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Interactions of 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene with Mitochondrial Oxidative Phosphorylation

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ABSTRACT. The effects of DDE (2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene), the major metabolite of DDT (2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane), on rat liver mitochondrial bioenergetic activities were examined. The approach developed by M. D. Brand (*Biochim Biophys Acta* **1018:** 128–133, 1990) was used to assess the effects of DDE because it is possible to discriminate the sites of action of compounds having pleiotypic effects on oxidative phosphorylation. Data were further confirmed using a "classical" approach, including measurements of transmembrane potential, respiratory indexes, enzymatic activities and membrane permeability to protons. DDE up to 40 nmol/mg protein affected the proton motive force generating system. In fact, DDE interacted with succinate dehydrogenase (complex II), decreasing respiration and membrane potential. In this concentration range, the permeability of the inner membrane to protons remained intact. Only higher concentrations (≥80 nmol/mg) increased permeability to protons, uncoupling oxidation from phosphorylation. The phosphorylative system was not affected because the rate of ATP synthesis was unchanged. In addition, data from carbonyl cyanide *m*-chlorophenylhydrazone–uncoupled rotenone-inhibited preparations or submitochondrial particles indicated that F_0F_1 ATPase activity is not affected by DDE. Therefore, DDE inhibition of complex II and putative inhibition of succinate translocation explain the depression of mitochondrial respiration. The use of appropriate substrates and assay conditions indicates that complexes I, III and IV were not affected by DDE. The uncoupling of oxidative phosphorylation at high concentrations (>80 nmol DDE/mg protein) was probably related to deleterious effects on the integrity of the mitochondrial membrane. We confirmed that the technique originally proposed by Brand is useful for characterizing the effects of xenobiotics on oxidative phosphorylation. In addition, data provided by this technique closely agree with data from classical studies. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:299–308, 1997.

KEY WORDS. *in vitro* toxicology; oxidative phosphorylation; DDE; mitochondria; submitochondrial particles

Beginning in the 1940s and for a period of approximately 30 years, DDT[§] was the common pesticide used in agriculture for the control of insect pests and vector-borne diseases, e.g., yellow fever, typhus and malaria [1, 2]. However, the spread and uncontrolled use of DDT brought risks to the environment as a consequence of its long residual lifetime and its accumulation in food chains, along with the appearance of DDE, a major intermediate on DDT metabolism [1, 3]. In spite of the ban of DDT in most industrialized countries, the insecticide is still commonly used in developing countries, causing environmental contamination and health problems to humans, useful animals and insects. Even after its application has ceased, residues persisting in soils continue to contaminate the environment for long periods; furthermore, its high lipophilic character promotes accumulation in adipose tissues. These residues can continuously be circulated to target organs by the blood [4]. Residues of DDE and DDT in human tissues and breast milk are often detected in developing and industrialized countries [5–10].

DDE, an end product of DDT degradation, is structurally similar to the parental insecticide, but its insecticidal activity is comparatively low. Moreover, its acute toxicity to mammals, including man, is believed to be relatively low, although this belief has not been established. However, due to its lipophilic nature, long-term effects cannot be excluded; *p,p'*-DDE is also persistent and accumulates in food chains [3]. A reported risk of the bioaccumulation of *p,p'*-DDE is the eggshell thinning observed in a number of predator birds [1, 10, 11] as a consequence of inhibition of prostaglandin synthesis in the avian eggshell gland mucosa [12, 13]. In mammals (bats), DDE crossed placental mem-

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§ *Abbreviations:* DDE, 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene; DDT, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane; TPP^+, tetraphenylphosphonium ion; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Δp, proton motive force; ΔΨ, mitochondrial membrane potential; SMP, submitochondrial particles; BSA, bovine serum albumin; PMS, phenazine methosulphate; EOTA, ethylenediaminetetraacetic acid; EGTH, ethyleneglycol-bis (β-aminoethyl ether).

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branes [14]. In humans, *p,p'*-DDE interacts with receptors of sexual hormones and may be involved in the increase in the amount of estrogen receptors found in some breast cancers [15]. *p,p'*-DDE has been reported a potent antagonist of androgen receptors [16], causing abnormal male sexual development both in humans or wildlife.

The strong hydrophobic character of DDE also explains its disturbing effects in native membranes and liposomes. It affects their fluidity [17] and induces thermotropic and fluorescence polarization changes in phospholipid dispersions [18, 19].

In the present study, we describe the interaction of *p,p'*-DDE with mitochondrial functions. These studies are relevant to toxicology because most of the energy involved in cellular metabolism and intermediary metabolic compounds are generated at the expense of mitochondrial respiration. Interactions of chemical compounds with mitochondrial functions can therefore result in severe impairment to the general metabolism because any perturbation of these functions will affect the energy transduction processes that require ATP. Furthermore, mitochondria is a good model for studying cell toxicity of many xenobiotics, because data obtained from mitochondrial studies are generally correlated with cytotoxicity parameters reported in cell cultures and whole organisms [20–22].

MATERIALS AND METHODS

Chemicals

p,p'-DDE (chromatographic grade) was obtained from Riedel-de-Häen (Seelze, Germany) and dissolved in absolute ethanol. Equal volumes of ethanol solvent added to controls (up to 4 μL) were without effect on measured activities. ATP and ADP were obtained from Sigma (St. Louis, MO, USA). All the other chemicals were of the highest grade.

Preparation of Mitochondria

Wistar rats (200–300 g) were fasted overnight before being killed by cervical displacement, and mitochondria were isolated by conventional methods [23], with slight modifications. Briefly, liver mitochondria were isolated in a medium containing 210 mM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.4), 0.2 mM EGTA, 0.1 mM EDTA and 0.1% defatted BSA. EDTA, EGTA and defatted BSA were omitted from the final washing medium, adjusted at pH 7.2. The mitochondrial pellet was washed twice, suspended in the washing medium and immediately used for the determination of the oxygen uptake and measurement of $\Delta\Psi$, ATPase and ATP-synthase activities. For the determination of the activity of the enzymes linked to the respiratory chain, the preparations were divided into aliquots and frozen in liquid nitrogen for 1–7 days. At the time of use, each aliquot was freeze thawed three times and maintained at 0°C. Protein was determined by the biuret method, using BSA as standard [24].

Preparation of SMP

Nonphosphorylation SMP were prepared according to Lee and Ernster [25], with some modifications. Isolated rat liver mitochondria were suspended in a small volume of medium (210 mM mannitol, 70 mM sucrose, 2 mM EDTA, pH 8.6) and frozen at −80°C for approximately 7 days. The mitochondria were thawed and disrupted in a Branson sonifer cell disrupter (model B-12) at maximum power for about 1 min at intervals of 20 sec. The sample was diluted to a final volume of 25–30 mL and centrifuged at 10,000*g* for 10 min. The mitochondrial pellet was discarded, and the sample was ultracentrifuged at 105,000*g* for 40 min. The pellet was suspended in washing medium (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.2) and ultracentrifuged twice at 105,000*g* for 40 min. The final pellet was resuspended in a small volume of washing medium, and the isolated particles were stored at −70°C for 1–6 months, without loss of activity.

$\Delta\Psi$ *Measurements*

The mitochondrial transmembrane potential was estimated by calculating transmembrane distribution of TPP^+ with a TPP^+-selective electrode prepared according to Kamo *et al.* [26], using a calomel electrode as the reference. TPP^+ uptake was measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective and reference electrodes was measured with an electrometer and recorded continuously. The voltage response of the TPP^+ electrode to log[TPP^+] was linear, with a slope of 59 ± 1, in good agreement with the Nernst equation. Calibration runs in the presence of DDE excluded any direct interference of the metabolite with the electrode signal. Reactions were carried out in an open vessel at 25°C, with magnetic stirring in 1 mL of standard respiration medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 5 mM HEPES, pH 7.2) supplemented with 3 μM TPP^+. This TPP^+ concentration was chosen to achieve high sensitivity in measurements and to avoid possible toxic effects on mitochondria [27, 28]. The $\Delta\Psi$ was estimated from the following equation (at 25°C):

$$\Delta\Psi(\text{mV}) = 59 \log(v/V) - 59 \log(10^{\Delta E/59} - 1)$$

as previously described [26, 29]. v, V, and ΔE stand for mitochondrial volume, volume of the incubation medium, and the deflection of the electrode potential from the baseline, respectively. This equation was derived assuming that TPP^+ distribution between the mitochondria and the medium follows the Nernst equation and that the law of mass conservation is applicable. A matrix volume of 1.1 μL/mg protein was assumed. No correction was made for the "passive" binding contribution of TPP^+ to the mitochondrial membranes because the purpose of the experiments was to show relative changes in potential rather than absolute values. As a consequence, we can anticipate an overestimation

of the $\Delta\Psi$ values that may reach approximately 20 mV. However, the overestimation is only significant at $\Delta\Psi$ values below 90 mV and, therefore, does not affect our measurements. From data summarized in Fig. 2B, it can be clearly stated that DDE does not affect TPP^+ binding. DDE was added in ethanolic solutions (up to 4 μL) to the reaction medium supplemented with 1 mg mitochondria protein and incubated for 5 min before the addition of respiratory substrates. This time was chosen because DDE effects were maximal at 5 min.

Mitochondrial Respiration

Oxygen consumption of isolated mitochondria was measured polarographically using a Clark-type oxygen electrode, connected to a suitable recorder, in a water-jacketed closed chamber with magnetic stirring at 25°C [30]. The respiratory control ratio (state 3 respiration/state 4 respiration, RCR) and P/O ratio were calculated according to Chance and Williams [31]. Respiration rates were calculated assuming an oxygen concentration of 450 nAt O/mL in the experimental medium at 25°C. DDE was added in ethanolic solutions (up to 4 μL) to 1 mL of standard reaction medium supplemented with mitochondria (1 mg protein) and 2 mM of rotenone (when succinate was used) and incubated for 5 min.

Simultaneous Measurement of Respiration Rate and Membrane Potential

The TPP^+ electrode was inserted through the glass lid of the oxygen electrode chamber, enabling the simultaneous measurement of $\Delta\Psi$ and respiration rate for titration experiments. All experiments were carried out in the presence of the ionophore nigericin and 130 mM KCl, which camps the pH gradient across the mitochondrial inner membrane [32, 33], making the $\Delta\Psi$ the sole component of Δp. Other details are specified in the captions to the figures.

Enzymatic Activities

ATP-synthase activity was determined by measuring the pH changes associated with ATP synthesis, as reported previously [34]. The reaction was performed at 25°C in an open vessel with 2 mL of reaction medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 2 mM KH_2PO_4, 2 μM rotenone and 5 mM succinate or reduced TMPD, pH 7.2). DDE was injected into the medium after the addition of 1 mg mitochondria protein and allowed to incubate for 5 min. The reaction was started by adding 150 μM ADP to the mitochondrial suspension. The pH change was evaluated with a Crison pH meter connected to a Perkin-Elmer recorder. The H^+ consumption was calculated by adding known amounts of standard HCl to the reaction medium. The addition of oligomycin (1 μg) to the medium completely abolished H^+ consumption.

ATPase activity was determined by monitoring the pH change in association with ATP hydrolysis [35]. The reaction was carried out at 25°C in 2 mL of reaction medium (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM HEPES, 2 μM rotenone, pH 7.2) supplemented with submitochondrial particles (0.3 mg protein) or mitochondria (1 mg protein). The reaction was initiated by the addition of 2 mM Mg-ATP. DDE was added in ethanolic solution (up to 4 μL) and allowed to incubate for 5 in. The addition of oligomycin (1 μg/mg protein) to the medium completely abolished the production of protons.

Succinate dehydrogenase activity was measured polarographically [36] at 25°C in 1 mL of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 0.1 mg antimycin A, 1 mM KCN and 0.5 mg protein of broken mitochondria, with two cycles of freezing and thawing. The reaction was initiated by the addition of 1 mM PMS, used as an artificial electron acceptor.

Succinate cytochrome c reductase activity was measured spectrophotometrically [37] at 25°C by following the reduction of oxidized cytochrome c by the increase in absorbance at 550 nm. The reaction was initiated by the addition of 5 mM succinate to 2.5 mL of the standard reaction medium supplemented with 2 μM rotenone, 1 mM KCN, 54 μM of cytochrome c and 0.3 mg protein of broken mitochondria.

Cytochrome c oxidase activity was measured polarographically [38] at 25°C in 1 mL of the standard reaction medium supplemented with 5 mM succinate, 2 μM rotenone, 10 μM cytochrome c and 0.5 mg protein of broken mitochondria. The reaction was initiated by the addition of 5 mM ascorbate plus 0.25 mM TMPD.

Mitochondrial Swelling

Mitochondrial osmotic volume changes were followed by the apparent absorbance changes at 540 nm with a suitable spectrophotometer recorder setup. The reactions were carried out at 25°C in 2.5 mL of NH_4NO_3 medium (135 mM NH_4NO_3, 0.1 mM EDTA, 5 mM HEPES, pH 7.2), supplemented with 2 μM rotenone and 0.3 mg mitochondria. DDE was added in ethanolic solution and incubated for 5 min.

Determination of Adenine Nucleotides

Adenine nucleotides (ATP, ADP and AMP) were extracted using an acidic extraction procedure and separated by reverse-phase liquid chromatography, as described previously [39]. Briefly, acidic extraction was performed as follows: 1 mg protein mitochondria was incubated in 1 mL standard respiration medium supplemented with 2 μM rotenone, 3 μM TPP^+ and malonate. DDE was added in the concentrations of 20 and 50 nmol/mg protein. A control in the absence of DDE was carried out. After the incubation time (5 min), succinate was added to induce state 4, and 2 min later 300 nmol ADP were added to the reaction vessel to induce state 3; 1 min later, 500 μL of the suspension

were added to 500 μL ice-cooled $HClO_4$ 0.6 M. After 5 min, the mixture was vortexed, centrifuged for 2 min at 10,000 rpm in a Eppendorf table centrifuge at 0°C, and the pellet was discarded. The supernatant was neutralized with KOH 3 M and centrifuged again (10,000 rpm, 2 min) to precipitate all $KClO_4$ produced. The supernatant was filtered through Millipore filters (0.22 μm diameter) and stored at −80°C for further chromatographic analysis. All the extraction procedure was carried out at 0–4°C to minimize degradation of nucleotides. The chromatographic apparatus was a Beckman System-Gold, consisting of a Binary Pump Model and a 166 Variable UV detector, controlled by a computer. The detection wavelength was 254 nm and the column used was a Lichrospher 100 RP-18 (5 μm) from Merck (Darmstadt, Germany). During each run, an isocratic elution with 100 mM potassium phosphate buffer, pH 6.5, and 1% methanol was performed for 10 min, followed by a 5-min linear gradient up to 10% methanol at a flow rate of 1.25 mL/min. The time required for each analysis was 5 min. The detection limit for each analyte was 3–5 pmol.

Statistics

The results are presented as mean ± SD of the number of experiments indicated in the legends to the figures. Statistical significance was determined by using two-tailed Student's t test.

RESULTS

The effects of DDE were evaluated on the Δp generating system (i.e. electron transport chain and substrate transporters) according to Brand [40]. Nonphosphorylating mitochondria were titrated with CCCP to promote an increase in respiratory rate and to decrease ΔΨ; ΔΨ was plotted against respiration rate to describe the kinetic response of the Δp generators [40]. Consequently, if a compound inhibits any component of the redox chain, this curve will be displaced downward to the left. Figure 1 shows ΔΨ against the respiratory rate obtained in the presence of several concentrations of DDE (0, 20 and 50 nmol DDE/mg protein). The displacement of the curve induced by DDE is concentration dependent and indicates that one or more components of the Δp generating system are inhibited by the pesticide.

To localize the exact action of DDE on the redox chain and phosphorylative system, the alterations of the transmembrane potential (ΔΨ) developed by mitochondria upon substrate oxidation were followed, as previously described (Fig. 2). After addition of succinate, mitochondria developed a potential of about −212 mV (negative inside). With the addition of ADP, the potential dropped to −175 mV because ATP-synthase uses ΔΨ to phosphorylate ADP. After a short lag phase, when ADP phosphorylation takes place, the transmembrane potential repolarized close to the initial value. DDE progressively depressed ΔΨ and decreased depolarization following addition of ADP. Moreover, the lag phase preceding repolarization increased and the rate of repolarization decreased progressively (Fig. 2A). However, using ascorbate + TMPD as the respiratory substrate, the developed ΔΨ was not affected even at the highest DDE concentration studied (i.e. 80 nmol DDE/mg protein; see Fig 2B). Also, the drop in potential due to the phosphorylation of ADP in the presence of reduced TMPD was not affected by DDE. Potentials from energization with pyruvate + malate (10 mM + 2.5 mM) were unchanged by DDE and fully recovered after the phosphorylation cycle (unpublished data). Therefore, DDE clearly does not affect complex I activity. In addition, this observation indicates that complexes III and IV are not affected.

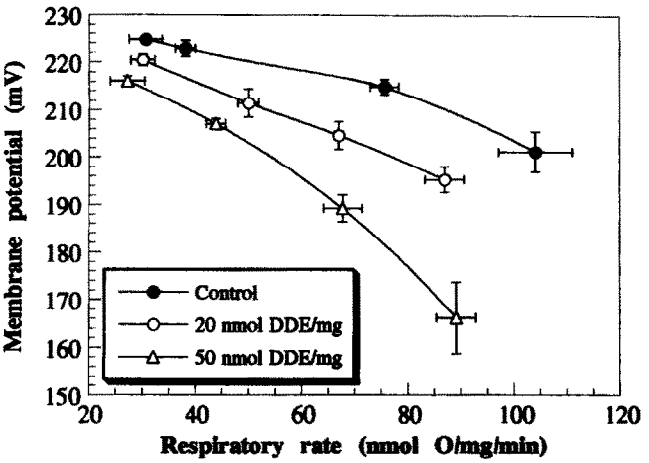

FIG. 1. Effect of DDE on the Δp generators. Nonphosphorylating mitochondria were titrated with an uncoupler (CCCP), and ΔΨ and respiratory rate were measured simultaneously. After 5 min of incubation with DDE at the indicated concentrations, succinate (5 mM) was added to 1 mg protein mitochondria to induce state 4. Two minutes later, 10 nM CCCP was added, followed by three further additions. Media (1 mL) were supplemented with 1.5 μg oligomycin. The data are mean ± SD for six different experiments performed with three different mitochondrial preparations. All other experimental details are described in the text.

Respiratory rates of state 4, state 3, CCCP-stimulated (uncoupled) respiration and respiratory indexes (RCR and P/O ratios) were studied, in the presence of succinate or reduced TMPD as respiratory substrates (Table 1). State 4 respiration was not significantly affected, either in the presence of succinate or of ascorbate + TMPD. Using ascorbate + TMPD as electron donor, state 3, CCCP-stimulated respiration and the respiratory indexes were not affected. Conversely, using succinate as electron donor, DDE inhibited state 3 and CCCP-stimulated respiration and decreased respiratory indexes ADP/O and RCR. Therefore neither the terminal segment of the respiratory chain nor the phosphorylatory system were affected by DDE.

Studies of enzymatic activities of respiratory complexes II, III and IV localized the components of the mitochondrial respiratory chain affected by DDE (see Fig. 3). The terminal segment of the chain, cytochrome c oxidase (com-

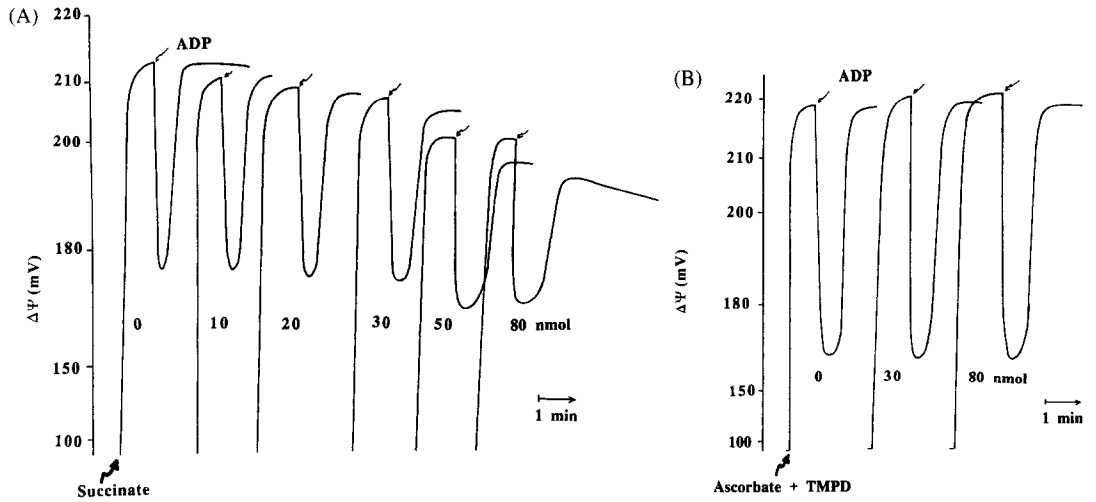

FIG. 2. Effect of DDE on ΔΨ. Mitochondria (0.8 mg protein) were incubated for 5 min in 1 mL standard respiratory medium supplemented with 2 μM rotenone and 3 μM TPP^+. Succinate (A) or reduced TMPD (B) were used as respiratory substrates. Addition of ADP (0.12 mM) induces the state 3 condition. In the presence of succinate (A), DDE depresses the total developed ΔΨ and the depolarization induced by ADP and reduces the level and the rate of repolarization. However, in the presence of reduced TMPD (B), DDE is without effect. The different concentrations of DDE are indicated on the traces. The traces represent typical recordings from several experiments with different mitochondrial preparations.

plex IV), was not affected by DDE; however, succinate dehydrogenase (complex II) and succinate cytochrome c reductase were partially inhibited, indicating that DDE interacts with electron transfer at the level of complex II. Because state 3 respiration was inhibited by about 60% at approximately 50 nmol DDE/mg protein and complex II was inhibited by 15%, succinate translocation across the inner membrane is apparently impaired by DDE.

Putative proton leaks induced by DDE through the mitochondrial inner membrane were investigated in nonphosphorylating mitochondria titrated with malonate, a respiratory inhibitor, and Δp was plotted against respiration rate (Fig. 4). In a steady state, the proton efflux must equal the proton leak, assuming that no slip in the proton pumps occurs [41]. In addition, any secondary effect on leak due to alterations in the value of Δp is eliminated. Therefore, if a given compound increases the proton leak across the mitochondrial inner membrane, the curve will be displaced downward and to the right. DDE did not increase the proton leak at the concentrations of 20 and 50 nmol/mg protein. The absence of proton leak was further confirmed by monitoring the mitochondrial swelling in isoosmotic NH_4NO_3 (Fig. 5). In fact, only for 80 nmol DDE/mg protein was the permeability of mitochondria to protons slightly increased, a small effect compared with that of CCCP.

Figure 6 shows that DDE did not affect the phosphorylation system, as predicted previously. The kinetic depen-

TABLE 1. Effect of DDE on respiratory indexes

Condition	Control	20 nmol/mg	30 nmol/mg	50 nmol/mg	80 nmol/mg
Succinate					
State 4	11.58 ± 1.02	10.31 ± 0.98	8.94 ± 1.23	11.26 ± 1.23	6.26 ± 0.69
State 3	78.83 ± 6.31	60.84 ± 6.43	34.27 ± 3.47	29.51 ± 2.80	22.25 ± 2.11
CCCP s.r.	50.63 ± 1.08	48.49 ± 1.29	44.83 ± 1.89	27.42 ± 1.42	22.21 ± 4.08
RCR	7.33 ± 0.57	6.52 ± 0.35	4.30 ± 0.39	2.93 ± 0.33	2.29 ± 0.27
ADP/O	1.95 ± 0.02	1.93 ± 0.02	1.80 ± 0.02	1.80 ± 0.02	1.49 ± 0.08
Asc + TMPD					
State 4	33.18 ± 0.97	n.d.	33.19 ± 1.19	32.05 ± 0.09	39.70 ± 0.59
State 3	45.82 ± 1.43	n.d.	48.10 ± 0.60	46.81 ± 0.59	50.37 ± 0.61
CCCP s.r.	47.41 ± 1.89	n.d.	48.00 ± 0.59	n.d.	50.39 ± 0.62
RCR	1.44 ± 0.03	n.d.	1.45 ± 0.35	1.43 ± 0.55	1.27 ± 0.32
P/O	1.08 ± 0.09	n.d.	1.12 ± 0.06	1.13 ± 0.08	1.10 ± 0.04

Mitochondria (1 mg protein) were incubated in 1 mL of the respiratory standard medium. State 3 respiration was initiated by the addition of 1.2 mM ADP. CCCP respiration (CCCP s.r.) was initiated by the addition of 2 μM CCCP. State 4 respiration was initiated by the addition of 5 mM succinate or ascorbate + TMPD after 5 min of incubation. ADP or CCCP was added 2 min after the initiation of state 4 respiration. RCR and P/O were calculated as described previously [33]. Values are expressed in nAt O/mg protein/min. Data are mean ± SD of three to six independent experiments. n.d., not determined.

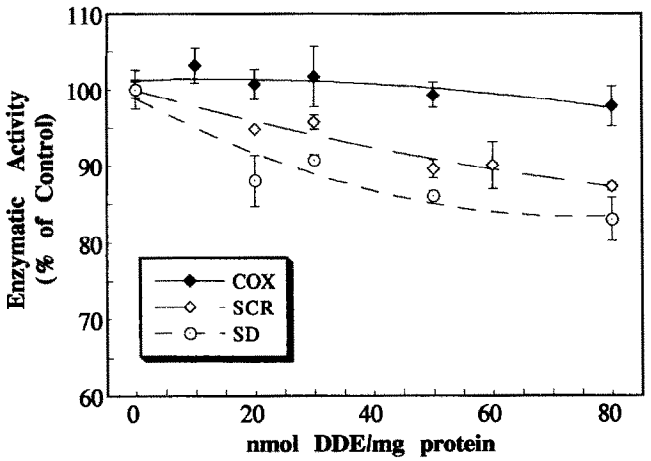

FIG. 3. Effect of DDE on succinate dehydrogenase (SD), succinate cytochrome c reductase (SCR) and cytochrome c oxidase (COX). Values are means ± SD of three to six independent experiments (when the error bars are absent, SD is encompassed by the size of the symbols).

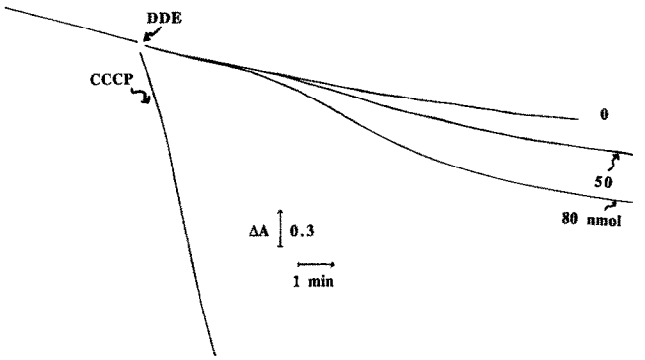

FIG. 5. Effect of DDE on mitochondrial swelling in isoosmotic NH_4NO_3 medium. Mitochondria (0.3 mg) were incubated for 5 min with DDE at the indicated concentrations in 2.5 mL of NH_4NO_3 medium (135 mM NH_4NO_3, 0.1 mM EDTA, 5 mM HEPES, pH 7.2) supplemented with 2 μM rotenone. The reactions were carried out at 25°C. The traces represent typical recordings.

dence of phosphorylation on Δp might be appraised from a plot of Δp against the respiratory rate of phosphorylating mitochondria titrated with a respiratory inhibitor. The respiratory rate measured was higher than required to balance the influx of protons through F_0F_1–ATP-synthase. Because the proton leak through the mitochondrial inner membrane depends on Δp [41], the plot of Δp against respiratory rate can be easily transformed into the proton current driving the phosphorylation system. Using this approach, we eliminate any secondary kinetic effect of DDE on the phosphorylation system by producing alterations in Δp. These results were further confirmed by studying the effect of DDE on the synthesis and hydrolysis of ATP.

Figures 7 and 8 show the effect of DDE on ATP-synthase activity, using either the novel or the classical approach, respectively. Figure 7 shows the dependence of the rate of ATP synthesis on membrane potential under the effect of DDE. The adenine nucleotides were extracted, as previously described, and determined by high-pressure liquid chromography. ATP synthesis against ΔΨ was practically insensitive to DDE.

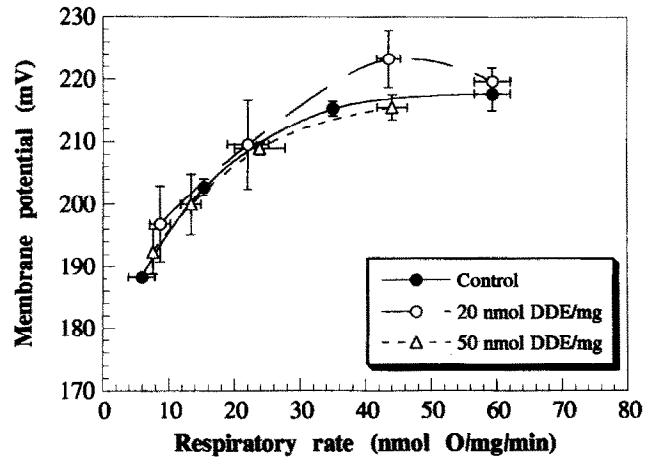

FIG. 4. Effect of DDE on the proton leak of the mitochondrial inner membrane. Nonphosphorylating mitochondria were titrated with a respiratory inhibitor (malonate), and ΔΨ and respiratory rate were measured simultaneously. After 5 min of incubation with DDE at the indicated concentrations, succinate (5 mM) was added to 1 mg protein mitochondria (in 1 mL of reaction medium) to induce state 4. Two minutes later, malonate (0.3 mM) was added, followed by three further additions. For this experiment, 1.5 μg oligomycin were present. The data shown here are mean ± SD of six different experiments performed with three different mitochondrial preparations. All other experimental details are as described in the text.

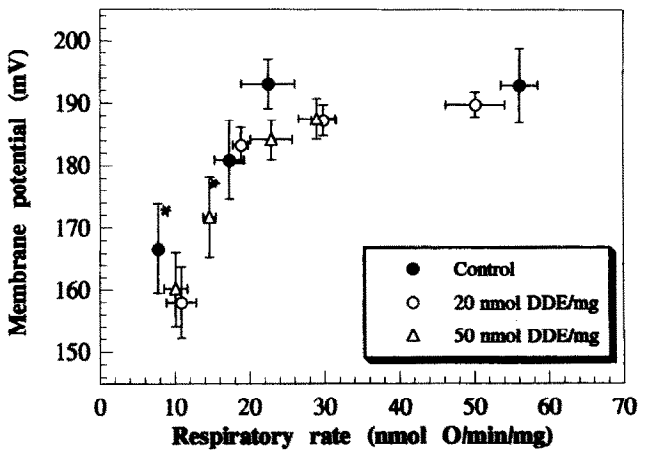

FIG. 6. Effect of DDE on the phosphorylation system. Phosphorylating mitochondria were titrated with a respiratory inhibitor, malonate, and ΔΨ and respiratory rate were measured simultaneously. After 5 min of incubation with DDE at the indicated concentrations and 1 min of incubation with malonate (0.3, 0.6, 0.9 and 1.2 mM), succinate (5 mM) was added to 1 mL reaction medium containing 1 mg protein mitochondria to induce state 4. Two minutes later, the reaction was stopped by the addition of oligomycin (1.5 μg). The data shown here are mean ± SD of six different experiments performed with three different mitochondrial preparations. *$P > 0.05$ (referred to control). All other experimental details are as described in the text.

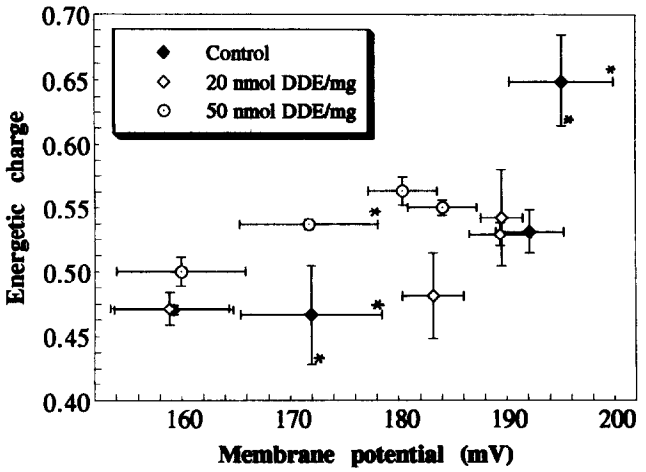

FIG. 7. Synthesis of ATP as a function of the membrane potential as affected by DDE. Data were obtained from mitochondria used in the experiment described in Fig. 6. The adenine nucleotides were extracted as previously described and determined by high-pressure liquid chromography. Energetic charge was calculated as {[ATP] + 0.5[ADP]}/{[ATP] + [ADP] + AMP]}. Different steady-state values of $\Delta\Psi$ were established by incubating with different concentrations of malonate, a respiratory inhibitor. Data are means ± S of six different experiments performed with three different mitochondrial preparations. *$P > 0.05$ (referred to control). All other experimental details are as described in the text.

Figure 8 shows the effect of DDE on ATP-synthase activity, determined by H^+ removal from the medium [34], in the presence of succinate (Fig. 8A) or ascorbate + TMPD (Fig. 8B) as respiratory substrates. With succinate, ATP-synthase was clearly inhibited by DDE at concentrations below 50 nmol/mg protein. With TMPD as electron donor, a small inhibition was detected only at higher concentrations (80–120 nmol/mg; not shown). The different effects are explained by the selective inhibition of complex II by DDE, as also documented by data in Figs. 2 and 7, and by significant effects on the translocation of succinate across the inner membrane.

Table 2 summarizes the effect of DDE on ATPase activity. DDE stimulated the ATPase of intact mitochondria (in the presence of an uncoupler) only at high concentrations (80 nmol DDE/mg protein). However, the ATPase of submitochondrial particles was not affected. Therefore, the effect does not result from a direct action of DDE on the F_0F_1–ATPase complex but rather from putative disruptive effects on mitochondrial inner membrane, as inferred from permeabilization to H^+ induced by high concentrations of the metabolite (see Fig. 5).

DISCUSSION

The present work addresses the *in vitro* effect of *p,p'*-DDE on mitochondrial oxidative phosphorylation. Several studies have reported noxious effects of DDE on wildlife and humans, thereby warranting the interest of this toxicological study in terms of bioenergetic injuries [9–15]. Because DDE is a metabolite of DDT, this study is also relevant to DDT toxicity.

Liver mitochondria were used for several important reasons: mitochondrial fractions are easy to obtain, with a high degree of purity and intactness; the control of respiration in isolated mitochondria is well documented [42, 43]; and the organization and function of electron transport chain and oxidative phosphorylation within mammalian species are highly conservative in the course of evolution. Therefore, liver mitochondria preparation is a very convenient method for studying bioenergetic toxicities of xenobiotics [44–50]; moreover, data from mitochondrial studies can generally be correlated with cytotoxicity parameters evaluated by other methods [20–21].

In this work, the approach of Brand [40] was used to determine the effects of DDE on mitochondrial bioenergetics. Data and interpretations were further confirmed using classical approaches, including studies of transmembrane potential, respiratory indexes, enzymatic activities and membrane permeability to protons.

DDE, up to 50 nmol DDE/mg protein, affects the Δp generating system when succinate is the oxidable substrate. This action occurs as a consequence of succinate dehydrogenase inhibition, which in turn decreases respiration rate and consequently transmembrane potential. In this range of concentrations, the permeability of the inner membrane to protons is not affected. However, the permeability increases above 80 nmol DDE/mg protein, thus partially uncoupling oxidation from phosphorylation. Even at high concentrations, DDE does not affect the phosphorylation system itself because the rate of ATP synthesis is not affected using ascorbate + TMPD as substrate. In CCCP-uncoupled rotenone-inhibited preparations or submitochondrial particles, ATPase activity is not affected by DDE, confirming the absence of direct effects on the mitochondrial ATP-synthase (ATPase) system. This result is at variance with that for DDT because this compound strongly affects the function of the ATP-synthase complex [44]. When the energization is provided by pyruvate + malate, the transmembrane potentials are unchanged by DDE and fully recover after the phosphorylation cycle, indicating that DDE does not affect the activity of complex I. This observation further confirms that activities of complexes III and IV are also not affected. Therefore, DDE specifically interacts at the level of succinate dehydrogenase (complex II) and the translocation system of the metabolite.

The capacity of DDE to inhibit mitochondrial respiration results in a reduced ATP synthesis. Therefore, the reduced energetic efficiency of mitochondria may account for some toxic effects resulting from the impairment of the energy requirements of the cell and from the crucial importance of energy metabolism in active tissues, e.g. liver. In addition to a partial inhibition of respiration, DDE probably exerts significant effect on succinate translocation across the inner mitochondrial membrane because state 3 respiration is inhibited by about 60% but succinate cyto-

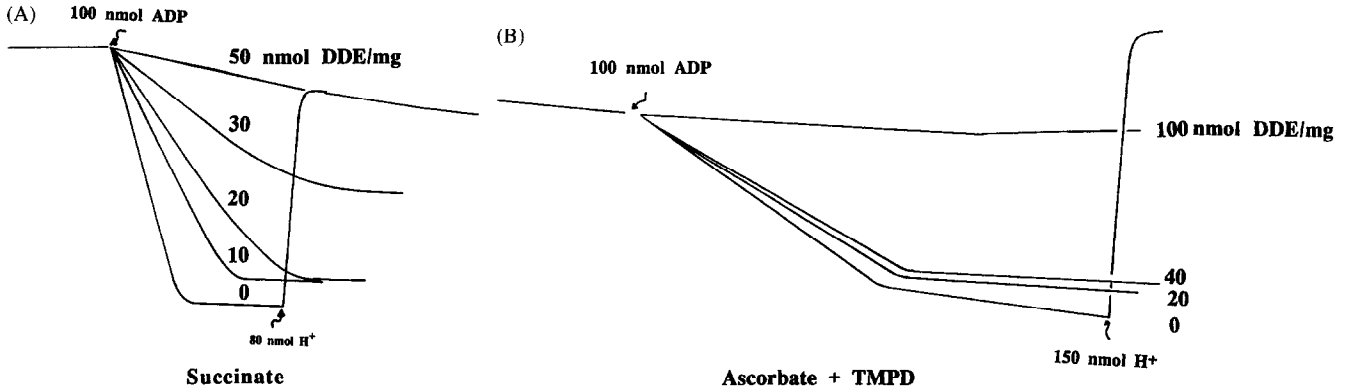

FIG. 8. Effect of DDE on ATP-synthase activity using succinate (A) or ascorbate + TMPD (B) as respiratory substrates. Experimental conditions are described in Materials and Methods. The traces represent typical recordings from several data experiments with different mitochondrial preparations.

chrome c reductase is depressed by a mere 15% at 50 nmol DDE/mg protein.

As a consequence of its high partition coefficient (Kp ≈ 160,000 at 24°C in mitochondria [51]), DDE is highly incorporated into the mitochondrial membranes, preferentially on the central core of the bilayer. Therefore, the observed effects depend on the DDE:protein ratio rather than on the absolute concentrations of DDE or protein. Thus, the DDE:protein ratio is always stated rather than the molar concentration of DDE, which may be irrelevant. In other words, standardized data are produced only when the relative amount of lipophilic xenobiotic is stated relative to the amount of biomaterial, as currently done in this and other related works [44, 45]. The effects of DDE are putatively committed to its partition in the inner mitochondrial membrane because there is no reason to propose any active uptake of DDE as it is devoid of electric charge at variance with TPP^+.

The inhibitory effects of DDE on mitochondrial oxidative phosphorylation may account for its chronic toxicity, although the concentrations used in our study cannot be readily related to the concentrations in tissues [4–14] because the distribution of DDE depends on second parameters, namely the partition coefficients [51] and DDT metabolism. Because liver is the main tissue where DDT metabolism takes place, liver mitochondria may be particularly exposed to DDE, which may reach local concentrations of the order of those used in our studies.

Thus, the technique developed by Brand [40] is useful in determining the multiple effects of a xenobiotic (in the present case, DDE) on oxidative phosphorylation because the obtained data correlate well with the results of classical studies.

TABLE 2. Effect of DDE on ATPase activity

nmol DDE/mg protein	ATPase (+CCCP)	ATPase (SMP)
Control	44.94 ± 0.23	577.13 ± 32.90
20	50.41 ± 1.25	n.d.
30	n.d.	564.28 ± 29.34
40	50.83 ± 0.51	n.d.
50	48.15 ± 1.25	587.03 ± 28.12
80	73.60 ± 7.03	585.78 ± 26.76

The reaction medium (2 mL) was supplemented with submitochondrial particles (0.3 mg protein) or mitochondria (1 mg protein) for the study of ATPase activity of submitochondrial particles or CCCP-uncoupled rotenone-inhibited mitochondrial preparations. DDE was added in ethanolic solution (up to 4 μL) and allowed to incubate for 5 min. The reaction was initiated by the addition of 2 mM Mg-ATP; H^+ production is given for a period of 3 min from the start of the reactions by adding known amounts of HCl. Values are expressed as nmol H^+/(mg protein^{-1}min^{-1}). ATPase activity of submitochondrial particles is higher because the enzyme is substantially purified. Data are means ±SD of four to six independent experiments. n.d., not determined.

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