The DPPC + DOPC liposome is probably not an adequate model of the more complex biological bilayer. However, the formation of separate lipid domains, as in the DPPC + DOPC liposomes [28], may represent lipid–lipid relationships characteristic of some biomembranes [24, 29–31]. The fact that tricyclic antidepressants interact differently with DPPC + DOPC liposomes than with either DPPC or DOPC liposomes suggests the possible importance of phase boundary regions as possible sites for drug effects.

The results of our study indicate that the tricyclic antidepressants, nortriptyline and protriptyline, perturb the DPH-labeled bilayers of DPPC + DOPC liposomes but do not perturb the DPH-labeled bilayers of DPPC, DPC, or egg PC liposomes. Nortriptyline- and protriptyline-induced perturbations of the bilayers of DPPC + DOPC liposomes occurred at concentrations that are similar to the concentrations of nortriptyline and protriptyline that perturb lymphocyte membranes [11]. In addition, nortriptyline and protriptyline decreased the transition temperature of the lipids in DPPC + DOPC liposomes.

In contrast, desipramine and imipramine quenched the fluorescence emission of DPH in DPPC + DOPC and egg PC liposomes at concentrations similar to those concentrations of desipramine and imipramine that quenched DPH fluorescence emission in murine lymphocytes. The concentrations of desipramine and imipramine that quenched DPH fluorescence were also similar to the concentrations of nortriptyline and protriptyline that perturbed the phospholipid bilayers of liposomes and lymphocytes. Other methods will be required to determine the presence of desipramine and imipramine effects on the lipids of DPPC + DOPC liposome and lymphocyte membranes. Finally, desipramine and imipramine did not alter the fluorescence anisotropy or lifetime of DPH in DPPC liposomes.

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REFERENCES

1. H. Rasmussen, O. Fontaine and T. Matsumoto, *Ann. N.Y. Acad. Sci.* **372**, 518 (1981).
2. F. Hirata and J. Axelrod, *Science* **209**, 1082 (1980).
3. P. F. Pilch, P. A. Thompson and M. P. Czech, *Proc. natn. Acad. Sci. U.S.A.* **77**, 915 (1980).
4. D. B. P. Goodman, M. Wong and H. Rasmussen, *Biochemistry* **14**, 2803 (1975).
5. K. L. Audus and M. A. Gordon, *J. Immunopharmac.* **4**, 13 (1982).
6. G. G. Nahas, B. Desoize and C. Leger, *Proc. Soc. exp. Biol. Med.* **160**, 344 (1979).
7. C. I. E. Smith, L. L. G. Hammarstrom and J. D. Waterfield, *Scand. J. Immun.* **7**, 145 (1978).
8. J. D. Waterfield, L. Hammarstrom and E. Smith, *J. exp. Med.* **144**, 562 (1976).
9. G. G. Haydu, L. Goldschmidt and A. D. Drymiotis, *Ann. rheum. Dis.* **33**, 273 (1974).
10. J. T. Leyberg and J. C. Denmark, *J. ment. Sci.* **33**, 1123 (1959).
11. K. L. Audus and M. A. Gordon, *J. Immunopharmac.* **6**, 105 (1984).
12. A. D. Bangham, *Ann. N.Y. Acad. Sci.* **308**, 2 (1978).
13. T. R. Tritton, S. A. Murphree and A. C. Sartorelli, *Biochem. Pharmac.* **26**, 2319 (1977).
14. M. Shinitzky and Y. Barenholz, *J. biol. Chem.* **249**, 2652 (1974).
15. A. D. Bangham and R. W. Horne, *J. molec. Biol.* **8**, 660 (1964).
16. K. L. Audus, D. L. Johnson and M. A. Gordon, *J. Immunopharmac.* **4**, 329 (1983).
17. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*. Plenum Press, New York (1983).
18. J. R. Lakowicz, F. G. Prendergast and D. Hogen, *Biochemistry* **18**, 50 (1979).
19. J. R. Lakowicz and F. G. Prendergast, *Science* **200**, 1399 (1978).
20. J. R. Lakowicz and F. G. Prendergast, *Biophys. J.* **24**, 213 (1978).
21. J. Bermejo, A. Barbadillo, F. Tato and D. Chapman, *Fedn Eur. Biochem. Soc. Lett.* **52**, 69 (1975).
22. B. R. Cater, D. Chapman, S. M. Hawes and J. Saville, *Biochem. biophys. Acta* **363**, 54 (1974).
23. J. Bermejo, P. Fernandez, F. Tato and A. Belmonte, *Res. Commun. Chem. Path. Pharmac.* **8**, 101 (1974).
24. B. R. Lentz, Y. Barenholz and T. E. Thompson, *Biochemistry* **20**, 4529 (1976).
25. B. R. Lentz, Y. Barenholz and T. E. Thompson, *Biochemistry* **20**, 4521 (1976).
26. C. D. Stubbs, T. Kouyama, K. Kinosita, Jr. and A. Ikegami, *Biochemistry* **20**, 4257 (1981).
27. R. A. Demel, W. S. M. Querts van Kessel and L. L. M. van Deenen, *Biochim. biophys. Acta* **266**, 26 (1972).
28. T. E. Thompson, B. R. Lentz and Y. Barenholz, in *Biochemistry of Membrane Transport*, FEBS-Symposium No. 42 (Eds. G. Semenza and E. Carafoli), p. 47. Springer, Berlin (1977).
29. R. D. Klausner, A. M. Kleinfeld, R. L. Hoover and M. J. Karnovsky, *J. biol. Chem.* **255**, 1286 (1980).
30. P. R. Cullis and B. DeKruijff, *Biochim. biophys. Acta* **599**, 399 (1979).
31. M. K. Jain and H. B. White, *Adv. Lipid Res.* **15**, 1 (1977).

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## Effect of uncharged anesthetics on ion binding to liposome surfaces

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Calcium binds more strongly to negatively charged lipid surfaces (phosphatidylserine) than to electrostatically neutral phospholipids (phosphatidylcholine) [1]. Trivalent praseodymium, however, strongly binds to both types of lipids. Binding probably occurs via electrostatic interactions between the positively charged ion and the negatively charged phosphate oxygen of the phospholipid or the carboxylic group of phosphatidylserine. As reported recently,

positively charged drugs when incorporated into liposomes decrease binding of calcium by electrostatic repulsion [2]. By NMR techniques we observed that uncharged benzocaine, known as a surface local anesthetic, also influences praseodymium binding to phosphatidylcholine liposomes [3]. In this communication we compared the effect of the uncharged local anesthetic benzocaine and of the uncharged inhalation anesthetics halothane and enflurane on ion binding to liposome surfaces, using laser Doppler electrophoresis.

*Materials and methods*

Phosphatidylcholine was purified by column chromatography, phosphatidyl-l-serine was purchased from Serdary. Benzocaine and halothane were from Hoechst and enflurane from Abbott. For the preparation of benzocaine containing liposomes the drug was dissolved in chloroform and added to the phospholipid sample (also in chloroform) before evaporation of the solvent. The concentration was 5 mM in the final buffer solution. Halothane and enflurance (5 mM) were equilibrated in the buffer (1 mM KCl, 1 mM glycine, pH 7) for one hour by vigorous stirring in a stoppered flask. This medium then was added to the dried phospholipids. The procedure for the preparation of the liposomes as well as the method for measuring electrophoretic mobilities and all other technical details have been described previously [4].

Values are means of three experiments with a maximum variation of 4%.

*Results and discussion*

The results of this study are summarized in Fig. 1. $Ca^{2+}$ and $Pr^{3+}$ decrease the electrophoretic mobility of charged liposomes by binding to the surface and neutralizing negative surface charges. The effect of $Pr^{3+}$ is stronger than that of $Ca^{2+}$. At small ion concentrations surface charges are neutralized and at higher concentrations of $Pr^{3+}$ the liposomes become positively charged. Binding of $Pr^{3+}$ to electrostatically neutral liposomes (phosphatidylcholine) is stronger than of $Ca^{2+}$ (the effects of $Ca^{2+}$ are not shown here, see also ref. 1). Benzocaine antagonizes binding of $Pr^{3+}$ and $Ca^{2+}$ to phosphatidylcholine liposomes, containing 10% phosphatidylserine. The same effect is observed for $Pr^{3+}$ in phosphatidylcholine liposomes. Higher concentrations of the ions are needed to obtain the same change in electrophoretic mobility. Similar effects have been observed with charged local anesthetic drugs [4]. Benzocaine with a pK of 2.5 is more than 99.99% uncharged at pH 7. Therefore the results cannot be related to a charge effect. With a lipid/buffer partition coefficient of 145 (phosphatidylcholine plus 10% phosphatidylserine in 1 mM KCl, 1 mM glycine, measured spectrophotometrically according to [5]) benzocaine is strongly dissolved into the lipid phase. From the partition coefficient value one benzocaine molecule per 5 lipid molecules in the liposomes was calculated. In a previous NMR study we came to the conclusion that benzocaine must be located near the glycerol backbone between the lipid acyl chain at $C_1$ and $C_2$ of the phospholipids [3]. Altering intramolecular energetics, the phospholipid head group configuration is changed and binding of di- and trivalent ions is affected [6].

The two inhalation anesthetics halothane and enflurane act in a different way. With concentrations of 5 mM we could not observe any effect on drug binding to charged or uncharged liposomes. Also different application methods (either directly added to the $N_2$ dried lipids before emulsion, or added to the buffer suspended liposomes before sonication) gave the same results. The partition coefficients could not be measured by spectrophotometric methods but are found in the literature to be for enflurane 98 [7] or 120 [8] and for halothane 224 [7] or 330 [8], determined for oil/water. These partition coefficients are in the range of that of benzocaine or even higher so that the effects of both groups of drugs can be compared. The oil/gas partition coefficients of the inhalation anesthetics are similar to those given above, which clearly demonstrates that most of the drug is still retained in the lipid phase [8]. The lack of effect therefore cannot be attributed to a low lipid concentration of the inhalation anesthetics. As the liposome size distribution is not changed by benzocaine or inhalation anaesthetics when measured by laser light scattering (in the absence of electric field) the distinct effects also cannot be due to different changes in the physical properties of the liposomes.

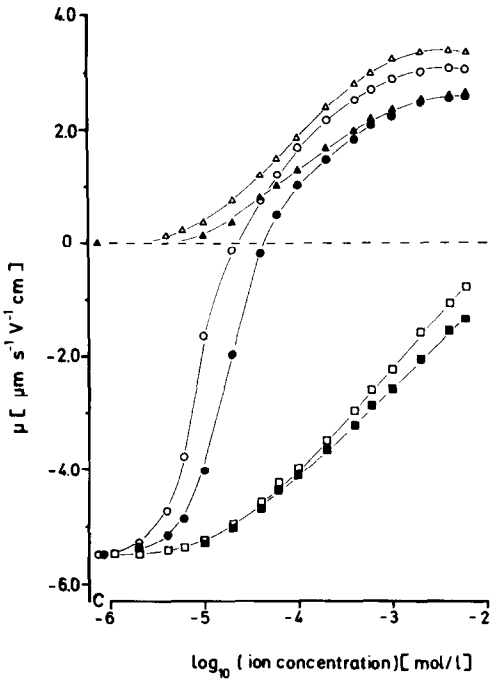


Fig. 1. Electrophoretic mobility of phosphatidylcholine and phosphatidylcholine plus 10 mole% phosphatidylserine (0.3 mg lipid/ml buffer) liposomes as a function of ion concentration. △, phosphatidylcholine – $Pr^{3+}$; ○, phosphatidylcholine plus 10% phosphatidylserine – $Pr^{3+}$; □, phosphatidylcholine plus 10% phosphatidylserine – $Ca^{2+}$. The filled symbols represent the data for benzocaine containing liposomes. Buffer: 1 mM KCl, 1 mM glycine, pH 7.

Benzocaine and halothane are about equieffective in blocking nerve conduction [9]. This effect can be antagonized by pressure. Our results with liposomes indicate different mechanisms of action than membrane expansion, which is postulated for biological tissues. Inhalation anaesthetics are known to partition preferentially into the hydrocarbon core of the lipid bilayer [10]. Being further away from the polar head group region than benzocaine their effect on head group configuration and therefore on ion binding is less.

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REFERENCES

1. S. McLaughlin, N. Mulrine, T. Gresalfi, G. Vaio and A. McLaughlin, *J. gen. Physiol.* **77**, 445 (1981).
2. P. Schlieper and R. Steiner, *Biochem. Pharmac.* **32**, 799 (1983).
3. P. Schlieper and L. Michaelis, *Biophys. Struct. Mech.* **10**, 1 (1983).
4. P. Schlieper, P. K. Medda and R. Kaufmann, *Biochim. biophys. Acta* **644**, 273 (1981).
5. P. Schlieper and R. Steiner, *Chem. Phys. Lipids* **34**, 81 (1983).
6. H. Hauser, W. Guyer, B. Levine, P. Skrabal and R. J. P. Williams, *Biochim. biophys. Acta* **508**, 450 (1978).
7. A. Stewart, P. R. Allott, A. L. Cowles and W. W. Mapleson, *Br. J. Anaesth.* **45**, 282 (1973).
8. W. Forth, D. Henschler and W. Rummel, *Pharmakologie und Toxikologie*, Wissenschaftsverlag, Mannheim (1983).
9. J. J. Kendig and E. N. Cohen, *Anesthesiology* **47**, 6 (1977).
10. P. Seeman, *Pharmac. Rev.* **24**, 581 (1972).

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# Localization of diethylstilbestrol metabolites in the mouse genital tract

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Diethylstilbestrol (DES*), a potent estrogen and reported transplacental carcinogen [2], is biotransformed to a variety of metabolites in several animal species and also in humans [3]. The oxidative metabolism of DES affects its hormonal activity [4] and may also be of importance for its carcinogenicity, as it has been proposed that DES is bioactivated to reactive intermediates in analogy to chemical carcinogens [5, 6]. Of particular interest in this respect is the peroxidase-catalysed metabolism of DES, which leads to Z,Z-dienestrol (Z,Z-DIES) via a reactive semiquinone and quinone [7]. Peroxidative activity has been demonstrated in tissues depending on estrogens for growth, and these enzymes can be induced by estrogens [8]. Recently, it has been shown that the mouse fetal genital tract, a known *in vivo* target organ for the hormonal and carcinogenic activity of DES, can metabolize DES to Z,Z-DIES when maintained in organ culture [9]. However, it is unknown whether Z,Z-DIES or other oxidative DES metabolites are formed in the mouse genital tract under *in vivo* conditions. Therefore, the present communication reports on a study of the metabolites of DES in the mouse genital tract *in situ*.

## *Materials and methods*

Monoethyl-2-$^{14}$C-DES (sp. act. 56 mCi/mmole, The Radiochemical Centre, Amersham, U.K.) consisted of 66.9% E-isomer and 33.1% Z-isomer with no impurities detectable at a level of 0.2% by radio-HPLC and radio-TLC. Reference compounds E-DES and its monoglucuronide and monosulphate, Z-DES, 1-HO-E-DES, 4′-O-$CH_3$-E-DES, Z,Z-DIES and 1-HO-Z,Z-DIES [9–11] were characterized by mass spectrometry and their retention times in HPLC and GLC as previously described [3, 12].

Female CD-1 mice (Charles River France S.A., Cleon, France) were at the age of 6 weeks. Ovariectomy was carried out under anesthesia with Nembutal (Abbot AG, Ingelheim, F.R.G.) 5 days prior to the experiments. For estrogen pretreatment, DES (40 μg/kg b.w.) was injected s.c. as a solution in tricaprylin (Carl Roth OHG, Karlsruhe, F.R.G.) daily for 3 days prior to the experiment. Controls received tricaprylin only. All animals were injected i.v. (tail vein) with $^{14}$C-DES dissolved in propan-1,2-diol (1 μg/μl) at a dose of 1.6 μg/g b.w.

The animals were sacrificed by decapitation and the vagina, cervix, and uterus removed. Half of the tissue from each animal was used for the extraction of DES metabolites, the other half to determine peroxidase activity. Metabolites were extracted from the tissue homogenate by precipitation with ethanol and successive washing of the precipitate with ethanol, diethyl ether/ethanol 3:1 (v/v), and diethyl ether as previously described [5, 7]. The residue of the combined extracts was dissolved in 40 μl ethanol for HPLC analysis. Non-extractable radioactivity bound to the sediment was determined by liquid scintillation counting after combusting an aliquot of the air-dried precipitate in a Packard 306 Sample Oxidizer. Peroxidase activity was solubilized from the tissues and determined as described by Lyttle and DeSombre [8], using guaiacol as the substrate.

The tissue extracts containing the metabolites were separated by reverse-phase HPLC as reported previously [12]. The retention times were 5–8 min for conjugated metabolites, 15.8 min for 1-HO-Z,Z-DIES, 18.3 min for 1-HO-E-DES, 23.2 min for E-DES, 24.6 min for Z,Z-DIES, 26.4 min for Z-DES, and 29.1 min for 4′-O-$CH_3$-E-DES. The column eluate was collected in 0.4 ml fractions and an aliquot of each fraction used for radioactivity measurement in a Packard Tricarb 3390 liquid scintillation counter with automatic external standard (Packard Instruments, Frankfurt, F.R.G.).

## *Results*

In order to identify the DES metabolites present in the uterus of the intact, estrogen-induced mouse *in situ* and to determine their concentrations at different time points, a single dose of $^{14}$C-DES was i.v. injected into 6-week-old intact mice pretreated with unlabelled DES for 4 days. After 15, 30 and 60 min, the animals were sacrificed, the uteri removed, and DES and its metabolites extracted. The highest amount of extractable uterine radioactivity was found after 15 min (Table 1). The amount of non-extractable radioactivity did not exceed 4 pmole/g wet weight and did not differ significantly between animals sacrificed at different time points.

The extractable radioactivity was analyzed by radio-HPLC (Table 1). Besides small amounts of glucuronide or sulphate conjugates, which were not further identified, two metabolites of DES were present in the uteri as identified by cochromatography with authentic reference compounds: Z,Z-DIES and 4′-O-$CH_3$-DES. Both metabolites were found in the same relative amounts (approximately 9% and 5%, respectively) at all time points studied.

In a control experiment, $^{14}$C-DES (200 pmole) was added to a freshly obtained mouse uterus prior to homogenization, and the tissue processed as usual. Traces of Z,Z-DIES (1.5%) but no other metabolites (limit of detection 0.05%)

* Nomenclature of DES and metabolites according to the system of Metzler and McLachlan [1].