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THE EFFECT OF DDT ON K^+ TRANSPORT IN MOUSE LIVER MITOCHONDRIA

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Summary

This study describes DDT-induced changes in membrane permeability of mitochondria and erythrocytes to K^+ as monitored by a K^+-selective electrode. DDT is a strong inhibitor of valinomycin-mediated K^+ uptake and the corresponding H^+ efflux and an inducer of K^+ leakage out of mitochondria but not to any significant extent out of erythrocytes. The inhibition of K^+ uptake and H^+ efflux was a function of (a) preincubation time between mitochondria and DDT, (b) mitochondrial concentration, (c) the nature of the carrier solvent and (d) temperature. The kinetics of inhibition of K^+ uptake showed that DDT is an uncompetitive inhibitor with respect to valinomycin and a competitive inhibitor with respect to K^+. The efflux of endogenous K^+ showed a sigmoid dependency on DDT concentration and was reduced to endogenous rates when the temperature was lowered below the gel-liquid crystalline phase transition of the lipids. It is suggested that the DDT-induced changes in membrane permeability are due to perturbation of the lipid phase and that its toxicity may be due in part to hyperpolarization of subcellular membranes.

Introduction

Although the chemical synthesis of 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT) was first announced over 100 years ago and its remarkable selective insecticidal activity about 40 years ago, there is little information on the critical lesion(s) responsible for its toxicity [1]. This lack of knowledge

Abbreviation: DDT, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane.

has been a barrier to overcoming the development of resistance of pests to this insecticide, to reducing its environmental persistence and to the rational development of analogs with greater biological selectivity.

To date, the target site for DDT is presumed to be the central and sensory nervous systems of invertebrate animals. An axon challenged with DDT displays intense volleys of repetitive discharges and an increased negative after-potential. Both effects are due to a modification of the Na^+ and K^+ conductance of the axon membrane [2,3]. This effect suggests that DDT induces a redistribution of ions across excitable membranes of nerves. Since ionic gradients and, hence, membrane potentials also determine many membrane-linked functions of non-neural tissues, it becomes of considerable importance to determine the biomembrane specificity of this DDT effect. This report is the first of a number from this laboratory directed at elucidating the lesion(s) induced by organochlorine insecticides in non-excitable biomembranes.

Methods and Materials

Preparation of mitochondria

Mouse liver mitochondria were prepared from young female mice (BB4-I, CR Swiss albino) in 0.25 M sucrose [4]. The mitochondrial pellet was washed twice and suspended in 0.25 M sucrose, unless stated otherwise, by gentle homogenization to a concentration of about 35—45 mg protein per ml. The preparation was used within 2—3 h.

Preparation of human red blood cells

Human blood collected in citrate was obtained from a local hospital. The blood was centrifuged at $5900 \times g$ for 5 min at about 2° C. The plasma and buffy layer were carefully removed by aspiration. The packed cells were resuspended in 0.25 M sucrose/0.04 M Tris-HCl buffer (pH 7.4) and were washed by three cycles of centrifugation and resuspension in the buffered sucrose solution. The final suspension of cells was made at a concentration equivalent to about 45 mg protein per ml.

Assay methods

Changes in H^+ and K^+ activities were monitored by a pH electrode (Radiometer GK 2321 C) and a potassium-glass electrode (Beckman 39047). The signals were amplified by a pH meter (Radiometer PHM 28) and recorded continuously on a Sargent SRLG or Varian G14 Recorder. In the case of K^+ measurements, the initial signal was suppressed by a zero-voltage supressor connected to the recorder. Calibration of the electrodes was made by adding known amounts of either KCl or HCl at the beginning and the end of each run.

The 3 ml reaction mixture used in the K^+-uptake experiments contained 250 mM sucrose, 10 mM Tris-succinate (pH 7.2), 1.4 mM KCl, 10 mM Tris-acetate (pH 7.2) and 2.5 μM rotenone. Measurements of efflux of endogenous K^+ were performed in the same reaction mixture except for the absence of KCl.

The 6 ml reaction mixture used in the H^+-flux experiments contained 250

mM sucrose, 10 mM sodium succinate (pH 7.2), 9 mM sodium acetate (pH 7.2), 1.8 mM KCl and 5.8 μM rotenone.

All reactions were carried out at 23°C in a thermostatically controlled water-jacketed reaction vessel. The reaction mixture was stirred vigorously by a magnetic flea and each electrode was stabilized in the reaction mixture for several minutes before initiation of the reaction. Further additions (in microliter amounts) as well as the order of addition are indicated in the legends to each figure.

Oxygen consumption by mitochondria was measured polarographically with the Clark-type membrane-coated electrode (Yellow Springs, Model 53) connected to an SRG Sargent recorder. The reaction medium was identical to that used in the K^+-uptake experiments. Total volume was 3.0 ml and the temperature was 23°C. A dimethylformamide solution of DDT was added to the reaction medium containing 9.5—10.0 mg mitochondrial protein. Following a 0.5 min preincubation, state 4 respiration [5] was initiated by addition of Tris-succinate.

Protein was determined by using the method of Lowry et al. [6] using bovine serum albumin as standard.

The K^+ content of the mitochondria and red blood cells was determined on 10% trichloroacetic acid extracts using the Jarrell-Ash Atomic Absorptiometer.

Chemicals

Rotenone and valinomycin were purchased from Sigma Chemical Co. and added as ethanolic solutions. Albumin was obtained from Pentex Corp. All other reagents were of analytical grade. *p,p'*-DDT (purity greater than 99%) was obtained from Nagatuck Chemicals and only one peak was detected by gas chromatography. It was dissolved in either absolute ethanol or, where indicated, in dimethylformamide. Glass-distilled water, passed through a cartridge of a mixed ion-exchange resin (Rockford, IL), was used throughout.

Results

Valinomycin-mediated K^+ uptake

Preliminary experiments showed that the two carrier solvents, ethanol and dimethylformamide, at 1% (v/v) incurred zero inhibition, and at 3.3% about 5—10% inhibition of the rate and extent of valinomycin-mediated K^+ uptake and the associated H^+ efflux.

Since experiments were often performed over a period of 3 h, precaution was taken to correct for any loss of activity during this time period by interspersing several controls between the test runs. The decline in the K^+ uptake and H^+ efflux over this time span was about 5—10%.

The data in Fig. 1 (trace a) show that addition of valinomycin to mouse liver mitochondria oxidizing succinate produced a rapid uptake of K^+. The rate of uptake was dependent on the external KCl concentration (not shown, see also Ref. 7) and on the presence of a 'permeant' anion [7] which in these experiments was acetate. When varying concentrations of DDT were added to

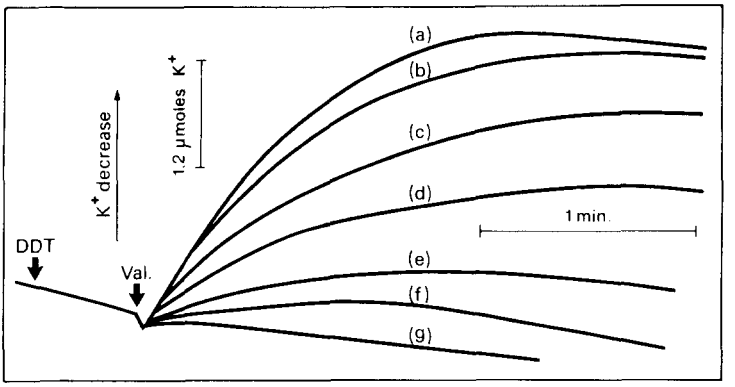
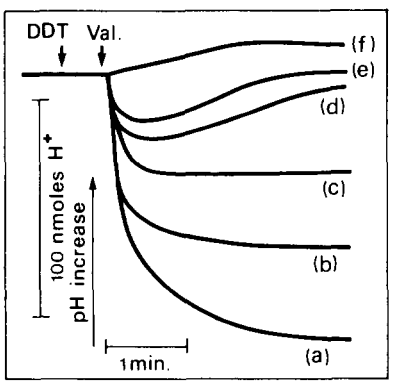


Fig. 1. The blocking of valinomycin-mediated K^+ uptake by DDT. K^+ uptake by mitochondria (6.7 mg protein) in the 3 ml reaction mixture (see text) was initiated by 1.0 μg valinomycin (Val) 0.5 min after addition of the following concentrations of DDT (nmol/mg protein): (a) none, (b) 43, (c) 65, (d) 108, (e) 151, (f) 194, (g) 323. The trace upward indicates uptake of K^+ by the mitochondria, i.e., decrease of K^+ in the reaction medium.

the reaction mixture about 0.5 min prior to the addition of valinomycin, both the rate and extent of K^+ uptake were progressively inhibited (Fig. 1, traces b–g). Complete inhibition was produced by about 194 nmol/mg protein (trace f). Higher concentrations (trace g) induced a slow efflux of endogenous K^+.

Carrier-mediated uptake of K^+ is associated with an efflux of H^+ (Fig. 2, trace a) at a ratio of 32 : 1 in these experiments. This is in line with the masking effect of H^+ efflux by permeant anions causing a deviation from the usual



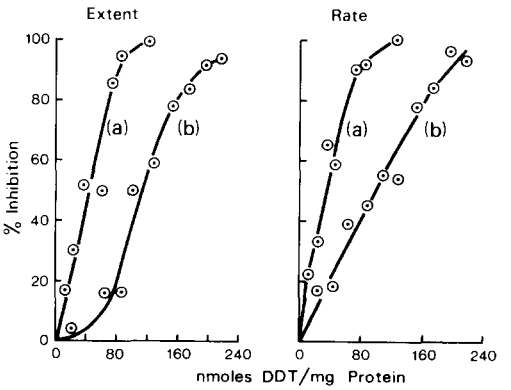


Fig. 2. The blocking of H^+ efflux by DDT. The H^+ efflux out of mitochondria (14.1 mg protein) in the 6 ml reaction mixture (see text) was initiated by addition of 2.0 μg valinomycin (Val). The downward trace indicates efflux of H^+ out of the mitochondria, i.e., decrease in pH of the reaction medium. DDT was dissolved in dimethylformamide and added at the following concentrations (nmol/mg protein): (a) none (control), (b) 50, (c) 66, (d) 83, (e) 108, (f) 176.

Fig. 3. Inhibition of the extent and rate of valinomycin-mediated K^+ uptake by DDT dissolved in (a) dimethylformamide and (b) absolute ethanol. The 3 ml reaction mixture (see text) contained 6.7 mg mitochondrial protein. K^+ uptake was initiated by 1.0 μg valinomycin. Rate = nmol K^+/min per mg protein; Extent = nmol K^+/mg protein.

stoichiometry of 1 : 1 [8]. Not suprisingly, the inhibition of K^+ uptake by DDT was associated with an inhihibition of extrusion of H^+ (Fig. 2 traces b—e). Complete inhibition was achieved by about 137 nmol/mg protein (trace f). It should also be noted that at higher concentrations of DDT (traces d and e) the acidification of the reaction medium was transient, suggesting movement of H^+ back into the mitochondrial matrix.

Optimizing the inhibition of K^+ uptake by DDT. (i) The effectiveness of DDT was dependent on the nature of the carrier solvent (Fig. 3). DDT dissolved in dimethylformamide produced a 50% inhibition of the rate and extent of K^+ uptake by about 40 nmol/mg protein. In contrast, in ethanol 100 nmol/mg protein were necessary to achieve this same level of inhibition.

(ii) Inhibition by DDT was a function of the preincubation time with the mitochondria. The preincubation was carried out in the reaction mixture containing all the components for varying times prior to initiation of the reaction by valinomycin. At a concentration of 90 nmol/mg protein, the inhibition of the extent of K^+ uptake increased from 35% after a 0.5 min preincubation to about 85% after a 5 min preincubation. The extent of K^+ uptake by controls (no DDT) preincubated under identical conditions was not affected. Preincubation of mitochondria with DDT also enhanced the inhibition of H^+ efflux. A 5 min preincubation with 54 nmol/mg protein completely abolished the release of H^+. In view of the lipophilic nature of DDT, these data suggest that the inhibition depends on the extent of partition of DDT into the lipid phase of the inner membrane.

(iii) The ratio of DDT to mitochondrial protein was also an important parameter determining the inhibition of K^+ uptake. The inhibition by 705 nmol DDT was reduced from 100% for 2 mg mitochondrial protein to about 20% for 9 mg mitochondrial protein.

Kinetics of inhibition. The inhibition of K^+ uptake was counteracted by increasing concentrations of K^+. The Lineaweaver-Burk plot (Fig. 4A) shows that DDT acts as a competitive inhibitor of K^+ uptake. The K_i value of DDT for K^+ uptake was about 7 nmol/mg protein. In contrast, this inhibition was not relieved by increasing concentrations of valinomycin. A Lineweaver-Burk plot (Fig. 4B) shows a change in the intercept on the $1/v$ ordinate without changing the slope of the lines, indicating a typical uncompetitive inhibition.

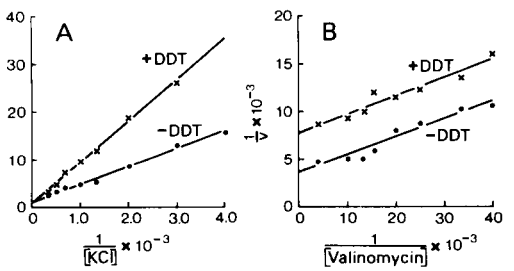


Fig. 4. Double-reciprocal plots of effect of DDT on the rate of valinomycin-mediated K^+ uptake. The reaction mixture (see text) contained 5.9 mg mitochondrial protein. The reaction rates were calculated over the 2 min period after initiation of K^+ uptake by valinomycin. The DDT dose was 15 nmol/mg protein. (A) Reaction initiated by 0.25 μg valinomycin; (B) KCl concentration was 1.4 mM.

Effect of temperature. The data in Fig. 5 show some interesting effects of temperature on the inhibition of K^+ uptake. Firstly, the rate of K^+ uptake was a linear function of temperature. No discontinuity was evident, indicating that the valinomycin-K^+ complex was mobile at temperatures below the lipid phase transition which for liver mitochondria occurs in the range of 8—23°C [9,10]. In contrast, the valinomycin-mediated K^+ conductance through bilayers of glyceryldipalmitate was abruptly obliterated below its transition temperature [11]. We assume that because of the heterogenity of the mitochondrial lipids, some fluid domains persist even below the bulk transition temperature. Secondly, although DDT reduced the rate of K^+ uptake, the temperature dependence was unchanged up to 20°C. Above 20°C there was a sharp increase in inhibition.

Efflux of K^+ from K^+-loaded mitochondria

The data in Fig. 6A show that the addition of DDT to K^+-loaded mitochon-

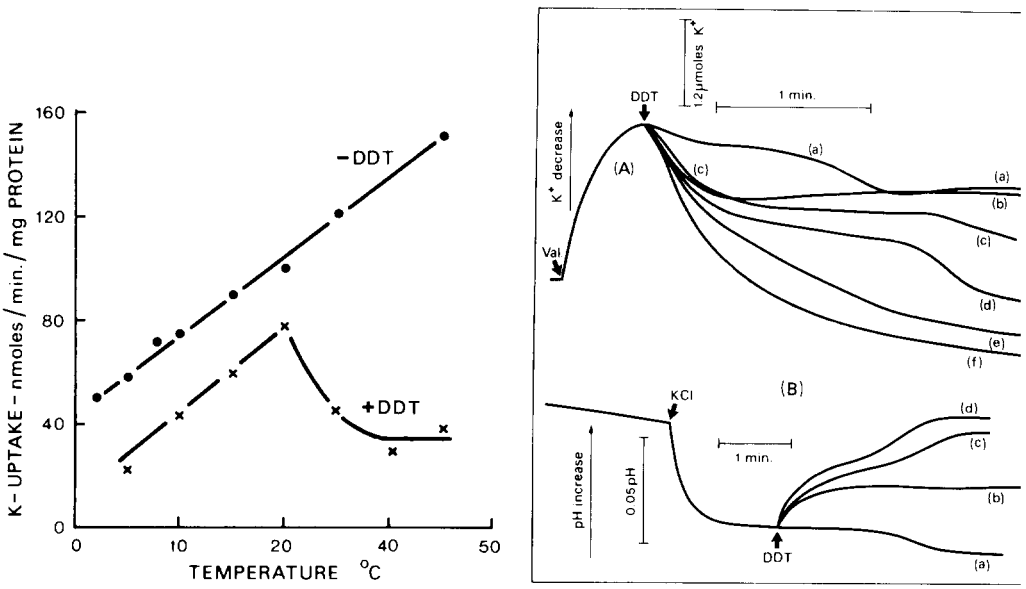


Fig. 5. Effect of temperature on DDT inhibition of valinomycin-mediated K^+ uptake. The reaction mixture (see text) contained 10.3 mg mitochondrial protein. The DDT dose was 33 nmol/mg protein. The temperature was monitored in the reaction vessel by a thermocouple probe connected to a digital thermometer (Cole-Palmer Intern., Chicago, IL).

Fig. 6. Effect of DDT on K^+-loaded mitochondria. (A) Efflux of K^+. Reaction medium (see text) contained 7.5 mg mitochondrial protein. K^+ uptake was initiated by 1.0 μg valinomycin. DDT in dimethylformamide was added at the indicated concentrations (nmol/mg protein): (a) none, (b) 23, (c) 47, (d) 70, (e) 94, (f) 117, The trace downward indicates an efflux of K^+ out of the mitochondria, i.e., increase in K^+ content of the reaction medium. Dimethylformamide added alone up to 35 μl, which was the maximum volume used in these experiments, had no effect. (B) Uptake of H^+. The 3 ml reaction medium contained 250 mM sucrose, 4 mM sodium succinate, 0.2 ml mitochondria (10.3 mg protein) in 0.04 mM Tris-HCl/0.25 M sucrose (pH 7.4) and 0.44 μg valinomycin. Total volume was 2.9 ml. K^+ uptake was initiated by addition of 2 mM KCl. The downward trace indicates a decrease in pH of the reaction medium due to efflux of H^+ in response to the valinomycin-mediated K^+ uptake. When K^+ uptake was complete, DDT dissolved in ethanol was added at the indicated concentrations (nmol/mg protein): (a) none, (b) 24, (c) 48, (d) 97.

dria produced a rapid efflux of K^+. Maximum rate of efflux was induced by about 120 nmol DDT/mg protein. Addition of dimethylformamide up to 1% (v/v), which was the maximum concentration used in these experiments, had no effect on the rate of K^+ release.

The extent of efflux was also dependent on the concentration of DDT (Fig. 6A). Low concentrations (traces b and c) caused an efflux of K^+ which reached a plateau equivalent to 40—50% of the accumulated K^+. As the concentration of DDT was increased (traces c and d), this plateau was followed by another burst of K^+ release. This stepwise release of K^+ was not evident at high concentrations of DDT (trace f) which released monotonically all the accumulated K^+ as well as an additional quantity which amounts to about 30% more than was accumulated. This additional amount probably represents the endogenous K^+.

Fig. 6B shows that the concomitant uptake of H^+ was also monotonic or biphasic depending on the DDT concentration.

Since the valinomycin-mediated uptake and retention of K^+ by mitochondria is an energy-dependent process [12], it was important to eliminate the possibility that these effects of DDT were related to its ability to inhibit the respiratory chain. This is unlikely since 96 nmol DDT/mg protein, a concentration which caused a complete inhibition of uptake (Fig. 3) or complete release of K^+ (Fig. 6), had only a weak inhibitory effect (approx. 30%) on state 4 respiration.

Release of endogenous K^+

The release of endogenous K^+ from mitochondria treated with DDT (Fig. 6A, trace f) was investigated in greater detail in this section. In order that this effect be maximized, KCl was omitted from the reaction mixture. Varying concentrations of DDT in a constant volume of dimethylformamide were added to the reaction mixture containing energized mitochondria (succinate + rotenone).

The rate of release of endogenous K^+ was increased from an endogenous rate of about 2 to about 30 nmol/min per mg protein in the presence of 106 nmol DDT/mg protein. This release was preceded by a lag period, the length of which was dependent on the DDT concentration. The activity-concentration curve showed no effect of DDT up to a concentration of about 40 nmol/mg protein. Above this concentration, the leakage rate increased progressively with increasing DDT concentrations reaching a maximum rate at about 160 nmol/mg protein. This concentration of DDT also caused the complete release of the endogenous content of K^+ (about 120 nmol/mg protein). The sigmoid nature of the concentration-activity curve suggests that a critical quantity of DDT must partition into the lipid phase of the inner membrane before the membrane is rendered permeable to K^+.

The data in Fig. 7 show the effect of temperature on the spontaneous and DDT-induced release of K^+. The spontaneous release of K^+ demonstrates a negligible increase with temperature. However, starting at 10°C, the DDT-induced efflux of K^+ was strongly enhanced by temperature. Since this coincides with the phase transition of mitochondrial lipids [9,10], we conclude that DDT is unable to stimulate K^+ transport across membranes, the lipids of

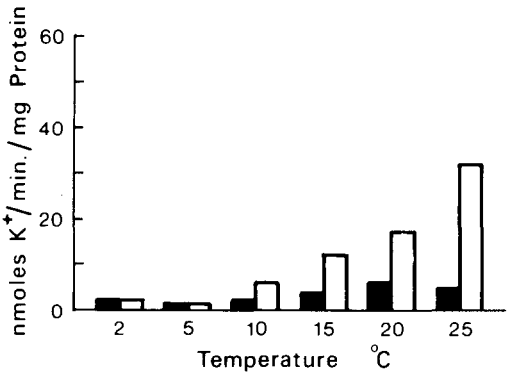


Fig. 7. Effect of temperature on the spontaneous and DDT-induced efflux of K^+. Mitochondria (5.3 mg protein) were added to the reaction mixture (see text) and the spontaneous efflux of K^+ was monitored for 1 min. DDT (132 nmol/mg protein) was then added and the efflux rate was calculated from the linear portion of the curve. Filled bars, spontaneous efflux; unfilled bars, DDT-induced efflux.

which are in the gel (solid) phase. This is further supported by the fact that although DDT at 23° C induced an efflux of the total K^+ content of mitochondria, erythrocytes which show no transition temperature [13] and have a higher order parameter [14] lost only 20% of their K^+ content.

Discussion

The results presented in this study show that DDT causes a redistribution of K^+ across the inner mitochondrial membrane either by inhibition of induced K^+ permeability or by activation of endogenous K^+ permeability. The inhibition of carrier-mediated K^+ transport is a consequence of competition with K^+ at some step in the transport system. The exact mechanism of inhibition is at present not clear. However, the findings presented here indicate competition for the same site(s) which bind K^+ in the transport process. On the other hand, the weakly acidic nature of DDT [15—17] may increase the positive charge of the membrane surface and so interfere with the formation of the valinomycin-K^+ complex. The increase in fluorescence of 8-anilino-1-naphthalenesulfonate following treatment of submitochondrial particles with DDT is probably a consequence of increased binding of the anionic dye to the more positive surface of the membrane (Hijazi, A.N. and Chefurka, W., unpublished observations).

A number of parameters determine the degree of inhibition of the carrier-mediated K^+ transport by DDT, presumably because they determine the concentration of DDT in the lipid phase. The increase of inhibition of K^+ efflux by DDT with increasing temperature may also be related to an increase in DDT concentration in the lipid phase as a consequence of increased fluidity of the lipids. It should be noted that these effects of DDT are most pronounced within the temperature range (10—25° C) that coincides with the reported transition range (8—23° C) for mitochondrial lipids [9,10]. Furthermore, several studies have reported that dipalmitoyl phosphatidylcholine dispersions show an increased binding capacity for small molecules through the

transition temperature [18,19] due to increased partition into the more fluid hydrophobic interior of the bilayer.

The observation that DDT had a negligible effect on the flux of K^+ across the erythrocyte membrane may be related to the highly ordered lipid phase, as suggested by the high order parameter and lack of transition temperature [13,14]. Since a fluid bilayer is obligatory for incorporation of DDT-like chemicals into lecithin vesicles [15,20], one would expect negligible partition of DDT into the lipid phase of the erythrocyte membrane. In contrast, partition of DDT into the fluid lipids of the mitochondrial membrane may further increase the disorder of the lipid phase, thus increasing it permeability to K^+. Alterations of the lipid phase have recently been invoked to explain the increased permeability of DDT-treated liposomes to electrolytes and non-electrolytes [21].

The DDT-induced permeability to endogenous K^+ would be expected to promote movement of K^+ down its concentration gradient and so remove positive charges from the matrix. Since under the conditions of these experiments, the opposing H^+ influx would only partially compensate for K^+ efflux ($K^+/H^+ = 32 : 1$), this DDT-induced movement of K^+ would be expected to hyperpolarize the mitochondrial membrane. The present study therefore throws into question the idea that the nervous system of invertebrates is the only target site in DDT poisoning.

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