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PARTITION OF PARATHION IN SYNTHETIC AND NATIVE MEMBRANES

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Partition coefficients of [^{14}C]parathion were determined in several types of membranes. Model membranes of egg phosphatidylcholine, dimyristoyl- (DMPC), dipalmitoyl- (DPPC) and distearoyl- (DSPC) phosphatidylcholines, and native membranes of mitochondria, sarcoplasmic reticulum, brain microsomes, myelin and erythrocytes were investigated. Parathion partition is variable among the membranes under study and depends on temperature and cholesterol content. First-order transition of membrane lipids from the gel to the liquid crystalline state is accompanied by a sharp increase in the partition coefficient of parathion. The insecticide is easily accommodated in bilayers of short-chain lipids, since the partitions were 1950, 650 and 270 in DMPC, DPPC and DSPC, respectively, at temperatures 10°C below the midpoint of their transitions. Preferential interaction with short-chain lipids promotes phase separation in heterogeneous bilayers, favouring segregation of lateral domains enriched in insecticide-phospholipid complexes. Cholesterol incorporation in membranes prevents the binding of the insecticide either through competition for similar interacting sites or as a consequence of changes in structural organization of phospholipids.

Introduction

Since World War II, parathion has been widely used in control of insect pests. Unfortunately, the benefits of its use were accompanied by undesirable toxic effects on useful insects, other animals and man [1–3]. Thus, the exact knowledge of the effects of the insecticide is an imperative task. Consequently, several attempts have been carried out to understand the physiological effects, but the precise biochemical mechanisms are not yet completely understood.

The organophosphorus insecticides are powerful inhibitors of acetylcholinesterase. This target is responsible for most of the physiological effects, e.g., hyperexcitability, convulsions and muscular paralysis, events which precede death in poisoned animals [2,4–7]. Additionally, other markers of toxicity (memory and visual disturbances, schizophrenia and depression) not related to the inhibition of acetylcholinesterase may chronically develop in poisoned individuals [2,7,8]. Effects of organophosphorus insecticides on other membrane enzymes have been reported [9–13], but the molecular mechanisms of action are unknown.

The above findings, the lipophilic character of most organophosphates and the dynamic functions of membranes led us to envisage biomembranes as candidates for target and sites of immediate and chronic insecticide action. Consequently, the interaction of the xenobiotics with biomembranes has been studied in our laboratory

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Abbreviations: Parathion, *O,O*-diethyl-*O*-*p*-nitrophenylphosphorothioate; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; T_m, midpoint temperature of thermotropic transition.

in the past few years [14–17]. Organophosphorus insecticides dramatically modify intrinsic interactions in model and native membranes; these molecular effects induce perturbations of membrane permeability and enzyme dynamics [14–17]. Among the compounds under study, parathion, the most powerful toxic, was also the most effective in inducing membrane perturbations.

To characterize further the nature of parathion interaction, we report here its partitioning in model membranes of egg phosphatidylcholine, synthetic lipids (DMPC, DPPC and DSPC) and several native membranes.

Though the observations in simple synthetic model lipids cannot be readily extrapolated to complex native membranes, they often facilitate the interpretation of results in clear physicochemical terms. Therefore, artificial membranes were used here as model operational systems.

Materials and Methods

Preparation of materials

Liposomes. Liposomes were prepared as previously described [15], except that buffer comprised 50 mM KCl/10 mM Tris-maleate (pH 7.0). Cholesterol-containing liposomes were obtained by supplementing original lipid solutions with appropriate amounts of cholesterol. After hydration, the final lipid concentration was 20 mM.

Native membranes. Mitochondrial fraction from rat liver was isolated according to Hogeboom [18]. Sarcoplasmic reticulum was prepared from rabbit white muscles as described by Carvalho and Mota [19]. Brain microsomes and crude myelin were obtained from sheep brain according to Hajós [20]. Ghost membranes from pig erythrocytes were prepared by the method of Buckley and Hawthorn [21]. Protein concentration was measured by the biuret method [22] using bovine serum albumin as standards. The membrane suspensions were rapidly frozen in liquid air and kept at −80°C till use.

Determination of partition coefficients

Incorporation of [^{14}C]parathion was determined by a filtration procedure in fibre-glass filters [23] and the filters were counted for radioactivity. The retention of membrane materials was always controlled by measuring the amount of phospholipid retained in filters. After filtration of a suitable amount of membrane suspension, the filters were treated at 180°C with 1.0 ml 70% $HClO_4$ [24] for 2 h. Released lipid phosphate was then measured according to Bartlett [25]. Generally, the amount of retained lipid was about 80% of the total filtered.

For studies of parathion incorporation, [^{14}C]parathion ($1 \cdot 10^{-6}$ M) was added to membrane suspensions in buffer (132 μM in lipid). After 4 h equilibration, aliquots (0.5 ml) were rapidly filtered through Whatman GF/B filters, under vacuum. The excess radioactive buffer in filters was washed out by filtering 5 ml of nonradioactive buffer. The filters were then transferred into vials containing 8 ml Triton X-100-supplemented scintillation fluid [26]. After several hours of equilibration, the radioactivity was counted in a Packard 460 spectrometer programmed for automatic quenching correction computed from an efficiency correlation determined for ^{14}C-quenched standards, by the external standardization method.

The partition coefficient was calculated from the fraction of insecticide retained in membranes (p) according to the equation [27]:

$$p = \frac{K_p(V_l/V_a)}{K_p(V_l/V_a)+1}$$

where K_p is the partition coefficient, V_l and V_a are the volumes of the lipid and aqueous phases, respectively. In our experimental conditions, the equation can be rewritten as:

$$K_p = \frac{p}{1.22L(1-p)} \cdot 10^6$$

where L is the amount of lipid in nmol.

Data were statistically analysed. The average of at least six independent measurements was calculated. Standard deviations were calculated with $n-1$ weight. Linear regressions were determined by the least-squares method and the correlation coefficients were calculated. Significance coefficient is defined as the square of correlation coefficient.

Thermotropic lipid phase transitions

Thermotropic transitions were estimated by turbidimetry, as previously described [15]. The

pseudo-absorbances of liposome suspensions (about 1 mM in lipid) were recorded at 430 nm in a set-up composed of a Spectronic 20 spectrophotometer (cell holder specially modified to permit thermoregulation) and a log recorder. Parathion was added at a final concentration of $1 \cdot 10^{-4}$ M.

Reagents

Egg phosphatidylcholine (type V-E), cholesterol, DMPC, DPPC, and DSPC, at least 98% pure, were obtained from Sigma. [*ring*-2,6-^{14}C]Parathion (21 mCi/mmol) was obtained from Amersham, International, U.K.

Results

Partition coefficients of [^{14}C]parathion in model membranes

The organophosphorus insecticide, parathion, concentrates 700–1000-fold in egg phosphatidylcholine bilayers relatively to the buffer phase, over the temperature range of 10–40°C (Fig. 1). Egg phosphatidylcholine bilayers remain in the fluid state over this temperature range, since the phase transition is centered at −5°C [28]. The partition of the insecticide decreases dramatically to about 50 in bilayers supplemented with 50 mol% cholesterol. This dramatic effect led us to study the effect of the relative concentrations of cholesterol incorporated in liposomes, as documented in Fig. 2. The partition coefficient of parathion decreases linearly with the molar ratio of cholesterol. This linear dependence is statistically significant (correlation coefficient −0.97), meaning, an absolute significance coefficient of 0.94. Extrapolation of the theoretical curve to abscissa predicts a zero partition at about 50 mol% cholesterol. Nevertheless, a limited amount of parathion incorporates in the membrane at this high cholesterol content. This 'residual' amount may be related either with unspecific absorption to lipid lamellae surfaces or may represent the compound entrapped between lamellae of multilayered liposomes used in this study. In any case, 'residual' binding does not represent filter adsorption which was conveniently controlled with appropriate blanks.

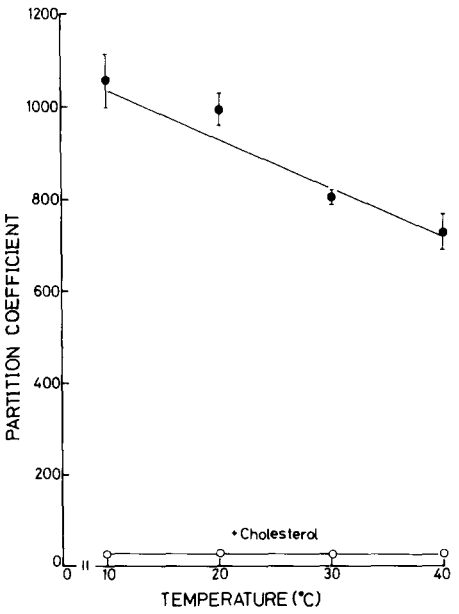

Fig. 1. Partition coefficient of parathion in egg phosphatidylcholine membranes, as affected by temperature. When the membranes contain equimolar mixtures of phosphatidylcholine and cholesterol (open symbols), the partition is dramatically decreased. Note that partition of parathion has a negative dependence on temperature. This dependence is not apparent when cholesterol is incorporated. The regression line was calculated by the least-squares method; each point represents the average of at least six independent measurements (vertical lines indicate ± S.D.).

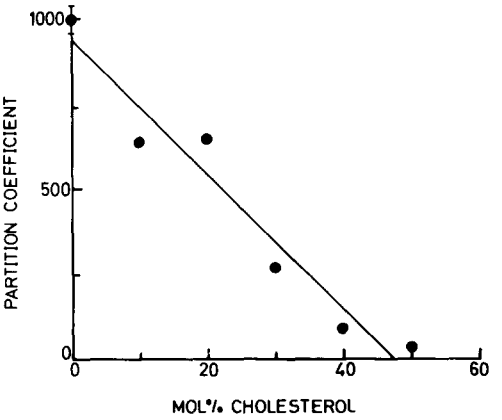

Fig. 2. Effect of cholesterol on partition coefficient of parathion in egg phosphatidylcholine liposomes. The regression line was calculated as described in Fig. 1. Except for control (zero mol% cholesterol), the standard deviations are within the space covered by the symbols. The correlation coefficient is −0.97. Note that, in theory, the expected partition would approach zero for about 50 mol% cholesterol (abscissa intersection).

In order to understand the dependence of partition coefficient on the fluidity and structure of lipid bilayers, studies have been carried out in liposomes reconstituted with well-defined synthetic lipid species, e.g., DMPC, DPPC and DSPC (Fig. 3). In all cases, it is clear that partitions of parathion are increased within the temperature range of cooperative transition from the gel to the liquid crystalline state. Within this temperature range, gel and liquid-crystalline domains coexist laterally in the bilayer and oscillation between the two phases is expected to occur at high rates [29,30]. Therefore, the phase oscillation appears to favour the incorporation of parathion.

Fig. 3 also indicates that parathion incorporates better in bilayers of short-aliphatic-chain lipids than in those prepared with long-chain species. Thus, maximal partitions obtained in DMPC, DPPC and DSPC bilayers were about 2200, 1000 and 500, respectively. Therefore, an increase in chain length by two carbon atoms results in a decrease of partition to about one-half. Since short-chain lipids produce more fluid membranes as compared with those formed with long-chain lipids [31], we are tempted to conclude that membrane fluidity is one of the factors which control the insecticide incorporation. However, other factors may as well contribute since, for DMPC, the partition is significantly increased in the gel state (10–20°C), as opposed to the fluid condition (30°C), by a factor of about 4.

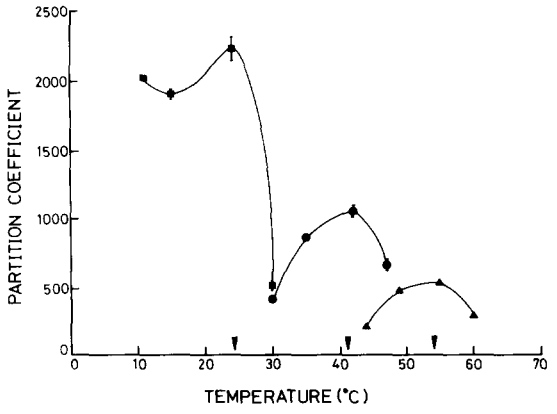

Fig. 3. Partition coefficients of parathion in synthetic bilayers of DMPC (■———■), DPPC (●———●) and DSPC (▲———▲). Note the significant increase of partition in the temperature range of thermotropic phase transition (T_m values for DMPC, DPPC and DSPC are 24, 41 and 54°C, respectively, as indicated by arrows).

Effect of parathion in thermotropic lipid phase transitions

Parathion displaces the midpoint transition temperatures (T_m) of bilayers reconstituted with several lipid species (Fig. 4; also see Ref. 15). When added to pure DMPC and DSPC bilayers (transitions centered at 24 and 54°C), the insecticide lowers T_m by about 5 and 3°C, respectively. This difference in effect is expected as a consequence of higher partition of the insecticide in DMPC bilayers.

Bilayers formed with binary mixtures of the two lipids (equimolar concentrations) exhibit a single phase transition within a temperature range comprised between those of pure lipids, i.e., centered at 42°C (Fig. 4). This transition is, however, broader than those of individual lipids, and the binary system tends to deviate from the behaviour of an ideal solution due to the difference in chain lengths by 4 carbon atoms [32]. Parathion converts the single transition of the binary system into a clear biphasic transition. In particular, the high temperature component is only slightly modified

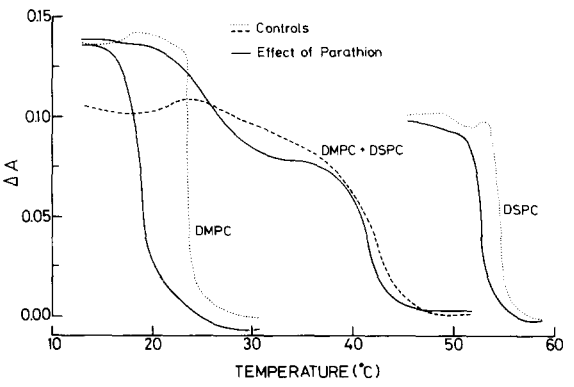

Fig. 4. Effect of parathion on thermotropic transitions of liposomes containing DMPC, DSPC or equimolar mixtures of the two lipid species. Parathion shifts the midpoint transitions for DMPC and DSPC by about 5 and 3°C, respectively. The insecticide induces a biphasic transition in mixed bilayers (DMPC + DSPC). The lower transition is centered at 26°C, a value close to T_m of pure DMPC. The higher temperature transition of the mixture (T_m = 42°C) is not significantly affected by parathion. The curves represent heating cycles. For the sake of clarity, experimental points (about 1°C intervals) were deleted.

upon addition of the insecticide, but a distinct transition centered at 26°C is now apparent (Fig. 4). This temperature closely approaches T_m of pure DMPC bilayers (24°C), but the new transition is relatively broader, beginning at about 22°C and ending up at about 30°C. Though less pronounced, a similar effect of parathion has been observed in binary mixtures of DMPC plus DPPC [15]. These effects are presumably a consequence of preferential partition of parathion in DMPC, thus disturbing the random lateral distribution of lipid species in the bilayer.

Partition of parathion in native membranes

Incorporation of parathion was studied in a variety of biomembranes isolated from cellular homogenates, namely, mitochondria, sarcoplasmic reticulum, brain microsomes, erythrocyte ghosts and myelin. Incorporation is limited in myelin as compared to brain microsomes. In its turn, partition in this fraction is lower than in sarcoplasmic reticulum and mitochondria, where the insecticide incorporates to a greater extent (Fig. 5). Data clearly show (Fig. 6) that partitioning of parathion decreases with the increase of the relative amount of 'native' cholesterol, similar to the observations found in artificial membranes reconstituted with synthetic lipids (Fig. 2). A linear relationship of partition decrease with increase in cholesterol is also apparent. Surprisingly, the determined correlation coefficient is also close to that found for synthetic membranes, i.e., −0.97. Therefore, cholesterol is the main regulator of insecticide partition in native membranes. Thus, myelin and erythrocyte membranes with high cholesterol contents (e.g., cholesterol/phospholipid molar ratios of 33 and 37 mol%, respectively) have limited and similar partitionings of about 60. Partition increases to about 100 in brain microsomes where cholesterol is less abundant (25%), and to 120 and 150 in sarcoplasmic reticulum and mitochondria, respectively, i.e., membranes with minimal cholesterol contents.

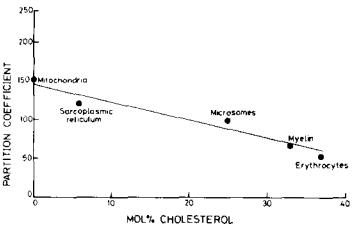

Fig. 6. Partition of parathion in native membranes as a function of intrinsic cholesterol content. These data were taken from Fig. 5 (partitions at 24°C). Regression line was calculated as described for Fig. 1. The correlation coefficient is −0.97. Intrinsic membrane cholesterol was determined in lipid extracts by the Lieberman-Bürchard technique as described elsewhere (Huang et al. [33]) and ratioed with the phospholipid content.

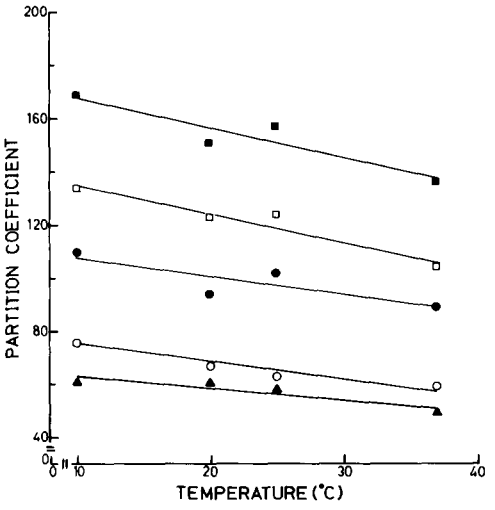

Fig. 5. Partition coefficients of parathion in native membranes. Regression lines were calculated as described for Fig. 1. ■, Mitochondria; □, sarcoplasmic reticulum; ●, microsomes; ○, myelin; ▲, erythrocytes.

Discussion

Accumulation of parathion in biological structures is often estimated on the basis of the classical octanol/water partitioning (141, cf. Ref. 34). Our data indicate that such estimations are very rough, since the actual partitions in membranes depend on several physical parameters and the type of biomembrane.

A consistent factor controlling incorporation of parathion is temperature. Generally, the partition decreases as temperature is increased. Since the major effect of temperature on membrane lipids is to decrease the structural order, it appears that parathion preferentially accommodates in ordered structures. On the other hand, data obtained with

synthetic phosphatidylcholines suggest that incorporation is improved in membranes of short-chain lipids, i.e., with higher degree of fluidity as compared with those formed with longer-chain species [31]. Apparently, as the length of fatty acid hydrocarbon chain increases, the voids which accommodate the insecticide decrease in number. Therefore, fluidity and membrane order may both play a role on parathion incorporation; however, the apparent contradiction arising from the effect of temperature and chain length suggests that other factors, indirectly related with these structural parameters, may also be important. Thus, the partition in DMPC is significantly increased in the gel state (temperature range from 10 to 20°C) relative to the liquid-crystalline state (30°C). Therefore, complementary fitness of insecticide accommodation is improved by short-chain lipids in the gel phase. In addition to membrane fluidity, geometrical factors imposed by molecular structure determine the partition of parathion.

Partitions of parathion in synthetic membranes are maximal within the temperature range of cooperative phase transition (Fig. 3). At the phase transition, gel and liquid-crystalline phases coexist and oscillate at a high rate. Ordering oscillation in adjacent phases may create transient defects between disordered and ordered domains [29,30] which would favour incorporation of extraneous molecules. Additionally, enhancement of lateral compressibility and extensibility at the phase transition [35,36] may also contribute to the increased incorporation of parathion.

Preferential accommodation of parathion in short-chain lipid bilayers explains the biphasic transitions induced in binary systems of DMPC plus DSPC (Fig. 4). This effect interpreted as a phase separation is substantiated by the finding that parathion preferentially interacts with DMPC. Some components of the mixture undergo a phase transition in a lower temperature range while other remain in the gel state. Since the lower temperature transition is centered at a T_m close to that of DMPC, it appears that parathion moves part of DMPC lipids into separate membrane domains, favouring lateral phase separation. In the temperature range of 30–35°C, the bilayer contains two separated domains, one containing fluid DMPC species associated with parathion and the other containing an insecticide-free solution of DMPC plus DSPC in the gel state.

These findings and the increased permeability of liposomes and erythrocytes promoted by parathion [14,16] are apparently in contrast with the conclusions of other investigators [12] who claimed that parathion decreases the membrane fluidity. However, these conclusions were derived from indirect evidence, i.e., *n*-values for allosteric inhibition of acetylcholinesterase and fluidity were not precisely defined. Hence, other physical parameters such as geometry, structure and molecular shape of lipids may as well contribute for the reported observations.

Cholesterol content of membranes also determines to a large extent the partition coefficient of parathion. Apparently, cholesterol prevents insecticide interaction, either by modifying the membrane structural organization or competitively opposing the entry of parathion. Cholesterol from 7 to 33 mol% progressively increases molecular order in the liquid-crystalline state [37–39]. At 33 mol% in DMPC or DPPC, observed broad transitions have been assigned to 'uncooperative melting' of loosely associated phospholipid with 1 : 1 cholesterol-phospholipid complexes [40]. This arrangement leaves little intermolecular void in the bilayer. At 50 mol%, there are strong interactions between all cholesterol and phospholipid molecules (1 : 1 binding stoichiometry), allowing for maximal van der Waal's contacts in the hydrocarbon region.

Our data summarized in Figs. 1 and 2 concur well with the above proposals. Indeed, membranes containing 33 mol% cholesterol only incorporate a small amount of parathion. Furthermore, extrapolation of the theoretical curve predicts a zero partition at 50 mol% cholesterol; strong interactions between sterol and phospholipids prevent void volumes for parathion accommodation. Therefore, it is concluded that the main effect of cholesterol is mediated by the alteration of the overall structure of the bilayer, rather than by a decrease in fluidity.

* Regarding parathion incorporation, native membranes behave similarly to liposomes. Dependence of partition on temperature also displays negative correlation. Similarly, linear decrease of insecticide partition is observed with the increase

of cholesterol molar concentration, with a correlation coefficient of 0.97. Perturbations induced by cholesterol in native membranes are presumably similar to those described for synthetic bilayers; therefore, incorporation of parathion in native membranes is probably conditioned by the long-range bilayer structure affected by cholesterol interaction. In conclusion, the cholesterol content is the main factor controlling partition of parathion in native membranes. Similar behaviour has been observed for other drugs [41] whose partition coefficients also decrease with the cholesterol content.

Variability of incorporation in native membranes indicates that distribution of parathion in poisoned organisms is presumably rather heterogeneous. According to the measured partitions in vitro, we are tempted to predict that the insecticide would preferentially accumulate in highly functional membranes of organelles, namely, mitochondria and microsomes. Incorporated parathion perturbs several basic mechanisms operating at the membrane level, as shown before for the increased permeability of liposomes and native membranes to nonelectrolytes and increased facilitated diffusion of electrolytes [14,16]. It was also shown that the insecticide perturbs the action of the Ca^{2+} pump of sarcoplasmic reticulum membranes by increasing its efficiency and overall activity [17]. Apparently, this effect may result from insecticide interaction with the Ca^{2+} pump boundary lipid; consequently, this would perturb normal lipid-protein interactions involved in modulation of enzyme activity. Likewise, similar effects are expected for other membrane enzymes, including acetylcholinesterase.

Therefore, the interaction of organophosphates with biomembranes must be considered in the process of toxic action of these compounds.

Acknowledgement

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