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HYDROCARBON SOLVENTS: EFFECT ON CALCIUM MEDIATED EVENTS IN LIPOSOMES*

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The effect of hydrocarbon solvents on membranes was investigated by measuring the inhibition of Ca^{2+}-induced turbidity in liposomes. Chloroform, carbontetrachloride, 1,1,1-trichloroethane, hexane, cyclohexane and benzene protected against Ca^{2+}-induced turbidity, and the concentration of each solvent required for a 50% decrease was in the millimolar range. Double-reciprocal plot analysis was consistent with the presence of solvent binding sites on the membrane.

Keywords: liposomes; calcium; solvents.

Introduction

To better understand the contribution of phospholipids to membrane mediated processes, liposomes have been employed as a model system [1]. Examples of membrane related phenomena studied with this approach are permeability [2], drug delivery [3] and Ca^{2+}-mediated membrane fusion [4]. During our studies on membrane-toxicant interactions, we observed a number of widely used organic solvents affected synthetic phospholipid membranes in a similar manner. We describe, herein, the reversal by some halogenated hydrocarbon and 6-carbon solvents on Ca^{2+}-induced turbidity of anionic liposomes and the dependence on solvent concentration. We will also show the results are consistent with a binding mechanism for the solvent molecules to site(s) on the membrane.

Methods

Phospholipid bilayers composed of dimyristoylphosphatidylcholine (DMPC) plus 4 mol% dicetylphosphate (DCP) were prepared by sonication and maintained as

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Abbreviations: DMPC, dimyristoylphosphatidycholine; DCP, dicetylphosphate; Tris, 2-hydroxymethyl,2-amino,1,3-propandiol, CCl_4, carbontetrachloride, $CHCl_3$, chloroform, TCE, 1,1,1-trichloroethane.

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described [5]. (DCP provided the anionic component of the membrane; in its absence, no effect by Ca^{2+} was observed). DMPC was a product of Sigma Chemical Company (St. Louis, MO) and was judged pure by TLC before and after liposome preparation. Phospholipid concentration was based on total phosphate analysis, using Elon as a reducing agent [6]. Turbidity, which may reflect aggregation or fusion processes [4], was measured at 360 nm. All experiments were carried out in 10^{-2} M Tris, pH 8 + 10^{-1} M KCl at 22–23°C, the transition temperature for DMPC [7] and the temperature at which we observed a maximal effect by Ca^{2+}. Samples (1 ml) were incubated 16–18 h under conditions stated in the figure legends. Shorter times could not be used; in one experiment significant turbidity was not observed for four hours and increased linearly for 20 h. Liposomes, maintained at 35–40°C, were added last and when volatile solvents (prepared in 95% EtOH) were studied, glass stoppered tubes were used to prevent evaporation. Liposomes were visualized with an electron microscope (Zeiss, Model EM 9 S-2). Samples were stained with 1% sodium phosphotungstate and viewed at a magnification of 63 000 [8].

Results and Discussion

The maximal effect of Ca^{2+} on anionic DMPC liposomes occurred between 0.5 and 1.0 mM Ca^{2+} (Ca^{2+}/phosphate = 0.2–0.3) and represented a 10–20-fold increase in A_{360}. (A_{360} in the absence of Ca^{2+} was <0.1). At higher Ca^{2+} concentrations (5–100 mM) the A_{360}-value decreased but did not drop below 50% of the maximum. The effect of Ca^{2+} was specific in that Mg^{2+} did not induce the same increase in turbidity at similar concentrations. Even at 2 mM Mg^{2+}, the A_{360} increase was only 27% of that observed in the presence of 2 mM Ca^{2+}. Turbidity measurements monitor aggregation as well as fusion processes [4], and our results cannot distinguish between the two phenomena. Data reported by Wilshut et al. [9], however, suggest aggregation occurs at high Ca^{2+} concentration while Ca^{2+}-induced fusion plays a role at lower concentrations. The specificity of Ca^{2+} over Mg^{2+} in our study is in conformity with their results [9] as well as those obtained from Ca^{2+}-stimulated fusion of phosphatidylcholinephosphatidyserine vesicles [10]. We do not, however, draw strict analogies with the results just described, because effects of divalent ions on liposomes depend on many parameters, including size and composition [9,11].

The effect of halogenated aliphatic and 6-carbon solvents on the Ca^{2+}-induced aggregation of anionic DMPC-liposomes is shown in Fig. 1. We also determined the curve generated with 1,1,1-trichloroethane (TCE) in the presence of 1 mM Ca^{2+} was similar to that observed in the presence of 50 mM Ca^{2+}. The solvents may act by completely disrupting the vesicles or preventing the formation of the larger structures. The data in Fig. 2 suggest the latter process may be operative because discrete structures, similar in size but not identical to DMPC-liposome, were observed in the electron microscope. EtOH used as a carrier for the organic solvents did not effect

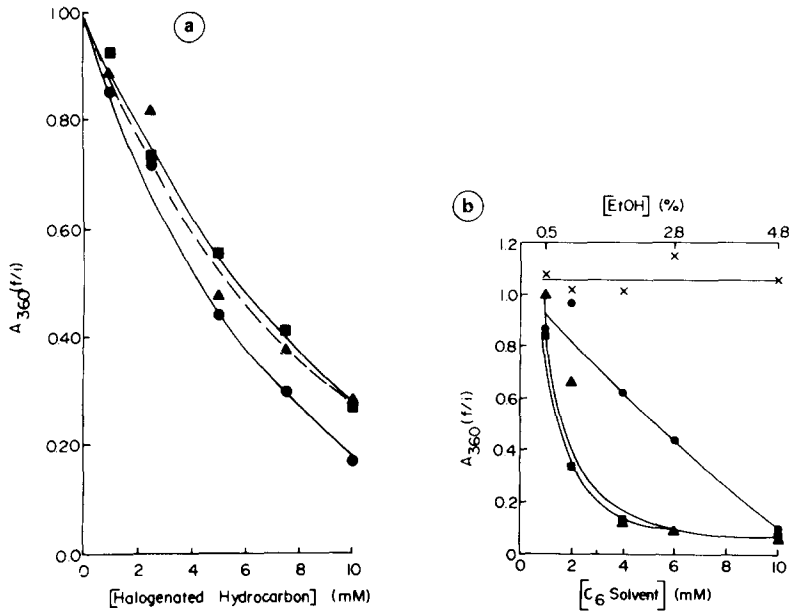

Fig. 1. Effect of hydrocarbon solvents on calcium-induced aggregation of DML-liposomes: A_{360} f/i is the ratio of A_{360} in the presence of hydrocarbon to A_{360} in its absence. A: Effect of halogenated aliphatic solvents. DMPC, 3.6 mM; $CaCl_2$, 50 mM; temperature, 23°C; incubation time, 16 h. All tubes contained EtOH at a final concentration of 1.9%. At this EtOH and *without* $CaCl_2$ no aggregation of DML-liposomes was observed. ●——●, CCl_4; ▲——▲, TCE; ■——■, $CHCl_3$. B: Effect of 6-carbon solvents: DMPC, 3.0 mM; $CaCl_2$, 1 mM; temperature, 22°C; incubation time, 16 h. Note that unlike the experiment described in Fig. 3A, EtOH was also varied. The effect of EtOH alone at each concentration is indicated by X——X. Each X represents the EtOH concentration present with the solute whose concentration is indicated on the lower axis. ■——■, cyclohexane; ●——●, hexane; ▲——▲, benzene.

the turbidity of liposome preparations up to a concentration of 4.8%. Furthermore, protection against Ca^{2+}-induced aggregation by 10 mM benzene and cyclohexane was not significantly different in the presence of 1.9% EtOH.

Double reciprocal plots [12,13] were constructed to determine if the data were consistent with the presence of solvent binding sites on the membrane. We assumed a change in turbidity (Δ_{360}) reflected the change in concentration of a complex and the data in Fig. 3 support our assumption. Furthermore, the rapid equilibrium condition [13] which is a basis for the equations used in double reciprocal plot analysis assumes the presence of a complex. Conformity of our data (Fig. 4) to double reciprocal plot analysis therefore suggests turbidity changes may reflect complex formation and shows solvent binding sites on the membrane are possible. Apparent association constants (K_a-values) were calculated as: CCl_4, 0.11 mM^{-1}, $CHCl_3$, 0.084 mM^{-1}; TCE, 0.059 mM^{-1} with 50 mM Ca^{2+} and 0.055 mM^{-1} in the

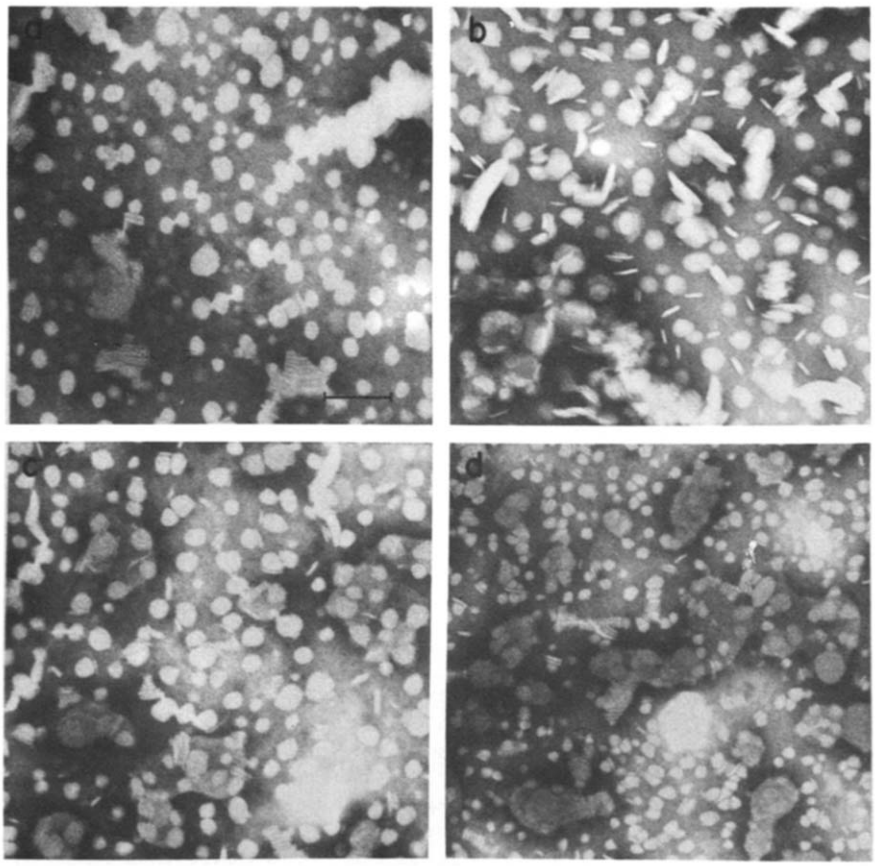

Fig. 2. Election micrographs of DML-liposomes treated with chlorinated hydrocarbon solvents in the presence of Ca^{2+}. Samples were incubated at 23°C for 16 h DMPC, 3.6 mM. (a) DMPC liposomes incubated with no additions, (b) 10 mM CCl_4 + 50 mM Ca^{2+}; (c) 10 mM $CHCl_3$ + 50 mM Ca^{2+}; (d) 10 mM TCE + 50 mM Ca^{2+}. In the presence of 50 mM Ca^{2+} alone, large aggregates formed. The samples were stained and visualized as described in Methods. The bar represents 100 nm.

presence of 1 mM Ca^{2+}. The data of Fig. 3 were further analyzed by constructing a log-log plot, from which a rate constant was calculated as 0.00935 h^{-1}.

The experiments described in this paper describe the reversal, by hydrocarbon solvents, of Ca^{2+}-induced turbidity of liposomes; they do not describe the mechanism by which the reversal occurs. Results obtained from double-recipocal plot analysis suggest, however, the three halogenated aliphatic solvents bind weakly to site(s) on the membrane. Jain and Wu [14] observed a common effect of structurally unrelated solvents on a model membrane system and suggested the compounds were imbedded close to the terminal methyl groups within the bilayer. In their

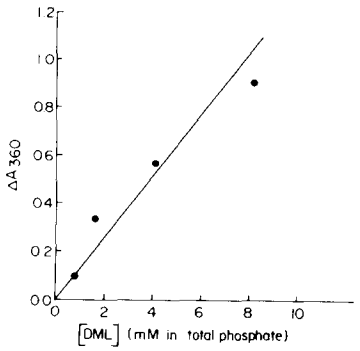

Fig. 3. Effect of liposome concentration on Turbidity increase (Δ_{360}) in the presence of 50 mM Ca^{2+}. DMPC concentration was determined as total phosphate. Temperature, 22°C; incubation time, 21 h.

case, however, higher solvent concentrations were used and it would be premature to extrapolate their conclusions to our data.

Detailed studies on Ca^{2+}-mediated aggregation and fusion showed that Ca^{2+} is bound to the phosphate groups of two opposed membranes [4,9–11,15]. Studies with apolar hydrocarbons have led to the suggestion these substances are located

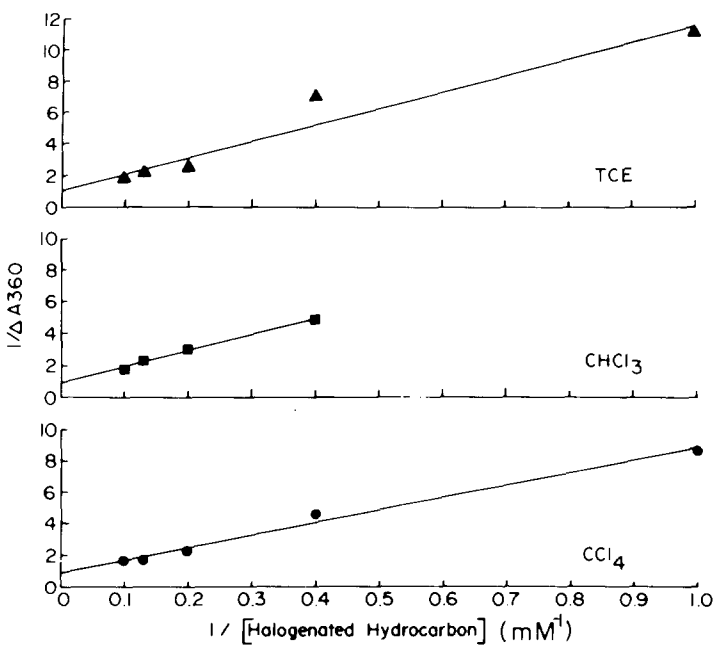

Fig. 4. Double reciprocal plots of the effect of halogenated hydrocarbons on A_{360} of DML-liposomes in the presence of 50 mM $CaCl_2$.

within the bilayer, perhaps close to the terminal methyl groups [14,16]. The presence of the different binding sites on the phospholipid implies interference by the solvents with the Ca^{2+} effect, observed with our study, cannot be attributed to simple competition. A possible explanation may be found in the decreased transition temperatures observed in the presence of many solvents [14,16]. Such a change suggests a distortion in membrane structure that may affect subsequent complex formation with Ca^{2+}.

Our results on the reversal by the hydrocarbon solvents on a Ca^{2+}-effect on liposomes would suggest that in vivo the regulatory action of Ca^{2+} may be subject to alterations by similar substances such as halogenated hydrocarbons associated with contaminated environments. A number of workers have found that Ca^{2+}-transport and mobilization were affected by hydrocarbon solvents including $CHCl_3$ and CCl_4 [17,18]. Hence, the liposome system should be useful in understanding the role of the phospholipid component in carrying out such processes. The current investigation is a start in that direction and additional studies should reveal the details by which the hydrocarbon solvents are able to regulate such membrane mediated processes.

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