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Different effects of propofol and nitrosopropofol on DMPC multilamellar liposomes

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Abstract

The mechanisms of reaction of propofol with nitrosoglutathione lead to the formation of an active species which was identified, and then synthesised, as 2,6-diisopropyl-4-nitrosophenol. In the present work, we demonstrate the in vitro formation of 2,6-diisopropyl-4-nitrosophenol, then we discuss the interaction of propofol and 2,6-diisopropyl-4-nitrosophenol with dimyristoylphosphatidylcholine and egg yolk phosphatidylcholine multilamellar liposomes using differential scanning calorimetry and spin labelling techniques. It was demonstrated that both molecules are highly lipophylic and absorb almost entirely in the lipid phase. The thermotropic profiles showed that these molecules affect the temperature and the co-operativity of the gel-to-fluid state transition of the liposomes differently: the effects of 2,6-diisopropylphenol on the lipid organisation are quite similar to phenol and coherently interpretable in terms of the disorder produced in the membrane by a bulky group; 2,6-diisopropyl-4-nitrosophenol is a stronger perturbing agent, and ESR spectra suggest that this is due to a relative accumulation of the molecule into the interfacial region of the bilayer.

Keywords: Propofol; Nitrosopropofol; Liposomes

1. Introduction

In a recent study, we investigated the possible involvement of the NO system in the mitochondrial effects of 2,6-diisopropylphenol (DPP), a widely used anaesthetic known as propofol [1].

In our mitochondrial preparations, DPP was added diluted in ethanol and NO was supplied by using nitrosoglutathione (GSNO) as a donor. It was found that, in the presence of either 0.15–0.2 mM DPP or 0.25 mM GSNO alone, mitochondria were clearly suffering, but maintained an acceptable amount of energetic activity. On the contrary, when both 0.2 mM DPP and 0.25 mM GSNO were simultaneously present in the incubation medium, mitochondrial respiration was completely insensitive both to ADP and CCCP additions, ATP synthesis was substantially not detectable and also

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oligomycin-sensitive ATP lysis undergoes an apparent and strong inhibition.

From experimental evidence, it appeared that a synergism exists between NO and the anaesthetic, which leads to the full abolition of respiration and we suggested that the effects might be ascribed to little amounts of a new molecule possibly derived from the interaction of DPP and GSNO in the mitochondrial environment.

These results could also have relevant implications in the pharmacological field, so we investigated the mechanisms of reaction of DPP with NO and GSNO and the possible formation of active species, one of which was identified, and then synthesised, as 2,6-diisopropyl-4-nitrosophenol (DPPNO).

In the present work, we demonstrate the in vitro formation of DPPNO, then we discuss that side of the problem concerning the interaction of DPP and DPPNO with the membrane, in order to obtain information about the drugs distributions and the modifications they produce in the lipid organisation. We have also determined the pK_a values and the partition coefficients, which are important parameters for evaluating drug toxicity and enter into the activity evaluation [2]; in particular, there is a good correlation between the activity of anaesthetics and their lipid/water partition coefficients [3].

Dimyristoylphosphatidylcholine (DMPC) and egg yolk phosphatidylcholine (EYPC) multilamellar liposomes were used as membrane model systems and studied by means of differential scanning calorimetry (DSC) and spin labelling techniques. The experiments were repeated with phenol (POH) for propofol to verify if DPP and DPPNO have any specific behaviour with respect to the lipid bilayers.

It was found that the effects of phenol and DPP on the lipid organisation are quite similar and coherently interpretable in terms of the disorder produced in the membrane by small bulky groups, while DPPNO is also a stronger membrane perturbing agent.

These results agree, in part, with the data reported by Tsuchiya [4] in a very recent work where it was observed that propofol lowered the phase transition of liposomal model membranes and increased their fluidity. Moreover, by comparison with a number of alkyl and dialkylphenols and benzenes, a structure-specific action of propofol was stated. Our data demonstrate that the addition of NO to the propofol molecule greatly enhances its effects on model membranes and (data submitted for publication) mitochondrial respiration.

2. Materials and methods

2.1. Chemicals

All chemicals, of the highest available quality, were obtained from the Sigma Chemical Co. (St. Louis, USA), while the solutions were prepared with quality milliQ water. NO gas, with a purity grade of 99.9%, was purchased from SIAD S.p.A. (Milano).

2.2. 2,6-diisopropyl-4-nitrosophenol synthesis

The synthesis of 2,6-diisopropyl-4-nitrosophenol (DPPNO) was carried out following the same procedure as for nitrosophenol [5]. In particular, 2 ml of concentrated hydrochloric acid were slowly added to a solution of 3 g of 2,6-diisopropylphenol (DPP) in 25 ml ethanol cooled to -5 °C. A solution of 1.1 g sodium nitrite dissolved in 5 ml water was gradually added to the mixture under vigorous agitation, maintaining the temperature below 0 °C. After the addition of all the nitrite, taking approximately 15 min, the yellow product was agitated for 30 min and then poured into iced water. The yellow precipitate was crystallised from toluene and characterised by GC/MS analysis. A purity grade higher than 95% was confirmed by thin layer chromatography.

2.3. S-nitrosoglutathione synthesis

S-nitrosoglutathione was prepared according to the procedure of Hart [6].

2.4. GC/MS procedure

Gas chromatographic analyses were carried out with a Shimadzu gas chromatograph GC 17A equipped with a QP5000 mass spectrometer. A

capillary column, 25 m×25 μm WCOT CP-SIL 8CB low bleed purchased by Chrompack, was used. For the identification of the products, the Database Library Editor NIST 62 was used.

2.5. Liposome preparation

Multilamellar vesicles were prepared following the method of Kusumi et al. [7]. Phospholipids were dissolved in a 2:1 chloroform/methanol mixture, then dried with a stream of nitrogen gas and kept under vacuum for at least 14 h. The dried lipids were suspended, when not otherwise specified, in a HEPES 0.1M, pH 7.2, buffer. The lipid dispersion, with a 101 mM final lipid concentration, was warmed at approximately 40 °C, mixed vigorously with a vortex for 30 s and used just as obtained for DSC measurements. When required, spin labels were added to the chloroform methanol mixture.

2.6. Determination of the partition coefficients

The octanol–water partition coefficients of the phenols were experimentally determined by simply shaking a solute with the two immiscible solvents and then analysing the solute concentration in both phases [8] through their absorbance values: for propofol $\lambda = 270$ nm, $\varepsilon = 1100$; for phenol $\lambda = 270$ nm, $\varepsilon = 1300$; for DPPNO $\lambda_1 = 310$ nm, $\varepsilon_1 = 18\,000$, $\lambda_2 = 386$ nm, $\varepsilon_2 = 25\,000$.

For the determination of the liposome–water partition coefficient, the lipid dispersion, maintained for 1 h at 20 °C, was then centrifuged at $12\,000 \times g$ for 15 min at the same temperature; the supernatant was carefully drained from the pellets, and the amount of the phenols in the solution was calculated from their absorbance values.

2.7. UV-Vis

Spectrophotometric measurements were recorded on a UV-Vis Beckman DU 640 instrument. GSNO decay and products formation were monitored following the absorbance changes at specified wavelengths in closed thermostated quartz cell. All measurements were taken in triplicate.

2.8. DSC measurements

Calorimetric measurements were performed on a Setaram DSC 92.

Approximately 50 mg of phospholipid dispersion was placed in an aluminium crucible. An identical crucible was filled with an equivalent weight of HEPES solution and placed in the reference cell.

The temperature scanning rate was 0.5 °C min^{-1}. The transition temperature T_c from the L_β to L_α phase of DMPC was taken at the peak of the DSC profiles.

2.9. ESR measurements

ESR measurements were performed on a Bruker ER 200 D, 9 GHz spectrometer at microwave power range from 0.1 to 220 mW. Samples were placed in a gas permeable TPX tube, 1 mm i.d. (Wilmad, NJ, USA) and centred in the resonant cavity, then deoxygenated under nitrogen flow.

ESR spectra are interpreted in terms of the experimental order parameter S which is experimentally defined as: $S = 0.5407 \times [3(A_{max} - A_{min})/(A_{max} + 2A_{min})]$, where A_{max} and A_{min} are suitable peak positions in the spectra.

3. Results and discussion

3.1. DPPNO formation

When a 250-μM GSNO solution in 15 mM MOPS, pH 7.0 was incubated for several hours in aerobic conditions with an equimolar concentration of propofol, no change of the UV-Vis absorption spectra was observed and the nitrosoglutathione absorption band at 336 nm remained unchanged.

After the addition of 100 μM Cu^{II}, a yellowish hue slowly appeared. UV-Vis spectra, recorded at 60 min after the mixing of the reactants, evidenced the formation of an absorption band at 310 nm as a consequence of a chemical reaction. When the dissolved oxygen was excluded, the peak at 336 nm, related to the GSNO, disappeared but no new bands were formed.

The chemical nature of the products was determined by carrying out the reaction on a micropre-

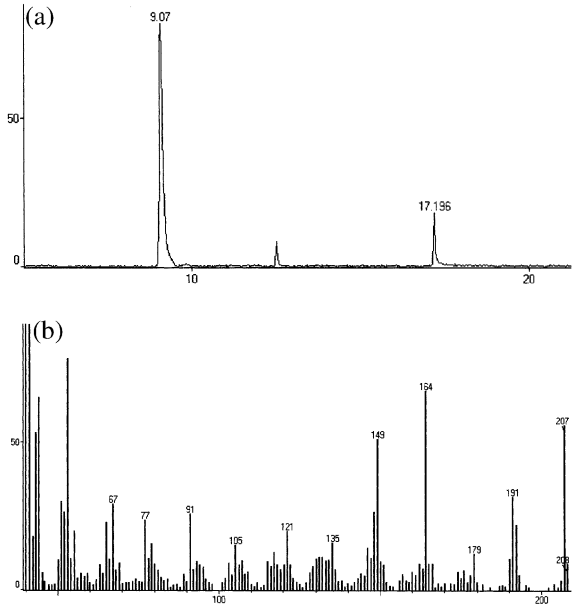


Fig. 1. (a) Gas chromatographic profile of the reaction products of propofol and GSNO in the presence of Cu^{2+}, in MOPS-buffered solution pH 7.0. The two GC peaks at 9 and 13 min are due to propofol and to a stabilising agent of the ether solution; the peak with a retention time of 17.2 min is characterised by a mass/charge m/z ratio of 207 according to the molecular weight of DPPNO. (b) Mass spectrum of the reaction product with a retention time of 17.2 min.

parative scale. In particular, an air-equilibrated solution of 250 μM GSNO, 250 μM propofol and 100 μM copper sulfate in MOPS, 15 mM, pH 7.0, was allowed to react at room temperature for several hours in the dark. The resulting yellow solution was extracted with ethylic ether and the organic phase dried on magnesium sulfate.

GC/MS analysis of the ether solution showed a peak characterised by a retention time of 17.2 and a mass/charge m/z ratio of 207 (Fig. 1), which is consistent with the hypothesis of the formation of 2,6-diisopropyl-4-nitrosophenol.

To confirm this possibility, we synthesised 2,6-diisopropyl-4-nitrosophenol on the basis of the procedure for the preparation of 4-nitrosophenol reported by Kharasch [5] and described in Section 2.

The gas chromatographic retention time and the mass spectra of 2,6-diisopropyl-4-nitrosophenol turned out to be identical to those obtained from the analysis of the compounds obtained by the reaction between propofol and GSNO in the presence of copper and characterised by a m/z ratio of 207.

The identification of the compound is also confirmed by UV-Vis spectra; in fact, in aqueous buffered solution, the two absorption bands of DPPNO are analogous to the spectrum reported for nitrosophenol [5], and compatible with the peak wavelengths and the spectrum shape obtained from the reaction solution.

The initial rate V_o of product formation as a function of the copper concentration is plotted in Fig. 2. V_o values were calculated from the variations in absorbance at 310 nm. The initial lags, sometimes observed, were ignored and the initial rate values were taken on the interval of higher slope of the curve of absorbance vs. time.

Appreciable amounts of DPPNO were only obtained at copper concentrations ≥ 50 μM; from 50 μM to approximately 0.2 mM, the DPPNO concentration is linear depending on the copper concentration; above this value, the slope decreases significantly.

The direct reaction between nitric oxide and propofol was also investigated at the different experimental conditions. In particular, the experiments were carried out bubbling pure NO gas in test tubes containing 250 μM DPP, in 15 mM MOPS buffered solutions at pH 7.0 and 22 °C

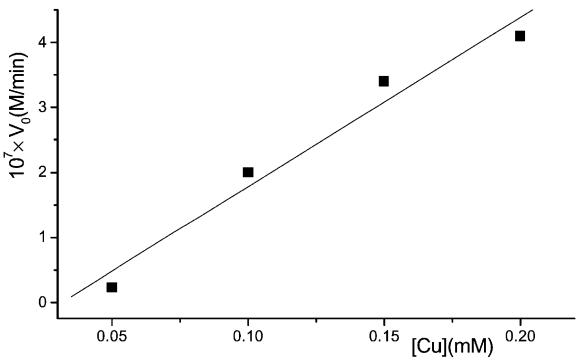


Fig. 2. Influence of Cu^{2+} concentration on the initial rate of DPPNO formation in solutions containing 0.25 mM GSNO, 0.25 mM DPP and 15 mM MOPS buffer, pH 7.0, $T = 22$ °C.

Table 1
Values of log P at 20 °C for the various systems

	Octanol–water	Liposomes–water	
		DMPC	EYPC
POH	1.48 [28]	1.81	2.17
DPP-NO	1.97	2.42	2.53
DPP	2.84	2.89	2.60

temperature under anaerobic and aerobic conditions.

UV-Vis spectra and GC/MS analysis did not show appreciable amounts of DPPNO; its presence in a solution of 100 μM Cu^{2+} did not change the results significantly.

3.2. Partition coefficients

The logarithm of the octanol–water partition coefficient (log P) is used as a measure of the hydrophobicity of compounds and it is assumed to represent the general tendency of a chemical to partition between an aqueous and an organic phase. P is defined as $P=(n_{oct}/V_{oct})/(n_w/V_w)$, where n denotes the number of substituted phenols moles, V is the volume and $_{oct}$ and $_w$ refer to octanol and water phase, respectively. For the liposomes–water system $P=(n_{lip}/V_{lip})/(n_w/V_w)$, which requires the evaluation of the total liposome volume V_{lip}; it can be expressed as $V_{lip}=\beta(C_{lip})_0V_0$ [9], where β is the molar volume of lipids (l/mol), $(C_{lip})_0$ is the total lipid concentration and V_0 is the total volume. In the majority of experiments, $V_{lip}\ll V_0$, hence $V_0\approx V_w$; β depends on the nature of phospholipids and on which mesophase is present: we used $\beta=0.630$ for the L_β gel phase of DMPC [10], and $\beta=0.757$ for EYPC [11].

In Table 1, the values of log P, measured at 20 °C, are quoted for the various systems.

As is shown, the hydrophobicity is dependent on the polarity of the NO group and on the presence of hydrocarbon chains linked to the aromatic ring, so that the more polar POH is also the less hydrophobic, because the OH groups of DPPNO and DPP are screened by two highly hydrophobic isopropyl residues in positions 2 and 6. This makes DPP and DPPNO two highly lipophylic molecules, which, in a water dispersion of multilamellar liposomes, are almost exclusively present in the lipid phase. The log P values are systematically higher in liposomes than in octanol, as already observed for a number of different substituted phenols [12].

3.3. Dissociation constant

In Fig. 3a, the UV/Vis spectrum of DPPNO is reported. At neutral pH, DPPNO shows two absorption bands at 310 and 386 nm in accordance with the spectrum of nitrosophenol [5]. The two bands identify the non-dissociated and dissociated forms of the phenolic –OH group, as was confirmed by UV/Vis spectra at acid and alkaline pH. A pK_a of 7.5 was determined from the experimental dissociation curve of DPPNO (Fig. 3b) calculated through the absorbances at different pH values. It is more than three orders lower that the pK_a of propofol ($pK_a\approx11$), the difference being

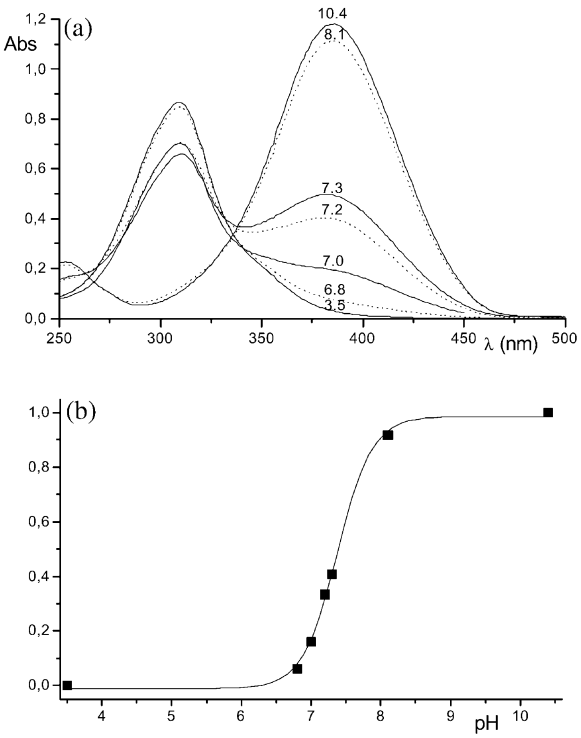


Fig. 3. (a) UV-Vis spectrum of DPPNO at different pH values. The values of pH are reported for each curve in the figure. (b) Dissociation curve of DPPNO vs. pH values.

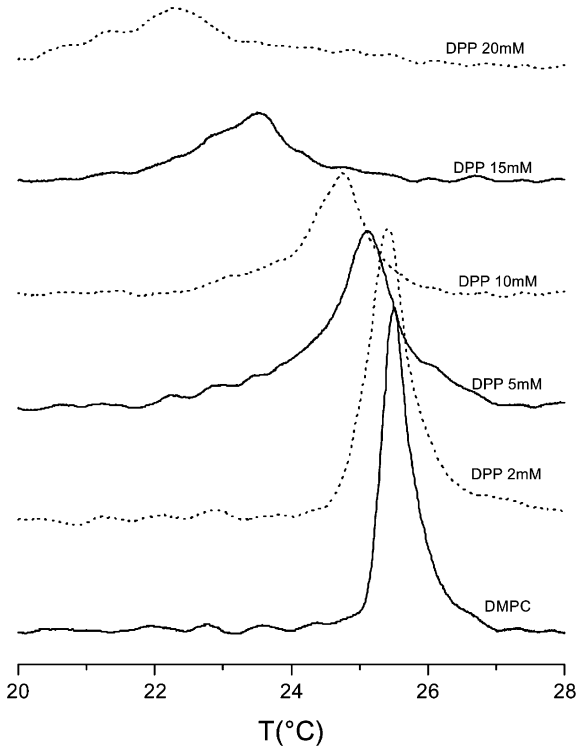


Fig. 4. DSC profiles of the gel-to-fluid state transition of DMPC multilamellar liposomes at increasing DPP concentrations. *y* Axis: dH/dT in arbitrary units; *x* axis: temperature T in °C.

due to the electron-withdrawing effect of the –NO group in the para position to the –OH group, which increases its acidity. As expected, the pK_a of DPP is similar to the value of p$K_a = 10$ reported for phenol [13].

3.4. Differential scanning calorimetry

The DSC profiles of the liposomes (Figs. 4–6) are broadened and shifted towards lower temperatures (Fig. 7) at increasing dopant concentrations, while no phase separation phenomenon was observed; the effects of DPP and POH are widely comparable, but at low and intermediate concentrations, the shift of T_c is larger in the presence of POH. The broadened profiles and the lowered transition temperatures demonstrate that both the size and the packing of the co-operative units undergoing the transition are modified by DPP and POH and indicate, coherently, with the results of ESR measurements, that the ordered organisation of phospholipids in the gel state is perturbed at any depth [14].

The T_c dependence on concentration is strikingly different in the case of DPPNO, which, at concentrations above 10 mM, destroys any detectable transition.

According to the equation $dT_c/d[P] = (K_{gel} - K_{liq})/[a + b(K_{gel} + K_{liq})]$, where $[P]$, K_{gel}, K_{liq}, a and b are the total concentration of the solutes, their partition coefficients in the gel and fluid phase, and two constants, respectively [15]; the decrease of T_c is determined by a greater partitioning of the molecules into the liquid than into the gel phases of DMPC. The partitioning of phenols is an entropy-driven process below T_c, while it is an enthalpy-driven process above it; the entropy gain originates mainly from the removal of hydro-

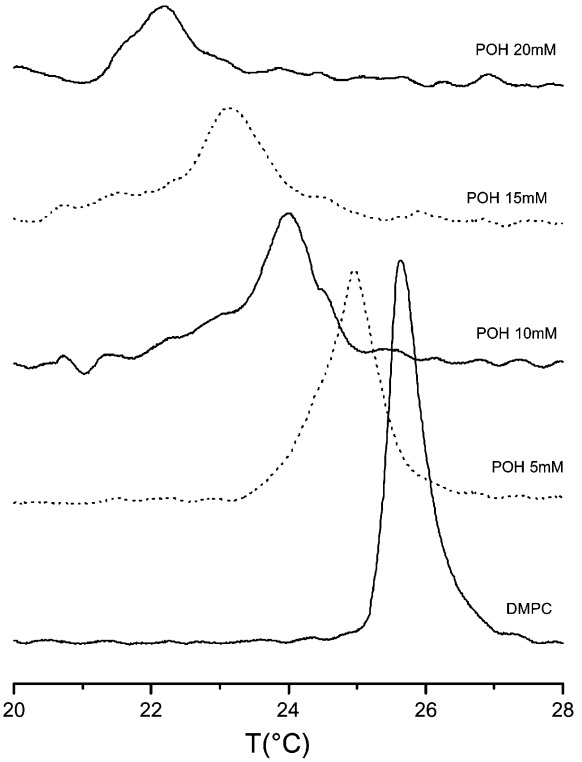


Fig. 5. DSC profiles of the gel-to-fluid state transition of DMPC multilamellar liposomes at increasing POH concentrations.

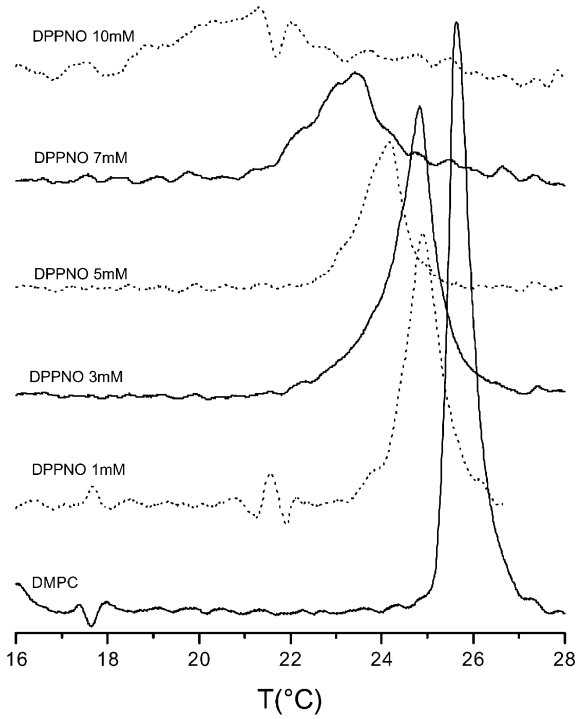


Fig. 6. DSC profiles of the gel-to-fluid state transition of DMPC multilamellar liposomes at increasing DPPNO concentrations.

carbons from water [16] while the enthalpy contribution, which, from the sign of ΔT_c, seems to predominate, is determined by attractive forces. In the case of POH and DPP, these forces may be of van der Waals type or may arise from the hydrogen bonding of phenolic OH with phospholipids. Instead, the strong preference of DPPNO for the fluid phase suggests, as will be discussed later, that other forces play a role in the partitioning of this molecule.

3.5. Order parameter S

ESR spectra have been collected for stearic acids, spin labelled at the fifth, seventh and 10th carbon positions, incorporated in DMPC and EYPC liposomes, with and without dopants, as described in sample preparation. The *S*-parameter was measured over (27.5 °C) and below (20.0 °C) the gel to fluid state transition temperature of

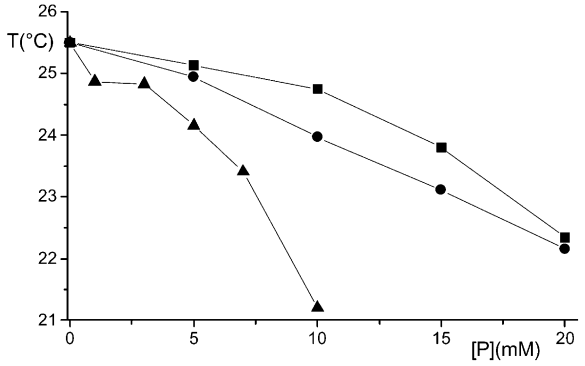


Fig. 7. Phase transition temperatures of DMPC liposomes vs. the [*P*] concentrations in mM of: ■ — DPP; ● — POH; and ▲ — DPPNO. The temperatures are taken at the peak of the DSC profile.

DMPC, and at 25 °C in the case of EYPC, which does not have a definite transition temperature. In the following, we will only discuss the effects on the gel state of DMPC, because in other cases, *S* did not change by addition of the dopants. Evidently the lipid matrices, in the fluid state, can accommodate rather high (up to 20 mM) concentrations of the dopants without aggravating the disordered motions of the lipid chains, as it was already described, for example, in [17].

The values of *S* in DMPC liposomes at various DPP concentration are shown in Fig. 8; in Fig. 9a,b, *S* is plotted for the three dopants at 5 and 10 mM concentrations. In the gel state, the order

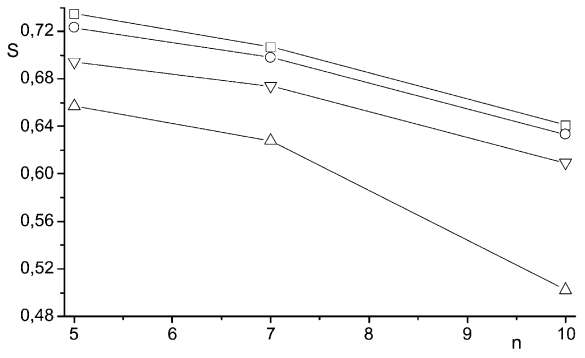


Fig. 8. Order parameter *S* values vs. spin label position (*n*) at increasing DPP content: □ — 0 mM; ○ — 5 mM; ▽ — 10 mM; and △ — 20 mM.

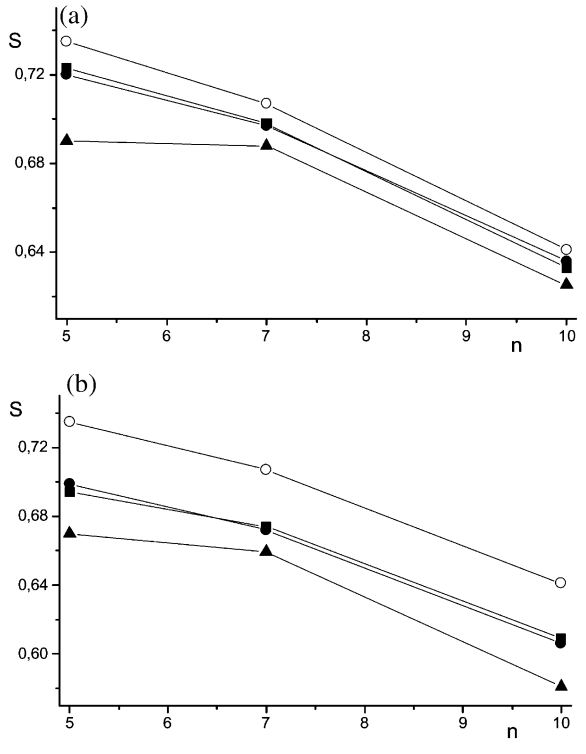


Fig. 9. Order parameter S values vs. spin label position (n) for the three dopants at (a) 5 mM and (b) 10 mM concentrations: ○ — without dopants; ■ — DPP; ● — POH; and ▲ — DPPNO.

parameter S for n-SASL's in pure DMPC liposomes is only slightly decreasing from the surface to the centre of the bilayer, and Fig. 8 evidences how the S values, at all DPP concentrations, are shifted by approximately the same amount, irrespective of the position n of the label; deviations from this regular trend can be observed only at the highest concentration (20 mM) in correspondence with 10 SASL. Moreover, from the data (Fig. 9a,b), it appears that there are no meaningful differences between DPP and POH.

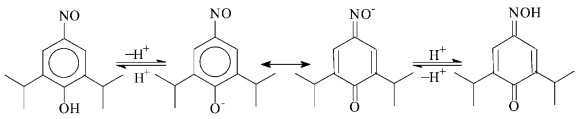
DPPNO lowers the order of the lipid chains more than POH and DPP at all depths, but the perturbation is stronger in correspondence with the fifth position, indicating a possible accumulation near the interfacial region.

If we look at the results from DSC and ESR, we can recognise some coherency. POH and DPP behave in exactly the same way: they accumulate in the lipid region of the bilayer and, at least at the tested depths, from the fifth to the 10th spin-labelled position, the changes in the order parameter S indicate that they diffuse almost uniformly in this region. They could be seen as bulky groups which lower both the temperature and the cooperativity of the transition because, when intercalated in the lipid moiety, through their steric hindrance and collisions, they are able to reduce the van der Waals forces between the ordered hydrocarbon chains.

On this simple basis, it is difficult to explain the stronger perturbing action of DPPNO because the presence of the NO group does not justify by itself any really different hydrophobic interactions of DPPNO with the lipid moiety of the bilayer. The experimental results can instead be interpreted on the hypothesis that DPPNO is distributed both in the lipid phase of the bilayer, with effects similar to the other phenols, and in the interfacial region, where it produces a relevant destabilising action. In this sense, the pK_a value, surprisingly near to the pH physiological value, would strongly suggest a role for the deprotonated and negatively charged form of DPPNO, which, like other dissociated phenols [12], in addition to hydrophobic contributions, interact with the positively charged moiety of the membrane through specific, electrostatic binding forces.

The validity of this hypothesis is not confirmed by DSC experiments at a lower pH condition. At pH = 6, in fact, the deprotonated DPPNO component should be strongly reduced both in the solution and in the membrane, but we did not observe any corresponding change in the T_c plot (Fig. 7). In the light of these considerations, we would be led to conclude that the relevant effects of DPPNO on the membranes are due to its partitioning in the interface where it can form different bonds with the glycerol, the phosphate and amino residues of the lipid head.

In fact, considering the DPPNO pK_a value (7.5) we suppose that, at physiological pH, the following different forms of the molecule can be simultaneously present in the interfacial region [18]:



In addition to the electrostatic interaction between the dissociate phenolic hydroxyl and the positive charge of the choline amino group, all these forms, due to the presence of –OH, $=O^-$, –N=O, $=N–O^-$, =N–OH groups, can interact through hydrogen bonds with the polar heads of the phospholipids.

Before concluding, another point deserves further discussion. The reason why the membrane effects of propofol may be responsible for its mode of anaesthetic action, are clearly stated in [4], and we will assume that they are valid for DPPNO as well. Anyway, the dopant concentration is a crucial point, because, in many cases, it has been noted that the membrane effects of anaesthetics are very small at clinical concentrations. As an example, in DSC and ESR measurements on liposomes, we used concentrations in the 1/20-mM range, while mitochondria were clearly suffering in the presence of 100/200 μM DPP. The apparent discrepancy is reasonably resolved, considering that DPP and DPPNO, due to their high partition coefficients, accumulate preferentially in the lipid phase, and that, when the effects on the lipid moiety of the membranes are accounted for, the most important parameter is the dopant/lipid molar ratio. Looking back on our results, they demonstrate that a strong modification of the transition temperature and co-operativity of the liposomal membranes are obtained in the presence of a 1:10 DPPNO/lipid ratio, but limited effects are observed even at 1:100 ratios. These values are comparable with the dopant/lipid ratio which affects the mitochondrial respiration, approximately 1:20, and those quoted, for example, by Tsuchiya [4].

4. Conclusions

Although recent literature on the interaction of phenols with lipid phases exists, the interest lies mainly in the way they partition between an organic or lipid phase and water, for the implications it may have in toxicological and environmental fields [2,12,14,19–22]. Little is reported about the thermotropic behaviour of membrane models in the presence of phenol or simple phenolic compounds [23] and propofol [4] and, even more so, nothing about DPPNO, which we are studying for the first time. It resulted that propofol and phenol behave roughly like small, weakly or non-polar molecules, which interact non-specifically with the membrane. In the case of DPPNO, which produces an evident change with respect to the other molecules in the ordered organisation of lipids, a different mechanism can be hypothesised and the proposed explanation is that DPPNO partitions both in the lipid and in the interfacial region of the bilayer; the effects of its presence in the inner region would be analogous to the effects of DPP and phenol, while its interaction with the polar headgroups would account for the greatest part of the membrane destabilisation. These arguments do not imply, of course, that the interfacial region is forbidden to a molecule like phenol, but rather suggest more specific interactions of DPPNO. In this sense, the presence of the –NO group assumes some relevance and makes its role worth further investigation.

It is interesting to recall that the stronger perturbation observed on membranes correlates with a stronger inhibition of mitochondrial respiration by DPPNO. In the past few years, high concentrations of propofol have been reported to alter the energetic behaviour of liver and heart mitochondria [24–26], and most recently, we demonstrated that propofol and GSNO, a physiological NO donor, show a sort of co-operativity in decreasing energetic mitochondrial efficiency [27]. We have observed (data submitted for publication) that the addition of NO to the propofol molecule greatly enhances its effects on mitochondrial respiration and oxidative phosphorylation, the effective concentrations being shifted to values lower than those previously reported for propofol. As an example, 50 μM propofol did not show apparent effects on energetic parameters of mitochondrial metabolism, while 50 μM DPPNO has dramatic consequences for mitochondrial respiration, transmembrane potential and ATP synthesis, and consequently, also for cellular energy availability.

The metabolic evidence on isolated mitochondria fit well with the observations on model systems, reported in the present paper, which demonstrate the different possibilities of interactions with the membrane phospholipid moiety of DPP and DPPNO, as well as with the shift of the pK_a of the phenolic hydroxyl to a near-physiological pH, due to presence of NO bound to the aromatic ring. At this time, we cannot assert that the different physico-chemical features of the two molecules completely explain the different intensities of the mitochondrial effects of propofol and DPPNO; however, they certainly play an important role in a mechanism which requires further investigation on potential direct interactions.

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