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A STUDY OF THE PERMEATION OF DIHEXADECYL PHOSPHATE VESICLES BY VARIOUS ANESTHETICS

Pierre E. MÉNASSA * and Camille SANDORFY

Département de Chimie, Université de Montréal, Montréal, Québec H3C 3J7, Canada

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A simple spectroscopic method for the evaluation of the effect that perturbers may have on a membrane model is described. The model was made from dihexadecyl phosphate (DHP) bilayers. The perturbers used were unconventional anesthetics (*n*-alcohols C_1–C_8; *n*-hexane and *n*-pentane) and conventional anesthetics (chloroform, methoxyflurane, halothane and enflurane). The results show a correlation between vesicle permeation by anesthetics and their clinical potency. Two modes of perturbation by which the anesthetics may induce vesicle permeation are proposed.

1. Introduction

Anesthesia can be induced by a wide variety of chemical substances without any emphasis on a particular functional group or molecular structure. This shows that anesthesia is not due to a chemical reaction but rather to an alteration of the pattern of intermolecular forces. Anesthetics may act at the synapse or on the axonal membrane. Nonetheless in both cases it appears to be a matter of membrane permeability.

Aliphatic alcohols are among the substances that possess anesthetic potency at least up to C_{13} [1–3]. At C_{14} this anesthetic potency disappears. In an attempt to explain this cutoff we studied the interaction of inverted micelles of sodium di(2-ethylhexyl)sulfosuccinate (AOT) with a number of aliphatic alcohols [4,5]. The results we obtained showed a dependence of the association constants for AOT-*n*-alcohol on the hydrocarbon chain length of these *n*-alcohols. This dependence levelled off at C_{10}. Thus, a parallelism exists between the permeability of AOT in the presence of *n*-alcohols and their anesthetic potency. Although inverted micelles mimic to some extent lipid membranes surrounding a protein, they can only be considered as a remote model for actual cell membranes. Single-compartment vesicles represent a more realistic model for the study of membrane permeability. Indeed, like real membranes they are made from bilayers. Furthermore, ions may be entrapped in their cavity. In this work we used single-compartment vesicles made from dihexadecyl phosphate (DHP) into which we encapsulated the marker tris(2,2′-bipyridyl) ruthenium(II) chloride hexahydrate ($Ru(bpy)_3^{2+}$). We then studied the permeation of DHP vesicles by different anesthetics. This was done by monitoring $Ru(bpy)_3^{2+}$ leakage across the bilayer, using electronic absorption spectroscopy.

2. Materials and methods

2.1. Materials

Most of the chemicals used were stated to be 99% pure or more: methanol, ethanol, *n*-propanol,

* Present address: Concordia University, Chemistry Dept., 1455 De Maisonneuve Blvd. W., Montreal, Quebec H3G 1M8, Canada.

n-hexanol, *n*-octanol and chloroform (American Chemicals); *n*-butanol (Anachemia Chemicals); *n*-pentanol (Baker Chemicals); *n*-heptanol (BDH Chemicals); *n*-hexane and $Ru(bpy)_3^{2+}$ (Aldrich Chemicals); *n*-heptane (Fisher Chemicals) and DHP (Sigma Chemicals); methoxyflurane (Abbott Laboratories), halothane (Ayerst Laboratories) and enflurane (Ohio Medical Laboratories). Triply distilled deionized water ($R = 18$ MΩ/cm) was used. The analytical grade cation-exchange resin AG 50W-X2 (100–200 mesh, hydrogen form) was purchased from Biorad Laboratories.

2.2. Methods

2.2.1. Preparation of vesicles

DHP vesicles were chosen for this work because of their great stability (a few months) and ease of preparation. The entrapment of $Ru(bpy)_3^{2+}$ in DHP vesicles was extensively studied by Tricot et al. [8]. Based on their data we estimated the optimal concentration of DHP to be 2×10^{-3} M l^{-1}, that of $Ru(bpy)_3^{2+}$ 3.33×10^{-4} M l^{-1} and the pH at sonication 5.7. Vesicles were prepared by sonicating at 80°C the required amount of DHP in triply distilled deionized water. NaOH was then added to bring the solution to the required pH. Finally, $Ru(bpy)_3^{2+}$ was injected and sonication was carried on for approx. 45 min. The solution was then filtered to remove the titanium particles released from the sonicating probe. The solution prepared this way contained single-compartment vesicles with $Ru(bpy)_3^{2+}$ adsorbed on the outer and inner surfaces. Further details on the preparation of DHP vesicles were given by Tricot and Fendler [9].

2.2.2. Calculations

Visible absorption spectroscopy was used, and the band monitored was that of $Ru(bpy)_3^{2+}$ located at approx. 455 nm. First, the solution containing the vesicles was passed over a cation-exchange resin. This operation exchanged the $Ru(bpy)_3^{2+}$ adsorbed on the outer surface or free in the solution with the H^+ of the resin. Thus, the collected solution contained markers entrapped only inside the vesicles. The absorption spectrum of a solution sample was then recorded on a Cary 17 spectrometer (Varian/Instrument Division, CA). The absorption contribution of the vesicles (i.e., vesicles without markers) was then subtracted. The resulting spectrum was our standard. Next, the perturb-

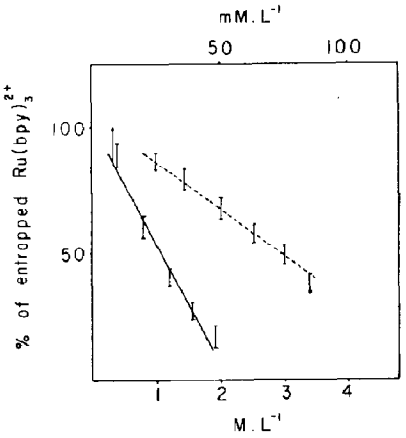

Fig. 1. The percentage of entrapped $Ru(bpy)_3^{2+}$ as a function of the molarity of added ethanol (— — —) and *n*-hexanol (———).

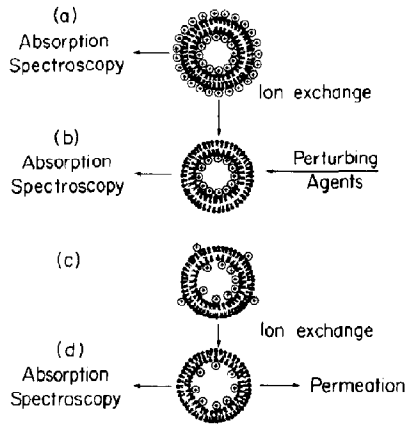

Fig. 2. Major steps determining vesicle permeation: (a) After preparation vesicles have $Ru(bpy)_3^{2+}$ adsorbed on the inner and outer surfaces. (b) After passage over the cation-exchange resin, the vesicles are left with $Ru(bpy)_3^{2+}$ adsorbed on the inner surface only. The absorption spectra of the entrapped $Ru(bpy)_3^{2+}$ constitute the standard. At this stage perturbing agents are added. (c) After perturbation some of the $Ru(bpy)_3^{2+}$ will leak to the outside. (d) After passage over the cation-exchange resin, vesicles will retain only the $Ru(bpy)_3^{2+}$ still adsorbed on the inner surface. Comparison of the absorption spectrum of the latter with the standard provides a means for measuring vesicle permeation.

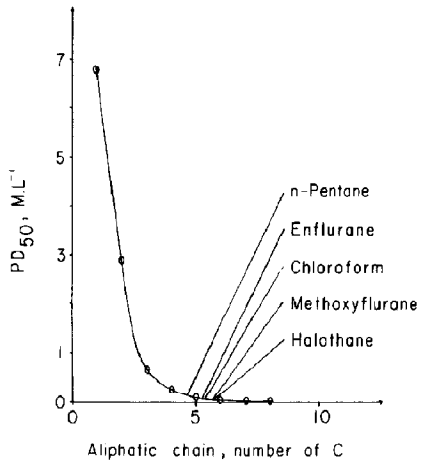

Fig. 3. PD_{50} of various aliphatic alcohols as a function of chain length. The PD_{50} values of different anesthetics are also shown for comparison.

ing agents (i.e., anesthetics, alkanes...) were injected into the eluted solution. This perturbed the membrane and some of the markers leaked to the outside of the vesicles. The 'perturbed' solution was then passed over the cation-exchange resin. The markers that leaked to the outside of the vesicles were thus exchanged with H^+. The collected solution contained the same amount of vesicles but entrapping less $Ru(bpy)_3^{2+}$ than the unperturbed ones. The absorption spectrum was then recorded and the contribution of the vesicles subtracted. The resulting spectrum was compared to the standard. The difference in their absorption band was used to calculate the quantity of the remaining entrapped $Ru(bpy)_3^{2+}$ which was expressed as a percentage of the standard. Plots of the percentage of trapped $Ru(bpy)_3^{2+}$ as a function of the concentration of the added perturbers are shown in fig. 1. The concentration of perturbers needed to permeate the vesicles to 50% of the original unperturbed ones was named PD_{50} (permeation dose at 50%). It provided a useful means of comparison between different perturbers. The main steps of the procedure are summarized in fig. 2.

3. Results

The aliphatic alcohols studied ranged from C_1 to C_8. Alcohols with a chain length of more than 8 carbons were not soluble in the vesicle solutions. Increasing the chain length of the *n*-alcohols resulted in a decrease in their PD_{50}. This is illustrated in fig. 1 where the slope of the straight line representing *n*-hexanol is much steeper than that depicting ethanol. The relation between the chain length and the respective PD_{50} is shown in fig. 3. It shows a pronounced dependence of the PD_{50} of the *n*-alcohols on their chain length. Conventional anesthetics (halothane, methoxyflurane, etc.) were also studied and their PD_{50} determined. They all fall within the same range, between the PD_{50} of *n*-pentanol and that of *n*-hexanol. The effect of *n*-pentane and *n*-hexane ont he permeation of vesicles was also determined. The PD_{50} of *n*-pentane was found to be higher than that of *n*-pentanol. It was not possible to solubilize more than 20 mM *n*-hexane per l vesicle solution. Such concentrations did not have any effect on DHP vesicles.

4. Discussion

It is clear from the experimental data that anesthetics affect the permeability of the bilayer. Furthermore, this effect is more pronounced for the potent anesthetics. The PD_{50} of methanol was found to be 6.75 M l^{-1}. Increasing the length of the aliphatic alcohols resulted in a decrease of the corresponding PD_{50}. *n*-Octanol has a PD_{50} of 15×10^{-3} M l^{-1}. This can be understood in terms of membrane/buffer partition coefficients [10]. The longer the aliphatic chain the larger the partition coefficient [10] and the smaller is the PD_{50}. In fact, when the partition coefficient is large (e.g., *n*-octanol), most of the *n*-octanol injected into the vesicle solution tends to go inside the bilayer, hence a smaller quantity of *n*-octanol is needed to permeate the membrane. This remains true if one considers the conventional anesthetics: chloroform, methoxyflurane, halothane and enflurane. Their respective PD_{50} values were found to be 51×10^{-3}, 34×10^{-3}, 20×10^{-3} and 76×10^{-3}

M l^{-1}. From their relatively low PD_{50} one would expect a relatively high membrane/buffer partition coefficient. This is indeed the case, their membrane buffer partition coefficients being close to that of *n*-hexanol [10]. The PD_{50} obtained with the vesicles were also compared (table 1) with the nerve-blocking concentrations *c* (frog sciatic nerve at 22°C). The correlation between the two sets of values is remarkable. For example, the *c* values of chloroform and halothane were found to lie between those of *n*-pentanol and *n*-hexanol. The PD_{50} values of chloroform and halothane were also between those of *n*-pentanol and *n*-hexanol. The results obtained so far are in line with the Meyer-Overton rule [11,12], which relates anesthetic potency with solubility in lipids. The alkanes *n*-pentane and *n*-hexane were also studied. The PD_{50} of *n*-pentane was found to be 154×10^{-3} M l^{-1} and that of *n*-hexane larger than 20×10^{-3} M l^{-1}. Although their membrane/buffer partition coeffcients are larger than those of the corresponding alkanols (i.e., *n*-pentanol and *n*-hexanol), their anesthetic potencies are weaker [13]. This is well reflected by the PD_{50} of *n*-pentane which is larger than that of *n*-pentanol. The same would probably apply to *n*-hexane and *n*-hexanol if higher concentration of *n*-hexane could be used.

Table 1

Comparison between the PD_{50} values of different perturbers and their nerve-blocking concentrations [a]

Perturber	PD_{50} (M l^{-1})	Nerve-blocking concentration [b] (M l^{-1} H_2O)
Methanol	6.75	2.4
Ethanol	2.90	5.0×10^{-1}
n-Propanol	6.70×10^{-1}	2.18×10^{-1}
n-Butanol	2.85×10^{-1}	6.8×10^{-1}
n-Pentane	1.54×10^{-1}	–
n-Pentanol	8.20×10^{-2}	2.1×10^{-2}
Enflurane	7.60×10^{-2}	–
Chloroform	5.10×10^{-2}	5×10^{-3}
Methoxyflurane	3.40×10^{-2}	–
Halothane	3.00×10^{-2}	5×10^{-3}
n-Hexane	$> 2.00 \times 10^{-2}$	–
n-Hexanol	2.70×10^{-2}	6×10^{-3}
n-Heptanol	2.00×10^{-2}	1.75×10^{-3}
n-Octanol	1.50×10^{-2}	–

[a] From ref. 10.
[b] Frog sciatic nerve (22°C).

Entrapment measurements of $Ru(bpy)_3^{2+}$ and methyl viologen (MV^{2+}) were carried out by Tricot et al. [14]. They showed that the entrapments of $Ru(bpy)_3^{2+}$ and MV^{2+} were charge-controlled. Indeed, the ratio of entrapped $Ru(bpy)_3^{2+}$ was found to be 42%. This corresponds closely to the inner surface fraction (ratio of inner to total surface). Furthermore, they showed that, although $Ru(bpy)_3^{2+}$ is bulkier than MV^{2+}, it diffuses more easily since MV^{2+} is more strongly bound than $Ru(bpy)_3^{2+}$ to the anionic vesicle. Then, for $Ru(bpy)_3^{2+}$ to diffuse from the inside of the vesicle to the outside, the electrostatic binding force must be overcome. Once this has been achieved entrapment will no longer be charge-controlled but volume-controlled. Then the entrapped ions will be forced out of the vesicles since at such DHP concentrations the inner volume fraction amounts only to about 0.2% [14].

It is not known by what mechanism this electrostatic barrier is overcome. One possibility would be to consider bulky $Ru(bpy)_3^{2+}$ electrostatically bound to the anionic vesicles via hydration water molecules. The perturber would slightly affect the bridging water molecules. It is conceivable that the alcohols lie with their hydrophobic chains parallel to the alkyl chains of the DHP with the polar part of the alcohol facing the phosphate head groups. The vesicles would thus suffer two perturbations: the first due to the hydrophobic forces interacting between the alkyl chains of the vesicle and the aliphatic chain of the alcohols and the second due to the interaction of the OH group of the alcohol with the phosphate groups of DHP and/or the hydration water of the marker. Similar results were obtained by Shibata et al. [15]. They showed that anesthetics release counterions from the surface of ionic surfactant micelles. Furthermore, in a subsequent paper Yoshida et al. [16] showed that anesthetics weakened the hydrogen bond between water and glycerol-α-monooleate (a surfactant). The explanation given above may thus account for the differences between the PD_{50} values of *n*-pentanol and *n*-hexanol with respect to *n*-pentane and *n*-hexane. With *n*-hexanol and *n*-pentanol the two modes of perturbations are present while with *n*-hexane and *n*-pentane only one can exist and is of hydrophobic nature. One would

then expect *n*-hexanol and *n*-pentanol to have lower PD_{50} values than those of the corresponding alkanes. The experimental data obtained seem to support this hypothesis. Finally, it is not known to us if the perturbation affects the outer surface of the bilayer before the inner one. Indeed, there is experimental evidence in the literature that anesthetics direct their action mainly to the water/phospholipid interfacial region [17]. In our case no $Ru(bpy)_3^{2+}$ was left adsorbed on the outer surface after the initial passage of the solution through the resin. It was thus not possible to measure the effect that perturbers may have had on the vesicles' outer surface.

5. Conclusions

In summary, this work describes the use of a simple membrane model system for the study of anesthetic-induced vesicle permeation. The results obtained showed a correspondance between the clinical potency of anesthetics and vesicle permeation. The latter is sought to be the effect of two perturbations: the first due to the hydrophobic interaction between the alkyl chains of the vesicles and the non-polar part of the anesthetic, the second to the interaction between the proton donor group of the anesthetic and the phosphate group of the vesicle.

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