Piracetam-induced changes to membrane physical properties

ARTICLE *in* BIOCHEMICAL PHARMACOLOGY · OCTOBER 1995

Impact Factor: 5.01 · DOI: 10.1016/0006-2952(95)00225-O

4 AUTHORS, INCLUDING:

Michel Deleers
Université Libre de Bruxelles
116 PUBLICATIONS **1,438** CITATIONS
SEE PROFILE

Robert Brasseur
University of Liège
360 PUBLICATIONS **11,227** CITATIONS
SEE PROFILE

Pergamon

Biochemical Pharmacology, Vol. 50, No. 8, pp. 1129–1134, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00225-1

PIRACETAM-INDUCED CHANGES TO MEMBRANE PHYSICAL PROPERTIES

A COMBINED APPROACH BY ^{31}P NUCLEAR MAGNETIC RESONANCE AND CONFORMATIONAL ANALYSIS

JACQUES PEUVOT,* ANDRÉ SCHANCK,† MICHEL DELEERS‡ and ROBERT BRASSEUR§‖

* Département médical UCB s.a. B-1420-Braine-l'Alleud, Belgique; † Laboratoire de Chimie Physique et de Cristallographie and Research Center for Advanced Materials, Université Catholique de Louvain, B-1348-Louvain-la-Neuve, Belgique; ‡ Secteur Pharmaceutique, UCB s.a., B-1420-Braine-l'Alleud, Belgique; and §Centre de Biophysique Moléculaire Numérique, Faculté des Sciences Agronomiques de Gembloux, Passage des Déportés no. 2, B-5030-Gembloux, Belgique

(*Received* 1 *December* 1994; *accepted* 17 *May* 1995)

Abstract—Piracetam, Nootropil® (2-oxo-1-pyrrolidine acetamide), is a drug promoting erythrocyte deformability. To establish the mode of action of this compound, we have investigated its influence on the organization of model phospholipid membranes. ^{31}P NMR data show that the drug induces a structural modification in liposomes made of phosphatidylcholine and phosphatidylethanolamine. Our conformational analysis results have allowed the interpretation of the effect of piracetam on these model membranes: the specific interaction between the drug molecules and the phosphate headgroups induces a new organization of the lipids favouring formation of mobile drug-phospholipid complexes that exhibit an isotropic-type signal in the ^{31}P NMR spectra.

Key words: piracetam; model membrane; ^{31}P NMR; conformational analysis; isotropic structure; physical properties

In most cases, all drugs that act in the human body interact with certain metabolic pathways and are transformed into one or more metabolites. However, piracetam, which is very soluble in water and only poorly soluble in organic media, is neither ionized nor bound by proteins. It is not metabolized; no metabolite of any sort has been evidenced (even when starting from radioactive carbon) in the excretory products (urine, faeces), the blood, the liver, or the brain [1]. In addition, piracetam improves red blood cell deformability *in vitro* and restores the impaired deformability of physiologically deoxygenated sickle cell anemia cells [2].

Given that this drug is not metabolized yet improves erythrocyte deformability, we hypothesized that it might have a physical effect on some membrane components, specifically, a reorganization of membrane phospholipids. To verify this hypothesis, appropriate physical and theoretical approaches to answer these questions have been used.

The ability of hydrated lipids to adopt a variety of phases in addition to the bilayer phase is well documented in the literature. Early significant contributions to this research have been made by using X-ray techniques [3], freeze-fracture techniques [4], and ^{31}P NMR [5].

^{31}P NMR is a particularly useful analytical tool for the study of the polymorphic phase behavior of hydrated phospholipids. It reveals membrane structure without labelling or isotopic enrichment and without perturbing the membrane. This physical method senses the behavior and environment of the phosphorus atom in the phospholipid headgroup, and reports on the conformation and structural dynamics of the phosphate headgroup. The observed ^{31}P spectra are characteristic of the different possible organizations adopted by the phospholipid molecules: bilayer structures, hexagonal phases, and phases where isotropic motion can occur. In this investigation, ^{31}P NMR has been used to examine the polymorphic behavior of aqueous dispersions of DPPC,¶ DPPE, BBPE, and CL/PC mixture in the presence of piracetam. ^{31}P NMR data have revealed that the drug induces structural modification in lipid organization.

The results of our conformational analysis have enabled us to explain our NMR observations. Conformational analysis is also a very helpful method for the study of the interaction between phospholipids and drugs [6], thus allowing a molecular understanding of the mechanism of action of piracetam on the phospholipids. Using this theoretical approach, the assembling mode of DPPC and DPPE has been analysed in the presence of piracetam. The results show that piracetam molecules surround the polar head of the phospholipids, and thus are able to modify lipid organization.

MATERIALS AND METHODS

DPPC, DPPE, BBPE, EYPC, and CL were purchased from Sigma Chem. Co. (St. Louis, MO) and Serva Chem. (Heidelberg, Germany).

‖ Corresponding author. Tel. 32-81-62.25.21; FAX 32-81-62.25.22.

¶ Abbreviations: DPPC, dipalmitoyl phosphatidyl choline; DPPE, dipalmitoyl phosphatidyl ethanolamine; BBPE, bovine brain phosphatidyl ethanolamine; EYPC, egg yolk phosphatidyl choline; CL, cardiolipine; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine.

NMR

The powder spectrum of phospholipids organized in bilayers results from the rapid rotation along the molecular axis, which provokes a partial average of the chemical shift tensor [7]. The observed spectrum shows a peak at high field and a shoulder at low field; the difference between these two edges is called effective chemical shift anisotropy $\Delta\sigma_B$ (the index B is for bilayer). It is known that $\Delta\sigma_B$ of phospholipids in the bilayer phase is of the order of −40 ppm for PC [8] and PE [9]. In non-bilayer phases, additional motional averaging mechanisms result in distinctive ^{31}P NMR spectra for lipids in the hexagonal (H_{II}) phase or phases where isotropic motion occurs (cubic, rhombic, micellar, inverted micellar, and small vesicles). Such lipids in the hexagonal H_{II} phase exhibit lineshapes with a low field peak and a high field shoulder where $\Delta\sigma_H \cong 20$ ppm (the index H is for hexagonal) because of the rapid rotation of the cylinders [7]. Phases where phosphate heads undergo rapid isotropic averaging motion produce a narrow symmetrical ^{31}P NMR spectrum at the chemical shift value σi (isotropic shift), characteristic of sonicated vesicles. Thus, ^{31}P NMR easily allows detection of these different phases.

Approximately 40 mg of phospholipid was prepared from a chloroform-methanol solution. Solvent was then evaporated under vacuum in a dessicator and the residual lipid film on the glass wall allowed to swell with a buffer containing 15% D_20 (for field-frequency stabilization on the deuterium signal), 100 mM NaCl, and 17 mM Tris-HCl, pH 7.4. Piracetam and/or $CaCl_2$ were added as aliquots of stock solution (200 mM). The samples were subjected to several freeze-thaw cycles and vortexed for several minutes. ^{31}P NMR spectra were acquired at 101.3 MHz on a Bruker WM 250 spectrometer. Ten-mm NMR tubes were used. Typical Fourier transform parameters were: 3000–5000 scans; 60° (10 μsec) flip angle; 30 kHz spectral width; 8K data points; 0.5 sec recycle time. Proton decoupled spectra were obtained by using powergated decoupling. A line broadening of 50 Hz was applied to the free induction decay before Fourier transformation. The chemical shifts were measured relative to H_3PO_4 (85%) as an external reference.

Molecular modelling

The conformational analysis procedure was based on the technique developed by Brasseur [6].

A simplex minimization procedure [10] was used to further reduce the total internal energy by rotation of torsional axes of piracetam conformations. The molecule was oriented at the air-water interface, taking into account the positions of the hydrophobic and hydrophilic centres [11].

The procedure (the Hypermatrix Method) used to surround one molecule (A) with other molecules (B) is a modification of the sequential method used to surround one drug with lipid molecules [12]. This method is based on a non-relaxed strategy in which the molecular structure of all compounds is fixed through the hypermatrix procedure. In essence, this consists of fixing the position and orientation of molecule A after orientation at the air/water interface. A second molecule B was oriented at the interface and allowed to move along the x-axis in 0.05-nm steps. At each position, the second molecule was rotated in steps of 30° around its long axis z' and around the first molecule.

l is the number of positions along the x-axis; m, the number of rotations of the second molecule around the first; and n, the number of rotations of the molecule itself. For each set of values of l, m, and n, the intermolecular energy of interaction was calculated as the sum of the London-Van der Waals' energy of interactions (E^{vdw}), the electrostatic interaction (E^{cb}), and the transfer energy of atoms or groups of atoms from a hydrophobic to a hydrophilic phase (E^{tr}). Then, the second molecule was moved in steps of 0.05 nm along the z'-axis perpendicular to the interface, and the position of the z'-axis varied in steps of 5° with respect to the z-axis, thus obtaining the lowest interaction energy state for each set of values l, m, and n.

The energy values together with the coordinates associated with each set of l, m, and n were stored in a hypermatrix and classified according to decreasing values of the interaction energy. The position of the third molecule C was defined as the first energetically favorable orientation stored in the hypermatrix, taking into account the sterical and energetic constraints imposed by the presence of the second molecule. Thus, orientations were disregarded where overlap of atomic coordinates of two molecules occurred and where the interaction energy between the two molecules was positive. To minimize the conformational energy further, the position of the second and third molecules was then alternatively modified in steps according to the energy classification of the hypermatrix. For the fourth molecule, the same process was repeated, but the positions of the three surrounding molecules were modified alternatively to find the lowest energy state. In the calculation, the interaction energy among all monomers in the aggregate was considered and reduced to a minimum until the lowest energy state of the entire aggregate was reached. We limited this approach to the number of molecules sufficient to surround one central molecule.

All calculations were performed using a Pentium processor. The software used was PC-TAMMO+ (Theoretical Analysis of Molecular Membrane Organization) and PC-MSA+ (Molecular Structure Analysis) [6]. Graphs were drawn with the PC-MGM+ (Molecular Graphics Manipulation) program.

RESULTS

The effect of piracetam is shown on the ^{31}P NMR spectra of DPPC (Fig. 1) and DPPE (Fig. 2) organized in bilayer phases (giving a typical lineshape with low field shoulder and high field peak). A narrow signal at the isotropic chemical shift already appears at low concentrations of piracetam, and increases with larger amounts of added compound.

The BBPE undergoes a bilayer to hexagonal transformation at ~12°C, as illustrated in Fig. 3 (A series); by increasing the temperature, the bilayer phase (bottom spectra of Fig. 3) is progressively replaced by a hexagonal H_{II} structure, giving rise to a spectral component of reduced chemical shift anisotropy with low field peak and high field shoulder. Further successive additions of piracetam influence phospholipid organization; below the transition temperature, the isotropic peak indicated by an asterisk (*) in B and C series spectra of Fig. 3 increases with piracetam concentration.

We also studied the influence of the drug on mixtures of phospholipids undergoing bilayer to hexagonal phase transition induced by Ca^{++} ions. An equimolar mixture

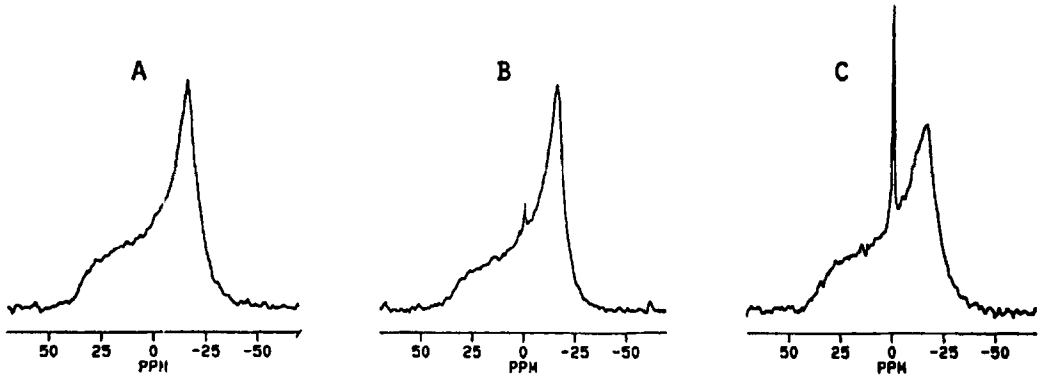

Fig. 1. 101.3 MHz ^{31}P NMR spectra of DPPC at 308K; (A) control and after addition of piracetam up to a drug/DPPC ratio of (B) 0.3 and (C) 3.

CL/EYPC is organized in bilayers as shown in Fig. 4, spectrum A. Piracetam alone has no effect on CL/EYPC organization; a high concentration of this compound does not modify the lineshape at all. The stability of the bilayer organization with piracetam was followed for more than 24 hours (Fig. 4, spectrum B). Subsequent addition of Ca^{++} induces the formation of hexagonal H_{II} and isotropic phases, as seen in Fig. 4, spectrum C. The other spectra of Fig. 4 correspond to the observations of the piracetam effect after incubation of the CL/EYPC mixture with Ca^{++}. Figure 4, spectrum D shows the Ca^{++} effect on the lipid mixture: namely, a partial transformation of the bilayer phase in hexagonal and isotropic phases. After addition of piracetam at low concentration, the proportion of hexagonal H_{II} phase is slightly reduced (Fig. 4, spectrum E). In the presence of higher proportions of piracetam, the importance of the isotropic peak substantially increases (Fig. 4, spectrum F). Conformational analyses of DPPC [12] and DPPE [6] have been run previously. For each phospholipid, the assembling mode of interaction with piracetam was analysed. The structures shown in Figs. 5 and 6 correspond to the high probability of an interaction between DPPC and DPPE with piracetam. Piracetam is arranged around the polar head group of each phospholipid, and is largely below the plane of the interface, as described in ref. [6].

The total conformational energy of the molecule at the air-water interface was empirically calculated as the sum of all contributions resulting from local interactions (i.e. Van der Waal's energy, torsional potential, electrostatic interactions, and transfer energy). Electrostatic energy was calculated as a function of the dielectric constant. The values of this dielectric constant (ϵ) at the interface, established experimentally and theoretically, vary from 10 to 40 [6]. In this study, to simulate the interface, the dielectric constants of the hydrophobic and hydrophilic media were taken as 3 and 30, respectively. The dielectric constant was assumed to increase linearly between these two media. The transfer of energy for distinct moieties of the molecule has been determined experimentally by numerous authors, and summarized by Tanford [13]. The values used for the valence angles, bond lengths, and atomic charges are those currently used in conformational analysis studies [14], and are similar to those more recently used in computer modelling [15–17].

In the calculation procedure, changes of 60° were first imposed on each of n torsional angles, yielding 6^n conformers. The internal energy was calculated for each of these conformers. The most probable configurations were taken as those yielding the lowest internal energy; such a selection was based on one statistical weight, associated with the energy of each individual configuration, taking into account the Boltzmann relationship. The mean interaction energy between one drug surrounded by lipids was equal to the sum of lipid-drug and lipid-lipid interaction energies divided by the number of surrounding lipids [12, 18].

DISCUSSION

The main feature of the effect of the drug on ^{31}P NMR spectra revealed by our observations is the narrow, dose-dependent piracetam signal. This phenomenon is clearly visible in Figs. 1 and 2 for DPPC and DPPE samples influenced by different additions of the drug.

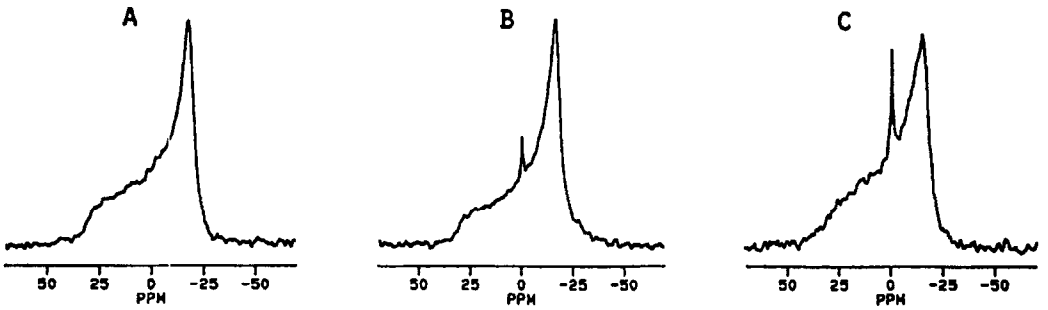

Fig. 2. 101.3 MHz ^{31}P NMR spectra of DPPE at 333K; (A) control and after addition of piracetam up to a drug/DPPE ratio of (B) 1 and (C) 2.5.

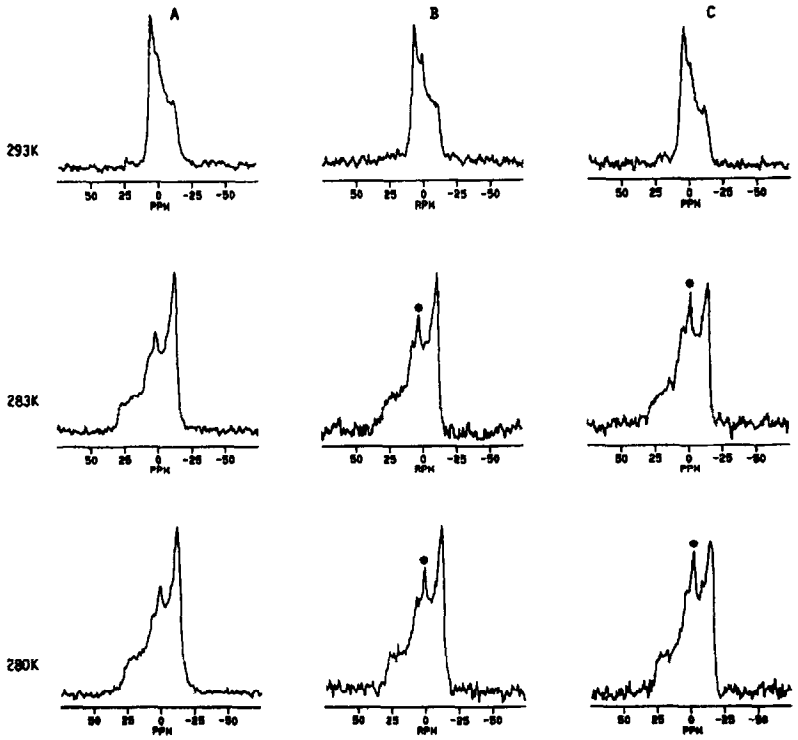

Fig. 3. 101.3 MHz ^{31}P NMR spectra of BBPE as a function of temperature: control spectra (A series) and after addition of piracetam up to a drug/BBPE ratio of 0.25 (B series) and 0.5 (C series).

Narrow signals at the isotropic shift values have been observed by different authors under various conditions. These isotropic type resonances have been attributed to the presence of lipidic particles or other intermediary phases, which may have cubic, rhombic, or tetragonal structures [19, 20]. However, to our knowledge, this observation had not yet been made on pure DPPC or DPPE.

The change in molecular shape is considered the main driving force behind the bilayer-nonbilayer transformation [20]. Our conformational analysis results help interpret the ^{31}P NMR observations.

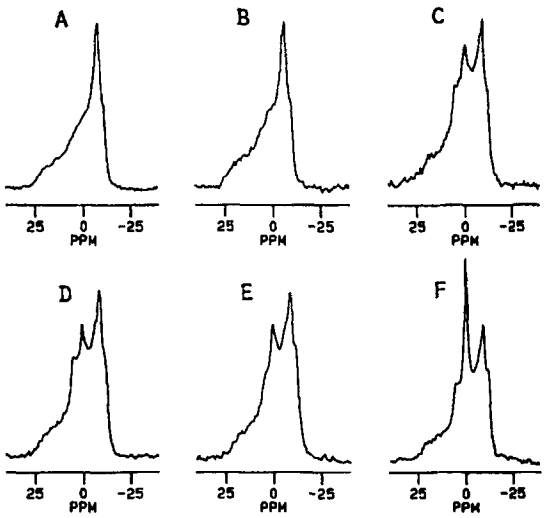

Fig. 4. 101.3 MHz ^{31}P NMR spectra of CL/EYPC mixture: (A) control; (B) sample A + piracetam (drug/EYPC ratio of 1.2); (C) sample B + Ca^{++} (Ca^{++}/CL ratio of 0.25); (D) sample A + Ca^{++} (Ca^{++}/CL ratio of 0.25); (E) sample D + piracetam (drug/EYPC ratio of 0.4); (F) sample E + piracetam (drug/EYPC ratio of 1.2).

As shown in Figs. 5 and 6, piracetam molecules, by surrounding the polar heads, modify the shape of the phospholipids complexed with the drug. An interface curvature is induced by the piracetam molecules inserted between the phospholipids, preferentially at the level of the polar headgroups. The phospholipid-piracetam complexes adopt a more conical shape. It is possible that several headgroups completely "solvated" by the drug favour the formation of micelles or small vesicles that produce an isotropic-type signal because of their greater mobility.

This effect is confirmed by our observations on BBPE spectra. The temperature behavior of this phospholipid reveals a lamellar-to-hexagonal phase transition. This transformation of unsaturated PE had previously been evidenced by Cullis and de Kruijff [9].

We have observed that in the presence of piracetam, the isotropic signal increases in a dose-dependent manner with drug content (see spectra B and C at 280 and 283 K in Fig. 3) as long as the transformation of BBPE from bilayer to hexagonal phase is not too advanced. At 293 K (Fig. 3), when the hexagonal phase is predominant, the effect of the drug on the spectrum shape is no longer significant. These observations could be explained by a greater stability of the hexagonal phase compared to the PE-drug complexes organized in micelles or small vesicles. At 280 and 283 K, when BBPE adopts a bilayer structure, the piracetam-PE interaction is probably strong enough to enhance the formation of small entities leading to the increase of the narrow component.

The influence of the tested drug on CL/PC mixture is

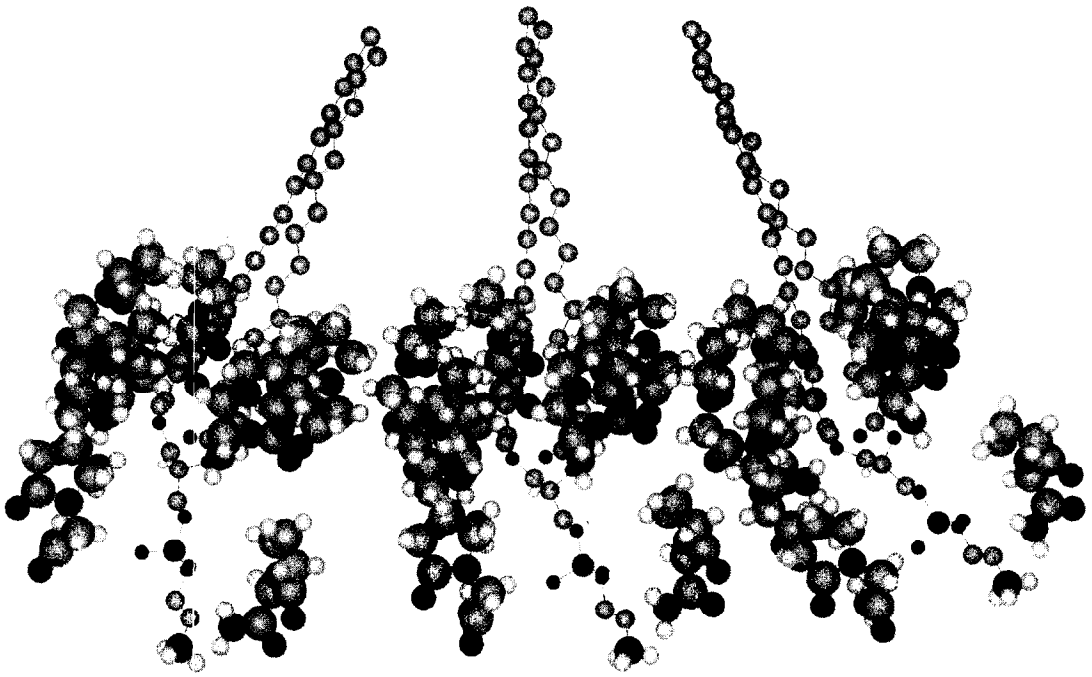

Fig. 5. Ball sticks view of the mode of interaction of piracetam and DPPC. DPPC are represented by small balls, and piracetam by large balls. Carbon atoms in grey; oxygen atoms in red; nitrogen atoms in blue; hydrogen atoms in light grey.

more complicated to interpret. The drug alone does not influence the bilayer organization, probably because the interactions between the two phospholipids are stronger than the piracetam phospholipid interactions. However, at low concentrations of Ca^{++} (Ca^{++}/CL = 0.25), addition of a substantial amount of piracetam (drug/PC = 1.2) triggers the transformation to the isotropic type phase (Fig. 4, spectrum F). One can assume a combined influence of Ca^{++} and piracetam on this phospholipid mixture. The Ca^{++}, by inducing transformation in non bilayer structures, may favour the approach of the piracetam molecules to the polar headgroup of PC. As in pure DPPC, more mobile piracetam-PC complexes could then be formed, greatly enhancing the isotropic peak.

The complementary combination of an experimental tool, NMR, and of a theoretical method, conformational analysis, leads to a keen comprehension of important molecular phenomena. On the basis of ^{31}P NMR observations and theoretical calculations, we have shown that piracetam, by interacting with the phosphate headgroup

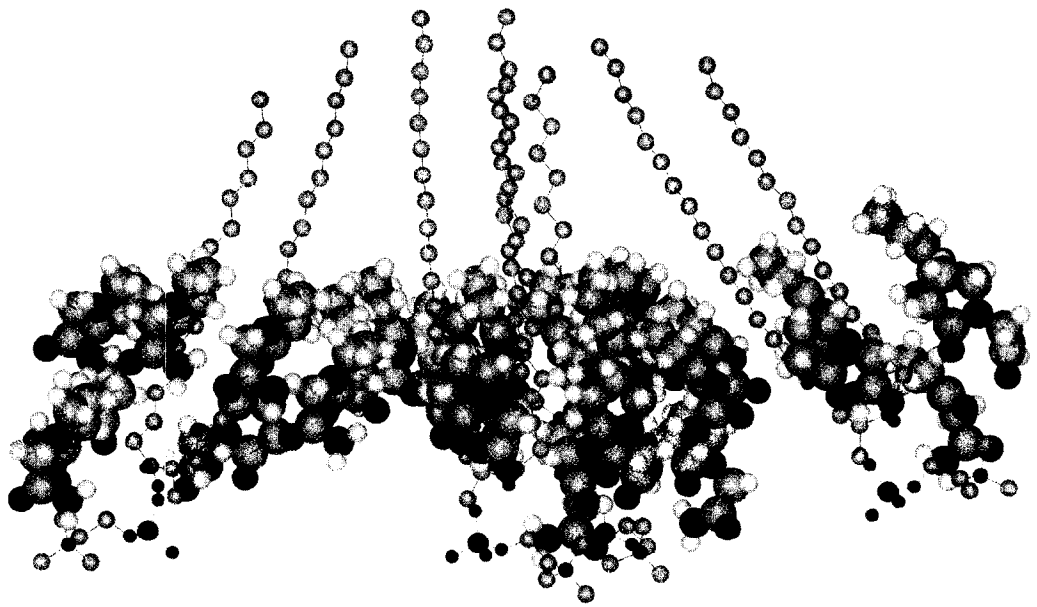

Fig. 6. Ball sticks view of the mode of interaction of piracetam and DPPE. DPPE are represented by small balls, and piracetam by large balls. Carbon atoms in grey; oxygen atoms in red; nitrogen atoms in blue; hydrogen atoms in light grey.

of PC and PE, induces formation of mobile drug-phospholipid complexes exhibiting an isotropic type signal in the ^{31}P NMR spectra.

This study shows that piracetam has a primarily physical effect on the phospholipids of the membrane, which may explain observed modifications in membrane physical properties.

It is tempting to speculate that this physical, aspecific action of the nonmetabolizable drug is related to various changes observed in cells, independent of their origin.

Work is in progress to address the question whether this new phospholipid-drug organization confers a better cell membrane viscosity, leading to improved cell function and better cell defence against aggression.

Acknowledgements—We gratefully thank Eric Cossement for his participation in the initial portion of this work.

The financial support of the Belgian Fonds National de la Recherche Scientifique and l'Association Française de Lutte contre la Mucoviscidose are gratefully acknowledged.

Robert Brasseur is Director of Research of the Belgian Fonds National de la Recherche Scientifique.

We thank Colleen Randall for her helpful suggestions on the manuscript, and Solange Favoreel for her secretarial assistance.

REFERENCES

1. Gobert JG and Baltes EL, Availability and plasma clearance of piracetam in man. *Il Farmaco (Ed. Pratica)* **32:** 84–91, 1977.
2. Gini EK and Sonnet J, Use of piracetam improves sickle cell deformability *in vitro* and *in vivo*. *J Clin Pathol* **40:** 99–102, 1987.
3. Luzzatti V, X-ray diffraction studies of lipid-water systems. In: *Biological Membranes* (Ed. Chapman D.), pp. 71–123. Academic Press, New York, 1968.
4. Cullis PR, Verkleij AJ and Ververgaert PHJT, Polymorphic phase behavior of cardiolipin as detected by ^{31}P NMR and freeze-fracture techniques. Effect of calcium, dibucaine and chlorpromazine. *Biochim Biophys Acta* **513:** 11–20, 1978.
5. Cullis PR and de Kruijff B, Polymorphic phase behavior of lipid mixtures as detected by ^{31}P NMR. Evidence that cholesterol may destabilize bilayer structure in membrane systems containing phosphalidylethanolamine. *Biochim Biophys Acta* **507:** 207–218, 1978.
6. Brasseur R, *Molecular Description of Biological Membrane Components by Computer Aided Conformational Analysis* (Ed. Brasseur R), Vol. I. CRC Press, Boca Raton, FL, 1990.
7. Seelig J, ^{31}P Nuclear Magnetic Resonance and the headgroup structure of phospholipids in membranes. *Biochim Biophys Acta* **515:** 105–140, 1978.
8. Cullis PR, Lateral diffusion rates of phosphatidyl choline in vesicles membranes. Effects of cholesterol and hydrocarbon phase transitions. *FEBS Lett* **70:** 223–228, 1976.
9. Cullis PR and de Kruijff B, ^{31}P NMR studies of unsonicated aqueous dispersions of neutral and acidic phospholipids. Effects of phase transition, p^2H and divalent cations on the motion in the phosphate region of the polar headgroup. *Biochim Biophys Acta* **436:** 523–540, 1976.
10. Nelder JA and Mead R, A simplex method for function minimization. *Computer J* **7:** 308–313, 1965.
11. Brasseur R, Deleers M and Ruysschaert J-M, Mode of organization of amphiphilic molecules at the lipid-water interface: A conformational analysis. *J Col Interf Sci* **114:** 277–282, 1986.
12. Brasseur R, Goormaghtigh E and Ruysschaert J-M, Theoretical conformational analysis of phospholipids bilayers. *Biochem Biophys Res Commun* **103:** 301–310, 1981.
13. Tanford C, *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*. John Wiley, New York, 1973.
14. Hopfinger AJ, *Conformational Properties of Macromolecules*. Academic Press, New York, 1973.
15. Weiner SJ, Kollman PA, Case DA, Singh UC, Ghio C, Alagona G, Profeta S and Weinger P. A new force field for molecular mechanical simulation of nucleic acids and proteins. *J Am Chem Soc* **106:** 765–784, 1984.
16. McCammon JA and Harvey SC, *Dynamics of Proteins and Nucleic Acids*. Cambridge University Press, 1987.
17. De Loof H, Harvey SC, Segrest JP and Pastor RW. Mean field stochastic boundary molecular dynamics simulation of a phospholipid in a membrane. *Biochemistry* **30:** 2099–2113, 1991.
18. Brasseur R, Killian JA, de Kruijff B and Ruysschaert J-M, Conformational analysis of gramicidin-gramicidin interaction at the air-water interface suggests that gramicidin aggregates into tube-like structures similar as found in the gramicidin-induced hexagonal HII phase. *Biochim Biophys Acta* **903:** 11–17, 1987.
19. de Kruijff B, Verkleij AJ, Van Echteld CJA, Gerritsen WJ, Mombers C, Noordam PC and de Gier J, The occurence of lipidic particles in lipid bilayers as seen by ^{31}P NMR and freeze-fracture electron-microscopy. *Biochim Biophys Acta* **555:** 200–209, 1979.
20. de Kruijff B, Verkleij AJ, Leunissen-Bijvelt J, Van Echteld CJA, Hille J and Rijnbout H, Further aspects of the Ca^{++} dependent polymorphism of bovine heart cardiolipin. *Biochim Biophys Acta* **693:** 1–12, 1982.