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A DILATOMETRIC INVESTIGATION OF THE EFFECTS OF GENERAL ANAESTHETICS, ALCOHOLS AND HYDROSTATIC PRESSURE ON THE PHASE TRANSITION IN SMECTIC MESOPHASES OF DIPALMITOYL PHOSPHATIDYLCHOLINE

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Summary

The effect of general anaesthetics, alcohols and hydrostatic pressure on the thermal transition in dipalmitoyl phosphatidylcholine multilayer liposomes has been measured using dilatometry. The volume increase at the transition (ΔV_t) is 0.0350 ± 0.003 ml/g, the transition temperature (T_t) 41.84 ± 0.09°C and the width of the transition 1.025 ± 0.18°C. ΔH calculated by the Clapeyron-Clausius equation is 8.4 kcal/mol. The *n*-alcohols C_3—C_5 reduced the transition temperature without affecting the transition width which was however, increased by *n*-hexanol. Trichloroethylene, the fluorescent probe *N*-phenyl-1-naphthylamine, and methoxyflurane all increased the transition width (reduced the cooperativity of the transition) with a simultaneous depression of T_t. Methoxyflurane caused a two-stage transition expansion. Diethyl ether's effect has similarities with both the C_3 and C_6 alcohols. Generally ΔV_t was unaffected by the agents.

Pressure increased T_t by 0.0238°C/atm linearly over the range 1—300 atm in both treated and untreated liposomes, and therefore cannot be said to antagonize anaesthetics. In both treated and untreated liposomes ΔV_t and the width of the transition were unaffected by pressure. Pressure thus reverses the effects of anaesthetics on T_t but not their spread of the transition width.

Introduction

In various physiological and physical preparations anaesthetics and hydrostatic pressure counteract each other's effects, suggesting that the distinctive molecular perturbations each can bring about are mutually opposed [1,2]. An example is the reduction in the height of the action potential in the giant axon of the squid by ethanol and its restoration by a pressure of 470 atm [3].

The endothermic phase transition in bilayers is also susceptible to the opposing effects of anaesthetics and hydrostatic pressure. The transition involves a marked increase in the fluidity of the hydrocarbon interior, a simultaneous increase in molar volume and an expansion in membrane area [4—6]. The transition is highly cooperative in nature, particularly in the case of pure dipalmitoyl phosphatidylcholine smectic mesophases (liposomes) which undergo a sharp transition at T_t = 41—42°C. The transition is affected by alien molecules which dissolve in or adsorb on, the bilayer. Anaesthetics and the shorter *n*-alcohols lower T_t [7]; conversely, because of the molar volume (ΔV_t) increase in the transition, pressure raises T_t.

Studies of anaesthetic-pressure interactions in the phase transition have so far been confined to experiments using electron spin resonance to detect the transition [2,8]. In the experiments reported here dilatometry has been used to observe the transition volume change in the presence of anaesthetics and at pressures up to 300 atm.

Methods

Measurements were made of the thermal expansion of a suspension of liposomes prepared from dipalmitoyl phosphatidylcholine (1,2-dihexadecyl-*sn*-glycero-3-phosphorylcholine) obtained from Koch-Light Ltd. A standard suspension was made from 0.1 g dipalmitoyl phosphatidylcholine in approx. 2 ml triple-distilled water purged oxygen-free with nitrogen. The suspension was shaken on a vortex mixer for 15 min at 50°C, stored overnight at 5°C and de-gassed before use. The thermal expansion of the suspension was measured in a simple U-tube capillary dilatometer of 0.6 ml volume. The temperature of the dilatometer was controlled to ±0.025°C by a water bath and the displacement of the meniscus in each of the limbs was read with a travelling microscope to an overall accuracy of $\pm 6 \cdot 10^{-5}$ ml. Calibration was carried out by measuring the expansion of a known weight of de-gassed water. Evaporation of either water or anaesthetics from the dilatometer was negligible.

Measurements at high hydrostatic pressure were carried out with a similar capillary dilatometer in the form of an inverted U, with each downward pointing limb filled with mercury, which in turn came into contact with the water which filled the pressure vessel. The displacement of the liposome suspension-mercury meniscus was read by a travelling microscope viewing through a high pressure window whose maximum safe working pressure was 300 atm at a maximum of 52°C. Pressure was generated by an air-powered hydraulic pump and measured to ±1 atm (1 atm = 101.325 kN/m^{-2}). The pressure vessel was kept at a controlled temperature ±0.025°C by water circulating through an enclosing water jacket where the temperature was measured by a thermometer. Temperature was normally increased by 1°C each 40 min in the pressure vessel and 1°C each 20 min at atmospheric pressure. The test suspension equilibrated within half of the time intervals and reproducible results were obtained on cooling. The application of high pressure took only a few seconds but 15 min were allowed for the dissipation of the heat of compression. Whereas temperature was usually progressively increased during an experiment, pressures were applied in a varied sequence, with no effect on the results. Anaesthetics

(clinical grade) and alcohols (analar grade) were added to the liposome suspension by weighing. The concentration of dipalmitoyl phosphatidylcholine in a given suspension was measured after the experiment by a dry weight determination to ±2%.

Results

A typical result of a dilatometry experiment is shown in Fig. 1 which illustrates the method for determining ΔV_t, T_t and the temperature range over which the transition occurs, i.e. its width, W_t. ΔV_t was independent of the concentration of the phospholipid suspension over the range 1—8%. The following results were obtained from not less than 12 experiments at atmospheric pressure; $\Delta V_t = 0.0350 \pm 0.003$ ml/g, $T_t = 41.84 \pm 0.09$°C, $W_t = 1.025 \pm 0.181$°C. The value of ΔV_t corresponds to 3.56% and may be compared with previously published data: 1.4% [5]; 2.56% [9]; 2.6% [10]; 4.0% [11]; 3.2% [12]; 3.26% [9]; 3.5% [9]; 3.5% [13].

Effects of n-alcohols on the transition

n-Propanol, *n*-butanol and *n*-pentanol reduced T_t with little effect on W_t whereas *n*-hexanol caused a marked increased in W_t (Fig. 2).

The concentration data are expressed as mM alcohol in the liposome suspension and so the actual membrane "concentration" of a particular alcohol is affected by the concentration of lipid in the suspension, normally between 4 and 6%. This variation is unimportant however, particularly as W_t and T_t are the important factors. Hypothetical concentrations of alcohols in or on the bilayers have not been calculated because the value of such data are entirely limited by the validity of the partition coefficient selected.

The concentration effect in the lower alcohols, particularly *n*-butanol, diminishes at high concentration, apparently saturating the bilayer, whilst *n*-hexanol becomes increasingly effective with increase in concentration (Fig. 3). ΔV_t was unaffected by a reduction in T_t in all cases.

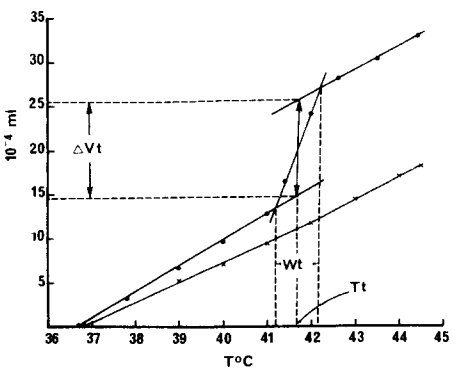

Fig. 1. Thermal expansion of a suspension of dipalmitoyl phosphatidylcholine liposomes showing the method for interpolating T_t, ΔV_t and W_t. The volume of the suspension was 0.624 ml and contained 5.11% dipalmitoyl phosphatidylcholine by weight. The thermal expansion of an equal volume of water is plotted (X).

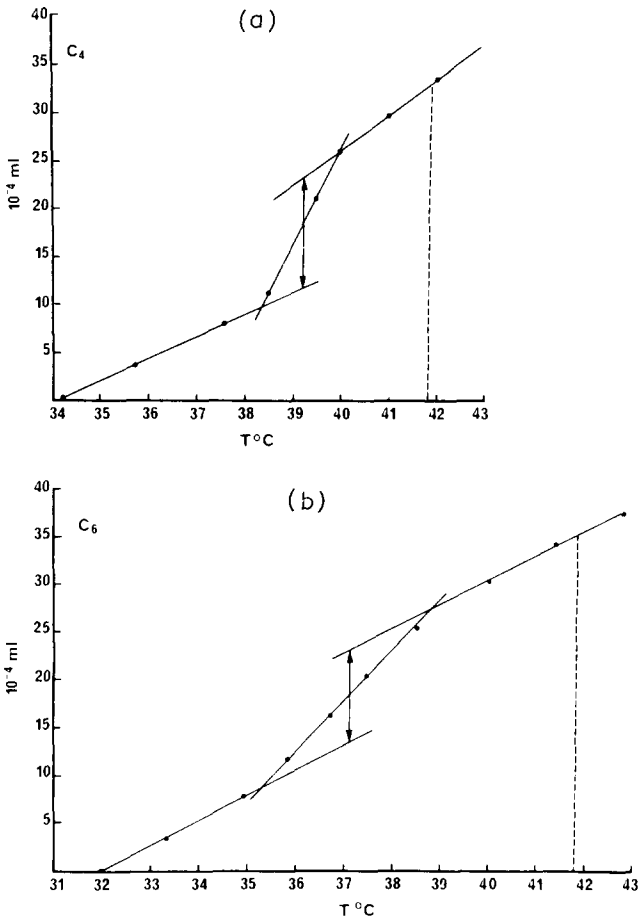

Fig. 2. Effect of *n*-alcohols on ΔV_t, W_t, and T_t. a, *n*-butanol, 78 mM; b, *n*-hexanol, 23 mM. Typical results are shown, the vertical dashed line indicates T_t for untreated liposomes.

Effect of general anaesthetics on the transition

Diethyl ether was added to the liposome suspension as a saturated aqueous solution prepared at 37°C, trichloroethylene and methoxyflurane were added directly by weighing.

Ether reduced T_t with little effect on W_t and no effect on ΔV_t. The effect of increasing concentration is similar to that of the lower alcohols (Figs. 4 and 5). Trichloroethylene lowered T_t with a marked increase in W_t and methoxyflurane caused a widespread, two-stage expansion at the transition, the second stage of which was responsible for most of the volume increase (Fig. 4). Neither anaesthetic affected ΔV_t which was measured with reduced accuracy in the broadened transition. The effect of varying the concentration of trichloroethylene and methoxyflurane is similar (Fig. 5).

The effects of ether and methoxyflurane were readily reversed by evaporating off the anaesthetic and the additives in trichloroethylene were without effect.

Addition of the non-volatile hydrophobic fluorescent probe *N*-phenyl-1-

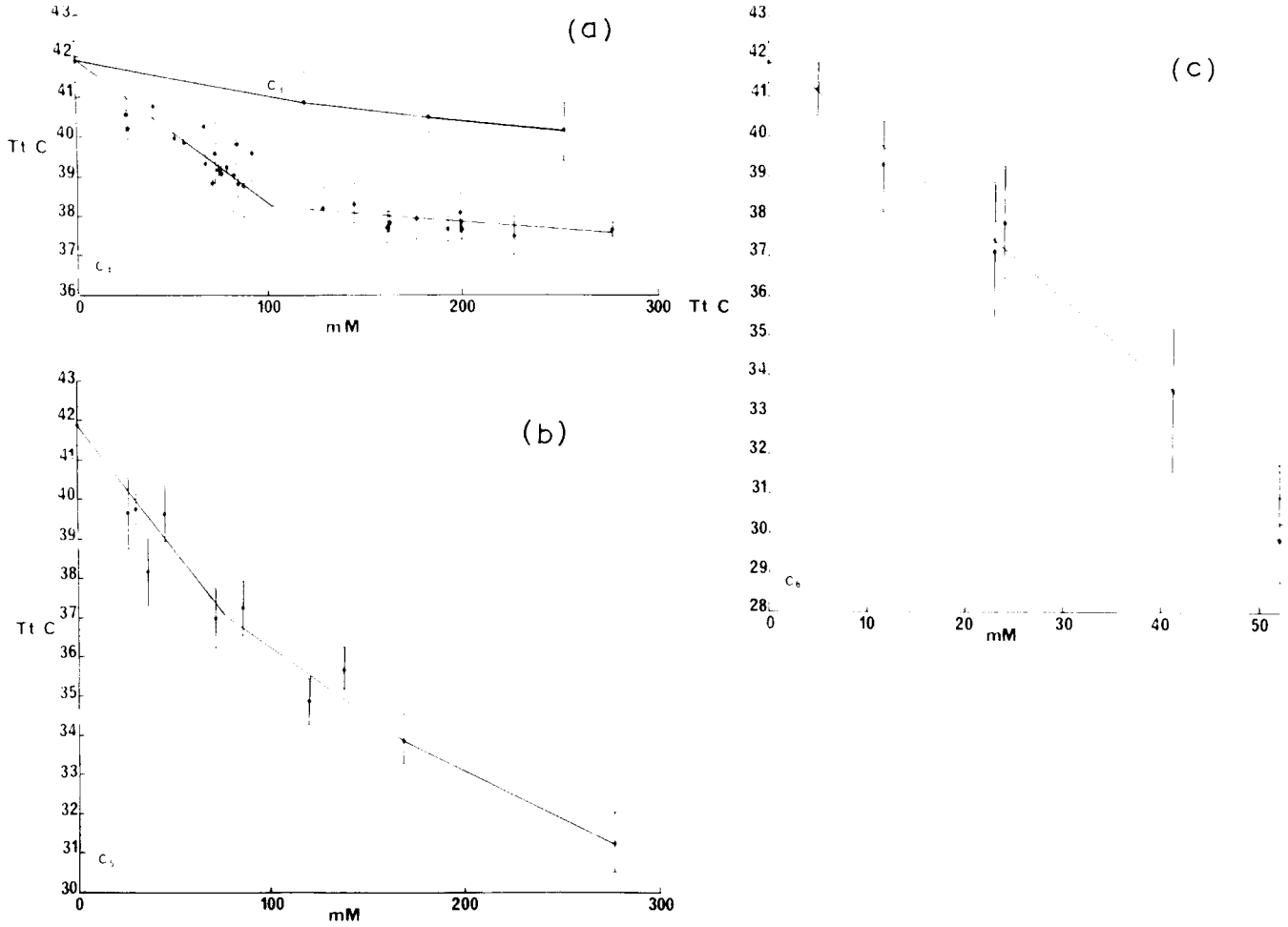

Fig. 3. Effects of different concentrations of propanolol, butanol (C_3, C_4, respectively in (a), pentanol (b), and hexanol (c) on T_t. In each graph the mean value of T_t and W_t are plotted for untreated liposomes, all other points and bars refer to individual values of T_t and W_t. The dotted bar in the case of hexanol indicates an alternative interpolation of W_t in a particular experiment. Concentration is mM alcohol per litre suspension containing 4—6% dipalmitoyl phosphatidylcholine by weight. The lines are drawn by inspection.

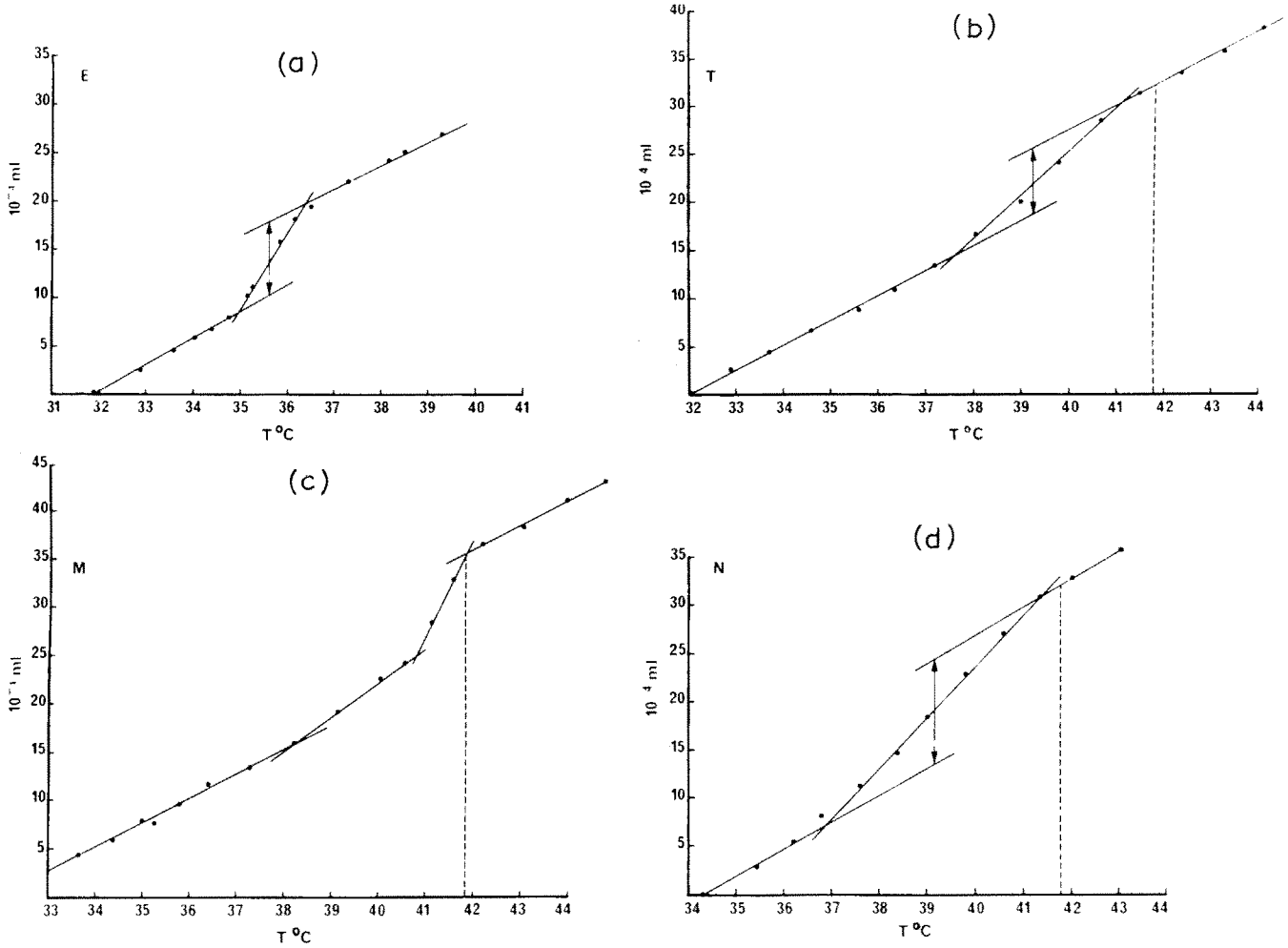

Fig. 4. Effects of anaesthetics on ΔV_t, W_t and T_t. a, diethyl ether, 472 mM; b, trichloroethylene, 39 mM; c, methoxyflurane, 7.8 mM; d, *N*-phenyl-1-naphthylamine, 11 mM. Typical results are shown, the vertical dashed line indicates T_t for untreated liposomes.

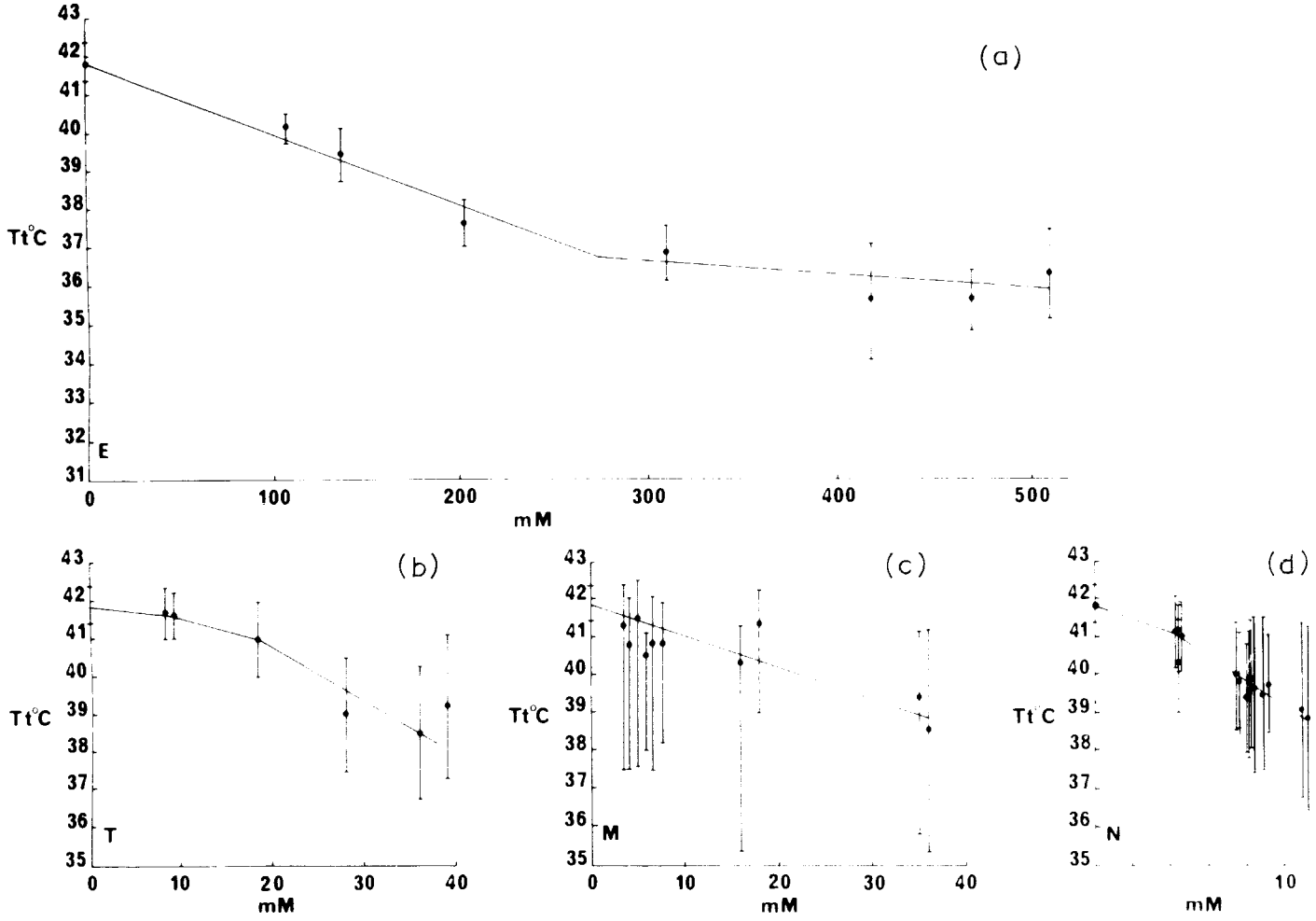

Fig. 5. Effects of different concentrations of anaesthetics on T_t. a, diethyl ether; b, trichloroethylene; c, methoxyflurane; d, *N*-phenyl-1-naphthylamine. In the case of methoxyflurane the inflexion point in the biphasic transition dilation is plotted instead of the mid point T_t.

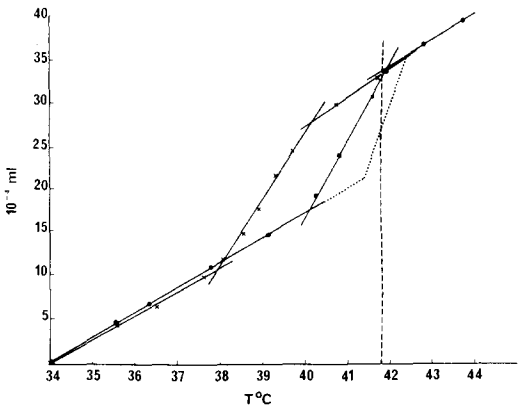

Fig. 6. Combined effects of *N*-phenyl-1-naphthylamine and *n*-butanol on ΔV_t, W_t and T_t. •———•, 4.5 mM *N*-phenyl-1-naphthylamine only (the dashed line indicates the transition of untreated liposomes); X———X, 4.5 mM *N*-phenyl-1-naphthylamine + 72 mM *n*-butanol.

naphthylamine to the liposomes depressed T_t and increased W_t in a manner similar to trichloroethylene. The effects of varying the concentration of *N*-phenyl-1-naphthylamine and trichloroethylene are also similar (Fig. 4 and 5). The combined effects of *N*-phenyl-1-naphthylamine and *n*-butanol are seen in Fig. 6. Liposomes in 4.5 mM *N*-phenyl-1-naphthylamine show a typically spread transition, T_t = 41.0°C, which is reduced to 39.05°C in the presence of 72 mM butanol with little effect on W_t. It appears that *N*-phenyl-1-naphthylamine and butanol act separately without modifying each others effect on the transition.

TABLE I

EFFECT OF HYDROSTATIC PRESSURE ON THE THERMAL TRANSITION OF DIPALMITOYL PHOSPHATIDYLCHOLINE LIPOSOMES TREATED WITH ANAESTHETICS

(1) *n*-Butanol. Three experiments, 72, 87, and 161 mM butanol.

Pressure (atm)	d*T*/d*P* (°C/atm)	Mean W_t (relative units)	Mean ΔV_t (relative units)
1		1	1
100	0.0239	0.93	1.01
300		1.29	0.98

(2) Methoxyflurane. Four experiments, 35, 42, 66, and 78 mM methoxyflurane. Second stage transition measured, see text.

1		1.0	1.0
100	0.0236	1.03 (mean of 2)	0.88 (mean of 2)
200		2.21 (mean of 2)	1.19 (mean of 2)
300		1.14 (mean of 2)	1.09 (mean of 2)

(3) *N*-Phenyl-1-naphtylamine. Two experiments, 77 and 88 mM *N*-phenyl-1-naphthylamine.

1		1.0	1.0
100	0.0241	0.86	1.0
300		0.96	0.93

Effects of pressure on the transition

Pressure increased T_t in both untreated and treated liposomes by 0.0238°C/atm linearly up to 300 atm, the maximum studied. ΔV_t and W_t were unaffected

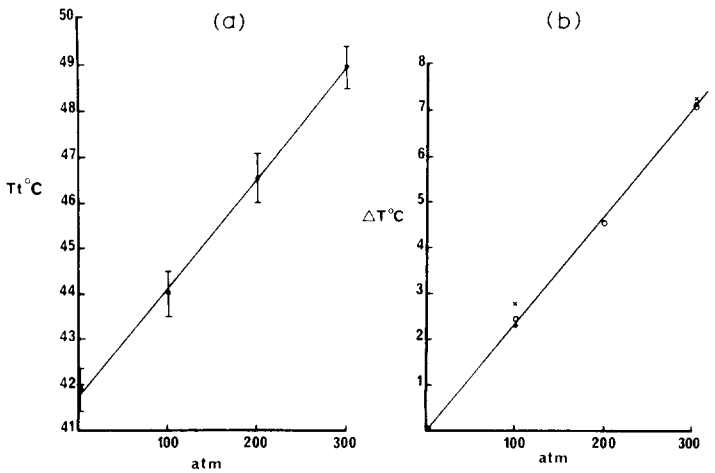

Fig. 7. The effect of hydrostatic pressure on T_t and W_t. (a) Untreated liposomes, the vertical bars correspond to W_t. (b) The increase in T_t caused by pressure on liposomes treated with *n*-butanol (●), mean of three experiments; methoxyflurane (○), based on four experiments, (second stage transition used, see text); (X), *N*-phenyl-1-naphthylamine, mean of two experiments. Various concentrations of anaesthetic were used to give values of T_t between 37.7 and 40.8°C at atmospheric pressure. See Table I for values of W_t.

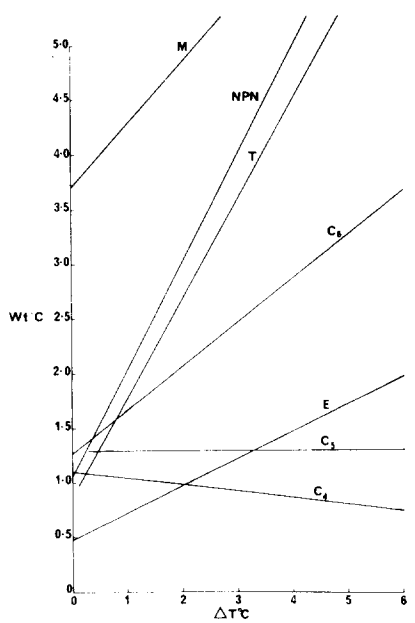

Fig. 8. The effect of alcohol and anaesthetics on T_t and W_t. The linear regression of W_t on the reduction in T_t (ΔT°C) calculated from data in Figs. 3 and 5 is plotted to show the different effects on W_t (cooperativity) and ΔT°C. W_t for untreated liposomes is 1.025°C. M, methoxyflurane; NPN, *N*-phenyl-1-naphthylamine; T, trichloroethylene; C6, hexanol, C5, pentanol; C4, butanol; E, diethyl ether.

by pressure (Fig. 7 Table I). Previously published data for untreated liposomes are 0.0218°C/atm (using ESR over 1—137 atm) [8]; 0.0262°C/atm (using dilatometry over 1—99 atm) [12] and 0.0238°C/atm (using ESR over 1—800 atm) [14].

The first part of the two-stage expansion in methoxyflurane-treated liposomes proved elusive at high pressure. Two experiments showed that it responded in the same way as the rest of the expansion, but one experiment showed it was unaffected by pressure and another was inconclusive. A more sensitive dilatometer is required to pursue this point. However, in all four experiments a transition was seen at high pressure, corresponding to the main, second stage expansion, the width of which was somewhat variable but certainly not reduced by pressure.

Discussion

The volume change occurring at the transition is a net change and the actual volume increase in the melting of the hydrocarbon chains may be partly masked by other changes [5,10,11]. Thoroughly sonicated suspensions, comprising very small vesicles whose bilayer organization is thought to be affected by the tight curvature of the membrane, showed no ΔV_t. Spectroscopic, calorimetric, fluorescent probe, and dilatometric measurements [11,15,16] have shown that sonicated vesicles exhibit a broadened transition at a reduced temperature. Since the present results show that alcohols and anaesthetics lower T_t and broaden the transition differentially, we have to consider whether they primarily affect the size distribution of the liposomes or act on the transition directly without affecting membrane curvature. Microscopic and light absorption observations show that the anaesthetics and alcohols do not affect the size distribution of the liposomes on a scale comparable to even mild sonication. The constancy of ΔV_t in treated liposomes shows that the total mass of bilayer which undergoes the transition remains constant. In this respect the treated liposomes differed from lightly sonicated suspensions which showed a slightly reduced T_t and a much reduced ΔV_t, which further sonication abolished altogether. It therefore seems most unlikely that anaesthetics and alcohols affect the transition by affecting the size distribution of the liposomes.

The different ways in which T_t and W_t are affected by the alcohols and anaesthetics are apparent in Figs. 3 and 5, and the linear regression of W_t on T_t is an arbitrary and convenient way of summarising these data (Fig. 8). The slope of the lines demonstrate that whereas *n*-butanol and *n*-pentanol have negligible effect on W_t, *n*-hexanol has a considerable effect. The contrast between the effect of varying the concentration of *n*-pentanol and *n*-hexanol has already been noted (Fig. 3). This distinction between the two alcohols is more marked than in previous studies using various other methods to detect the transition in dipalmitoyl phosphatidylcholine liposomes [7,16—20]. It corresponds to the different effects which the alcohols have on the neuro-muscular junction in which *n*-pentanol prolongs the decay of the miniature end plate current whilst *n*-hexanol accelerates it. Gage and his colleagues [21] argue that *n*-pentanol probably acts post-synaptically by changing the dielectric constant of the membrane, whilst *n*-hexanol accelerates the decay by increasing mem-

brane fluidity. The present work shows that *n*-hexanol decreases the cooperativity of the bilayer transition, probably by increasing inter-molecular spacing, which implies disordering, whilst *n*-pentanol exerts no such effect.

The anaesthetics and *N*-phenyl-1-naphthylamine also show interesting differential effects of T_t and W_t. Ether resembles *n*-hexanol and differs from trichloroethylene and *N*-phenyl-1-naphthylamine (Fig. 8). The line for methoxyflurane is displaced, reflecting methoxyflurane's potency in spreading the transition at low dose.

Hill's [7] colligative treatment of the depression of T_t by anaesthetics does not seem to offer any explanation for changes in the cooperativity of the transition process. The hydrophobic substance *N*-phenyl-1-naphthylamine, known from its fluorescence properties to occupy the hydrocarbon interior of a bilayer, initiated a melting dilation at reduced temperature and reduced the cooperativity of the transition. Trichloroethylene and methoxyflurane act similarly whereas more polar substances which are thought to act near the surface of the bilayer (*n*-propanol, *n*-butanol, *n*-pentanol) do not affect the cooperativity of the transition. *N*-Hexanol and diethyl ether are interesting intermediate cases suggesting sites of action between the polar surface and the apolar interior (Fig. 8).

Using the Clapeyron-Clausius equation

$$\frac{dT}{dP} = \frac{T_t \cdot \Delta V_t}{\Delta H}$$

and the measured values of dT/dP, T_t and ΔV_t for untreated liposomes, ΔH is calculated at 8.4 kcal/mol. This value is within the range of directly determined values for the normal transition. Spin label measurements of the analogous transition in the membranes of rabbit macrophages at pressures up to 272 atm yield $dT/dP = 0.0275°C/atm$, broadly similar to the value for liposomes [22].

The similar effects of pressure on both treated and untreated liposomes strongly suggest that pressure does not affect the partitioning of the anaesthetic between liposomes and water. Pressure raises T_t in untreated and treated liposomes without decreasing W_t, that is without affecting the cooperativity of the transition. Pressure thus reverses, but does not antagonize the effect of anaesthetics on T_t and it does not reverse their effect on W_t.

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