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INTERACTION OF INSECTICIDES WITH LIPID MEMBRANES

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Summary

The permeability of liposome membranes is increased by organophosphorus and organochlorinated insecticides at concentrations of 10^{-5}—10^{-4} M. The order of effectiveness is similar to the toxicity of the compounds to mammals, and is the following for permeation of non-electrolytes and for valinomycin-induced permeation of K^+: parathion > 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT) ≈ aldrin >> malathion > lindane. The degree of effectiveness for X-537A-induced permeation of Ca^{2+} was the following: aldrin ⩾ DDT > parathion >> malathion > lindane. The organophosphorus compound, ethyl azinphos (10^{-4} M), dramatically increases the permeability of liposome membranes to all the tested substances, probably as a consequence of surfactant effects. Some organochlorinated insecticides appear to react with cation ionophores and modulate their motion across lipid membranes.

It is suggested that the insecticides may exert some of their toxic actions by modifying certain mechanisms in the cell membrane.

Introduction

In recent years, chemical insecticides have been very widely used for reasons of health and to aid in food production. However, new problems have arisen, in particular the appearance of resistant races of pests, toxic effects on animals and man, and persistance of pesticides in environment [1—3].

Persistance of organochlorinated insecticides in the environment is particularly marked. These compounds are concentrated in lipid systems of organisms [4] and may exert their toxic actions on peripheral and also on central nervous system [1,3] by modifying the normal nerve conduction. Analogs of DDT are

Abbrevations: aldrin, 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-*endo*-5,8-*exo*-dimethano naphthalene; azinphos (ethyl), *O,O*-diethyl-*S*-[4-oxo-1,2,3-benzotriazin-3(4H)-ylmethyl]-phosphorothioate; lindane, 1,2,3,4,5,6-hexachlorocyclohexane; DDT, 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane; malation, *O,O*-dimethyl phosphorodithioate of diethyl mercaptosuccinate; parathion, *O,O*-diethyl *O*-*p*-nitrophenyl phosphorothioate; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone.

known to alter the normal patterns of the action potentials by modifying the conductance of axon membranes to Na^+ and K^+ [5,6]. These effects of chlorinated compounds are sometimes temperature dependent, being favored by low temperatures [3,7]. Therefore, it seems that the interaction of these compounds with biological systems is of a physical nature.

Organophosphorus insecticides are much less persistent in the environment and biosystems than chlorinated compounds, and there is no evidence of accumulation in animal tissues [2]. These compounds, in oxon form, are powerful inhibitors of acetylcholinesterase [2,8,9] and cause disturbances at nerve synapses and neuromuscular junctions. However, certain toxic effects do not appear to be related to inhibition of acetylcholinesterase [2]. An additional toxic effect of organophosphorus compounds is chronic neurotoxicity due to unknown mechanisms.

Though the precise mechanisms of insecticide action are unknown, the available data are consistent with the idea that most of the compounds interact presumably with membrane processes.

The present work was directed towards the elucidation of the action of insecticides on basic membrane mechanisms. Evidence is presented that insecticides increase the permeation rates of lipid membranes to non-electrolytes and to cation-ionophore complexes.

Materials and Methods

(1) Preparation of liposomes. Liposomes were prepared from egg phosphatidylcholine, dicetyl phosphate and cholesterol in 50 mM choline chloride as described elsewhere [10]. The molecular ratio of liposome contents was 96% phosphatidylcholine (or 66% phosphatidylcholine plus 30% cholesterol) and 4% dicetyl phosphate. A thin film of the dried mixture was shaken with 50 mM choline chloride to obtain a suspension of about 23 mM in lipid. The liposome suspensions were let to stand for several hours at room temperature before use.

(2) Turbidimetry measurements. The osmotic swelling of liposomes was followed in thermostatted conditions in a Spectronic 20 spectrophotometer attached to a recorder. The change in turbidity was followed at 450 nm after adding rapidly 20 μl of liposome suspension to 3.0 ml of isosmotic solutions containing the substances whose permeation rates were to be studied. The contents were mixed very efficiently by a magnetic flea rotating at 400 rev./min. Temperature measurements were made in the cuvette with a thermistor probe and readings were taken to within 0.1°C. The insecticides and the ionophores under test were added from ethanol solutions. Control experiments showed that the amounts of added ethanol were without effect on the reactions.

This methodology can be used to estimate permeation rates of externally added substances since the lipid membranes are very impermeable to choline ions (cf. ref. 11) which are present in the inner space of liposomes.

(3) Measurements of proton production. Measurements of proton production were made from pH trace recordings in conditions stated in the legends of figures. In order to obtain accurate data, 60 μl of liposome suspensions were added to 3.0 ml of assay media.

(4) Reagents. The reagents used were of analytical grade. Egg phosphatidyl-

choline (type V-E), CCCP, valinomycin, cholesterol (pure standard) and dicetyl phosphate were obtained from Sigma. The insecticides were obtained from Supelco, Inc. The compound ethyl azinphos was purified from the commercial material by recrystallization twice from ethanol. The antibiotic ionophore X-536A was generously supplied by Dr. Julius Berger (Hoffman-La Roche).

Results

(1) Permeability of liposomes to non-electrolytes

Organophosphorus and organochlorinated insecticides increase the permeability of liposome membranes to urea and to erythritol (Fig. 1). The most effective insecticide was ethyl azinphos which induced high permeation rates at a concentration of 10^{-4} M. However, this effect may be a consequence of a surfactant action, since similar results were observed for electrolytes which normally do not permeate lipid membranes. Therefore, we shall not emphasize herein the effect of this insecticide.

Fig. 1 shows that parathion has an effect even at concentrations as low as 10^{-5} M, but the other organophosphorus compound, malathion, shows a very limited effect even at concentrations of 10^{-4} M. These effects parallel the relative toxicity of these compounds in mammals [1].

The chlorinated compounds, DDT and aldrin, exert measurable effects at

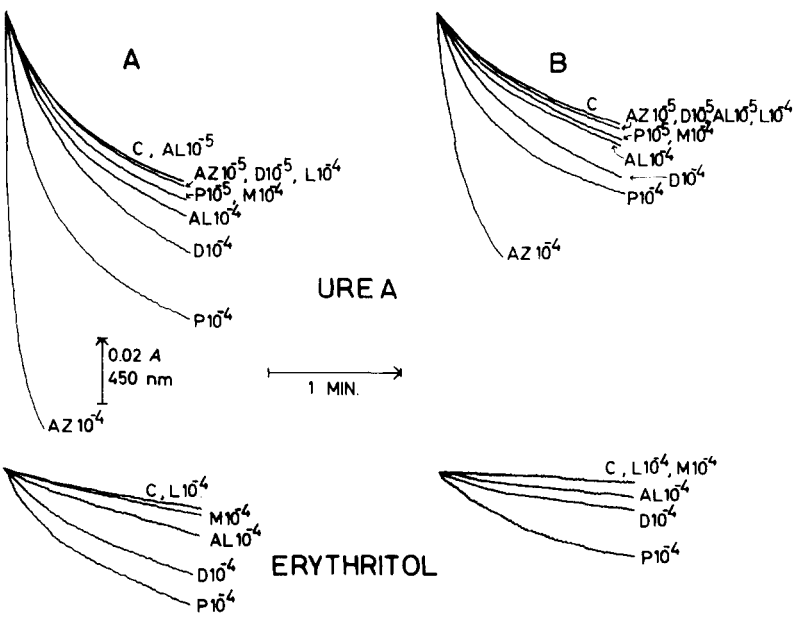

Fig. 1. Effect of insecticides on osmotic swelling of liposomes in isosmotic solutions of urea and erythritol. The insecticides were delivered from ethanolic solutions prior to the addition of liposomes. The amount of ethanol added (few μl) was without effect. The reactions were initiated by adding liposomes (20 μl) and changes in turbidity of the suspensions were continuously recorded at room temperature. A refers to normal liposomes and B to cholesterol-containing liposomes. Symbols: C, control; AZ, ethyl azinphos; P, parathion; M, malathion; D, DDT; AL, aldrin; L, lindane. The numbers refer to concentration (M).

concentrations greater than 10^{-5} M. In contrast, lindane has almost no effect at concentrations of 10^{-4} M. The order of effectiveness for the increase of permeation of non-electrolytes is parathion > DDT > aldrin >> malathion > lindane.

Fig. 1 also shows that liposomes containing cholesterol are less permeable than normal liposomes, in accordance with the results of other investigators [12,13]. However, the permeability of these liposomes is as affected by the insecticides under test as in liposomes without cholesterol.

The initial rates of osmotic swelling depend on temperature and linear Arrhenius plots were obtained. We consistently found that the incorporation of cholesterol in liposomes increases the energies of activation of urea permeation (Table I). These observations are not consistent with the conclusions of refs. 12 and 14, but we assume that the discrepancy is a result of the use of urea in this work rather than the use of polyols. Unfortunately, Arrhenius plots for the permeation of erythritol could not be elaborated, since the permeability of the liposomes used is very limited below 20°C and, thus, no accurate estimations of the initial rates of swelling could be made. Furthermore, both categories of insecticides, e.g. organophosphorus and organochlorinated compounds, lower the energies of activation of urea permeation by 4—8 kcal/mol in cholesterol-containing liposomes (Table I), but no significant effects were observed for control liposomes.

(2) Permeability of liposomes to electrolytes

The permeability of liposome membranes to electrolytes is rather low with the exception to ammonium acetate (Fig. 2). The insecticides under test increase the permeability of lipid membranes to this salt in a similar fashion as that described for non-electrolytes. Parathion appears to be the most effective compound in causing an increase of permeability. The effect of malathion is smaller than that of parathion and the chlorinated compounds, DDT and aldrin, have intermediate effects, lindane was almost without effect. Estimations of the energies of activation of the permeation process could not be made, since no linear Arrhenius plots could be obtained, presumably as a consequence of a complex permeation mechanism.

TABLE I

EFFECT OF INSECTICIDES ON THE ENERGIES OF ACTIVATION (cal/mol) OF INITIAL SWELLING RATES IN ISOSMOTIC SOLUTIONS OF UREA

The second number in each experiment refers to cholesterol-containing liposomes.

Experiment	Controls	Parathion (10^{-4} M)	Malathion (10^{-4} M)	DDT (10^{-4} M)	Aldrin (10^{-4} M)
1	12 888 18 900	9 140 10 785	13 393 13 527	—	—
2	10 877 17 711	—	—	10 237 13 829	—
3	13 041 17 941	—	—	—	13 436 14 245

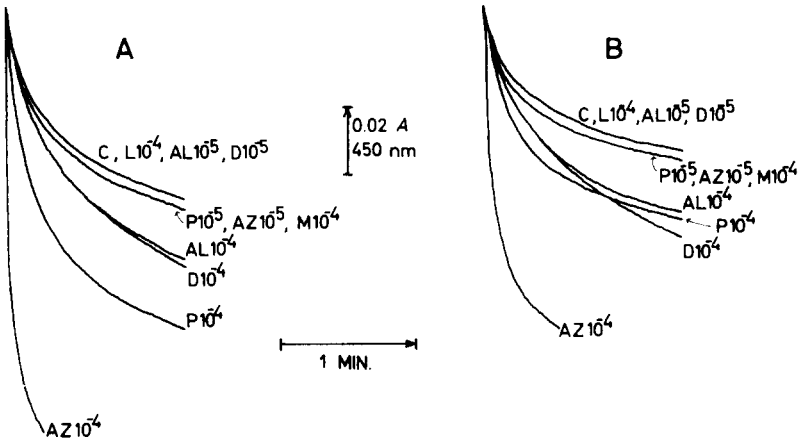

Fig. 2. Effect of insecticides on swelling of liposomes in isosmotic solutions of ammonium acetate. The experimental conditions were similar to those described for Fig. 1.

In accordance with previous work [11,15], we could only observe swelling of liposomes in isosmotic solutions of potassium acetate when a K^+ ionophore (valinomycin) and a proton carrier (CCCP) were added together. Neither valinomycin, nor CCCP alone could induce swelling of liposomes in potassium acetate media. Furthermore, ionophore-induced swelling was only observed in solutions of potassium acetate, but not in solutions of KCl (Fig. 3). Moreover, a net proton production dependent on pH of media, and, thus, on ΔpH across the membranes (pH of internal solution is 5.0), was observed in KCl but not in potassium acetate solutions. These findings suggest that K^+ exchange with protons and that acetate ions bind the protons released into the medium, thus, dissipating the proton gradient and the acetic acid so formed, passes the membranes. The overall effect of the process is the movement of K^+ and acetate ions into the inner aqueous space of the liposomes, thus causing osmotic swelling.

The insecticides under test exert significant effects on ionophore-induced swelling in potassium acetate solutions (Fig. 4) and the effectiveness of the

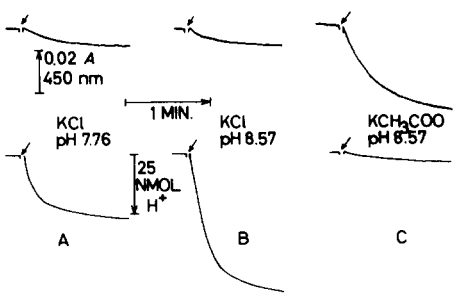

Fig. 3. Swelling of liposomes in isosmotic solutions of potassium salts (50 mM) and proton movements across the lipid membranes. Valinomycin and CCCP were added together (arrows) and the final concentrations were 2.4 and 13.3 μM, respectively. The upper traces are swelling recordings and the lower traces show proton movements. The reactions were carried out at room temperature. Internal solution of liposomes was 50 mM choline chloride at pH 5.0.

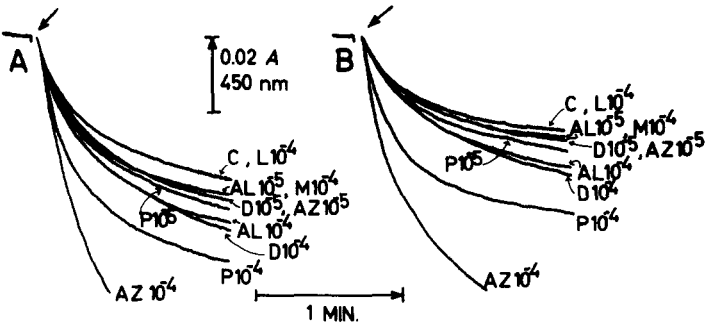

Fig. 4. Effect of insecticides on ionophore-induced swelling of liposomes in potassium acetate solutions. Swelling was induced by adding at arrows the ionophores CCCP (13.3 μM) and valinomycin (2.4 μM) to liposome suspensions containing the listed insecticides. The experimental conditions were similar to those of Fig. 1. The experiments with ethyl azinphos were particularly difficult to carry out, since the insecticide decreases the turbidity of liposome suspensions even in the absence of the ionophore.

compounds is similar to that described for non-electrolytes: parathion > DDT ≈ aldrin >> malathion > lindane. Furthermore, some of the compounds, viz. the organochlorinated insecticides DDT and aldrin, increase the energies of activation of carrier-mediated permeation of K^+ (Table II), suggesting a temperature-dependent interaction with the carrier system.

The effects of insecticides on osmotic swelling of liposomes in solutions of calcium acetate induced by the ionophore X-537A are summarized in Fig. 5. The mechanism of calcium acetate permeation into the liposomes is probably similar to that of potassium acetate, since X-537A act as exchanger for Ca^{2+} and protons.

In contrast to the results reported above, the chlorinated compounds, aldrin and DDT are the most effective and the relative effectiveness is as follows: aldrin ≥ DDT > parathion >> malathion > lindane. This different order of effectiveness may be a consequence of selective interaction of aldrin and DDT with the Ca^{2+}-ionophore complex which results in an increase of the permeation rate of the complex across the lipid membranes. However, the energies of activation of the permeation process are not significantly altered by these insecticides (Table III).

TABLE II

EFFECT OF INSECTICIDES ON THE ENERGIES OF ACTIVATION OF IONOPHORE-INDUCED INITIAL SWELLING RATES OF LIPOSOMES IN ISOSMOTIC SOLUTIONS OF POTASSIUM ACETATE

The second number in each experiment refers to cholesterol-containing liposomes.

Experiment	Controls	Parathion (10^{-4} M)	Malathion (10^{-4} M)	DDT (10^{-4} M)	Aldrin (10^{-4} M)
1	11 800 15 650	11 850 15 530	11 920 15 640	—	—
2	13 320 18 110	—	—	18 330 23 840	—
3	10 830 16 240	—	—	—	14 110 19 920

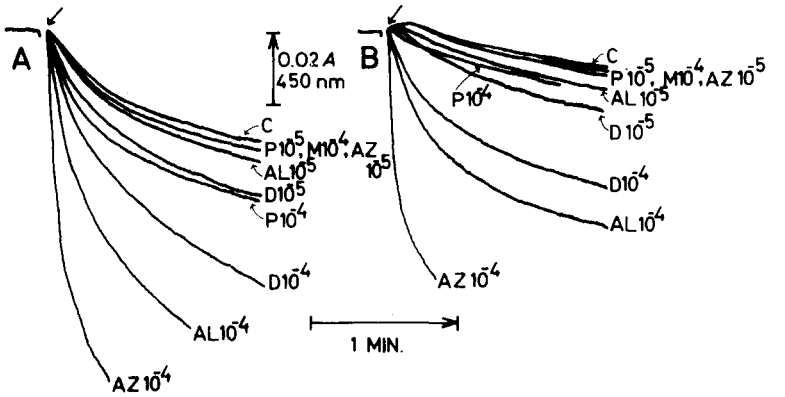

Fig. 5. Effect of insecticides on liposome swelling induced by X-537A in isosmotic solutions of calcium acetate. The experimental conditions were similar to those of Fig. 4. Swelling was induced by adding at arrows the ionophore X-537A (6.6 μM). As described in legend of Fig. 4, the experiments with ethyl azinphos were difficult to carry out.

TABLE III

EFFECT OF INSECTICIDES ON THE ENERGIES OF ACTIVATION ON X-537A-INDUCED INITIAL SWELLING RATES OF LIPOSOMES IN ISOSMOTIC SOLUTIONS OF CALCIUM ACETATE

The second number in each experiment refers to cholesterol-containing liposomes.

Experiment	Controls	Parathion (10^{-5} M)	Malathion (10^{-4} M)	DDT (10^{-5} M)	Aldrin (10^{-5} M)
1	17 520 17 730	18 110 17 340	—	—	—
2	17 810 17 720	—	18 120 17 320	—	—
3	15 420 18 210	—	—	17 330 18 320	—
4	14 720 15 640	—	—	—	17 100 16 410

Discussion

(1) Permeability of liposomes to non-electrolytes

Several investigators have shown that the permeability of lipid membranes to non-electrolytes is a function of the fluidity of membrane lipids [12—14]. The insecticides under test increase the permeability to urea and erythritol without apparent disruption of the lipid membranes, since the liposomes exhibit osmotic swelling in isosmotic solutions of non-electrolytes. Furthermore, no swelling was promoted by insecticides in solutions of non-permeant electrolytes such as salts of potassium, sodium and calcium. Furthermore, swelling in potassium and calcium electrolytes, was only observed in the presence of specific ionophores. Therefore, it appears that the insecticides do not induce detergent-like permanent damage of lipid membranes. Since it is rather difficult to assign carrier properties to the insecticides, it is suggested that the observed effects are a consequence of an increase of the fluidity of membrane lipids promoted

by the compounds. The results also suggest that the insecticides may perturb somewhat the interaction of cholesterol with other membrane lipids, since the energies of activation of urea permeation in cholesterol-containing liposomes are significantly lowered.

The detailed effects of insecticides on the physical state of membrane lipids are currently under study in our laboratory.

(2) Permeability of liposomes to electrolytes

The general behaviour of tested insecticides in the osmotic swelling of liposomes promoted by the ionophores is similar to that described for non-electrolytes and for "permeant" electrolytes such as ammonium acetate. Thus, it appears that the insecticides modify the permeability of the cation-ionophore complexes presumbaly by altering the fluidity of membrane lipids.

Surprisingly, the degree of effectiveness of insecticides on the permeation of Ca^{2+} promoted by X-537A is different from that described for K^+-valinomycin complex, ammonium acetate and non-electrolytes. Therefore, it is reasonable to suggest that the insecticides, DDT and aldrin, interact with the Ca^{2+}-ionophore complex.

The results obtained for energies of activation are difficult to interpret at the moment. The effects may be a consequence of temperature-dependent interactions of the insecticides with the membrane components and with the ionophores, occurring simultaneously. The results do not permit us to distinguish between the various components of interest. However, they provide evidence that membrane events depending on temperature are affected by the insecticides.

(3) Biological significance

The results permit us to assign to the insecticides general effects on membrane basic mechanisms, namely, permeability and interaction with transport mediated by simple carriers. Relatively low concentrations of the insecticides, varying from 10^{-5} to 10^{-4} M have significant effects. We realize that it is rather difficult to compare these effective concentrations with the doses reported to be toxic for rats and other mammals [1], since the partition of the compounds among the various biological systems is unknown. Furthermore, some insecticides accumulate preferentially in lipid domains of cells and organisms [3,4] so that the local concentrations near the membranes may be rather different than those obtained under the assumption of an homogeneous distribution of the compounds.

The available biochemical data about the mode of action of insecticides provide evidence that the organophosphorus compounds are potent inhibitors of acetylcholinesterase, but their delayed action, i.e. the chronic neurotoxic effect has not been yet explained. More and more, it is apparent that most of the compounds, organophosphorus or organochlorinated, interact with membrane events, including nerve conductance [5,6], plasma membrane [7,16,17] and organelle enzyme activities [18—22]. Furthermore, recent work provides evidence that organophosphorus insecticides, besides their typical action as inhibitors of acetylcholinesterase, they interfere as well with the allosteric behaviour of the enzyme through interaction with the membrane lipids [23]. Therefore,

the insecticides, besides particular modes of action, may exert their toxic effects at the membranes.

Our results provide evidence that insecticides modify basic membrane mechanisms, e.g. permeability to non-electrolytes and transport of cations mediated by ionophores. Furthermore, the effectiveness of the various compounds parallels quite well their relative degree of toxicity to mammals [1].

Studies of the effect of insecticides on membrane processes such us energetic transport of cations are under consideration for further studies.

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