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EFFECTS OF ENDOSULFAN AND ITS METABOLITES ON RAT LIVER MITOCHONDRIAL RESPIRATION AND ENZYME ACTIVITIES *IN VITRO*

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Abstract—Endosulfan (E) is an organochloric insecticide, which is quickly metabolized and eliminated from the body system. Toxic effects of E and its metabolites have been reported. The influence of E and its metabolites, viz. endosulfan sulfate (ES), endosulfan diol (ED) and endosulfan lactone (EL), has been examined on rat liver mitochondria *in vitro*. Endosulfan stimulated state-4 respiration at lower concentrations and inhibited it at higher ones, whereas state-3 respiration was inhibited at all the concentrations used, i.e. 5–100 μg/ml. A maximal 25-fold activation of latent Mg^{2+}-ATPase was achieved at a concentration that caused maximal stimulation of state-4 respiration. Activities of the respiratory chain-linked enzymes were inhibited at levels which corresponded to the concentrations of endosulfan used *in vitro*. Both the respiratory control ratio (RCR) and the ADP:O ratio fell sharply at endosulfan concentrations above 10 μg/ml. ES and ED exerted similar effects on mitochondrial oxidation of β-hydroxybutyrate, but at more than double the concentration of the parent compound, while EL proved least effective. The effects of the latter compound on mitochondrial enzyme activities were negligible. Our results suggest that endosulfan possesses dual properties, that of an uncoupler of oxidative phosphorylation and of an inhibitor of the electron transport chain, and that the *in vivo* cytotoxic/insecticidal effects of endosulfan and its metabolites might, therefore, be the consequence of impaired mitochondrial bioenergetics.

Endosulfan is an organochloric insecticide, and its metabolism has been studied thoroughly [1–3]. Its metabolite, endosulfan sulfate, is known to be more toxic than endosulfan itself [1]. Other metabolites, viz. endosulfan diol, endosulfan hydroxyether, endosulfan ether and endosulfan lactone, were found to be potentially less toxic [1]. Unlike other organochloric insecticides, endosulfan is not persistent *in vivo* and is found to be eliminated quickly from the body through fecal and urinary excretion. However, experimental studies *in vivo* indicated some toxic effects in various organs irrespective of its short stay in the body [4]. In insects, treatment with endosulfan resulted in increased respiration and body temperature [5]. The mechanism of such changes is not clearly understood. It is anticipated that endosulfan and its lipophilic metabolites might interact with membrane lipids of subcellular organelles like mitochondria and thereby manifest their toxicity through disruption of mitochondrial functions. In this preliminary study, we, therefore, report on the effects of endosulfan and its metabolites, -sulfate, -diol and -lactone, on mitochondrial activity *in vitro*.

MATERALS AND METHODS

Materials. Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) and its three metabolites were used. Endosulfan (E) was a gift from Hoechst, India. The metabolites of endosulfan namely, endosulfan lactone (EL), endosulfan diol (ED) and endosulfan sulfate (ES), were provided by Dr. I. Schuphan the Institute fur Pflanzenschutzmittelforschung Biologische Bundensanstalt, Berlin, F.R.G.

The chemicals obtained in the present study were from the indicated sources: DL-β-hydroxybutyric acid (β-OHB), succinate (SUC), rotenone (ROT), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), *N*-2-hydroxyethyl piperazine-*N*-2-ethane-sulfonic acid, sodium salt (Hepes), ethylenediamine tetraacetic acid (EDTA) and bovine serum albumin (BSA) from Sigma; cytochrome *c*, reduced nicotinamide adenine dinucleotide (NADH), adenosine diphosphate (ADP) and adenosine triphosphate (ATP) from the CSIR Center for Biochemicals, V.P. Chest Institute, Delhi; and 2,6-dichlorophenolindophenol (DCIP) from Loba Chemie, Bombay. All other reagents used were of analytical grade.

Preparation of mitochondria. Adult male albino rats weighing 150–180 g were used for the isolation of liver mitochondria according to Schnaitman and Greenawalt [6] with the following modifications.

The isolation medium contained 220 mM D-mannitol, 70 mM sucrose, 2 mM Hepes buffer (pH 7.4); 0.05% BSA; and 1 mM EDTA. Ten percent whole homogenate of the liver in isolation medium was centrifuged at 500 *g* for 15 min, and the resulting supernatant fraction at 7000 *g* for 15 min. The pellet was suspended in isolation medium and recentrifuged at 7000 *g* for 15 min. The pellet obtained was further taken into 20 vol. of BSA-free isolation medium and centrifuged at 8000 *g* for 15 min, and the mitochondrial pellet was finally suspended in the same medium to obtain 30–40 mg protein/ml.

The ADP:O ratio obtained with these mito-

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chondrial preparations was close to the theoretical value, suggesting their "tightly coupled" nature. Mitochondria exhibited transitions in respiratory states with substrates and ADP; they were deenergized with the classical uncoupler CCCP, releasing the respiratory controls. The mitochondrial protein was determined according to the biuret method [7], using bovine serum albumin as standard.

Measurement of respiration. Mitochondrial respiration employing succinate and β-hydroxybutyrate as substrates was measured polarographically using a Clark type oxygen electrode (YSI model) at 30° in 3 ml of air-saturated incubation medium as described by Estabrook [8]. The incubation medium contained 220 mM mannitol, 70 mM sucrose, 10 mM Hepes buffer, 2.5 mM KH_2PO_4, 5 mM $MgCl_2$, 0.05% BSA, and 20 mM KCl. The test compounds were delivered in 10 μl of alcohol. Respiratory control ratio (RCR) and ADP:O ratio were also measured according to Estabrook [8].

Assay of mitochondrial enzymes. The following enzyme activities were measured during the initial linear phase of the reaction with respect to time and protein: succinic dehydrogenase (SDH, EC 1.3.99.1) according to Slater and Bonner [9]; Mg^{2+}-adenosine triphosphate (Mg^{2+}-ATPase, EC 3.6.1.3) by the method of Ohyama *et al.* [10] and inorganic phosphorous liberated by the method of Taussky and Shorr [11]; cytochrome *c* oxidase (EC 1.9.3.1) according to Smith [12]; succinate–cytochrome *c* reductase (EC 1.3.99.1) using DCIP as an electron acceptor as described by King [13]; NADH-dehydrogenase (EC 1.6.2.1) using DCIP as an electron acceptor as described by Mahler [14] and monoamine oxidase (MAO, EC 1.4.3.4) by McEwen [15].

RESULTS

Effect of endosulfan on β-hydroxybutyrate and succinate-mediated mitochondrial respiration. The effect of various endosulfan concentrations on substrate oxidation was examined in the presence and absence of ADP. The rate of oxidation obtained with substrates and prior to the supplementation of ADP was denoted as state-4 and after ADP addition as state-3.

The polarographic tracings of succinate oxidation on treatment with endosulfan at different concentrations are given in Fig. 1. Endosulfan, up to 50 μg/ml, stimulated state-4 respiration with a maximal effect of 50% at 33 μg endosulfan. At 100 μg, state-4 respiration was reduced to the endogeneous level, i.e. without the addition of substrate. State-3 respiration, however, showed a dose-dependent inhibition. The rate of oxidation during state-3 respiration was the same as obtained in state-4 upon addition of endosulfan at concentrations above 25 μg/ml of incubation medium (Fig. 2a). At 5–10 μg endosulfan, the RCR ratio showed a signficant decrease of 25–35% and ADP:O of 10–25%, while at higher concentrations these criteria of coupled mitochondrial respiration were completely inhibited. Similar changes in bioenergetic indices were observed during β-OHB oxidation (Fig. 2b, Table 1).

Endosulfan above 50 ppm inhibited fully the CCCP-stimulated oxidation of either β-OHB or succinate. Similarly, the presence of endosulfan inhibited the rotenone-insensitive oxidation of succinate (Table 2), suggesting that the flow of reducing equivalents through the respiratory chain is impaired drastically by endosulfan.

Effect of endosulfan metabolites on mitochondrial respiration. The oxidation of β-hydroxybutyrate was studied with endosulfan metabolites in comparison with the parent compound. Three concentrations of the metabolites, viz. 10, 25 and 50 μg/ml, were used except with EL where a dose of 100 μg was also included. The data summarized in Table 1 indicate that metabolites of endosulfan share the same properties of inhibiting bioenergetics as that of the parent compound.

State-4 respiration was not affected by the two

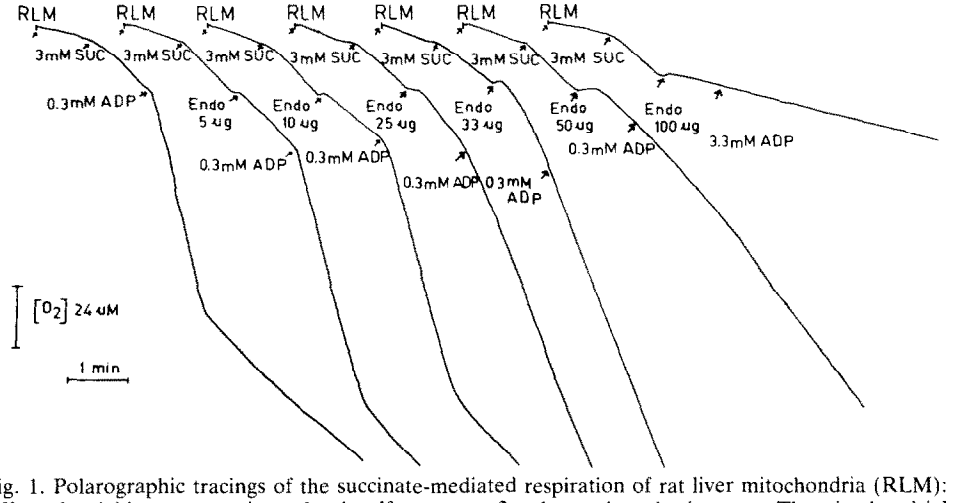

Fig. 1. Polarographic tracings of the succinate-mediated respiration of rat liver mitochondria (RLM): Effect of variable concentrations of endosulfan on state-3 and state-4 respiration rates. The mitochondrial respiration was measured with 3 mg of mitochondrial protein in 3 ml of air-saturated incubation medium at 30°. Endosulfan in 10 μl of ethanol was introduced into the incubation vessel to obtain microgram concentrations per milliliter of the medium as indicated in each tracing. Other conditions were the same as described under Materials and Methods.

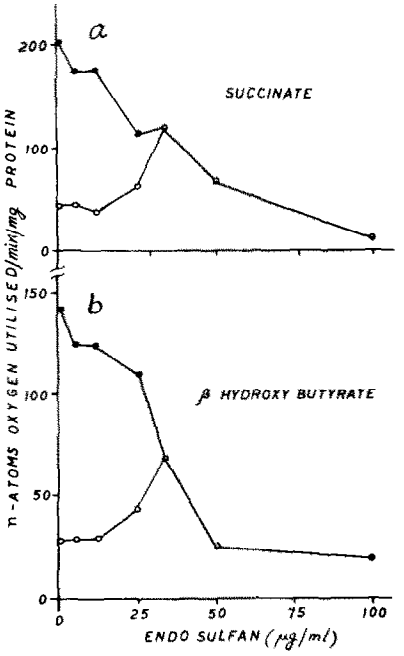

Fig. 2. Relationship between endosulfan concentrations and mitochondrial respiration. The values were plotted from the data, representative of at least two separate experiments, of tracings obtained with β-OHB and SUC as substrates. Procedures for the determination of state-3 (●—●) and state-4 (○—○) respiration were according to Estabrook [8]. Key: (a) succinate oxidation; and (b) β-hydroxybutyrate oxidation. Other conditions were the same as described under Materials and Methods. The coefficient of variation was less than 5%.

Table 1. Effects of endosulfan metabolites on the rate of β-hydroxybutyrate respiration in rat liver mitochondria*

Treatment (μg/ml)	Respiration rate† State-4	State-3	Respiratory ratios RCR	ADP:O
Control	26	132	5.0	2.8
Endosulfan				
5	26	124	3.70	2.5
10	26	114	3.25	2.0
25	40	102		
33	61	61		
50	22	22		
100	17	17		
Endosulfan sulfate				
10	26	119	4.0	2.6
25	26	120	3.0	2.3
50	43	43		
Endosulfan diol				
10	26	109	3.2	2.1
25	30	109	2.9	1.9
50	40	59		
Endosulfan lactone				
10	26	119	3.6	2.2
25	26	125	3.6	2.0
50	25	118	3.4	2.1
100	26	109	3.4	2.0

* The compounds at different concentrations were taken up in 10 μl ethanol and introduced into a 3-ml assay system containing 3 mg mitochondrial protein. The control received 10 μl ethanol. Other conditions are described in Materials and Methods. The data are from polarographic tracings representative of at least two separate experiments. The coefficient of variation was less than 5%.

† Expressed as natoms oxygen used·min^{-1}·(mg protein)$^{-1}$.

lower concentrations of ES and ED but was increased at 50 μg/ml. EL produced no effect on state-4 oxidation even at 100 ppm. The changes in mitochondrial respiration observed at 25 μg endosulfan were achieved by its metabolites, ES and ED, at about double this concentration.

Effect of endosulfan on mitochondrial enzyme activities in vitro. The data are recorded in Table 3. Treatment with endosulfan in general inhibited the flow of electron transport through the respiratory chain, as evidenced by the dose-related inhibition of respiratory chain linked enzymes *in vitro*. SDH and MAO exhibited a similar inhibition pattern. The concentration of endosulfan which caused 50% inhibition of the respiratory chain enzymes and SDH was found to be 50 μg which remained generally unaltered at 100 μg. In general, MAO, SDH and enzymes of the respiratory chain elicited a comparable dose-related inhibition, i.e. 10–50% inhibition with 5–50 μg endosulfan. However, terminal oxidase was more sensitive to endosulfan treatment because at 100 ppm of endosulfan only one-tenth of cytochrome *c* oxidase appeared operating, while other electron transport chain enzymes retained 50% of their efficiency.

Table 2. Effect of endosulfan on β-hydroxybutyrate or succinate-mediated mitochondrial respiration in the presence of CCCP or rotenone*

Experimental regimen	Oxygen used [natoms·min^{-1}·(mg protein)$^{-1}$]
β-OHB + CCCP	145
β-OHB + CCCP + ROT	2
β-OHB + endosulfan (33 μg) + CCCP	67
β-OHB + endosulfan (100 μg) + CCCP	12
β-OHB + CCCP + ROT + SUC	202
β-OHB + endosulfan (100 μg) + CCCP + ROT + SUC	11

* Data are from polarographic tracings representative of at least two separate experiments, conducted in duplicate. The coefficient of variation was less than 10%. Endosulfan was introduced in 10 μl alcohol; controls received the vehicle. Respiration rate presented is from the last addition shown in the experimental regimen. Other conditions are similar to those described in Materials and Methods. Concentrations of the compounds: 5 mM β-OHB; 3 mM SUC; 16 μM CCCP; and 10 μM ROT.

Table 3. Effect of endosulfan on mitochondrial enzyme activities *in vitro**

Endosulfan (μg/ml)	Succinate dehydrogenase†	Succinate–cytochrome *c* reductase‡	NADH-dehydrogenase‡	Cytochrome *c* oxidase§	Mg^{2+}-ATPase	Monoamine oxidase¶
0	171 ± 6.9	13.7 ± 2.1	27.8 ± 2.0	20.2 ± 2.1	7.7 ± 0.6	17.9 ± 2.5
5	145 ± 11.1**	11.5 ± 1.9	24.8 ± 2.7	19.7 ± 2.7	51.2 ± 7.0††	14.5 ± 4.5‡‡
10	132 ± 10.2‡‡	10.0 ± 1.8‡‡	22.0 ± 2.2**	18.5 ± 2.1‡‡	132.5 ± 10.2††	14.0 ± 3.0‡‡
25	112 ± 8.0††	9.0 ± 2.1**	18.5 ± 2.5††	13.6 ± 2.0††	190.4 ± 12.1††	9.2 ± 3.1††
50	92 ± 6.5††	7.0 ± 1.9††	14.8 ± 2.3††	8.4 ± 1.7††	76.1 ± 6.7††	9.2 ± 3.0††
100	73 ± 4.1††	7.0 ± 2.0††	14.7 ± 2.9††	2.3 ± 1.45††	31.4 ± 3.6††	9.0 ± 3.1††

* Each value is the mean (±S.D.) of three experiments, each determined in duplicate. Endosulfan was dissolved in ethanol and introduced in 10 μl into the assay system; controls received the same quantity of ethanol. Procedures for the measurement of enzyme activities are described in Materials and Methods.
† Expressed as nmoles $K_3Fe(CN)_6$ reduced · min^{-1} · (mg protein)$^{-1}$.
‡ Expressed as nmoles DCIP reduced · min^{-1} . (mg protein)$^{-1}$.
§ Expressed as nmoles cytochrome *c* oxidized · min^{-1} · (mg protein)$^{-1}$.
‖ Expressed as nmoles P_i liberated · min^{-1} · (mg protein)$^{-1}$.
¶ Expressed as nmoles benzaldehyde formed · min^{-1} · (mg protein)$^{-1}$.
**–‡‡ Significance of changes was measured by Student's *t*-test: **$P < 0.01$; ††$P < 0.001$; ‡‡$P < 0.05$.

Table 4. Effects of endosulfan metabolites on rat liver mitochondrial enzyme activities *in vitro**

Treatment (μg/ml)	Succinate dehydrogenase	Succinate–cytochrome *c* reductase	NADH-dehydrogenase	Cytochrome *c* oxidase	Mg^{2+}-Adenosine triphosphatase	Monoamine oxidase
Control	170 + 6.9	13.2 ± 2.1	28.5 ± 2.0	21.3 ± 2.1	7.96 ± 0.66	17.9 ± 2.5
Endosulfan lactone						
10	156.0 ± 6.0†	14.3 ± 2.1	28.2 ± 2.1	21.2 ± 2.0	6.63 ± 0.33	17.8 ± 1.7
25	148.0 ± 6.0‡	14.0 ± 2.2	28.0 ± 1.8	20.0 ± 2.1	6.66 ± 0.61	16.5 ± 1.6
50	139.2 ± 6.5§	14.1 ± 2.3	28.1 ± 2.0	20.1 ± 2.0	7.30 ± 0.40	14.8 ± 1.6
Endosulfan diol						
10	166.7 ± 6.05	14.0 ± 1.9	28.7 ± 2.2	21.3 ± 2.0	13.93 ± 1.63§	17.9 ± 2.2
25	166.6 ± 10.3	14.3 ± 2.1	29.0 ± 1.4	18.0 ± 2.5	11.94 ± 1.81§	17.5 ± 1.3
50	164.0 ± 9.1	15.3 ± 2.1	29.4 ± 2.1	10.6 ± 2.2§	11.28 ± 0.95§	14.7 ± 2.4
Endosulfan sulfate						
10	174.0 ± 6	14.0 ± 2.1	28.2 ± 2.2	21.3 ± 2.0	12.65 ± 1.20§	16.9 ± 1.8
25	167.7 ± 7.1	13.3 ± 2.3	18.5 ± 2.1§	24.6 ± 2.2	22.89 ± 1.01§	16.2 ± 1.2
50	161.4 ± 6.6	13.1 ± 2.0	15.2 ± 2.0§	32.6 ± 2.3§	14.26 ± 1.42§	14.0 ± 2.6

* Data represent the mean (±S.D.) of duplicate determinations from three batches of experiments. Endosulfan metabolites were introduced in 10 μl ethanol into the assay system; control received the same quantity of ethanol only. Other conditions and enzyme activities are similar to those described in Table 3.
†–§ Significance of changes was measured by Student's *t*-test: †$P < 0.05$; ‡$P < 0.01$; §$P < 0.001$.

Mg^{2+}-ATPase of mitochondria was most dramatically affected by endosulfan treatment. There was 7-, 17- and 25-fold activation of ATPase activity at 5, 10 and 25 μg concentrations respectively. A further increase in endosulfan resulted in a gradual decline of the activated ATPase activity. Both activation of Mg^{2+}-ATPase and stimulation of state-4 respiration were achieved at comparable concentrations of endosulfan.

Effects of metabolites on mitochondrial enzyme activities. The data are summarized in Table 4. The metabolites produced little change in SDH activity. NADH-dehydrogenase was inhibited by ES at 25 and 50 μg/ml, while ED and EL hardly produced any major alterations. Cytochrome *c* oxidase was activated by ES and inhibited by ED at 50 μg/ml. The latent Mg^{2+}-ATPase was activated by ES and ED but the degree of activation was far less than that of the parent compound. MAO was apparently not inhibited by the three metabolites.

DISCUSSION

The results of the present study indicate that endosulfan possesses the dual properties of an uncoupler of oxidative phosphorylation and an inhibitor of the electron transport chain. This is evidenced from our observations that, upon endosulfan treatment of mitochondria *in vitro*, the ADP-induced respiratory transitions were not manifested, ADP:O and respiratory control ratios decreased, mitochondrial oxidation of substrates was severely restricted with simultaneous inhibition of respiratory chain enzymes, and the latent Mg^{2+}-ATPase was activated while monoamine oxidase activity showed significant inhibition. In addition, endosulfan exerted biphasic effects on the *in vitro* respiration of rat liver mitochondria, causing stimulation of state-4 respiration at lower concentrations and inhibition at higher ones. The reasons for such a biphasic effect of endosulfan and its derangement of mitochondrial bioenergetics are not understood at present. However, it is conceivable that endosulfan, being lipophilic like other hydrochlorinated hydrocarbons [10, 16] might be interacting primarily with the mitochondrial lipoprotein surface, resulting in structural damage and changes in ionic permeability [17–20]. Inhibition of MAO by endosulfan would be indicative of such an altered outer mitochondrial membrane integrity, as indicated by others for certain compounds [21, 22].

The inhibition of mitochondrial respiration was found to occur in parallel to the inhibition in enzyme activities of the respiratory electron transport chain of mitochondria. These studies demonstrated that endosulfan is not a specific inhibitor for a particular site of the respiratory electron transport chain, and the effect exercised by endosulfan on mitochondrial respiration is a general one irrespective of whether succinate or a pyridine-linked substrate is used. Endosulfan thus diminished the availability of reducing equivalents to the terminal oxygen and hence the oxidation of substrates. Further lowered P:O ratio as well as the activation of latent Mg^{2+}-ATPase of mitochondria in the presence of endosulfan would be indicative of the uncoupling effect of this insecticide as is seen with known uncouplers [23, 24]. Unlike the DDE and Kethane type of compounds [10], the activation potential of the latent Mg^{2+}-ATPase by endosulfan was extremely high. The maximal activation of 25-fold at 25 μg endosulfan correlated with the maximal inhibition of MAO, suggesting again the interference of endosulfan with mitochondrial biomembranes.

The alterations of mitochondrial enzymes by the endosulfan metabolites were negligible in comparison to the effects seen with the parent compound which suggested that at the concentrations used they are relatively safer analogues with respect to the enzymes of the respiratory chain. Nevertheless, ES and ED are still possibilities in diminishing the mitochondrial oxidation of the substrates, possibly by restricting their entry into the mitochondria. Gorbach [25] and Dorough *et al.* [1] also demonstrated *in vivo* toxicity of endosulfan sulfate. Further studies are required to correlate the *in vitro* to *in vivo* damage to mitochondria.

From the foregoing observations it may also be postulated that mitochondrial respiration might offer a simple *in vitro* system of choice for monitoring cytotoxicity of direct acting compounds. However, limitations of such a system are quite apparent where metabolic activation of a test compound is prerequisite to its biological action, and in such cases measurement of respiration in intact cells *in vitro* would be the system of choice.

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