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Depth-Dependent Effects of DDT and Lindane on the Fluidity of Native Membranes and Extracted Lipids. Implications for Mechanisms of Toxicity

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In general the molecular mechanisms of insecticide action are poorly understood. Well-defined biochemical actions have been assigned only to organophosphorus and carbamate compounds as powerful inhibitors of acetylcholinesterase (Eto 1974; Doherty 1979). Organochlorine compounds of the DDT group may exert their actions by poisoning the axon membranes (Doherty 1979). These actions have been explained in terms of specific alterations of membrane permeability and conductance to the ions involved in axonic electrical events (Doherty 1979). Cyclodienes and lindane presumably act as presynaptic modulators of acetylcholine release (Doherty 1979). In addition to acute toxicity, insecticides can also cause chronic toxicity (Eto 1974) and, again, the molecular mechanisms of these effects are far from clear.

The above findings, the dynamic functions of biomembranes and the strong lipophilicity of most insecticides suggest that their effects, either acute or chronic, appear to be membrane connected. Consequently, the interaction of some popular insecticides with membranes has been studied in our laboratory in the past few years (Antunes-Madeira and Madeira 1979, 1982; Antunes-Madeira et al. 1980, 1981), as an attempt to understand the molecular basis of toxicity. To further characterize the membrane mechanisms affected by the insecticides, their partitions have been studied in model and native membranes (Antunes-Madeira and Madeira 1989a). Membrane fluidity and insecticide structure are main parameters affecting the partition. These findings and the postulated gradient of fluidity across the width of the bilayer (Chefurka et al. 1987) suggest different distributions of insecticides across the bilayer thickness and, consequently, different effects on membrane fluidity are expected (Antunes-Madeira and Madeira 1989b, 1990; Antunes-Madeira et al. 1990, 1991). In the sequence of the previous work, we compare the effects of DDT [2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane] and lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane) in the fluidity of native membranes and their extracted lipids probed by Py(3)Py [1,3-di(1-pyrenyl)propane].

MATERIALS AND METHODS

Various native membranes, namely, erythrocytes, brain microsomes, sarcoplasmic reticulum and mitochondria were prepared as described elsewhere (Antunes-Madeira and Madeira 1989a). Protein concentrations were determined by the biuret method (Gornall et al. 1949) calibrated with serum albumin. Membrane suspensions

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were rapidly frozen in liquid nitrogen and kept at - 80 °C until used. Membrane lipids were extracted according to Reed et al. (1960). Liposomes from membrane lipids were prepared by established procedures (Antunes-Madeira et al. 1980) except that the buffer contained 50 mM KCl and 10 mM Tris-maleate (pH=7.0). Preparation of liposomes with lipids from erythrocyte ghosts is facilitated at pH 8.5. In all cases, the final concentration of lipid used was normally 345 μM. Membranes and liposomes were briefly sonicated to get clear suspensions.

The incorporation of Py(3)Py was carried out as described previously (Almeida et al. 1982). Aliquots of an ethanolic solution of the probe (86 μM) were added to the membrane suspension (345 μM in lipid) to give a final lipid/probe molar ratio of about 900. The mixture was initially vigorously vortexed for 10 s and then incubated overnight. After the period of incubation with the probe, the insecticides were added from concentrated ethanolic solutions (50 mM). The period of equilibration varied from 1 to 2 h, according to the concentration used. It was ascertained that added concentrations of the insecticides were within the solubility range. Control samples received equivalent volumes of ethanol. Added solvent volumes always very small (few μl) had negligible effects in the measurements.

Fluorescence spectra were recorded in a Perkin-Elmer spectrofluorometer, Model MPF-3, provided with a thermostated cell holder. The temperature of the samples was checked to an accuracy of ± 0.1 °C with a thermistor thermometer. The excitation wavelength was 330 nm and the excitation and emission bandwidths were 4 and 6 nm, respectively. The intramolecular excimerization rate was evaluated as the excimer to monomer fluorescence intensity ratio obtained from the 490 to 378 nm signal ratio (I'/I), as previously described by Almeida et al. (1982). The excimer/monomer fluorescence intensity ratio, I'/I increases with membrane fluidity and decreases with membrane order. All the fluorescence measurements were corrected for the contribution of light scattering by using controls with membranes, but without added probe. Control experiments in the solvents alcohol and liquid paraffin, from 0 to 70 °C, have shown that no significant fluorescence quenching upon the fluorescence intensity ratio I'/I of Py(3)Py is exerted by DDT or lindane at concentrations used in this study.

The probe Py(3)Py was a gift of Dr. Zachariasse from Max-Planck--Institut für Biophysikalische Chemie, Göttingen, Germany. DDT and lindane (chromatografic grade) were obtained from Supelco.

Intramolecular excimer formation, resulting from association of an excited and an unexcited pyrene group belonging to the same molecule, may be described by the excimer to monomer fluorescence intensity ratio, I'/I (Almeida et al. 1982)

$$\frac{I'}{I} \approx \frac{K'_f}{K_f} \frac{K_a}{K_d + 1/\tau'_o} \qquad (1)$$

where K'_f, K_f, K_a and K_d are the rate constants for excimer fluorescence, monomer fluorescence, excimer formation and excimer dissociation, respectively, and τ'_o is the excimer lifetime in the absence of K_d. At relative low temperatures or at sufficiently high local viscosities, as in our membrane systems, $K_d << 1/\tau'_o$ (Zachariasse et al. 1980) and equation 1 can be written as follows:

$$\frac{I'}{I} \approx \frac{K'_f}{K_f} K_a \tau'_o \qquad (2)$$

Therefore, under our conditions, the fluorescence intensity ratio, I'/I is primarily determined by the rate constant of excimer formation, K_a, since the radiative constants (K'_f and K_f) and τ'_o have been found to be essentially independent of temperature (Zachariasse et al. 1980). Since I'/I of Py (3)Py has been shown to decrease with viscosity or to increase with fluidity, reflecting the rate of excimer formation (Zachariasse 1978), the technique is suitable to determine the fluidity degree of the probe environment in media such as membranes. To sum up, an increase in excimer formation (K_a) results in increased I'/I meaning an increase in membrane fluidity. Therefore, membrane fluidity increases with I'/I. This obviously means a decrease of viscosity, since fluidity is the reciprocal of viscosity.

The probe Py(3)Py is completely located inside the membrane away from the aqueous phase but not in the very hydrophobic core where other probes, such as DPH (1,6-diphenyl-1,3,5-hexatriene) distribute. The bispyrenyl propane probe is, therefore, displaced toward the polar head groups of phospholipid molecules, thus preferentially locating in the outer membrane regions (Almeida et al.1984). These regions, the so called cooperativity regions, i. e., the bilayer regions where the lipid packing is highly cooperative, are populated by membrane cholesterol (Oldfield and Chapman 1972). Therefore, cholesterol with its rigid planar structure, supresses bilayer cooperative phenomena with consequent modulation of fluidity.

RESULTS AND DISCUSSION

Representative native membranes, namely, sarcoplasmic reticulum, brain microsomes and erythrocytes and their lipid dispersions, differing in intrinsic cholesterol content were chosen to study the excimer to monomer fluorescence intensity ratio, I'/I, of Py(3)Py, over the temperature range from 10 to 40 °C (Figures 1, 2). Although in this range the native membranes are in the fluid state (Ohki 1988), the degree of fluidity depends on temperature and on the intrinsic cholesterol content (Figures 1, 2, solid lines). Membranes of sarcoplasmic reticulum with low cholesterol content (6 mol%) are significantly more fluid than those of brain microsomes and erythrocytes, where cholesterol accounts for 25 and 37 mol%, respectively. The degree of fluidity, in terms of I'/I ratio, follows the sequence: sarcoplasmic reticulum > brain microsomes > erythrocytes. Therefore, cholesterol orders fluid membranes at physiological temperatures according to several literature citations (Demel and Dekruyjff 1976). Although the membranes under study are on the fluid state, the temperature significantly affects the relative fluidity, since the ratio I'/I triplicates from 10 to 40 °C. Cholesterol is one of the major modulators of membrane physical properties and consequently may regulate the interaction of xenobiotics with biomembranes, namely insecticide compounds.

DDT and lindane (Figures 1, 2, dotted lines) induce ordering effects in fluid membranes such as sarcoplasmic reticulum and respective lipid dispersions, an effect identical to that observed in fluid systems of synthetic lipids without or with low cholesterol contents, i. e., less than 20 mol% (Antunes-Madeira et al. 1990a, 1991). Also in close agreement with the results obtained in models, DPH (1,6--diphenyl-1,3,5-hexatriene) does not detect any effect of DDT and lindane in fluid membranes (Antunes-Madeira and Madeira 1989b, 1990b). Apparently the fluid phase holds enough free volume to accommodate the insecticides without significantly disturbing the structure of the bilayer core where DPH distributes. A detailed examination of Figures 1 and 2 indicates that DDT, unlike lindane, induces ordering effects in cholesterol rich membranes and their extracted lipids. However, the magnitude of these ordering effects, evaluated by Py(3)Py, depends on the cholesterol content of the membrane and depends also on temperature. Therefore, the ordering effects of DDT are better noticed at high temperatures and at low

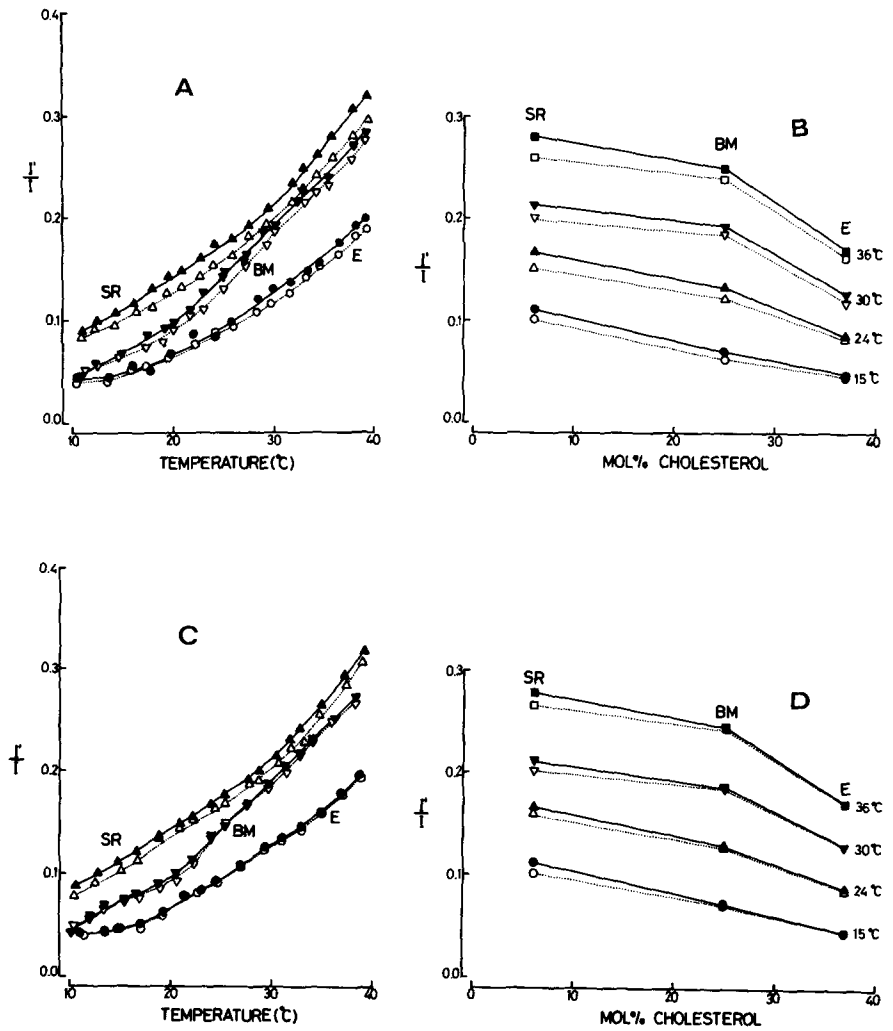

Figure 1.Intramolecular excimerization of Py(3)Py, i.e., excimer/monomer intensity ratio, I'/I, in native membranes as a function of temperature and intrinsic cholesterol content, in absence (solid symbols) and in the presence (open symbols) of 50 μM DDT (A, B) or lindane (C, D). Cholesterol/phospholipid molar ratios for sarcoplasmic reticulum (SR), brain microsomes (BM) and erythrocytes (E) are 6, 25 and 37 mol%, respectively. The small statistical errors for most experimental points fall within the size of the symbols.

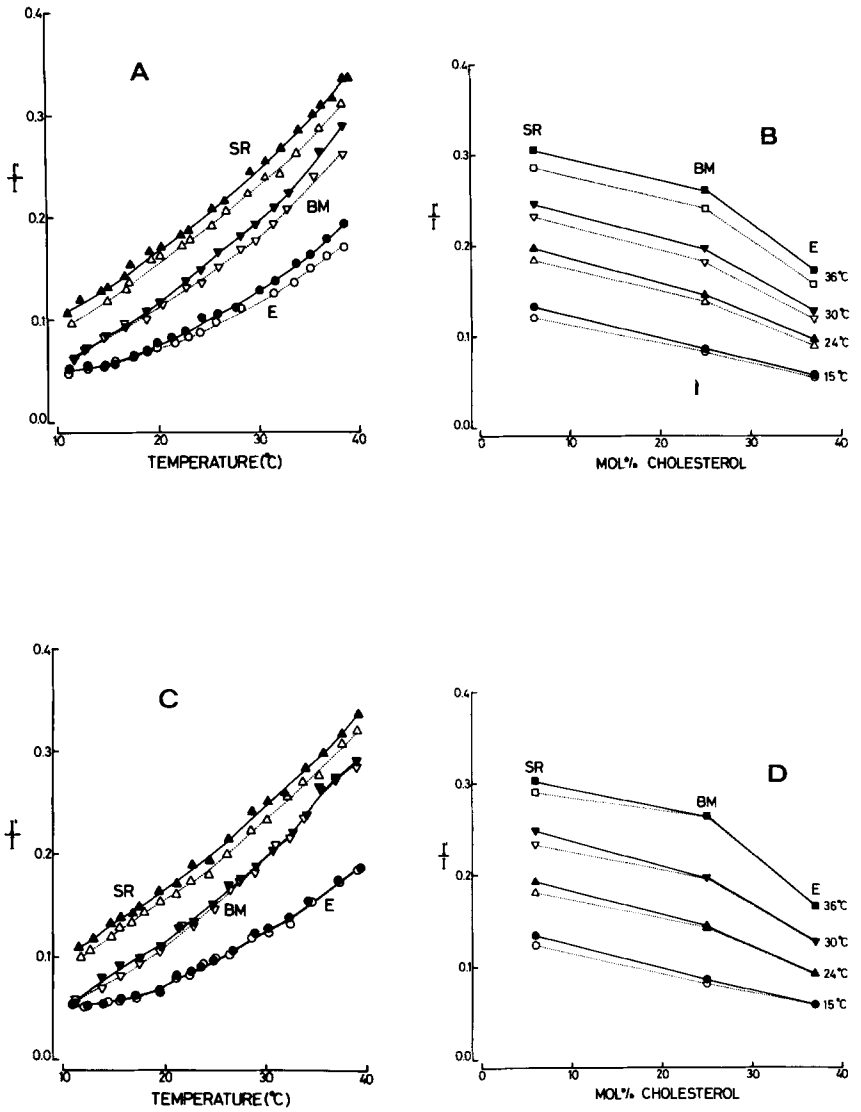

Figure 2.Intramolecular excimerization of Py(3)Py in total lipid dispersions of sarcoplasmic reticulum (SR), brain microsomes (BM) and erythrocytes (E) as a function of temperature and intrinsic cholesterol, in the absence (solid symbols) and in the presence (open symbols) of 50 μM DDT (A, B) or lindane (C, D).

cholesterol levels. In these conditions, the upper regions of the bilayer, where Py(3)Py apparently distributes, are more accessible to the insecticide. On the other hand, the increase in cholesterol and the decrease of temperature depress the condensing effects of DDT, probably as a consequence of a reduced accessibility of the insecticide to the cooperativity region of the bilayer. Additionally, for cholesterol levels as high as 37 mol% and for a temperature of about 15 °C, the condensing effects of DDT almost disappear, either in native membranes or in dispersions of extracted lipids as documented in Figures 1 and 2 (A, B). Conversely to DDT, Py(3)Py fails to detect any effect of lindane in cholesterol rich membranes, i.e., membranes of brain microsomes and erythrocytes and respective extracted lipids as indicated in Figures 1 and 2 (C, D). These findings closely agree with previous partition studies showing that lindane partitioning decreases linearly with the increase of intrinsic cholesterol. Actually, lindane partition would theoretically approach zero for about 47 and 61 mol% cholesterol in phospholipid dispersions and native membranes, respectively (Antunes-Madeira and Madeira 1985). The different behavior of DDT is related with significant DDT partition in cholesterol rich membranes. Cholesterol excludes DDT from bilayers less effectively than it does for lindane, as shown also in egg-lecithin liposomes enriched with cholesterol (Antunes-Madeira and Madeira 1989a). As previously postulated (Antunes-Madeira and Madeira 1989a), cholesterol presumably competes with lindane for identical interacting sites in the cooperativity region of the membrane. Such a competition with DDT is weaker owing the particular distribution of DDT in the bilayer core where it preferentially accommodates. Alternatively, similar effects would be produced if cholesterol could exclude DDT from the cooperativity region forcing it to distribute in the bilayer core, i.e., the domain of preferential accommodation. Also interesting is the fact that DDT, unlike lindane, induces moderate disordering effects into membranes of brain microsomes and erythrocytes, i.e., membranes with high cholesterol content, as detected by DPH (Antunes-Madeira and Madeira 1989b, 1990b). Clearly, the fluidizing effects of DDT do not correlate with the partition coefficients, ranging from 6×10^5 to 12×10^5, with the following sequence in the membranes under study: sarcoplasmic reticulum > mitochondria > brain microsomes > erythrocytes (Antunes-Madeira and Madeira 1989a). Although the incorporation of DDT in sarcoplasmic reticulum and mitochondria is significantly higher than in the other membranes, fluidizing effects, in the bilayer core, are noticed in the later but not in the former.

The experimental data in association with previous results (Antunes-Madeira and Madeira 1989b, 1990b; Antunes-Madeira et al 1990a, 1991) indicate that the localization of the insecticides across the bilayer thickness is a basic information to understand the effects in membrane fluidity. Considering very fluid membranes such as sarcoplasmic reticulum and mitochondria, the entire bilayer presents a high degree of fluidity although a fluidity gradient is still apparent (Chefurka 1987) with the interior of the bilayer more fluid than the outer regions. In these conditions, DDT would accommodate non selectively at any depth with a consequent wider distribution across the bilayer. Therefore, DPH, located in the very fluid bilayer core, detects no apparent effect of DDT but Py(3)Py displaced to the outer regions reports ordering effects of the insecticide due to an increase in packing density. As for DDT, only the upper region of the bilayer, less fluid, is affected by lindane, as detected by Py(3)Py. Disordering and ordering effects of DDT in cholesterol rich membranes and extracted lipids may also be understood on the basis of the fluidity gradient. Preferentially, the insecticide accumulates in the highly fluid hydrophobic core of the bilayer (Chefurka 1987) where the fluidity is further increased by its presence as probed by DPH (Antunes-Madeira and Madeira 1990b). The wave of perturbation propagates to the more ordered regions of the bilayer, presumably by weakening packing forces between lipid chains. Disordering effects generated

towards the upper region of the bilayer would promote a diffusion of DDT towards this region. Therefore, the cholesterol domains may be reached by DDT with consequent perturbation of phospholipid intermolecular contacts to accommodate cholesterol and DDT. Diffusion of DDT orders the upper regions close the phospholipid headgroups, as evaluated by Py(3)Py [Figures 1 and 2 (A, B)].

Data in native membranes and extracted lipids further support our previous hypothesis elaborated on the basis of studies with synthetic lipids (Antunes--Madeira et al. 1990a; Antunes-Madeira et al. 1991) that the differential effects of DDT and lindane on membrane fluidity depend on particular distributions across the bilayer thickness. The differential effects of DDT and lindane on membrane fluidity will certainly contribute for differential effects of these insecticides in membrane linked functions (Antunes-Madeira et al. 1981; Antunes-Madeira and Madeira 1982) and, consequently, for the specific toxicities (Metcalf 1971).

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REFERENCES

Almeida LM, Vaz WLC, Zachariasse KA, Madeira VMC (1982) Fluidity of sarcoplasmic reticulum membranes investigated with dipyrenylpropane, an intramolecular excimer probe. Biochemistry 21: 5972-5977

Almeida LM, Vaz WLC, Zachariasse KA, Madeira VMC (1984) Modulation of sarcoplasmic reticulum Ca^{++}-pump activity by membrane fluidity. Biochemistry 23: 4714-4720

Antunes-Madeira MC, Madeira VMC (1979) Interaction of insecticides with lipid membranes. Biochim Biophys Acta 550: 384-392

Antunes-Madeira MC, Carvalho AP, Madeira VMC (1980) Effects of insecticides on thermotropic lipid phase transitions. Pestic Biochem Physiol 14: 161-169

Antunes-Madeira MC, Carvalho AP, Madeira VMC (1981) Interactions of insecticides with erythrocyte membranes. Pestic Biochem Physiol 15: 79-89

Antunes-Madeira MC, Madeira VMC (1982) Interaction of insecticides with the Ca^{++}-pump activity of sarcoplasmic reticulum. Pestic Biochem Physiol 17: 185-190

Antunes-Madeira MC, Madeira VMC (1985) Partition of lindane in synthetic and native membranes. Biochim Biophys Acta 820: 165-172

Antunes-Madeira MC, Madeira VMC (1989a) Membrane partitioning of organophosphorus and organochlorine insecticides and its implications for mechanisms of toxicity. Pestic Sci 26: 167-179

Antunes-Madeira MC, Madeira VMC (1989b) Membrane fluidity as affected by the insecticide lindane. Biochim Biophys Acta 982: 161-166

Antunes-Madeira MC, Almeida LM, Madeira VMC (1990) Effects of lindane on membrane fluidity: intramolecular excimerization of a pyrene derivative and polarization of diphenylhexatriene. Biochim Biophys Acta 1022: 110-114

Antunes-Madeira MC, Madeira VMC (1990) Membrane fluidity as affected by the organochlorine insecticide DDT. Biochim Biophys Acta 1023: 469-474

Antunes-Madeira MC, Almeida LM, Madeira VMC (1991) DDT-membrane interactions studied with two fluorescent probes. Pestic Sci 33: 347-357

Chefurka W, Chatelier RC, Sawyer WH (1987) Perturbation of phospholipid bilayers by DDT. Biochim Biophys Acta 896: 181-186

Demel RA, Dekruyjff B (1976) The function of sterols in membranes. Biochim Biophys Acta 457: 109-132

Doherty JD (1979) Insecticides affecting ion transport. Pharmacol Therap 7: 123-151.

Eto M (1974) Organophosphorus pesticides:organic and biological chemistry. CRC Press, Cleveland, OH, pp 123-133

Gornall AG, Bardawill CJ, David MM (1949) Determination of serum proteins by means of the biuret reaction. J Biol Chem 177: 751-766

Metcalf RL (1971) The chemistry and biology of pesticides In: White-Stevens R (ed) Pesticides in the environment, vol 1. Marcel Dekker, N. Y., pp 67-124

Ohki K (1988) Ca^{++}- induced lateral phase separation in ternary mixtures of phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine inferred by calorimetry. J Biochem 104: 14-17

Oldfield E, Chapman D (1972) Dynamics of lipids in membranes: heterogeneity and the role of cholesterol.FEBS Letters 23: 285-297

Reed CF, Swisher SN, Marinetti GV, Eden EG (1960) Studies of the lipids of the erythrocyte. J Lab Clin Med 56: 281-289

Zachariasse KA (1978) Intramolecular excimer formation with diarylalkanes as a microfluidity probe for sodium dodecyl sulphate micelles. Chem Phys Lett 57: 429-432

Zachariasse KA, Kühnle W, Weller A (1980) Intramolecular excimer fluorescence as a probe of fluidity changes and phase transitions du phosphatidylcholine bilayers. Chem Phys Lett 73: 6-11

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