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ROLE OF PHOSPHOLIPIDS IN THE DDT-INDUCED EFFLUX OF POTASSIUM IN HUMAN ERYTHROCYTES

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Incubation of human erythrocytes for 1–2 h at 37°C in a suspension of dipalmitoylphosphatidylcholine (DPPC) liposomes results in a phospholipid enrichment of erythrocyte membranes by 45–55% and a depletion of cholesterol by 19–24%. The enrichment by DPPC was time and concentration dependent. By contrast, dioleoylphosphatidylcholine (DOPC) liposomes were less effective in enriching the membranes with phospholipid and in depleting the membranes of cholesterol. Concomitantly, the DDT-induced efflux of K^+ was reduced in the case of DPPC-enriched erythrocytes but enhanced in DOPC-enriched erythrocytes. These results suggest that DDT partitions more readily into the unsaturated than the saturated phospholipids of the erythrocyte membrane. It is concluded that the extent to which DDT affects the flux of K^+ across the membrane is dependent on the fluidity of the lipid phase. We also report here a rapid method for cholesterol depletion of red blood cells in comparison to previously reported methods.

Introduction

Many persistent lipophilic pesticides, e.g., DDT, are present in the environment and though they occur at low levels [1] they may be potentially hazardous because of their accumulation through the food chain [2] and possible accumulation in the lipid phase of cellular and subcellular membranes of the target organ(s). That cell membranes may be sites of action of a variety of toxicants is being increasingly emphasized (see Ref. 3).

Although the mechanism of DDT action is as yet poorly understood, its lipophilic character makes action at the membrane level very likely as indicated by several studies. Following exposure to DDT, marine teleosts show osmoregulatory failure and birds produce thin egg shells, both being directly related to the inhibition of the $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase, respectively [4,5].

More recently, it has been reported that DDT increases the K^+ permeability of the mitochondrial membrane. By contrast, the permeability of the erythrocyte membrane to K^+ is relatively refractory to DDT [6]. This differential effect was ascribed to differences in fluidity of the lipid phase of these membranes. It is well documented that the lipid phase of the erythrocyte membrane is highly ordered [7,8] while that of liver mitochondria is fluid [9,10]. Since a certain degree of fluidity is essential for the partition of DDT-like compounds into lipid bilayers [11,12], the lack of effect of DDT on the permeability of erythrocytes might be due to the inability of DDT to accumulate in this membrane.

Preincubation of erythrocytes with phospholipid dispersions results in an alteration of the

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Abbreviations: DDT, 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane; DPPC, 1,2-dipalmitoyl-3-phosphatidylcholine; DOPC, 1,2-dioleoyl-3-phosphatidylcholine.

cholesterol:phospholipid mole ratio [13] of the erythrocyte membrane, thus most likely changing the fluidity of the lipid phase of the membrane. This approach provides us with an opportunity to examine more directly the possible relationship between erythrocyte membrane fluidity and changes in its permeability to K^+ induced by DDT.

In this paper, the effect of cholesterol depletion and phospholipid enrichment of the erythrocyte membrane by liposomes made from DPPC and DOPC on the DDT-induced permeability of erythrocytes to K^+ is studied.

Methods and Materials

Preparation of erythrocytes

Erythrocytes were obtained from a healthy human donor by venipuncture. 30 ml of fresh blood were centrifuged at 2000 rpm in a Sorvall GLC-1 centrifuge for 5 min. The serum and buffy layer were carefully aspirated off and the packed cells were washed twice with 90-ml portions of isotonic KCl/sucrose (110 mM KCl + 60 mM sucrose, pH adjusted to pH 7.4 with Tris) by alternate centrifugation and resuspension. The washed erythrocytes were suspended in the isotonic KCl/sucrose buffer to the original blood volume, i.e., hematocrit of about 50%. Since the intracellular K^+ concentration of red blood cells is 105 mM [14], our medium of 110 mM KCl + 60 mM sucrose would stabilize the erythrocytes against extensive passive efflux of K^+ into the suspending medium.

Preparation of liposomes

300 mg phospholipid (DPPC or DOPC) were dissolved under N_2 in 5 ml chloroform in a 25-ml round-bottom flask and evaporated to dryness in a Buchi Rotavapor to obtain a thin coating of phospholipid on the inside wall of the flask. Following the addition of 10 ml of the istonic KCl/sucrose medium, the flask contents were warmed to 60°C in a water bath and vortex mixed for 3 min. The flask contents were cooled at room temperature (approx. 23°C), flushed with N_2 and sonicated in an icebath in an MSE Ultrasonicator (Measuring & Scientific Equipment Ltd., London, U.K., at a resonating setting of 1.6–1.8 A) for five 3-min periods, each separated by 1–2-min intervals to avoid overheating. After each sonication period more N_2 was introduced into the flask. The sample was then centrifuged at $15000 \times g$ in a Beckman model L2-65 ultracentrifuge and the translucent supernatant was used as the liposome preparation.

Incubation of the erythrocyte preparation with liposomes

The erythrocyte preparation (30 ml) was divided into two equal portions in 50-ml ground-joint, glass-stoppered conical flaks. To one aliquot was added an equal volume of the isotonic KCl/sucrose buffer (as control), and to the other an equal volume of the liposomes prepared in the same buffer. The preparations were incubated at 37°C in a Dubonoff water shaker at 40 cycles/min. Aliquots of each incubation mixture were withdrawn at intervals of 2 h and washed twice with 0.25 M sucrose (pH 7.4) by alternate centrifugation and resuspension and finally suspended in 3 ml of buffered isotonic sucrose to a hematocrit of about 50%. Aliquots (0.1 ml) of both the control and test sample were immediately used for measurement of K^+ efflux, and for the assay of total K^+, protein and cholesterol and phospholipid content.

Assays

(a) K^+ efflux was monitored with a K^+-glass electrode (Beckman 39047) as described by Chefurka et al. [6]. The reaction medium consisted of 250 mM sucrose, 10 mM Tris-acetate (pH 7.4), 10 mM Tris-succinate (pH 7.4), 15 μg rotenone, and a 0.1 ml aliquot of the appropriate sample. The total volume was 3.0 ml and the temperature 23°C. The efflux of K^+ was initiated by DDT (1 mg in 50 μl dimethylformamide) and monitored for 3 min. The passive leakage of K^+ measured over the 3 min period prior to addition of DDT was very low ranging from 22 to 32 nmol/min or 22 to 32 mmol/h per l of cells which is in good agreemwnt with similar rates already reported [15]. The carrier solvent had no effect on the K^+ flux. Calibration of the electrode was made by adding known amounts of KCl at the end of each run.

(b) Total K^+ was determined on trichloroacetic acid extracts of samples using a Jarrell-Ash Atomic Absorption Spectrophotometer (Model Atomsorb), against appropriate K^+ standards.

(c) Protein was assayed by the Folin-Ciocalteau method [16] using bovine serum albumin as standard.

(d) Cholesterol and phospholipid content of the red blood cell preparation was assayed as follows. 0.5-ml aliquots of the preparation were added to 7 ml of chloroform/isopropanol (7:11) solvent mixture [17], in ground-joint, glass-stoppered and acid-washed tubes. After standing for 3 h with occasional shaking (or overnight in the refrigerator), 2-ml aliquots of the organic phase were evaporated to dryness in the Buchi Rotavapor or Speed Vac Concentrator (Savant Instruments, Hicksville, NY). Cholesterol was assayed by the clinical method using the Cholesterol Colorimetric Assay Kit with appropriate cholesterol standards. Phospholipid was assayed by the method of Bartlett [18], with appropriate DPPC standards.

Chemicals

Spectral grade chloroform was obtained from Fisher Scientific Co. (Toronto, Canada). DPPC was purchased from U.S. Biochemical Corp. (Cleveland, U.S.A.). DOPC was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). All phospholipids were stored in a freezer (−20°C) and used within 3 weeks of their receipt. The purity of each batch of phospholipid was checked on silicic acid-precoated TLC plates using chloroform/methanol/water as the solvent system. Since the samples showed only one spot they were used as received from the supplier. Cholesterol of the highest purity was purchased from British Drug Houses (Toronto, Canada). The Cholesterol Colorimetric Assay Kit was obtained from Diagnostic Chemicals Ltd. (Charlottetown, Canada) and used within 2–3 weeks of receipt. This freeze-dried assay mixture was reconstituted fresh before use. Other chemicals and solvents were of analytical grade and obtained either from Fisher Scientific Co. (Toronto, Canada) or Sigma Chemical Co. (St. Louis, U.S.A.). DDT was obtained from Nagatuck Chemicals (Elmira, Canada) and was checked for purity (at least 99%) by gas-liquid chromatography. All glassware was acid-cleaned followed by thorough washing with deionized distilled water.

Results

Table I gives a comparison of the cholesterol and phospholipid content of human red blood cells as determined by us and seven other workers [19–25]. When the literature results are converted to the same units (nmol/ml), our results are in good agreement with those reported previously.

Preincubation of erythrocytes with DPPC and DOPC liposomes

The data in Fig. 1 show that preincubation of erythrocytes for 1 and 2 h with DPPC liposomes results in a phospholipid enrichment and cholesterol depletion of the erythrocyte membrane. Optimum phospholipid enrichment of the erythrocyte membrane was produced by 10 mg DPPC liposomes/ml medium, the increase being 45 and 55% after 1 and 2 h, respectively. The cholesterol content of these erythrocytes was reduced by 19 and 24% after 1 and 2 h.

The cholesterol/phospholipid ratio of these preparations (Fig. 2) decreased up to 10 mg phospholipid/ml and then stabilized. At this optimum concentration of DPPC, the reduction in the cholesterol/phospholipid ratio after 1 and 2 h preincubation was 41 and 55%, respectively. In order to make the experimental results independent of the DPPC liposome concentration, we used liposomes at 30 mg/ml medium in all the following experiments.

TABLE I

CHOLESTEROL AND PHOSPHOLIPID CONTENT OF RED BLOOD CELLS

Reference	Cholesterol (μmol/ml)	Phospholipid (μmol/ml)	Cholesterol/phospholipid ratio
19	1.75	2.08	0.84
20	1.72	2.30	0.75
21	1.63	2.00	0.82
22	1.73	2.27	0.76
23	1.87	2.20	0.85
24	1.51	1.92	0.81
25	1.71	2.10	0.81
Present work [a]	1.71	2.13	0.80

[a] Mean of 10 different preparations over a 6 month period; each sample was assayed in triplicate.

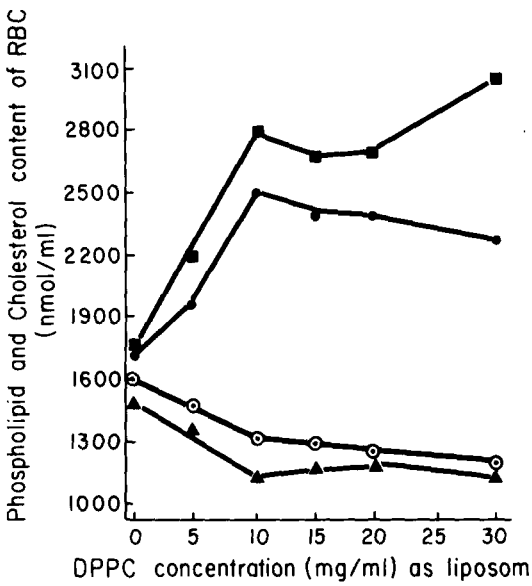

Fig. 1. The effect of liposome concentration on cholesterol depletion and phospholipid enrichment of erythrocytes: (■———■) phospholipid content afrer 2 h preincubation; (●———●) phospholipid content after 1 h preincubation; (⊙———⊙) cholesterol content after 1 h preincubation; (▲———▲) cholesterol content after 2 h preincubation. RBC, red blood cell.

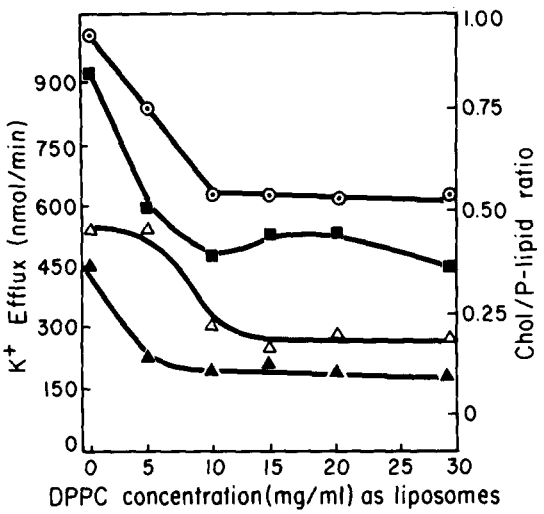

Fig. 2. Cholesterol/phospholipid (Chol/P-lipid) ratio and DDT-induced K^+ efflux as a function of DPPC liposome concentration. (⊙———⊙) cholesterol/phospholipid ratio after 1 h preincubation; (■———■) cholesterol/phospholipid ratio after 2 h preincubation; (△———△) K^+ efflux after 1 h preincubation; (▲———▲) K^+ efflux after 2 h preincubation.

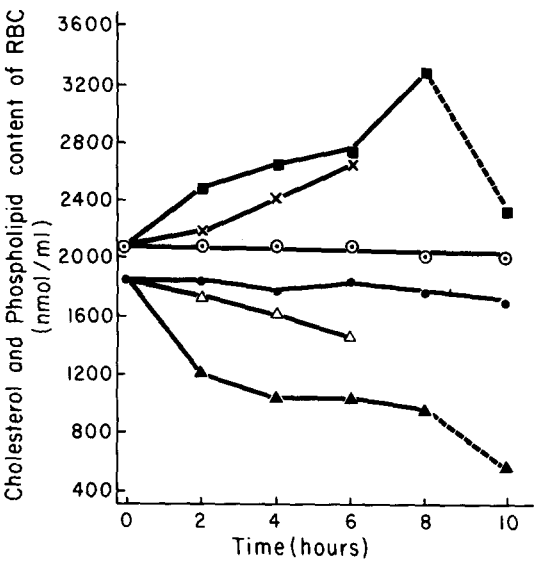

Fig. 3. Relative rates of cholesterol depletion and phospholipid enrichment of erythrocytes by DPPC and DOPC liposomes: (⊙———⊙) phospholipid content of controls; (×———×) phospholipid content of erythrocytes preincubated with 30 mg DOPC liposomes/ml; (■———■) phospholipid content of erythrocytes preincubated with 30 mg DPPC liposomes/ml; (●———●) cholesterol content of controls; (△———△) cholesterol content of erythrocytes preincubated with 30 mg DOPC liposomes/ml; (▲———▲) cholesterol content of erythrocytes preincubated with 30 mg DPPC liposomes/ml.

The data in Fig. 3 compare the relative capacity of DPPC and DOPC liposomes to enrich the erythrocyte membrane with phospholipid and deplete its cholesterol. Maximum enrichment of phospholipid by DPPC liposomes occurred after 8 h. Preincubation for 10 h resulted in a slight enrichment with phospholipid and a dramatic decrease in the cholesterol content. This may be related to the fact that this lengthy preincubation causes some hemolysis which may in an as yet unknown manner prevent loading of the membrane by phospholipid. By contrast, preincubation with DOPC liposomes caused both a lower enrichment with phospholipid and a lower loss of cholesterol than with DPPC liposomes.

The relative efficiency of the phospholipid enrichment and cholesterol depletion of the erythrocyte membrane by the two types of liposome is also seen in Fig. 4, where the cholesterol/phospholipid mole ratio is plotted as a function of time

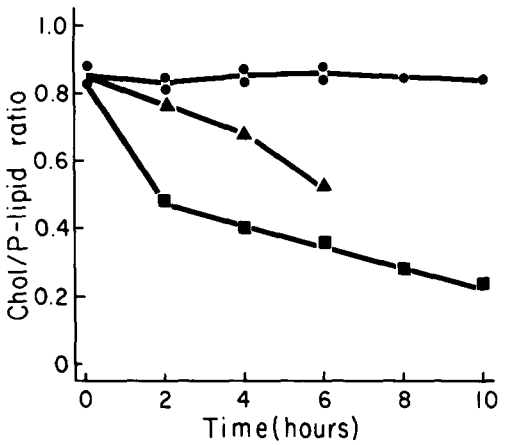

Fig. 4. Relative rates of cholesterol/phospholipid change of erythrocytes preincubated with 30 mg/ml of DOPC and DPPC liposomes: (●——●) control; (▲——▲) DOPC-treated erythrocytes; (■——■) DPPC-treated erythrocytes.

of preincubation. The cholesterol/phospholipid ratio of red blood cells that were preincubated with DPPC liposomes decreased 40 and 51% after 2 and 4 h preincubation, respectively. By contrast, pretreatment with DOPC liposomes for similar time periods reduced the cholesterol/phospholipid ratio by 7 and 18%. It appears that saturated phospholipids interact more rapidly than the unsaturated phospholipids with the erythrocyte membrane.

Table II gives a comparison of the extent of cholesterol depletion of red blood cells reported by eleven other workers [26–35], as compared to the rates found by us. All previous studies [25–34] have reported that a 30–35% and a 50% depletion of cholesterol of red blood cells required an incubation term of 6 and 10–16 h, respectively. By contrast, under the experimental conditions used

TABLE II

CHOLESTEROL DEPLETION OF RED BLOOD CELLS

References	% cholesterol depletion	Time required (h)	Medium used
26	33–39	6.5	Isotonic saline +Tris, pH 7.2
27	32–46	24	Phosphate-buffered saline, pH 7.4
28	29–34	6	Isotonic saline +Tris, pH 7.2
29	50	10	Phosphate-buffered saline, pH 7.4
30	28	14	Phosphate-buffered
	41	30	saline+antibiotics, pH 7.4
31	31	16	Serum (cholesterol
	46	25	free), pH 7.4
32	14	20	Saline/sucrose, pH 7.4
33	21	6	Isotonic saline+
	43	24	several salts, pH 7.2
34	50	24	Isotonic saline+ Tris, pH 7.4; outdated blood bank samples stored in citrate/dextrose bags
36	No depletion rate given		Serum albumin
	50	2.3	sonicate, pH 7.4
Present work [a]	35–38	2	Isotonic KCl/sucrose
	44–46	4	+Tris, pH 7.4,
	50–51	8	using DPPC liposomes

[a] Based on six different experiments; each sample assayed in triplicate.

by us, a 35–38% and a 50% depletion was achieved in 2 and 6–8 h, respectively. Clearly, the conditions used by us provide a rapid method for phospholipid enrichment and cholesterol depletion of erythrocyte membranes. The main difference in the experimental conditions used by us is the KCl/sucrose medium (110 mM KCl + 60 mM sucrose, pH 7.4, adjusted with Tris), which presumably enhances the rates of interaction and fusion of the erythrocyte membrane and the liposomes. Only two previous studies [35,36] have reported rates of cholesterol depletion similar to those reported here.

DDT-induced K^+ efflux

The data in Fig. 2 show that concomitant with a decrease in the cholesterol/phospholipid ratio of red blood cells pretreated with DPPC liposomes, the rate of efflux of K^+ induced by DDT was reduced, particularly for cells pretreated for 2 h. It is interesting to note that the rate of DDT-induced efflux of K^+ was not affected by pretreatment with 5 mg DPPC liposomes/ml for 1 h even though the cholesterol/phospholipid ratio was reduced by 15%. However, when the cholesterol/phospholipid ratio was reduced by 55% as a result of a 2 h treatment with 10 mg DPPC liposomes/ml, the DDT-induced K^+ efflux was suppressed by 57%. A regression analysis of the suppression of K^+ efflux vs. cholesterol/phospholipid ratio indicated a correlation coefficient (r) of 0.99 for 2 h preincubation. These data suggest that the ability of DDT to alter the permeability of the erythrocyte membrane to K^+ was reduced, probably because of its inability to partition into the highly saturated lipid phase of the enriched membranes.

The data in Fig. 5 show the relationship between the rate of DDT-induced K^+ efflux and the cholesterol/phospholipid ratio of red blood cells pretreated with DOPC liposomes (30 mg/ml at 37°C) for varying periods of time. While the rate of DDT-induced efflux of K^+ and the cholesterol/phospholipid ratio of controls (red blood cells pretreated in the KCl/sucrose medium) did not change significantly after 2 and 4 h preincubation, the rate of K^+ efflux induced by DDT from red blood cells enriched by a 4 h pretreatment with DOPC was increased by about 60%. This enhanced effectiveness of DDT to promote

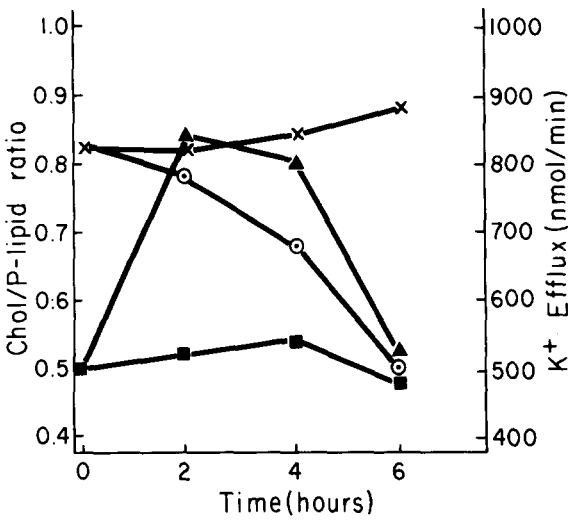

Fig. 5. Change in cholesterol/phospholipid (Chol/P-lipid) ratio and DDT-induced K^+ efflux of red blood cells: (× ——— ×) cholesterol/phospholipid ratio of control; (⊙ ——— ⊙) cholesterol/phospholipid ratio after preincubation with 30 mg DOPC liposomes/ml; (■ ——— ■) K^+ efflux from controls induced by DDT; K^+ efflux induced by DDT from red blood cells (▲ ——— ▲) preincubated with 30 mg DOPC liposomes/ml.

K^+ efflux was the result of a 17% decrease in the cholesterol/phospholipid ratio. Since the drop in cholesterol/phospholipid ratio reflects enrichment by unsaturated phospholipid as well as a small depletion of cholesterol, both events would tend to increase the fluidity of the membrane. Hence, these results suggest that increased fluidity enhanced the capacity of DDT to increase the membrane permeability to K^+. It is interesting, however, to note that folloqing prolonged pretreatment (6 h), the high permeability of the membrane to K^+ is reduced to near control levels. The reason for this decrease is at present not clear.

Discussion

The results presented in this paper show that the phospholipid and cholesterol content of the red blood cell membrane can be drastically changed by incubation with sonicated lipid dispersions. Although this method of cholesterol depletion of the erythrocyte membrane is now a routine procedure [13], the data presented here show that

under our experimental conditions levels of depletion which previosuly required 6 h or longer [26–34] were achieved in about 2 h. This study also provides evidence that conditions which lead to cholesterol depletion also enhance the phospholipid content of erythrocyte membranes. This is in agreement with the reports of Giraud and Claret [29] and Hope et al. [32] who used DPPC dispersions. It is, however, in contrast to several previous studies [30,33,36,37] which reported no phospholipid enrichment of erythrocytes when preincubated with egg lecithin or with DPPC. In view of the fact that our studies showed that DOPC was less effective than DPPC and that egg lecithin consists predominantly of unsaturated fatty acids [36], it is conceivable that the extent of lecithin unsaturation is an important factor in determining the extent of enrichment by phospholipids of the red blood cell membrane and may explain the lack of enrichment in those studies employing egg lecithin [30,37]. However, the reason for the discrepancy in those cases employing DPPC [31,36] is at present not evident.

The present study also established a strong correlation between saturation of the phospholipids of the membrane and the ability of DDT to increase its permeability to K^+. Erythrocyte membranes enriched with DPPC were much more refractory to DDT than were those enriched with DOPC. Since the fluidity of the lipid phase of the membrane is determined partially by the degree of unsaturation of the phospholipids, the results strongly suggest that DDT is less efficient in promoting the movement of K^+ across rigid (nonfluid) membranes. Presumably, this arises from the inability of DDT to partition into the nonfluid lipid phase. This is in line with reports that a certain level of fluidity is essential for DDT-like compounds [11,12] to partition into lipid bilayers. It is also in line with the fact that the DDT-induced K^+ efflux in the mitochondria is enhanced at temperatures that promote phase transition of the mitochondrial lipids [6,8,9]. We therefore conclude that an important parameter determining the response of the membrane to DDT is the degree of unsaturation of the enriching phospholipids and hence the degree of fluidity of the enriched membrane.

This correlation between fluidity and DDT action may have important physiological as well as toxicological consequences. As indicated in the Introduction, the dramatic insecticidal activity of DDT is well documented but poorly understood. The data presented here suggest that a partial basis for this activity may be the fluidity of the lipid phase of the sensitive target side.

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