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Interaction of mercury chloride with the primary amine group of model membranes containing phosphatidylserine and phosphatidylethanolamine

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The effects induced by inorganic mercury (Hg(II)) on the thermotropic properties of model membranes composed of a series of different phospholipids (DPPC, EPC, DMPA, PS, DMPS, DPPG) and mixtures of EPC/DMPE 1:1 or DPPC/SA 1:1, were studied by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). Concentrations of 0.5–1 mM Hg(II) abolished the phase transitions of model membranes containing only phospholipids bearing a primary amine group as PS, DMPS or DMPE. The transition temperature (T_m) of the mixture DPPC/SA 1:1 (50.9 °C) was shifted, in the presence of 2.5 mM $HgCl_2$, close to the T_m of pure DPPC. These findings indicate that: (a) the electrical charge of the phospholipids is not involved in the Hg(II)–phospholipid interactions as at the pH values used (5.8 and 8.0) the lipids affected by mercury were, respectively, neutral (DMPE), negatively (PS, DMPS), or positively (DPPC/SA) charged; (b) the only chemical function which may constitute a binding site for mercury seemed to be the primary amine group common to all phospholipids and to stearylamine which are strongly affected by Hg(II). These Hg(II)–phospholipid interactions suggest new mechanisms for the toxicological effects of mercurials at the membrane level.

Introduction

The contamination of cells by mercury compounds may be described as a sequence of processes starting with interactions between the metal and some ligands of the cell membrane, followed by the transport across this biological barrier and reactions with different cytoplasmic and nuclear components.

The toxicological properties of inorganic and organic mercury compounds are strongly dependent upon their chemical speciation which, in turn, is a function of the pH, p*Cl* and the concentration of other ligands with which mercury may form complexes [1,2].

Mercury is well known to be highly specific for sulfhydryl groups, this property being responsible for its fixation on proteins, which are potential targets at the membrane or cytoplasmic level [3–5]. In this way, mercury may affect several membrane functions such as transport and permeability of different ions and metabolites, enzyme activities; mercurials may also act on some structural properties of cellular membranes including deformability, osmotic fragility or protein solubility (for review, see Ref. 6). On the other hand, a number of studies have stressed that the amino groups of the base moiety of the nucleic acids (adenine, guanine, cytosine) are potential binding sites for Hg(II) and methylmercury [7–12], which may explain the chromosomal damage and the genotoxicity induced by this heavy metal [13,14]. Some observations indicate also the involvement of nitrogen in amino acids as binding site for mercury compounds [15,16].

The role of the lipidic bilayer of biological membranes in the transport of mercury has been little investigated and the binding processes even less. Flux and electrical measurements, as a function of pH and chloride concentration, indicate a high permeability of in-

Abbreviations: DPPC, dipalmitoylphosphatidylcholine; EPC, egg phosphatidylcholine; DMPA, dimyristoylphosphatidic acid; DPPG, dipalmitoylphosphatidylglycerol; PS, phosphatidylserine; DMPS, dimyristoylphosphatidylserine; DMPE, dimyristoylphosphatidylethanolamine; SA, stearylamine; T_m, transition temperature; *P*, fluorescence polarization ratio.

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organic mercury and methylmercury through planar bilayer membranes, due essentially to the diffusion of the neutral chloride species ($HgCl_2$, CH_3HgCl) [17,18]. The overall permeabilities are not apparently influenced by the different phospholipid constituents of the bilayers but are affected by diffusion through the aqueous unstirred layers adjacent to the membrane [18]. Using fluorescence quenching by methylmercury of a hydrophobic probe embedded in lipid bilayers, Lakowicz and Anderson [19] concluded that lipid membranes are highly permeable to this mercurial compound but that its partitioning into bilayers is small. With the same technique, it was shown that the accessibility of inorganic and organic mercury to the hydrophobic core of model membranes is highly influenced by the chemical forms and species of the metal, the pH of the medium and the different lipidic constituents of the membrane [20].

If the transport of mercury across lipid bilayers is actually better understood, few data are available concerning the effects of this metal on the physicochemical and structural properties of model membranes.

By fluorescence polarization measurements, Bevan et al. [21] showed that, in the presence of inorganic mercury and at neutral pH, the phase transition temperature (T_m) of vesicles composed of a mixture of a zwitterionic phospholipid (DPPC) and a negatively charged phospholipid (PS) was slightly but significantly increased by about 2 C°, while the T_m of pure DPPC vesicles was unaffected. These results were interpreted as revealing an electrostatic interaction between divalent mercury and the acidic headgroups of PS molecules. However, these data do not agree with the very weak effect of inorganic mercury, in contrast with the strong effects of Ca^{2+}, Zn^{2+} and Cd^{2+} ions, on the surface pressure and potential of stearic acid monolayers [22]. It was proposed that the different chloride concentrations used and also the ability of the metal to interact with phospholipids or fatty acids may explain this discrepancy [21].

In this paper we present a study concerning the effects induced by inorganic mercury (Hg(II)) on model membranes composed of phospholipids bearing different headgroups, the thermotropic properties being followed by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH). We demonstrate that Hg(II) can abolish the phase transitions of the model membranes containing phospholipids that have a primary amine group on their polar heads, such as PS and DMPE. Other zwitterionic or acidic phospholipids (DPPC, DMPA, DPPG) are not significantly affected by the presence of mercury. These results suggest a specific interaction between this metal and the amino groups of phospholipids, indicating a new kind of metal–lipid membrane interaction, without a significant contribution from the well-known electrostatic forces. These interactions may have an important physiological and toxicological significance.

Materials and Methods

DPPC, DMPA, DMPE and SA were purchased from Sigma (U.S.A.); DPPG from Medmark (F.R.G.); DMPS from Avanti Polar Lipids (U.K.) and PS from Lipids Products (U.K.). EPC was prepared according to the method of Singleton et al. [23] and DPH was obtained from Aldrich Chemical Co. (U.S.A.). Mercuric chloride was from Merck (F.R.G.). All reagents for buffer preparation (CH_3COOH, KH_2PO_4, NaOH) were divalent cation free (Prolabo, France) and deionized water (Millipore MQ system, U.S.A.) was used.

Sample preparation

For each series of experiments a stock of lipid dispersion in adequate buffer was prepared by vortexing the lipid (5 mg/ml) and the fluorescent probe DPH (10 μl of a stock solution $6 \cdot 10^{-3}$ M in tetrahydrofuran), above the transition temperature T_m of the lipids. For pH 4.0 to 6.0, CH_3COOH-NaOH buffer was used and for pH 6.0 to 8.5, KH_2PO_4-NaOH buffer, as these buffers do not bring chloride ions in the medium. Organic buffers, like Tris or Hepes, were not used as they strongly bind Hg(II) [24]. The pH was controlled before and after each fluorescence polarization measurement. Aliquots of a phospholipid-DPH stock solution were diluted in the appropriate buffer with or without inorganic mercury, the final volume being 3 ml and giving a lipid concentration of $2 \cdot 10^{-4}$M. The total mercury concentration (mM) in the different buffers was routinely checked by Atomic Absorption without flame (Varian spectrophotometer AA 475).

The DPH/lipid molar ratio did not exceed 1%, in order to minimize perturbations due to the fluorescent probe embedded in the bilayers. The final samples were homogenized above the T_m of the lipids. Control experiments indicated that an average time of incubation of three hours above T_m of the lipids is necessary in order to reach the equilibrium between Hg(II) and the phospholipid dispersions. The preparation of DMPE/EPC 1:1 and DPPC/SA 1:1 mixtures were obtained by co-lyophylization.

Fluorescence polarization measurements

The fluorescence polarization of DPH ($P = (I_v - I_h)/(I_v + I_h)$) was determined on a purpose-built entirely computerized spectrometer. The excitation wavelength was fixed at 360 nm and I_v and I_h intensities were observed simultaneously. Each data point was the average of ten measurements. The sample was cooled through the T_m, the temperature of the cuvette holder being regulated with thermoelectric heat pumps (±0.1 C°) and the cooling rate monitored automatically (30

C°/h). The transition temperatures T_m were well defined as the maxima from computer obtained derivatives of the temperature-dependent fluorescence polarization curves. We have checked by spectrofluorimetry (SLM 8000, Urbana, U.S.A.) that increasing concentrations of mercury had no effect upon the steady-state fluorescence intensity of DPH-labelled vesicles.

Results

Inorganic mercury, in a range of concentration between 0.05 mM and 2.5 mM, at pH 5.0 and 8.5, has no significant effect on the fluorescence polarization of DPH in vesicles of a synthetic zwitterionic phospholipid. Fig. 1 shows that identical fluorescence polarization curves are obtained from DPPC vesicles in the presence and absence of 2.5 mM of Hg(II), at pH 5.0, in

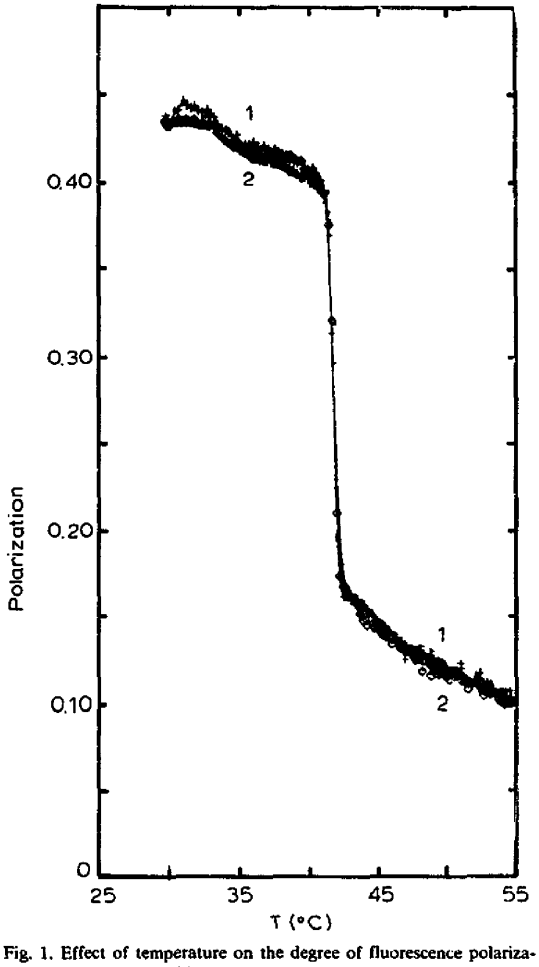

Fig. 1. Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of DPPC (0.2 mM), in the presence of $HgCl_2$, at pH 5.0 (acetate buffer). +, curve 1, control; ◇, curve 2, $HgCl_2$ 2.5 mM.

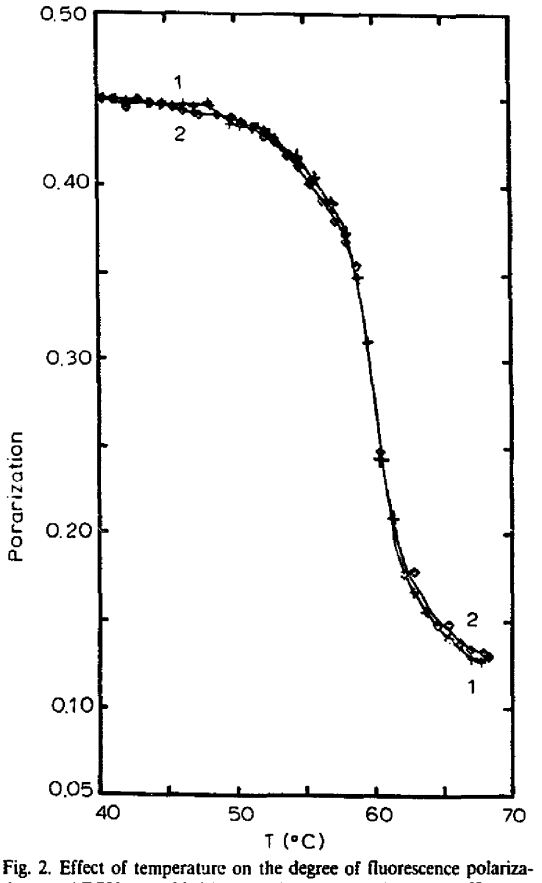

Fig. 2. Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of DMPA (0.2 mM), in the presence of $HgCl_2$, at pH 7.8 (phosphate buffer). +, curve 1, control; ◇, curve 2, $HgCl_2$ 2.5 mM.

all temperature ranges, in agreement with data published by Bevan et al. [21]. The same experiments done with the natural phospholipid, EPC, also reveal identical polarization curves in the presence and absence of the metal (data not shown).

The lack of effect of mercury, at both pH values already mentioned, is also observed in the thermotropic behaviour of a negatively charged phospholipid, DMPA, with a pK of about 3 [25], the T_m in the absence and presence of 2.5 mM Hg being 60.0°C (Fig. 2). With another negatively charged phospholipid, DPPG (pK = 2 [25]), inorganic mercury induces at 2.5 mM and pH 5.0 a very weak shift of the transition temperature which increases from 42.3°C to 43.4°C (Fig. 3). At pH 8.5, the metal no longer acts significantly on the transition temperature of DPPG (data not shown).

In contrast with the results described above, the effect of inorganic mercury on the thermotropic behaviour of PS, another negatively charged phospholipid

($pK = 3.7$ [26]), is very strong. In the experimental conditions used, as pH 5.8, the phase transition temperature of PS is quite smooth and centered at 18.7°C. As shown in Fig. 4, the metal (0.5 mM) induces an almost total disappearance of the lipid phase transition with an important increase in the degree of fluorescence polarization at temperatures above the T_m and a decrease of P at temperatures below the T_m. With variable Hg(II) concentrations, a small but significant effect is already observed at 0.05 mM (Fig. 5). The transition temperature vanishes above 0.5 mM of mercury but it should be stressed that the progressive disappearance of the phase transition occurs without a significant shift of the phosphatidylserine T_m (Fig. 5). The plot of changes of the DPH polarization P in the fluid phase (35°C), as a function of the mercury concentration (inset Fig. 5), indicates a saturation for metal concentrations > 0.5–1

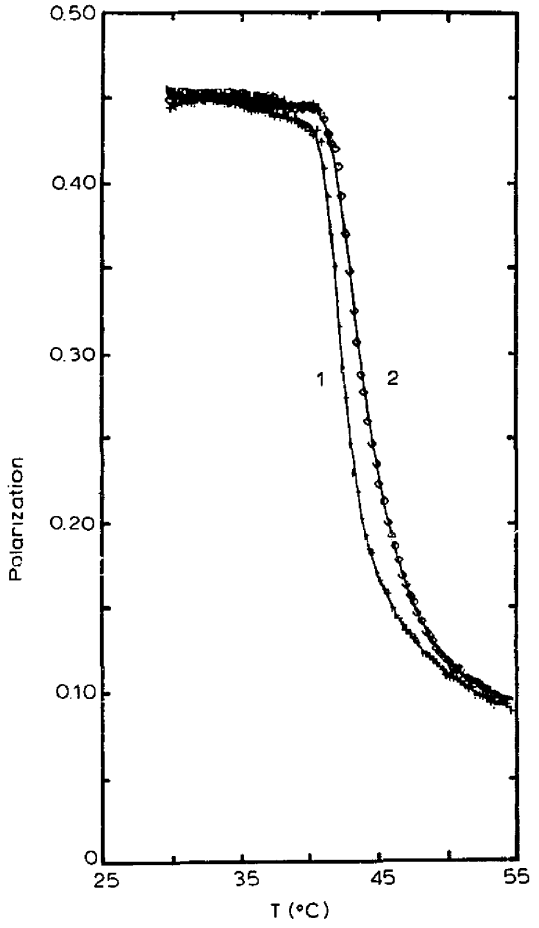

Fig. 3. Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of DPPG (0.2 mM), in the presence of $HgCl_2$, at pH 5.0 (acetate buffer). +, curve 1, control; ◇, curve 2, $HgCl_2$ 2.5 mM.

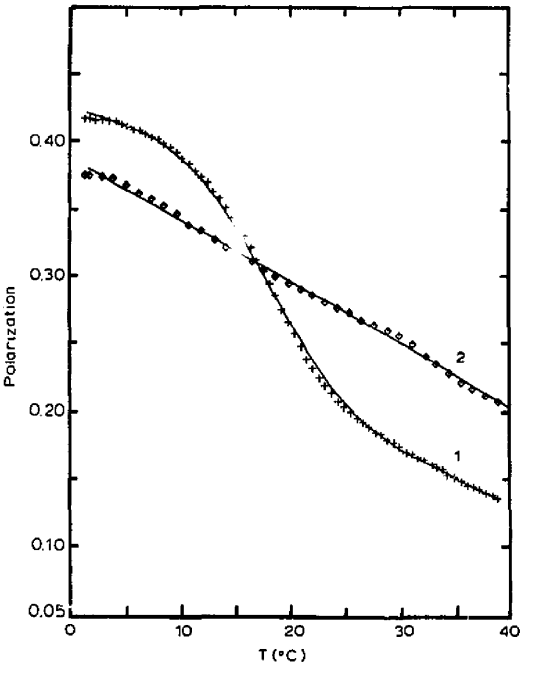

Fig. 4. Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of PS (0.2 mM), in the presence of $HgCl_2$, at pH 5.8 (acetate buffer). +, curve 1, PS; ◇, curve 2, $HgCl_2$ 0.5 mM.

mM. The same experiments were done with synthetic DMPS, which presents a well defined thermotropic behaviour and $T_m = 33$°C. As with PS, for 0.5 mM Hg(II) and pH 5.8, the phase transition of DMPS is abolished, the DPH polarization being decreased below the T_m and increased above the T_m (Fig. 6).

Since both the electrical charge of the phospholipids and the chemical speciation of mercury are pH dependent, we investigated the effect of a constant metal concentration (0.5 mM) on the thermotropic properties of PS vesicles when varying the pH from 4.0 to 8.0 (Fig. 7). At pH 4.0, Hg(II) induces a small decrease of P below the T_m without affecting the transition temperature of PS. In contrast, at pH 5.0 and particularly at pH 5.8, the phase transition is almost abolished as already described. For pH 7.0 and 8.0, the phase transitions are still present, even though their amplitudes are notably reduced and the fluorescence polarization values are increased above the T_m.

The two negatively charged phospholipids which are strongly affected by inorganic mercury (PS and DMPS) possess two putative binding sites for the metal: the carboxyl group and the primary amine group. The possible role of the amino group has therefore been tested by investigating the effects of Hg(II) on two different lipid mixtures: DMPE/EPC and DPPC/SA

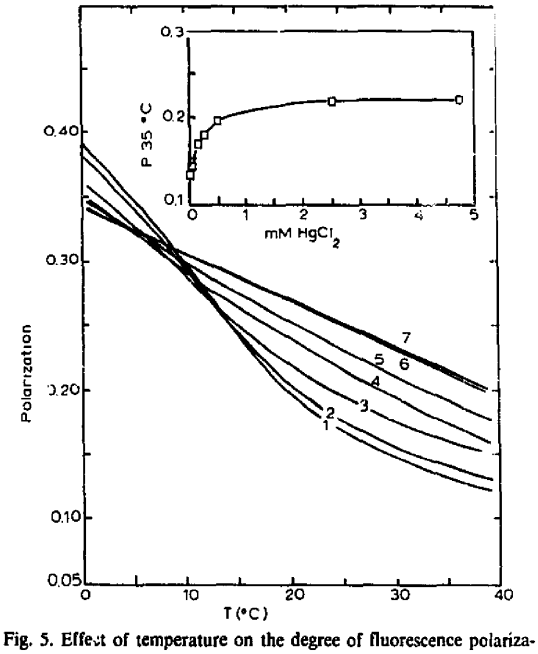

Fig. 5. Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of PS (0.2 mM), in the presence of increasing concentrations of $HgCl_2$, at pH 5.8 (acetate buffer). Curves: 1, control; 2–7, $HgCl_2$: 2, 0.05 mM; 3, 0.15 mM; 4, 0.25 mM; 5, 0.5 mM; 6, 2.5 mM; 7, 4.75 mM. Inset: Isotherm fluorescence polarization ratio (35 °C) of PS function of increased $HgCl_2$ concentrations.

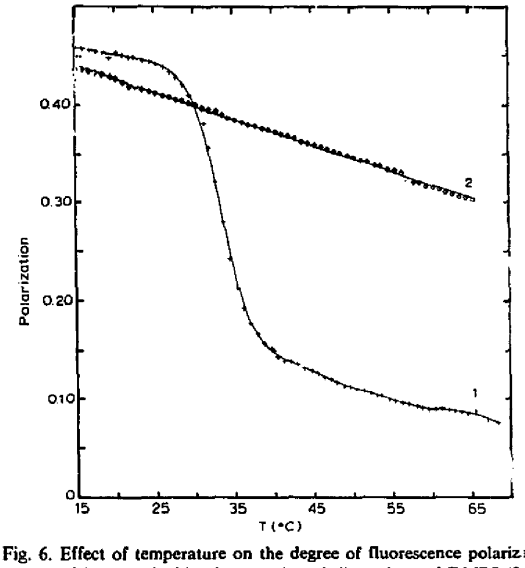

Fig. 6. Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of DMPS (0.2 mM), in the presence of $HgCl_2$, at pH 5.8 (acetate buffer). +, curve 1, control; ◇, curve 2, $HgCl_2$ 0.5 mM.

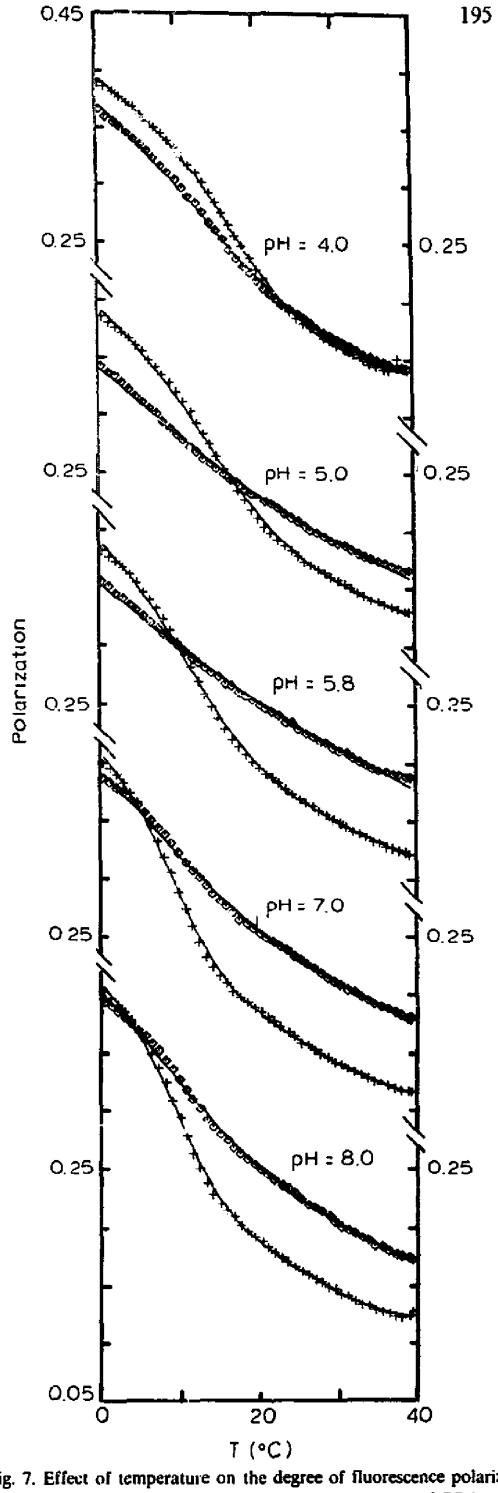

Fig. 7. Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of PS in the presence of $HgCl_2$, at different values of pH (acetate buffer for pH 4.0, 5.0 and 5.8; phosphate buffer for pH 7.0 and 8.0). +, control; ◇, $HgCl_2$ 0.5 mM.

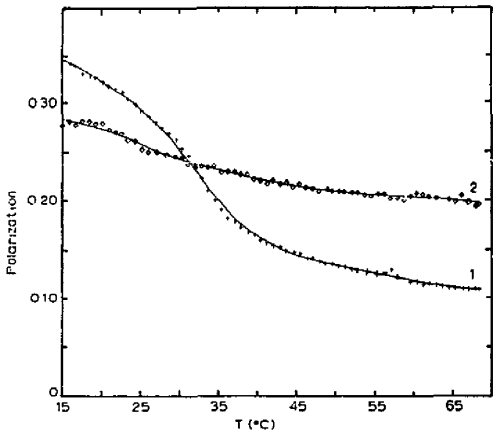

Fig. 8. Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of DMPE/EPC 1:1 (0.2 mM), in the presence of $HgCl_2$, at pH 5.8 (acetate buffer). +, curve 1, control; ◇, curve 2, $HgCl_2$ 0.5 mM.

vesicles, as the thermotropic properties of DPPC and EPC are not affected by the metal.

As a control, the fluorescence polarization of DPH with DMPE/EPC 1:1 vesicles in the absence of Hg(II) is shown in Fig. 8, in which a well defined transition occurs at 32.6°C. DMPE and EPC are zwitterionic lipids both having no net electrical charge at the pH values investigated [26]. Inorganic mercury induces, at a concentration of 0.5 mM and pH 5.8, as for PS and DMPS, a cancellation of the phase transition with a decrease of P at $T < T_m$ and an increase of P at $T > T_m$ (Fig. 8). The Hg(II) effect is qualitatively similar at pH 7.8 (data not shown), but a massive aggregation of vesicles did not allow a rigorous comparison with the effects obtained at pH 5.8.

DPPC/SA 1:1 vesicles are positively charged as the amine group of SA has a pK of about 9 [27]. The T_m of these vesicles is centered at 50.9°C and is also sensitive to the presence of Hg(II) (Fig. 9). Increasing concentrations of mercury induce a significative decrease of the T_m, the highest metal concentration used (2.5 mM) inducing a shift of T_m to 43.1°C (Fig. 9). The transition temperature of the mixture thus moves towards that of pure DPPC, with increase Hg(II) concentrations.

Discussion

The above results clearly document the fact that inorganic mercury has no effect on the thermotropic

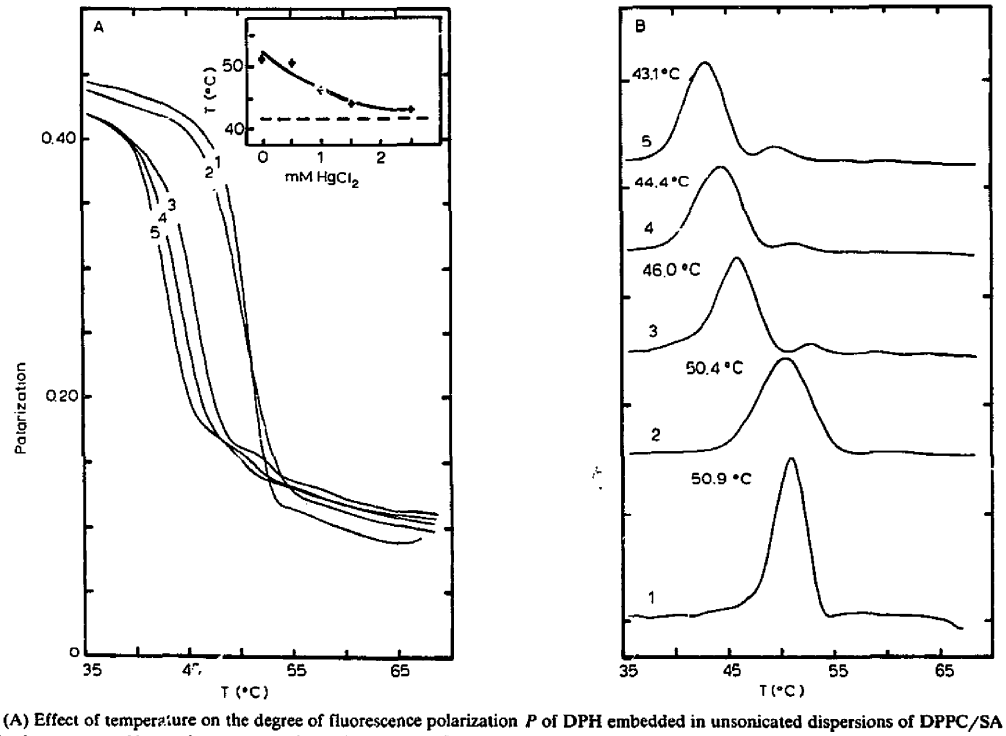

Fig. 9. (A) Effect of temperature on the degree of fluorescence polarization P of DPH embedded in unsonicated dispersions of DPPC/SA 1:1 (0.2 mM), in the presence of increasing concentrations of $HgCl_2$, at pH 5.8 (acetate buffer). 1, control; 2–5, $HgCl_2$: 2, 0.5 mM; 3, 1 mM; 4, 1.5 mM; 5, 2.5 mM. Inset: Transition temperatures T_m of DPPC/SA 1:1 (0.2 mM) in function of $HgCl_2$ concentrations at pH 5.8. (B) First derivatives of the curves shown in (A) indicating the shift of the transition temperature T_m as a function of the $HgCl_2$ concentrations. The numbers correspond to the conditions in (A).

behaviour of either the zwitterionic phospholipid DPPC or the negatively charged phospholipid DMPA, the temperature-dependent fluorescence polarization curves being practically identical, at all the pH values investigated. For another negatively charged phospholipid, DPPG, we observed, at the highest Hg(II) concentration used (2.5 mM) and only at pH 5.0, a very weak increase of the T_m (+1 C°), without significant effects on the polarization ratio above and below the transition temperature.

Surprisingly, Hg(II) induces drastic changes on the thermotropic behaviour of another zwitterionic phospholipid DMPE and negatively charged phospholipids, PS and DMPS, the transition of all these lipids being totally abolished at mercury concentrations of about 0.5 mM. It must be stressed that this occurs without any significant T_m shift during the dose-dependent decrease of the amplitude of the phase transition of PS. Moreover, the metal induces a strong increase of the fluorescence polarization parameter above T_m and a decrease below T_m. Thus, drastic changes of the thermotropism of these lipids occur independently of the net electric charge at the vesicle interface: since DMPE is zwitterionic, PS and DMPS are negatively charged and the mixture DPPC/SA has a positive net charge.

This puzzling behaviour of Hg(II)–phospholipid interactions may be understood if we take into account the chemical composition of the polar heads of the different phospholipids investigated. The only chemical function which may constitute a binding site for mercury seems to be the primary amine group which is common to both the serine and the ethanolamine headgroups of phospholipids that are strongly affected by the presence of the metal.

This working hypothesis is supported by the shift of the T_m of the DPPC/SA vesicles induced by Hg(II) which approaches in a dose-dependent manner the T_m of pure DPPC. This result clearly indicates a quite specific interaction between the metal and the nitrogen of the stearylamine, which at the membrane level probably results in a classical phase separation between SA-Hg(II) domains and pure DPPC, very similar to phase separations induced in phospholipid membranes by divalent cations [28,29]. So, we interpret the thermotropic behaviour of the phospholipids bearing a primary amine group in the presence of inorganic mercury as phase separations between Hg(II)-lipid complexes and the bulk lipid.

Our proposal that the phospholipidic primary amine groups could be a special binding site for mercury is supported by the following arguments: (a) major effects of the metal are seen only with phospholipids bearing this chemical group; (b) strong bonds are observed by nuclear magnetic resonance studies between Hg(II) and nitrogen bases [7–12] or the amino group of amino acids [15,16]; (c) the electrical charges of the polar heads seem to play a minor role in the interaction Hg(II)-phospholipids, but we cannot exclude a participation of the surface charges of lipid vesicles in this interaction, as revealed by the weak effect of mercury on the T_m of DPPG. Our results also suggest that the pH dependence of the effects of the metal on the thermotropic properties of the lipids is related essentially to the chemical speciation of the Hg(II). The presence or absence of a negatively electrical charge at pH 5.8 for PS and DMPE, respectively, and the positive charge borne by the stearylamine have no influence on the effects induced by the metal on these lipids. Thus, we argue that the interactions between Hg(II) and the amino group are not electrostatic in nature and hence are different from the extensively studied interactions between divalent or trivalent cations (Ca^{2+}, Zn^{2+}, Cd^{2+}, Al^{3+}) and negatively charged phospholipids [28–31].

Moreover, the lack of effect of mercury chloride on the surface pressure and surface potential of stearic acid monolayers which, in contrast, are strongly modified by other metal cations as Zn^{2+}, Cd^{2+} or Ca^{2+}, has been ascribed to the extremely low concentration of cationic mercury ions in the aqueous subphase, at pH 5.5, containing 154 mM NaCl [22]. Indeed, considering the theoretical chemical speciation diagrams of Hg(II) in aqueous phase, for pH between 5 and 8 and pCl between 0.8 and 3, the cationic species (Hg^{2+}, $HgCl^+$, $HgOH^+$) are practically absent, the only species apparently available in the aqueous solution being neutral or negatively charged ($HgCl_2$, $Hg(OH)_2$, $HgCl_3^-$, $HgCl_4^{2-}$) [32] and hence no significant effect may be expected on the acidic groups of fatty acids or phospholipids. So, the increase in the T_m of 60%/40% DPPC/PS vesicles in the presence of Hg(II), as described by Bevan et al. [21], may be interpreted as being caused by binding of mercury to the amino groups of PS and not as a classical cation–negative phospholipidic charge interaction.

The quite specific interaction between inorganic mercury and the NH_2 group of phospholipids which, in the pH range used in the experiments is essentially in the protonated form $-NH_3^+$-, may have important implications on the toxicological mechanisms of the metal at the cellular level. For instance, the binding and toxic effects of mercurials on red blood cells are well documented [5,6]. Hg(II) has a marked chemical affinity for erythrocytes and it has been estimated that about $4 \cdot 10^{-15}$ moles, which means $25 \cdot 10^8$ mercury atoms, are bound to a single cell. 90% of the binding sites were attributed to SH groups of hemoglobin, the remaining sites being distributed between the cell stroma and the membranes [5]. The figure of $22.5 \cdot 10^8$ metal atoms should correspond to 8.5 moles of Hg(II) per mole of hemoglobin, but the highest value available for the number of sulfhydryl content of hemoglobin was estimated at six cysteine residues per human hemoglobin

molecule [33]. So, the cellular binding of mercury in excess of 6 moles per mole of hemoglobin must be due to another class of binding sites, corresponding to about $6 \cdot 10^8$ mercury atoms per cell [5]. Qualitatively, the same conclusions were reached on work on the binding of mercurials to HeLa S3 suspension-culture cells, when Gruenwedel et al. [34] suggested the basic nitrogen of DNA and of chromatin as potential ligands.

The results presented here on interactions of Hg(II) with amino phospholipids support the proposal that at least some of the binding sites in cells other than the sulfhydryl groups, could be the primary amino groups of PS and PE. Taking into account the erythrocyte area, about 140 μm^2 [35], the area per phospholipid 60 $Å^2$ [36], the phospholipid/cholesterol molar ratio of 1 : 1, and assuming that membrane proteins occupy half of the membrane surface, one can estimate the number of phospholipids per red cell to be roughly $1.2 \cdot 10^8$. As PS and PE represent 45% of the total phospholipids [37], the erythrocyte membrane contains about $0.5 \cdot 10^8$ phospholipids bearing an amino group, of which 80% are in the inner layer [38].

Interestingly, this value lies in the same order of magnitude as the estimated value of binding sites for mercury other than the intracellular SH content in human erythrocytes [5]. Moreover, as lipidic membranes are highly permeable for neutral mercury species [17–19], we may reasonably assume that such amino phospholipids are easily accessible for the mercurials.

On the other hand, with the red cell, Hg(II) was shown to decrease osmotic fragility and K^+ leakage at low concentrations ($5.2 \cdot 10^7$–$6.5 \cdot 10^8$ Hg atoms per cell) [5]. These effects may be better understood by taking into account mercury–phospholipids interactions. If Hg(II) decreases fluidity and induces phase separations in cell membranes, as is shown here with PS and PE model membranes, such effects may be related to decreased osmotic fragility and K^+ leakage of red cells induced by the metal [5].

In conclusion, we propose that phosphatidylserine and phosphatidylethanolamine may represent a new class of binding sites for Hg(II) in cell membranes, and that the interaction of these lipids with mercury may be a new mechanism for the toxicological effects of mercurials at the membrane level. Structural data, by NMR spectroscopy, under current work in our laboratory, are, however, needed to better analyse, at the molecular level, the interactions between phospholipids and the mercury chemical species.

Acknowledgements

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