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# Lipid-dependent differential effects of stereoisomers of anesthetic alcohols

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**The *cis*- and *trans*-alkenols are equally potent general anesthetics but, respectively, lower and raise the gel-to-liquid crystalline phase transition temperature of saturated phosphatidylcholines (Pringle, M.J. and Miller, K.W. (1978) Biochem. Biophys. Res. Commun. 85, 1191–1198). Here we show that although this differential effect is somewhat reduced when a double bond is introduced into the *sn*-2 position of phosphatidylcholine, it is abolished when the ethanolamine head group is substituted for the choline head group in dimyristoyl lipids at neutral pH. At high pH, however, dimyristoylphosphatidylethanolamine assumes a negative charge, and its phase transition temperature drops to a value close to that for the corresponding phosphatidylcholine. Under these conditions the differential effect of the alkenol isomers is restored; the *cis*-alkenol lowers, while the *trans*-alkenol raises, the phase transition temperature of deprotonated dimyristoylphosphatidylethanolamine. Thus, the differential effects of *cis*- and *trans*-alkenols on the gel-to-liquid crystalline phase transition are dependent on the physical chemical characteristics of the polar region of the perturbed lipid species, but only weakly on that of the acyl region.**

## Introduction

The perturbing effects of alcohols on a broad range of membrane-related processes have been extensively investigated. The interest in these phenomena is two-fold. First, by incorporating into the membrane and providing a small, controlled disruption of its structure the alcohols can provide an insight into the forces responsible for the membrane's integrity [1–3]. Second, the pharmacological sites of action of alcohols probably reside in the lipid portion of biological membranes [4–7]. Specific models have been proposed in which perturbation of the lateral segregation of lipids in mixtures [8], or of the gel-to-liquid crystalline phase transition in a single lipid surrounding a protein [9], have been invoked to account for the production of general anesthesia by these agents. However, *cis*-alkenols lower, whereas *trans*-alkenols raise, both the temperature of the phase transition in phosphatidylcholine bilayers containing a single lipid [10] and the temperature of the lateral segregation in a mixture of two lipids [11]. However, these stereoisomers have been shown [12] to have equal anesthetic potency. While these ob-

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Abbreviations: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DMPE, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; ESR, electron spin resonance; *f*, spectroscopic solubility parameter; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; $T_m$, temperature of gel to liquid-crystalline phase transition; Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl; TLC, thin-layer chromatography; $W_{1/2}$, mean half-width of main transition.

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servations are inconsistent with established models of anesthetic action [6], it is probable that in model systems where either the contribution of the acyl chains to gel stability is already compromised (i.e. unsaturated lipids) or the head group makes a strong positive contribution to the gel phase's stability (e.g. phosphatidylethanolamines) (for a review see Ref. 13) the results might not be so inconsistent with anesthetic action.

Here we show that, while the differential effects of the *cis*- and *trans*-alkenols are maintained in stearoyloleoylphosphatidylcholine, they are abolished in dimyristoylphosphatidylethanolamine. Raising the pH of the aqueous environment surrounding DMPE re-establishes the differential effects of the alkenol stereoisomers. A preliminary account of this work has been published [14].

## Materials and Methods

DMPC, SOPC and DMPE (Avanti Biochemicals, Birmingham, AL) and *cis*- and *trans*-tetradecenol (NuCheck Prep, Elysian, MN) were used without further purification. Tempo was synthesized in our laboratory [10] following the method of Rozantsev (1970) [15].

Experimental samples were prepared by evaporating down lipids from chloroform solution under a stream of dry nitrogen. Alcohols were co-deposited from methanolic solutions to give the required final concentration and vacuum dried overnight. The vapor pressure of the alcohols used is so low, that data obtained by this new procedure did not differ from that obtained previously [10]. An aliquot of Tempo, 1 mM in 10 mM Hepes (pH 7.5), was then added, producing a final lipid concentration of 160 mM. The sample mixture was then heated in a water bath to 5 Cdeg above its phase transition temperature and repeatedly vortexed until a homogeneous dispersion was obtained.

In studies performed at a pH $\geq$ 12, lipid dispersions were cooled to 0°C in an ice water bath, and 1–3 $\mu$l of 10 M NaOH added and vortexed. The pH was measured by a glass microelectrode (Microelectrodes Inc., Londonderry, NH). Samples were kept at 0°C and pH $\geq$ 12 for no longer than 30 min prior to the performance of spectroscopy.

Following spectroscopy, samples were overloaded onto duplicate silica gel TLC plates (Whatman LK5DF, Clifton, NJ), chromatographed with a solvent of chloroform/methanol/*n*-propanol/0.25% KCl (w/v)/ethyl acetate (25 : 12 : 29 : 9 : 25, v/v), and developed in iodine vapor or 30% (w/w) $H_2SO_4$ spray. Samples incubated with strong alkali at room temperature and 60°C, and appropriate free fatty acids, served as controls to monitor for products of alkaline hydrolysis.

For ESR measurements, the lipid dispersion was introduced by Pasteur pipette into a glass-stoppered quartz flat cuvette (Wilmad Glass, Buena, NJ) and subsequently flushed with nitrogen. The cuvettes were then placed in a dewar situated in the cavity of a Varian E-109 or E-9 ESR spectrometer. Temperature was monitored by an external thermistor probe (Omega Engineering, Stamford, CT) placed just above the sample, and was kept constant to within 0.1 Cdeg of the desired temperature by heating or cooling a stream of nitrogen flowing through the dewar.

The spectroscopic solubility parameter, $f$, was determined for synthetic lipid dispersions by the method of Shimshick and McConnell [16], which is based upon the temperature-dependent partitioning of the spin label Tempo between lipid and water. A range of 10 Cdeg above and below the main transition was usually studied. Data was generally collected at half degree intervals during heating and further points were taken during cooling. The settle-time of the temperature controller was 3–5 min giving an average scan rate of 6 Cdeg/h. The transition temperature, $T_m$, was defined as the midpoint of the steep portion of the sigmoid-shaped melting curve. The upper and lower boundaries of the phase transition were defined as the points of intersection of the straight lines drawn through the steep section of the curve and the portions of the plot above and below the transition, respectively. The mean half-width, $W_{1/2}$, was half the temperature interval between these two points.

## Results

*Phosphatidylcholines*

The gel-to-liquid crystalline phase transition temperature, $T_m$, derived from the midpoint of plots of the spectroscopic solubility parameter, $f$,

against temperature were found to be 22.7°C for DMPC, within the range of values obtained by this method (for a tabulation see Ref. 17). The value for SOPC was 8.3 ± 0.3°C (± S.D., $n = 4$) at the upper end of the range reported in the literature [17,18]. Above the $T_m$ the temperature dependence of $f$ was greater for SOPC than for the saturated lipids (Fig. 1A).

The effects of the alkenols are shown in Fig. 1B and Table I. In both lipids, the *cis*-isomer lowered $T_m$ but the effect was more marked in SOPC than in DMPC. The *trans*-isomer either raised (DMPC), or had little effect (SOPC) on $T_m$.

*Phosphatidylethanolamines*

The $T_m$ for DMPE was found to be 47.3 ± 0.3°C ($n = 4$) (Fig. 2A). This value was consistent with those reviewed (47.5–49.5°C) [17] and, as would be expected for a probe technique, was slightly lower than those reported in recent high precision calorimetry and dilatometry studies [19,20].

In contrast to DMPC, both the alkenol isomers lowered the $T_m$ of DMPE by nearly 2 degrees at 10 mol%. Only at 20 mol% did the *cis*-isomer have a bigger effect than the *trans*-isomer (Fig. 2A and Table I).

The controlled pH increase caused a dramatic lowering of the main transition which fell by more than 20 Cdeg to 23.8°C, close to the value for DMPC. This behavior parallels exactly that reported for DPPE whose $T_m$ fell by 22° to that of DPPC when the pH was raised to 12.0 [21]. Along with the lowering of $T_m$ high pH also restored the differential effect of the alkenol geometric isomers. Table I shows that 20 mol% *cis*-tetradecenol lowered $T_m$ by 1.7 Cdeg whereas the *trans*-isomer raised $T_m$ by 5.1 Cdeg just as it did in DMPC (Compare Fig. 1B with Fig. 2B).

Care was needed in adjusting to high pH in order to avoid problems associated with hydroly-

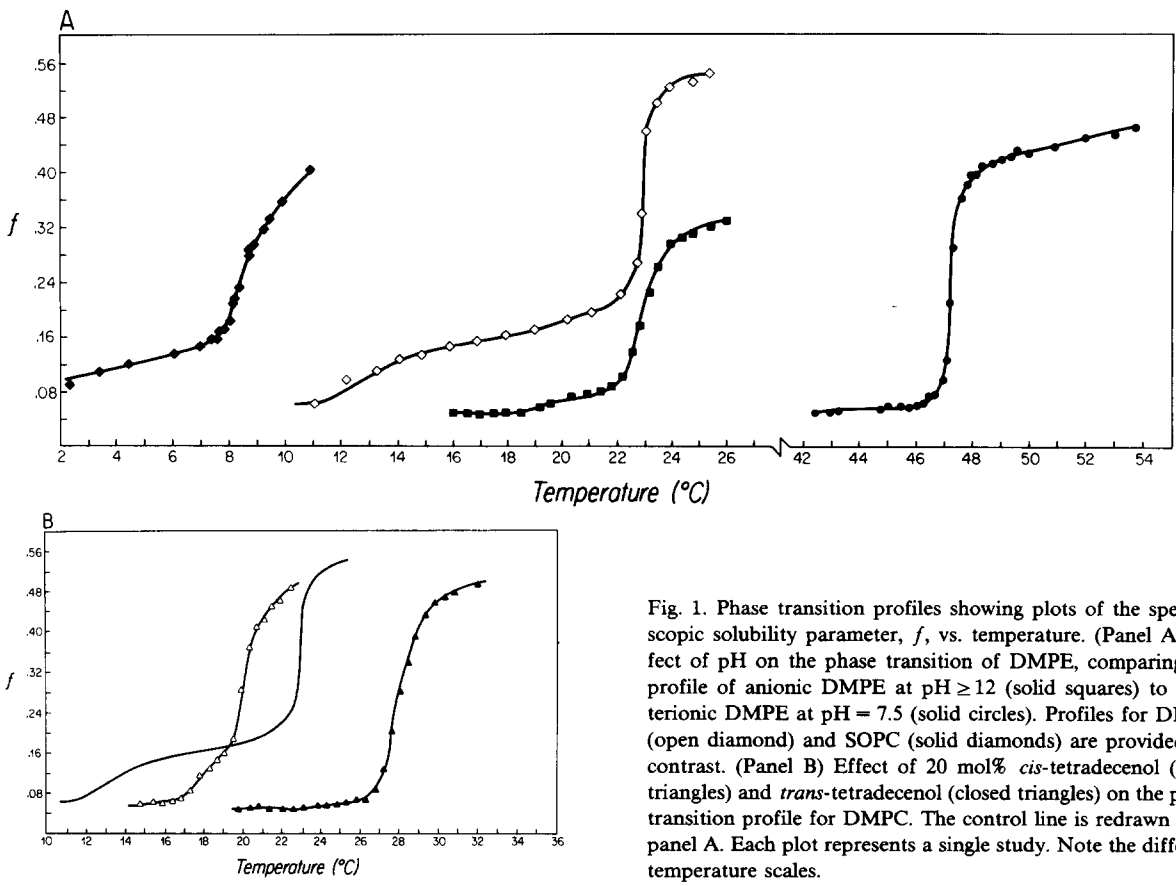


Fig. 1. Phase transition profiles showing plots of the spectroscopic solubility parameter, $f$, vs. temperature. (Panel A) Effect of pH on the phase transition of DMPE, comparing the profile of anionic DMPE at pH ≥ 12 (solid squares) to zwitterionic DMPE at pH = 7.5 (solid circles). Profiles for DMPC (open diamond) and SOPC (solid diamonds) are provided for contrast. (Panel B) Effect of 20 mol% *cis*-tetradecenol (open triangles) and *trans*-tetradecenol (closed triangles) on the phase transition profile for DMPC. The control line is redrawn from panel A. Each plot represents a single study. Note the different temperature scales.

TABLE I

CHANGE IN THE TRANSITION TEMPERATURE ($\Delta T_m$), AND TRANSITION HALF-WIDTH ($\Delta W_{1/2}$) FOR VARYING CONCENTRATIONS OF *cis*- AND *trans*-TETRADECENOLS INCORPORATED INTO LIPID BILAYERS

| | *cis*-Tetradecenol | | | *trans*-Tetradecenol | | |
|---|---|---|---|---|---|---|
| | mol% | $\Delta T_m$ | $\Delta W_{1/2}$ | mol% | $\Delta T_m$ | $\Delta W_{1/2}$ |
| DMPC | 20 | −2.7 | +0.2 | 20 | +5.2 | +0.8 |
| SOPC | 10 | −2.3 | +0.1 | 10 | +0.03 | +0.1 |
| | 20 | −3.7 | +0.4 | 20 | +0.33 | +0.1 |
| DMPE | 10 | −1.7 | +0.1 | 10 | −1.8 | +0.1 |
| (pH = 7.5) | 20 | −5.3 | +2.0 | 20 | −1.1 | +0.1 |
| DMPE | 20 | −1.7 | +0.9 | 20 | +5.1 | +0.5 |
| (pH ≥ 12) | | | | | | |

sis. When the pH was greater than thirteen, TLC revealed hydrolysis of DMPE within 30 min at room temperature, and under these conditions $T_m$ was poorly reproducible and $W_{1/2}$ increased to over 10°C. However, if care was taken to adjust the pH to twelve, or only just above, no hydrolysis was detected at 30°C even when incubations lasted 6 hours. At 50°C hydrolysis was not immediately apparent, but was present after 30 min. Our scans at this pH rarely went above 30°C and then only for a brief time. Thus, in practice, provided the pH was never allowed to go much above twelve $W_{1/2}$ remained within normal bounds (see below).

*Mean half-width of main transition*

Although the $W_{1/2}$ as defined by the spectroscopic solubility parameter does not correspond to that determined by calorimetry, it does provide a guide to relative changes. We determined $W_{1/2}$ as described with a precision of ±0.2 Cdeg.

In DMPC both alkenols increased $W_{1/2}$ from the control value of 0.3 with the *trans*-isomer having the biggest and only significant effect. This was the only occasion on which the *trans*-isomer had a bigger effect than the *cis*-isomer. In this it resembles the hexadecenols in DMPC [10].

SOPC had a relatively broad transition (0.8 Cdeg). This compares to values around 2 Cdeg reported by calorimetry at high scan rate [18]. The *trans*-isomer caused no additional increase. The *cis*-isomer caused a significant increase in $W_{1/2}$ but only at 20 mol% and this was small proportionately.

In DMPE at pH 7.5 (control $W_{1/2}$ = 0.5 Cdeg in accord with Refs. 19 and 20) the situation resembled that in SOPC but the *cis*-isomer at 20 mol% caused the biggest increase, proportionately and absolutely, observed in these experiments. The magnitude of this effect is double that of any observed in this work and is only equalled in the tetradecenols by the effect of the same isomer on DPPC [10]. In DMPE at high pH, $W_{1/2}$ was not significantly broader than at pH 7.5 when runs showing signs of hydrolysis were discarded (see

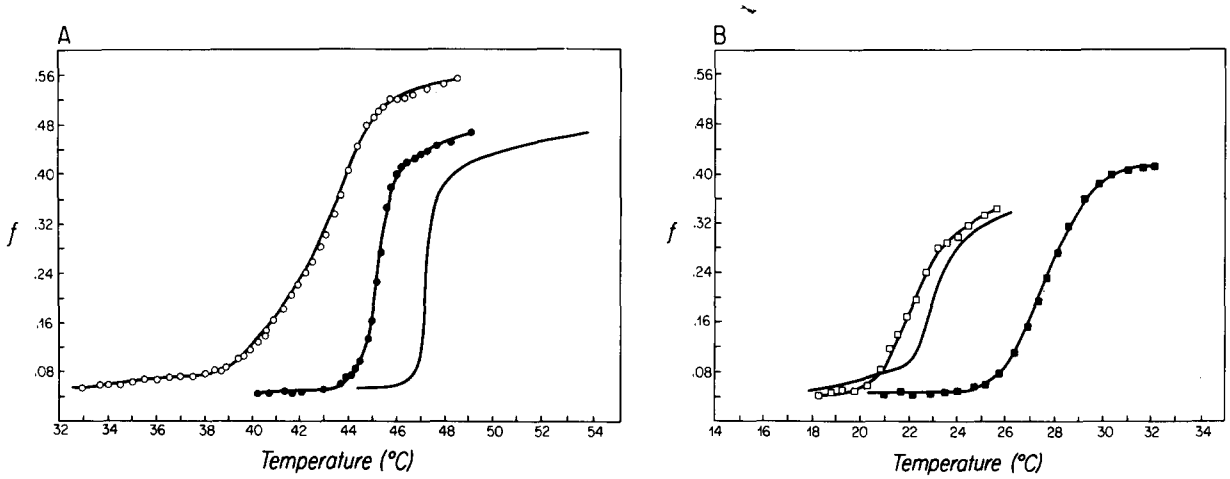


Fig. 2. Phase transition profiles showing plots of the spectroscopic solubility parameter, *f*, vs. temperature for DMPE containing 20 mol% *cis*-tetradecenol (open symbols) or *trans*-tetradecenol (closed symbols). (Panel A) Zwitterionic DMPE at pH = 7.5 (circles). (Panel B) Anionic DMPE at pH ≥ 12 (squares). Controls lines are redrawn from Fig. 1.

above). Both isomers caused an increase; that for the *cis*-isomer again was the most marked.

*The pre-transition*

A pre-transition was detected in DMPC at 12°C and was completed by 14°C (Fig. 1), in agreement with other workers [22]. This pre-transition temperature increased in the presence of alkenols. In the presence of 20 mol% *cis*-tetradecenol it was detected at 16.8°C and merged with the main transition at 19°C, but in the presence of the same concentration of the *trans*-isomer the pre-transition was not observed. In one experiment at 10 mol% *trans*-tetradecenol, which showed a $T_m$ of 24.3°C, a pre-transition extended from 19.5°C to 22.2°C and was distinct from the main transition.

In SOPC no pre-transition was observed but our scans commenced at 2°C, only 6 Cdeg below $T_m$ (Fig. 1). Other workers report similar findings in scans commencing at −8°C [18].

DMPE at pH 7.5 showed no pretransition above 42°C, in agreement with other work [19,20], but at high pH a weak pre-transition was detectable in the range 19 to 21°C (Fig. 2B). In the ether analog of DPPE a pre-transition has been noted at high, but not low, pH and its separation from the main transition was found to depend critically on salt concentration [23]. At 20 mol% this pretransition was not seen.

## Discussion

The new finding of this work is that the ability of the *cis*- and *trans*-isomers of tetradecenol to modulate the gel-to-liquid crystalline phase transition temperature of phospholipids depends on both the lipid head group and its aqueous environment. This effect is seen most dramatically when DMPC is compared to DMPE. At high pH DMPE behaves exactly like DMPC both in the value of $T_m$ and its response to the alkenol isomers. However, on lowering the pH a proton is added to the amine of the ethanolamine head group. This single change abolishes the ability of the *trans*-isomer to raise $T_m$, and at 10 mol% no *cis-trans* difference is seen. On the other hand, introducing a double bond in the phospholipid acyl region leaves the *cis-trans* difference intact, although it attenuates the ability of *trans*-tetradecenol to raise $T_m$.

The ability of the *trans*-alkenols to elevate $T_m$ reflects their ability to preferentially partition into, and stabilize, the gel phase. A detailed argument in support of this has been presented previously [10]. For our purposes, the stability of the gel phase can be thought of as arising from interactions both in the acyl region and in the head group region. Thus, in disaturated phosphatidylcholines, $T_m$ increases with acyl chain length because of increasing van der Waals attraction per mole. The fact that the choline head group has a larger cross-sectional area than the acyl region is accommodated in various ways (vertical displacement, tilting and rippling) in the different gel phases of DPPC but at some cost to overall stability [24]. Removal of *N*-methyl groups from the choline moiety causes successive increases in $T_m$, arising from the ability of the amine and phosphate groups to approach each other closely, forming an intermolecular hydrogen bond and salt bridge [13,17,21,26]. The importance of this bond is seen in saturated PEs on raising the pH above the p$K$ of the amine. This causes the $T_m$ to fall toward the value exhibited for DMPC (this work, and Refs. 13, 21, 26). Apparently such a bond is formed only if the correct relationship between neighboring phosphate and amine groups is maintained. Thus, in a head group with six instead of two methylenes between the phosphate and amine groups, removal of the *N*-methyl groups has no effect on the phase transition which is also insensitive to pH [21].

Incorporation of a long chain alcohol into lipid bilayers will both increase head group spacing and alter acyl packing. The differential effects of the tetradecenol isomers must originate in the stereochemistry of the double bond in the 9,10 position. The *trans*-isomer most closely resembles the all-*trans* configuration of the lipid's saturated acyl chains except for a small lateral jog of the long axis at the double bond, whereas the *cis* double bond effectively prevents the adoption of any configuration capable of packing closely with all-*trans* acyl chains. Thus both isomers will increase head group spacing, but the *cis*-isomer will be a little more effective than the *trans*-isomer. However, the *cis*-isomer will greatly disrupt the short range van der Waals attraction in the acyl region whereas the *trans*-isomer will only have a slight

destabilizing effect (compare corresponding alkanols and *trans*-alkenols in Ref. 10).

Accordingly, on insertion of the alcohol into the gel phase of disaturated phosphatidylcholine, $T_m$ is increased by *trans*-tetradecenol because the favorable relief of steric and coulombic repulsion in the head group region is not offset by a large reduction in acyl interactions as it is when the *cis*-isomer is inserted. In SOPC on the other hand, the unsaturated acyl chain of the phospholipid has already reduced head group repulsion at the expense of lowering acyl interactions ($T_m$ is lowered about 50 Cdeg compared to DSPC) and *trans*-tetradecenol only raises $T_m$ modestly, if at all (Table I). A further effect tending to attenuate the increase in $T_m$ in this case is the mismatching of chain length between the lipid and the alcohol. As we have commented on previously this increases the energetic price paid for insertion of even *trans*-alkenols because of reduction of methylene – methylene molecule interactions [10].

Unlike DMPC, DMPE at pH 7.5 exhibits strong attractive head group interactions which greatly stabilizes the gel phase. Because the juxtaposition of neighboring phosphate and amine is critical (see above) insertion of either alkenol isomer between phospholipids will tend to disrupt this stabilizing effect. Thus partitioning into the gel is no longer favored for even the *trans*-isomer, and the colligative lowering of $T_m$ dominates. Only at 20 mol% does the *cis*-isomer exert a greater effect than the *trans*-isomer, and this effect was accompanied by a marked broadening of the phase transition. *cis*-Tetradecenol caused no significant broadening at 10 mol%, but caused a 2 Cdeg increase in $W_{1/2}$ at 20 mol%. Thus the possibility of phase separation cannot be ruled out. At pH $\geq$ 12, however, a proton is removed from the amine group which destabilizes the gel phase, lowering its $T_m$ to that of DMPC. Now insertion of *trans*-tetradecenol is able to stabilize the gel just as effectively as it did in DMPC. This suggests that stabilizing the gel phase of DMPC involves relief of coulombic repulsion between phosphates rather than between quaternary ammonium ions. This is consistent with the fact that substitution of $-C(CH_3)_3$ for $=N^+(CH_3)_3$ causes no change in $T_m$ [21].

Our demonstration that stereoisomeric alkenols may exert quite different effects on phosphatidylcholines and phosphatidylethanolamines at pH 7.5 suggests their usefulness as probes of membrane organization. Indeed the fluorescent probes based on *cis*- and *trans*-parinaric acids clearly exploit the same principles [27]. The role of the more stable alkenols, however, would not be as reporter groups but as probes of the dependence of membrane function on lipid structure or organization. A number of workers have similarly made use of *cis*- and *trans*-fatty acids with some success [28].

*Implications for models of general anesthetic action*

This work shows that if one adopts a model of general anesthesia based on phase transitions of lipids [8,9], then the equal potencies of the *cis*- and *trans*-alkenols can only be accommodated if the lipids at the putative site of action behave like phosphatidylethanolamines rather than phosphatidylcholines. Our predictive model would suggest that any lipid in which head-group interactions lead to a significant stabilization of the gel phase relative to the equivalent phosphatidylcholine might participate in anesthetic action. It is interesting to note that it is just these lipids which have a strong tendency to form separate phases in lipid mixtures. Thus in a recent study PE showed a strong tendency to exclude cholesterol, which points to cholesterol-poor microregions of excitable membranes as possible sites of this type of anesthetic action. This prediction contrasts with that of alternative mechanisms based on concepts of lipid disordering, which suggest that cholesterol-rich regions of membranes best mimic the anesthetic site [6,10]. These contrasting predictions offer a means of distinguishing between the two types of theory.

Mechanisms by which changes in lateral separation of lipids might influence excitability have been discussed elsewhere and need not be reviewed here [8,9]. The important point is that such theories can now no longer be unequivocally rejected because of the equal potencies of the *cis*- and *trans*-alkenols; rather if one accepts these models the type of lipid which might be involved in anesthesia is restricted by our observations.

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