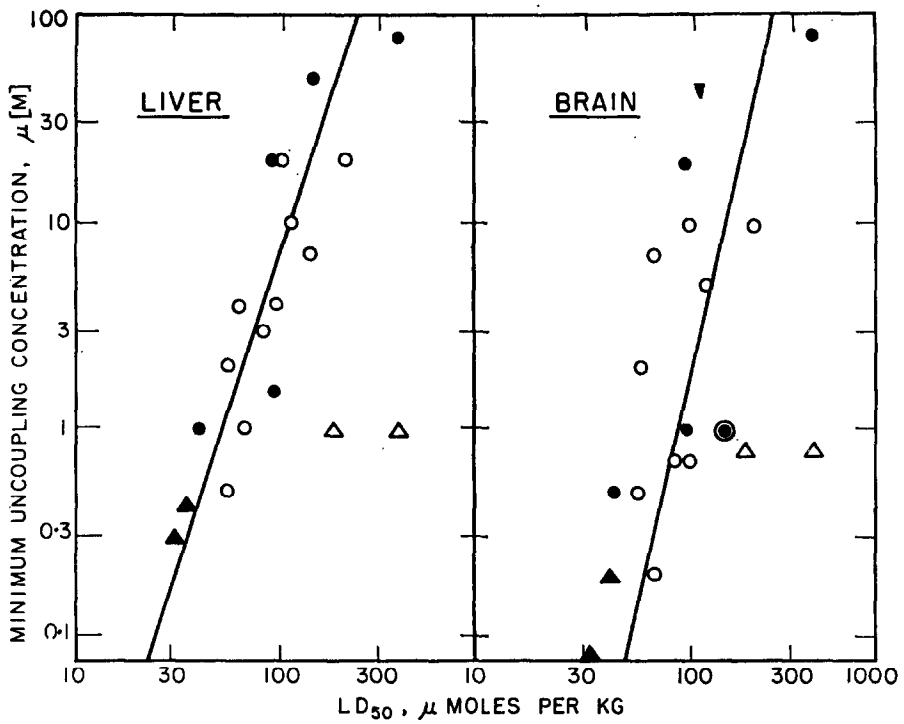

FIG. 2. Relationship with various uncouplers of oxidative phosphorylation of the minimum uncoupling concentration for liver and brain mitochondrial preparations from mice to their toxicity on intraperitoneal administration. Data from Table 1: open circles = TFB; closed circles = DN; open triangles = SA; closed triangles = CCP.

involved in the poisoning. The correlation of toxicity to mice with uncoupler poteney is somewhat better with liver mitochondria than with brain mitochondria; the salicylanilides deviate most from the anticipated correlation (Table 1, Fig. 2). The uncoupler potency vs. toxicity relationships with houseflies and honey bees (Table 1) are not presented as figures because the available data are limited. The greatest deviations in the correlation with honey bees are with DN-3 and TFB-6. Houseflies yield the poorest correlation among the species and preparations used.

The studies with living mice include representatives of the different types of uncouplers and involve doses and times selected, on the basis of preliminary tests, to give varying degrees of symptoms. The dosages administered result in severe symptoms of acute poisoning and/or death at the high dose, and mild or no symptoms, at one-half or one-third of the high dose. During the holding period, usually 20 min, the typical symptoms recorded by Burton *et al.*[4] are evident, including dyspnoea, weakness, occasionally salivation and death (in an extended position) with immediate *rigor mortis*. With one exception (DN-1), there is complete agreement between the uncoupling state of the brain mitochondria and the symptomatology of the animal when the poisoning manifestation is severe, but this is not true in the case of the liver mitochondria (Table 2). The nature of the results obtained for respiratory control during pyruvate oxidation is shown in Fig. 3, using TFB-10 as an example: entirely the same results are obtained with each of the uncouplers when the magnitude of

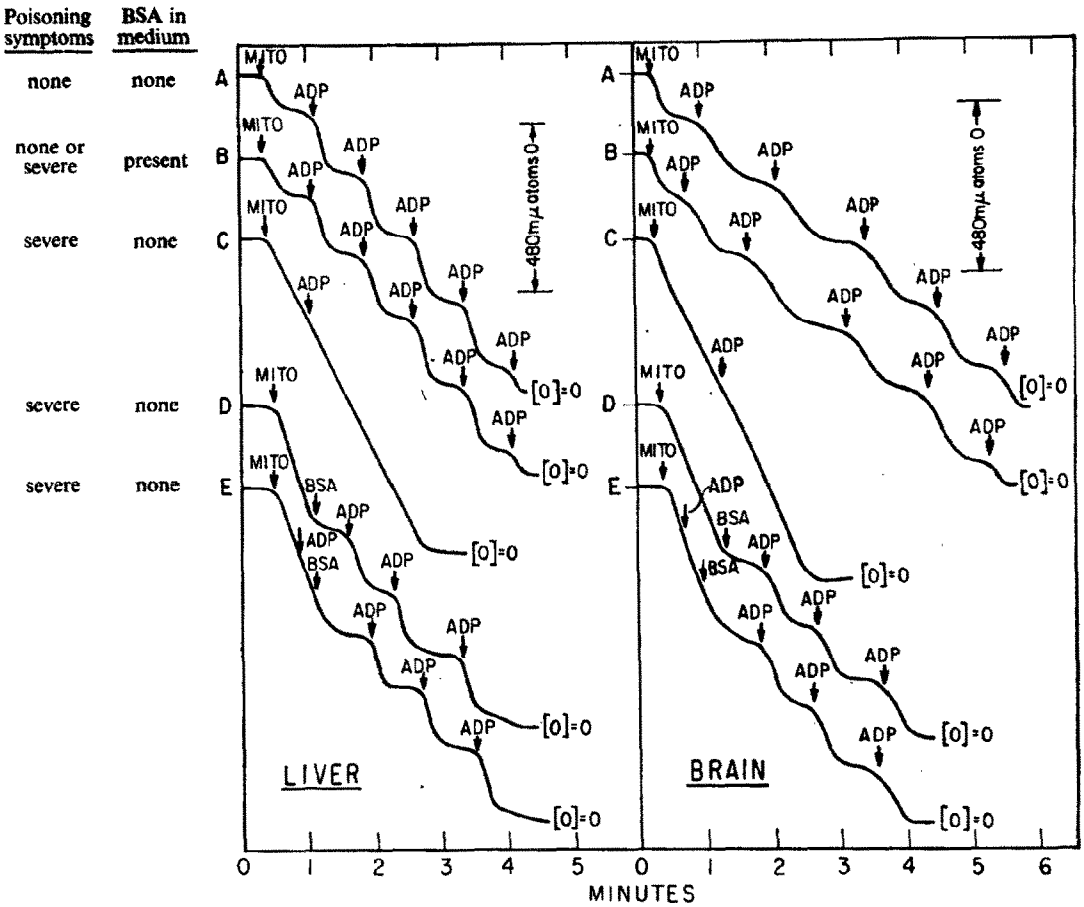

FIG. 3. Respiratory control during pyruvate oxidation by mitochondria prepared from the liver and brain of mice receiving injections of TFB-10 at 23 μmoles per kg (not any symptoms) or 115 μmoles per kg (severe poisoning symptoms), in typical experiments. Additions to the 2-ml reaction volume were as follows: ADP = 480 mμmoles; BSA = 60 mμmoles, added either before or after the mitochondria.

uncoupling noted in Table 2 is considered. With the exception of compound DN-1, which gives severe poisoning symptoms without detectable uncoupling, the brain mitochondria of mice are uncoupled at the higher dosage levels and sometimes the liver mitochondria are uncoupled as well (Fig. 3C). On the other hand, lower i.p. doses that produce only mild symptoms generally give partial uncoupling and those that give no symptoms at all do not affect the mitochondria because the mitochondria respond normally to ADP additions (Fig. 3A) and are also similar to normal mitochondria in sensitivity to uncouplers added *in vitro*. Uncoupled mitochondria isolated from poisoned animals fully recover their R.C. after addition of BSA to the incubation medium before or after the mitochondria and before or after the ADP (Fig. 3B, 3D and 3E); the presence of BSA in the incubation medium does not alter the normal mitochondrial activity (Fig. 3B compared with 3A), in confirmation of an

earlier report.[15] It is interesting to note that tetranitrocarbazole (C-1), although a very potent uncoupler *in vitro*, is not toxic to mice and does not give detectable uncoupling *in vivo* at the highest dosage which could be administered with this relatively insoluble compound; it is not known whether this is a result of penetration difficulties, a different pattern of distribution, or rapid metabolism of the carbazole.

Activity of brain and liver mitochondria prepared from mice intoxicated with respiratory inhibitors and other poisons. Certain compounds which are known to be, or are suspected to be, inhibitors of mitochondrial respiration were administered to mice to determine the correlation between dose, symptoms and respiratory activity and/or uncoupling of oxidative phosphorylation. As expected, brain mitochondria isolated from mice receiving doses of rotenone, piericidin A, and tri- and tetraethyltin, under conditions that yield severe toxic symptoms, are partially or completely inhibited towards the oxidation of pyruvate; this situation is not always found with liver mitochondria. An isomer of DN-4, 4-*sec*-butyl-2,6-dinitrophenol, acts as an inhibitor and not as an uncoupler; studies *in vitro* with liver mitochondria confirm this type of action for the 2,6-dinitro compound because not any concentration was found that would stimulate respiration but inhibition occurred at 0·1 μ[M]. At the same time, the brain and liver mitochondria, from animals poisoned with each of the aforementioned compounds, behave normally when tested for their phosphorylating capacities; so, neither their ADP/O ratios nor their R.C. indices differ from those of untreated mice. When BSA is added to mitochondria prepared from animals poisoned with these inhibitors, it is not effective in restoring the normal respiration rates, even when added at two to three times the concentration adequate to completely reverse the altered response of mitochondria prepared from animals poisoned with uncouplers. Concomitant with this difference in effect upon mitochondrial electron transport, the symptomatology of the mice treated with the various inhibitors differs greatly from that noted above for the uncouplers and varies somewhat with the various inhibitors studied; the symptoms include weakness and sometimes convulsions, but in no case is death followed by immediate *rigor mortis*.

Other compounds that do not affect the liver or brain mitochondria at doses resulting in severe symptoms of poisoning or death are as follows: callicarpone, Carzol, DDT, justicidin B, and Morestan (Table 2). Justicidin from the natural source, a mixture of A and B, gives the same effect at the same dose as noted in Table 2 for synthetic justicidin B.

DISCUSSION

The present results strongly support the hypothesis that uncoupling of oxidative phosphorylation may be the action of certain pesticide chemicals in the living animal and, thus, the cause of their toxicity. Although the uncouplers studied belong to different chemical series, all of the poisoned mice show similar and characteristic symptoms; these symptoms differ from those of animals poisoned with inhibitors of the electron transport system, such as rotenone, piericidin A, and organotins and from those produced by the other poisons examined which involve mechanisms of action different from those considered here.

There is some degree of correlation between the potency of the various compounds for uncoupling *in vitro* and their toxicity; however, this correlation is not a good one, possibly as a result of differences in elimination and detoxication processes that occur

in the intact cell and with the intact organism and/or differences in ease of approach to the site involved in the uncoupling when the mitochondria are in their normal environment in the intact cell. Unpublished studies in this laboratory indicate that some dinitrophenols (DN-1 and DN-4) and benzimidazoles (TFB-3 and TFB-6) are quickly and almost completely degraded by metabolism in houseflies and mice and that certain of these benzimidazole metabolites do not show uncoupling activity.* It is possible that the correlation between uncoupler potency *in vitro* and toxicity *in vivo* might be improved if the toxicity studies were made with organisms that metabolize the uncouplers less rapidly or, alternatively, if appropriate inhibitors of the uncoupler detoxification (i.e. synergists) were also administered.

Addition of BSA to an uncoupled preparation completely reverses the uncoupling, confirming the results of Weinbach and Garbus[15] and Ilivicky *et al.*[2] This appears to result from the ability of BSA to bind the uncoupling agents[15] and to remove them from both the uncoupled mitochondria and from an available form in the medium. The reversal effect of BSA is found not only in insect and liver preparations but it also occurs in brain preparations with each of the compounds studied. However, Menken *et al.*[21] found that uncoupling of brain mitochondria with bilirubin, which affects the structure of the mitochondria, does not involve a reaction which is reversible with BSA. The removal by BSA of the uncoupling agents seems to be nonspecific because dilution or washing of the mitochondria achieves the same effect of fully restoring the ADP/O and R.C. indices. Rotenone, an inhibitor instead of an uncoupler, is partially removed from its specific binding site in submitochondrial particles from beef heart by washing with BSA,[12] but this release is not evident on addition of BSA to inhibited mitochondria from rotenone-poisoned mice, possibly because the BSA does not come into contact with the rotenone at the specific site in these intact liver and brain mitochondria. Similar studies with liver and brain mitochondria from mice poisoned with piericidin A and the organotins show that BSA addition does not alleviate the magnitude of inhibition.

The action of the uncouplers used in the present study seems to be the same in each case, even with varying chemical types and varying tissue and species sources for the mitochondria; this confirms the finding of Wilson and Merz.[22] Uncoupling potency and adenosine triphosphatase induction, for compounds in the halogen- and mixed-halogen alkyl-substituted 2-trifluoromethylbenzimidazole series, are related by Jones and Watson[8] to the p*K*a of the compounds involved, with a high degree of correlation. This correlation is obviously not applicable to certain of the situations in the present study where considerable selectivity with mitochondrial source is evident. Many of the compounds studied, such as DN-3, DN-4, TFB-2 and C-1, offer little, if any, selective action; however, with others, such as DN-1, TFB-4, TFB-7 and TFB-10, a considerable degree of specificity is evident. Although there are some differences between insect preparations, the differences are more dramatic between insect and mouse preparations, and particularly between mouse-brain and mouse-liver mitochondria. Brain mitochondria are more sensitive than liver mitochondria towards uncoupling agents, as shown with sixteen of the twenty-one compounds active in the studies *in vitro* and with eight of the nine compounds active in the studies *in vivo*; with almost all other compounds, the 2 enzyme sources are of equal sensitivity. This sensitivity difference may be related to the greater lipid content of the brain mitochondria

* D. M. Bower, S. K. Bandal and J. E. Casida, unpublished results.

and, therefore, to an increased facility of the compounds to combine at the active site of enzymes in these mitochondria or, alternatively, to a higher capacity of the liver cells to degrade the uncouplers into inactive metabolites or bind them nonspecifically at sites other than the active coupling sites. The use of labeled uncouplers to determine the distribution pattern and metabolism of the respective compounds may contribute to the understanding of this phenomenon. The same factors may also contribute to the selectivity differences between mitochondria from other sources.

It is important to consider whether or not the uncoupling noted in the assays on mitochondria prepared from poisoned mice is indicative of, or related to, actual uncoupling *in vivo* or whether the uncoupling noted results from uncoupler released and recombining during mitochondrial preparation and assay. (As previously discussed, the release and recombination are readily achieved.) Uncoupling occurs with compounds and at doses which produce the same symptoms which, if extreme, result in death with immediate *rigor mortis*. In these cases, and in correlation with the symptoms, the brain mitochondria are always uncoupled, with the exception of animals poisoned with DN-1, although the liver mitochondria may or may not be uncoupled. These findings establish that the assays are actually indicative of the degree of uncoupling *in vivo* or, at least, of the existence of a critical level of compound in the tissue or in the mitochondria for uncoupling and for producing a biochemical lesion leading to poisoning. Poisoning alone is not enough because inhibitors of electron transport and other toxicants, even at lethal dosage levels, give no evident uncoupling of the preparations from poisoned animals. The actual uncoupler involved in the studies *in vivo* probably is not a normal cell constituent, such as a higher fatty acid, released in response to the uncoupler. This is so because such a product should bind in a manner independent of the uncoupler administered and this definitely is not the case with DN-1 which appears to dissociate during enzyme preparation and assay.

Assays *in vitro* with uncouplers added directly to the mitochondrial preparations show that the brain enzymes generally are more sensitive than the liver enzymes. The studies *in vivo* confirm the tendency towards greater sensitivity of the brain enzymes to uncouplers, and extend the relationship to include inhibitors of the respiratory chain. This suggests that, to be most meaningful, toxicological studies on uncouplers and inhibitors should consider brain mitochondria.

In certain of the results presented, the uncoupler or inhibitor involved is probably not the compound administered but, rather, it is a metabolite. For example, Dessin, dinocap, and TFB-11, important acaricides and/or fungicides, may be precursors of the more potent uncouplers. Also the inhibitor involved after tetraethyltin administration probably is triethyltin, formed by oxidative deethylation.[23] By appropriate use of studies *in vivo* in combination with studies *in vitro*, it is possible to determine whether the original compound or a metabolite is involved in the uncoupling or inhibition.

Acknowledgements—We wish to thank Mrs. Loretta Gaughan for skilled technical assistance and Dr. L. Lykken for helpful suggestions.

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