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INTERACTION OF THE MALONYLDIALDEHYDE MOLECULE WITH MEMBRANES

A DIFFERENTIAL SCANNING CALORIMETRY, ^{1}H-, ^{31}P-NMR AND ESR STUDY

J.-C. DEBOUZY,*†‡ F. FAUVELLE,*† H. VEZIN,*† B. BRASME† and Y. CHANCERELLE†
CRSSA, unités de *Biophysique et de †Radiobiochimie, 24 avenue du Grésivaudan, 38700 La Tronche, France

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Abstract—The membrane interactions of malonyldialdehyde (MDA), natural product of polyunsaturated fatty acids peroxidation were investigated by differential scanning calorimetry, and ESR or NMR spectroscopy. This component is located in the superficial part of the bilayer, where it increases the local fluidity. High concentrations of MDA induce major membrane damage. Similar consequences of MDA–membrane interactions were observed on erythrocyte ghosts.

Malonyldialdehyde (MDA§) (CHO-CH_2-CHO) was identified as a primary oxidation product of polyunsaturated fatty acids [1, 2]. The reactivity of functional groups of MDA with amino groups of aminosugars [3], proteins [4], purines [5] and pyrimidines [6] has been studied for a long time. It is generally admitted that the biological effects of MDA are related to its chemical reactivity. Thus, MDA exhibits *in vivo* mutagenic properties [1] and it is involved in cell ageing and food rancidity [7]. Large amounts of MDA are also produced in some pathological situations such as inflammatory diseases or irradiation. Previous studies pointed out the existence of chemical adducts of MDA [1–6]. Considering these biological effects, it is obvious that concomitant membrane alterations occur especially in ageing. If the partition coefficient measurement (performed at 267 nm) is considered, MDA solubility in water is three times that in octanol. This result shows that, besides the well-known hydrophilic properties of MDA, its solubility in hydrophobic solvent is not negligible. Thus, interactions of MDA with membranes required further investigation the results of which are presented in this paper.

First of all, the effect of MDA on the phospholipid phase transition was investigated by calorimetric measurements. ^{1}H-NMR experiments in the presence of synthetic membranes were then used to examine the location of MDA in the lipid bilayer. The consequences of the presence of MDA on membrane dynamics were then investigated using ESR techniques, using nitroxide-labelled fatty acids. ^{31}P-NMR experiments in phospholipid liposomes were used to study the membrane structure in the presence of MDA. Finally, a similar ^{31}P-NMR experiment was performed using human erythrocyte ghosts.

‡ Corresponding author.

§ Abbreviations: MDA, malonyldialdehyde; DMPC, dimyristoyl-phosphatidyl choline; DPPC, dipalmitoyl-phosphatidyl choline; SUV, small unilamellar vesicles; CSA, chemical shift anisotropy; DSC, differential scanning calorimetry.

MATERIALS AND METHODS

MDA and lipids

MDA was prepared according to the method of Protopopova and Skolnikov [8], Bertz and Dabbagh [9] or Lacombe *et al.*, [10] and was followed by ^{1}H-NMR control.

Egg L-α-phosphatidylcholine, dipalmitoyl-phosphatidylcholine (DPPC) and dimyristoyl-phosphatidylcholine (DMPC) were purchased from Sigma (La Verpillière, France). Before each sample preparation, the quality of the phospholipids used was checked by recording a ^{1}H-NMR spectrum in chloroformic solution.

Spin-labelled fatty acids (5-7-10-12-16 NS) for ESR experiments were purchased from Molecular Probes (Eugene, OR, U.S.A.). For each spin label, the number before NS corresponds to the location of the reporter group on the C16 acyl chain of the molecule with the acidic carbon counted as the first.

Samples for NMR, differential scanning calorimetry (DSC) and ESR experiments

Small unilamellar vesicles (SUV). Phosphatidylcholine was freeze-dried twice in D_2O and resuspended in pure D_2O to a final lipid concentration of 6 mM. SUV were formed by a 2 hr bath sonication. For MDA-containing SUV, MDA was added to the reference phospholipid SUV.

DMPC liposomes for ^{31}P-NMR experiments. The liposomes were prepared following the procedure described by Roux [11] and Hubell and McConnell [12]. The phospholipid chloroformic solution was firstly freeze-dried overnight, resuspended in pure D_2O and deoxygenated. The suspension was then introduced into NMR tubes which were then sealed. The liposomes were formed by five freezing and thawing cycles to a final lipid concentration of 50 mM. For MDA-containing liposomes, MDA and

the phospholipids solutions were mixed in the sample before freeze-drying.

Dispersions for DSC experiments. The DSC samples (containing about 2 mg of DPPC or DMPC, and MDA) were dispersed in 50 μL of 50 mM phosphate buffer pH 7.4. The microcaps were sealed and three heating–cooling cycles were performed before the scans were registered. Calorimetric scans were carried out on a Perkin–Elmer DSC4 spectrometer at a rate of 5°/min. Each sample yielded reproducible thermograms over three heating cycles from approximately 10 to 60°. Values for the enthalpy change of the transition were calculated by extending the baseline from the onset of the temperature transition to its completion and determining the enclosed area under the transition [13, 14].

Red blood cells

Red blood cells from human blood were first of all washed with 0.9% NaCl using three washing–centrifugation cycles (10 mn at 3000 rpm). Ghosts were formed by seven cycles, each one including the addition of an equal volume of 0.4% NaCl, 0.1 mM EDTA followed by a 30 min centrifugation at 4°, 20,000 rpm. At the end of the preparation, the ghosts were resuspended in pure D_2O.

ESR experiments

For ESR experiments, spin-labelled fatty acids were incorporated into SUV. The spectra were recorded on a Varian E109 spectrometer at a temperature range of 0°–50°. For 16NS experiments, the parameter measured was $\ln(H_0/H_{-1})$ versus the reciprocal absolute temperature, where Wo and H_0 are the central signal width and intensity, respectively, and H_{-1} the highfield signal intensity. $\ln(H_0/H_{-1})$ is related to the apparent label rotational frequency n (Hz) by the following equation [15]:

$$(n)^{-1} = 6.55 \times 10^{-10}\, Wo.[(H_0/H_{-1})^{1/2} - 1]^{-1}.$$

Since the above equation cannot be used for longer correlation times, the spectra recorded with 10NS and 12NS were analysed by plotting the inner ($2T'^{\perp}$) and outer ($2T'^{//}$) hyperfine splitting constants against temperature (°C). For 5NS and 7NS experiments a local order parameter dependence versus temperature (°C) was calculated. The outer and inner hyperfine splitting difference ($2T'^{//} - 2T'^{\perp}$) was measured and the local order parameter was obtained using the following equation:

$$S = (2T'^{//} - 2T'^{\perp})/(2T_0^{//} - 2T_0^{\perp})$$

(where $2T_0^{//}$ and $2T_0^{\perp}$ are the outer and inner hyperfine splitting values, respectively, measured on crystals [16]).

NMR experiments

^{1}H-NMR experiments were performed on a Bruker WH250 spectrometer and referenced in relation to internal tetramethylsilane. ^{31}P-NMR experiments in liposomes were performed at 161 MHz on a Bruker AM400 spectrometer, using a broadband proton decoupling and a standard dipolar echo sequence with a delay value of 15 μsec.

RESULTS AND DISCUSSION

Preliminary calorimetric experiments

On the heating thermograms obtained from dispersions of pure DPPC or DPPC enriched with MDA, a decrease in the enthalpy of the phase transition was observed, and a gradual drop in transition temperature with increasing MDA concentration was also found. The transition temperature of the pure lipid dispersion was found to be 41.8°. The addition of MDA gave rise to a reduction in the transition temperature to around 40.2° and 39.6° for dispersions containing 2% and 4% MDA (M/M), respectively, while the pretransition became unobservable. For the largest amounts of MDA neither the pretransition nor the main transition could be identified clearly. Only a very broad signal was observed on the thermogram that totally vanished after several heating cycles or with larger amounts of MDA (MDA : lipid molar ratio greater than 1 : 10). Similar results were obtained with DMPC, the transition temperature of which was lowered from 24° to 23.6° (2% MDA) and 23.4° (4% MDA). At this stage the existence of a MDA–membrane interaction was obvious, but no mechanism for this interaction could be proposed.

The location of MDA in the bilayer was then investigated by performing ^{1}H-NMR experiments in water and in the presence of synthetic vesicles.

NMR experiments in aqueous solution and in SUV

A ^{1}H-NMR spectrum of MDA (4 mM) in deuterated water was recorded at 250 MHz on a WM250 Bruker spectrometer. According to previous works [9, 10] MDA resonances (referenced to tetramethylsilane) were found at 5.68 (—CH_2—) and 8.50 ppm (—CHO). The observation of resonances of less than 1 Hz linewidth (Fig. 1B) is in full agreement with the hydrosolubility of MDA. The interaction of MDA with membranes was studied using SUV as model membranes. Such a system makes it possible to define the location of the drug (MDA) since it gives rise to resonances of 10–20 Hz linewidths. Thus, it can be expected to detect the MDA resonances. By way of contrast, other membrane systems (large unilamellar vesicle or multilayers) exhibit dramatically wider resonances which may preclude any peak attribution.

Figure 1A shows the 250 MHz ^{1}H-NMR spectrum of SUV recorded in the presence of MDA (MDA : lipids molar ratio 1 : 20). In comparison to a spectrum obtained from pure SUV, the phospholipid resonances are not affected by the presence of MDA. This makes it possible to observe the MDA resonances, and particularly the lowfield signal which is easily distinguished from the phospholipid resonances located in the highfield region. This component of the MDA spectrum was found at a chemical shift (around 8.5 ppm) close to those observed in aqueous solution (Fig. 1B). As far as the linewidth is concerned, this MDA resonance is broadened to a 9 Hz linewidth signal (Fig. 1C). This is related to the existence of a MDA–membrane interaction suggested by the calorimetric experiments. Moreover, this value is close to the 10 Hz linewidth measured for the protons of the most

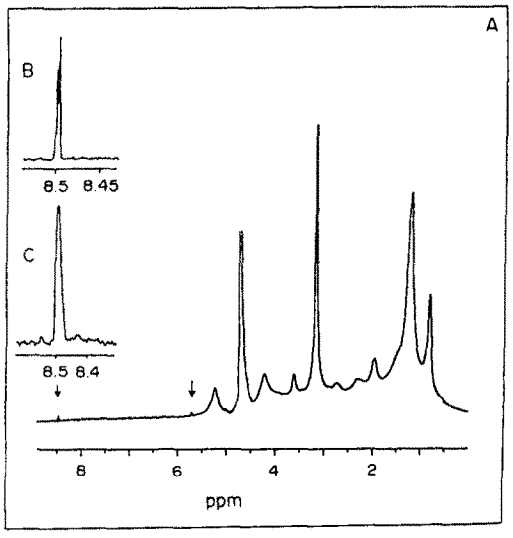

Fig. 1. 250 MHz ^{1}H-NMR. (A) Spectrum of MDA containing SUV. Arrows indicate MDA resonances. (B) Lowfield resonance of MDA in D_2O. (C) Expanded difference spectrum of MDA lowfield resonance obtained by subtraction from spectrum (A) of a spectrum of pure SUV. Lipids: MDA molar ratio of 20:1.

mobile part of the phospholipid molecule, i.e. methyl and choline protons. In order to obtain a more precise localization of the MDA molecule in the bilayer, we added progressive amounts of paramagnetic ions to SUV containing MDA.

It is important to recall some basic results concerning the effect induced by Mn^{2+} ions on the phospholipid resonances of SUV [17]. In the case of polar headgroups, for which each proton gives rise to a separate resonance for each layer, the addition of a small amount of Mn^{2+} ions is sufficient to broaden the signal corresponding to the external layer beyond detection, while the signal corresponding to the internal layer is poorly affected. On the other hand, the protons of the terminal methyl group appear as a single resonance which is gradually broadened on increasing the Mn^{2+} concentration. For a given resonance, the product of the peak height by its halfwidth is constant. Thus, it is easy to analyse a broadening effect by measuring the height of the peak for increasing amounts of paramagnetic ions. The result is plotted on Fig. 2, for MDA and phospholipid methylic, methylenic, methynic and choline resonances.

In order to follow the broadening of the MDA resonances upon the addition of Mn^{2+} ions, we carried out two sets of experiments on SUV in the absence and in the presence of MDA, and then observed the drug resonances by spectrum difference. In fact, only the lowfield resonance of MDA could be observed clearly.

Under these conditions, the MDA resonance linewidth variation against the Mn^{2+} concentration is practically superimposed upon that corresponding to the γ-choline resonance (Fig. 2). In both cases, the corresponding intensity of the peaks is reduced by a factor of 10. This suggests the presence of the MDA molecule at a superficial level of the bilayer where the choline group is located. However, the following points should be noted: (i) choline groups are located on both sides of the membrane distributed in the external–internal layer of SUV in approximately a 2:1 ratio. On a ^{1}H-NMR spectrum, the resonance observed at 3.23 ppm is attributed to both signals. After addition of paramagnetic ions, the external spectral component vanishes and the only remaining resonance observed is the internal choline signal: the corresponding intensity is $\frac{1}{3}$ of the total initial intensity and is not greatly affected by successive additions of paramagnetic ions. (ii) Under the hypothesis that MDA is located in the external part of the external layer of the membrane, a very small amount of Mn^{2+} should be sufficient to broaden the corresponding signal under detection.

Thus, the existence of identical paramagnetic dependence of MDA and choline resonances linewidths requires: (i) the presence of MDA on both sides of the bilayer and (ii) the presence of some Mn^{2+} ions in the intravesicular medium (or at

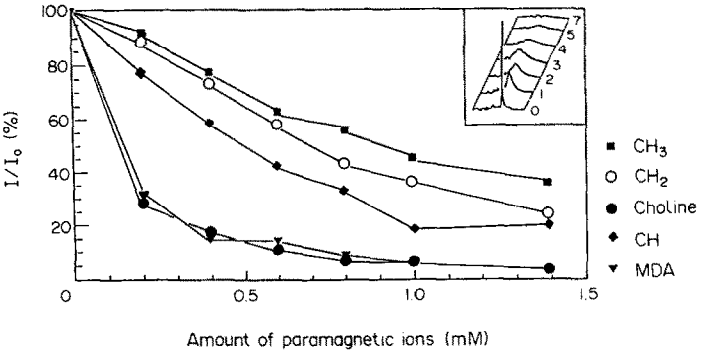

Fig. 2. 250 MHz ^{1}H-NMR of SUV. Relative peak height reduction (%) of (■) methyl, (○) methylene, (◆) methine, (●) choline and (▼) MDA protons plotted versus Mn^{2+} concentration. Inset shows the MDA highfield resonance broadening upon Mn^{2+} addition.

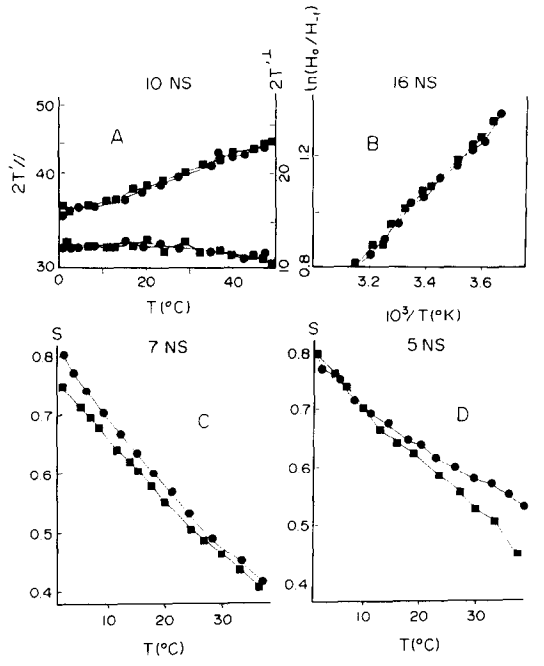

Fig. 3. ESR experiments in SUV. (A) 10NS experiment. Comparative plot of hyperfine constants $2T'//$ and $2T'^{\perp}$ versus temperature in °C. (B) 16NS experiment. Plot of $\ln(H_0/H_{-1})$ versus reciprocal absolute temperature. (C) 7NS experiment. Direct plot of the order parameter versus temperature (°C). (D) Same experiment as in (C) using 5NS spin-labelled fatty acid. ESR parameters are recorded in the absence (●) and in the presence of MDA (■). Lipid : MDA = 20 : 1 M/M.

the superficial part of the internal layer). A detergent effect could provide such results. For this molar ratio (MDA : lipid 1 : 20), this hypothesis must be rejected since the spectrum of the SUV is not affected by the addition of MDA (up to MDA : lipid molar ratio 1 : 10). In the absence of any evidence for MDA or Mn^{2+} transmembrane transport, this result suggests dynamic or structural modifications of the membrane which should be investigated.

Influence of MDA on phospholipid dynamics

Dynamic properties of the membrane in the presence of MDA at low molar ratio (MDA : lipid 1 : 20) were studied using phospholipid SUV of egg phosphatidylcholine. Nitroxide-labelled fatty acids were incorporated into membranes. By selecting the location of the nitroxide group along its C16 acyl chain, one can investigate the fluidity in the depth of the lipid bilayer (16NS) or obtain information on the hydrophilic part of the membrane (5NS–7NS), or study at an intermediate level (10NS–12NS). Since the typical correlation times are closely dependent on the membrane level studied, different methods of analysis are required (see Materials and Methods).

Experiments performed with 16NS spin label (Fig. 3B) showed no difference in the $\ln(H_0/H_{-1})$ against the reciprocal absolute temperature plot, when MDA was added. This is also the case for the $2T'//$ and $2T'^{\perp} = f(T°C)$ plots using 10NS and 12NS (not shown)-labelled fatty acids (Fig. 3A).

In the case of 5NS and 7NS the order parameter S plotted against temperature demonstrated: (i) for the 7NS label, a significant decrease in the order parameter (Fig. 3C) on all the temperatures studied is found when MDA is added (molar ratio 1 : 20). (ii) for 5NS label (Fig. 3D), a significant increase in the slope value of the $S = f(T°C)$ curve which leads to an important reduction of the order parameter at high temperatures, i.e. of 0.07 U at 30°. Moreover, the calculated thermal susceptibility is significantly reduced in the presence of MDA. Therefore, MDA appears to be a liquifying reagent that exhibits either a direct effect on the fluidity (7NS), or an increase in the temperature dependence of the bilayer fluidity (5NS) in its superficial part. These observations are consistent with the superficial location of MDA suggested by NMR experiments.

As discussed below, the influence of the MDA molecule on the bilayer structure was studied using liposomes (multilayers) as model membranes and ^{31}P-NMR spectroscopy. This technique is known to be effective for the study of the structural consequences of drug–membrane interactions [18].

Influence of MDA on membrane structure

The proton dipolar decoupled ^{31}P-NMR spectra of pure DMPC and of MDA–DMPC (molar ratio 1 : 10) recorded at different temperatures are shown in Fig. 4A. The chemical shift of a given ^{31}P nucleus depends on its orientation in the main field. This leads to contributions of the ^{31}P nuclei oriented parallel to the magnetic field B_0 (the $\sigma^{//}$ lowfield component) separate from those oriented in a direction perpendicular to B_0 (the σ_T highfield component). When the molecule is dissolved, i.e. in water, the rapid motion of all the ^{31}P nuclei gives rise to a single averaged resonance. This is not the case for anisotropic media such as membranes where the overall motion is restrained. In this case, each nucleus contributes separately to the spectrum. The position of the corresponding parallel and perpendicular resonances are closely dependent on the membrane structure (relative intensities of σ_T and $\sigma^{//}$ contributions) and fluidity [(from the measurement of the difference of the chemical shift anisotropy (CSA)].

Pure DMPC spectra show in the 25–65° thermal range a typical axially symmetric powder pattern with an effective CSA varying with temperature from −46 (25°) to −43 (65°) ppm.

When samples of DMPC–MDA mixtures are used (lipid : MDA 10 : 1), the low temperature spectra are also axially symmetric with a CSA close to that measured on pure DMPC samples (i.e. −45.7 ppm at 25° and −44.3 ppm at 35°). However, a significant increase in the σ_T spectral component can be observed. Since the same experiment was repeated at different delay values (from 10 to 25 μsec) the presence of oriented samples or artifacts due to echo truncation (which can give rise to such spectra) was refuted.

As the temperature is increased the corresponding CSA gradually decreases (to −30 ppm at 65°) and is

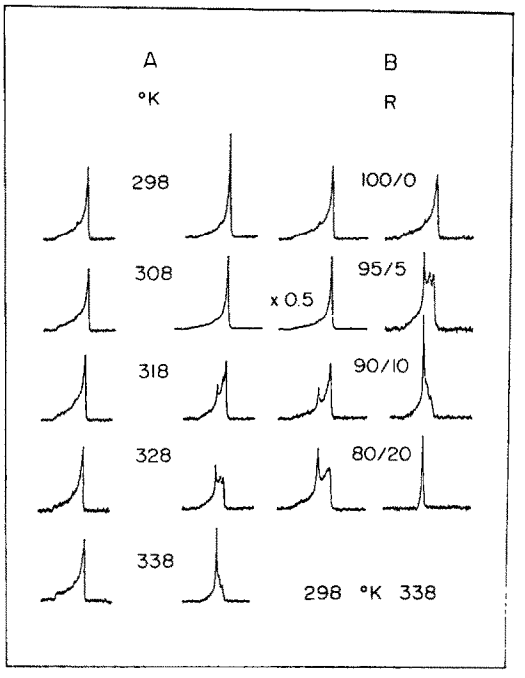

Fig. 4. 161 MHz ^{31}P-NMR in multilayers. (A) Temperature dependence of the ^{31}P-NMR spectra of pure DMPC (first column) and DMPC–MDA (10:1 M/M) dispersions (second column). (B) Concentration dependence of the ^{31}P-NMR spectra at 25° (third column) and 65° (fourth column). MDA : DMPC molar ratios are indicated between the last columns.

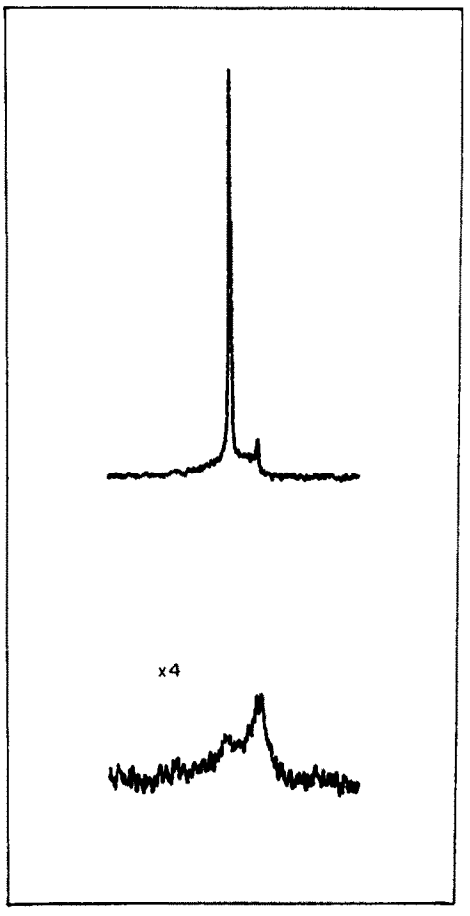

Fig. 5. 161 MHz ^{31}P-NMR of ghosts. Spectra recorded at 25° in the absence (bottom) and in the presence of MDA (top). Lipid : MDA ratio was evaluated at 5:1. For each spectrum 138,000 transients were acquired. A line broadening of 25 Hz was used.

superimposed upon two peaks located at $\delta = 16$ and $\delta = 8$ ppm, respectively (relative to the highfield edge of the bilayer spectrum).

As the temperature is increased further, the relative intensity of the axial symmetric component decreases with a concomitant increase in the intensity of the lowfield component while the intermediate peak remains unaffected.

Concentration dependence. When high ratio MDA–DMPC dispersions (MDA : lipid higher than 1:10) are used, the lowfield symmetric peak appears for spectra recorded at lower temperatures; (see Fig. 4B) with greater intensities the higher the ratio. This spectral component becomes exclusive at 25° for a MDA : lipid ratio of 1:4. In the 1:10–1:4 range the same isotropic aspect is obtained when successive spectra are recorded after several days at room temperature. In both cases, further spectra recorded by varying the temperature or after further time show no more spectral change.

Such a spectrum which only shows an isotropic symmetric resonance precludes the presence of a bilayer structure. This suggests considerable membrane damage consistent with the presence of a macroscopic gel observed at the end of the experiment.

Since similar results were also found with other phospholipids (DPPC), biological systems had to be tested. ^{31}P-NMR experiments were then performed on ghost membranes in the presence of MDA.

NMR experiments on ghosts

The ^{31}P-NMR spectra recorded on ghosts at 25° showed a typical axially symmetric powder pattern (Fig. 5). An important "isotropic" spectrum component was found and could not be removed by further washing. As MDA was added, spectrum modifications similar to those found in multilayers were observed, but larger amounts of MDA were required (about three times the MDA : lipid molar ratio used in synthetic membranes). This suggests that the other components of the ghost membranes, i.e. cytoskeleton or proteins, enhance the total membrane cohesion which limits the MDA effect.

Concluding remarks

This study shows that the MDA molecule in membranes is located in the superficial part of the layer, and induces a disordering effect on the hydrophilic core.

The MDA molecule is a potential mutagenic reagent both in bacterial and mammalian cells [1]. Strong nucleic acid alterations observed in the presence of MDA have been related to these properties, and to the observation of purine–MDA adducts in biological fluids, i.e. blood and urine. MDA adducts were also observed with other biological molecules, such as amino acids, aminosugars or other substrates like thiobarbituric acid. The latter reaction is still used to determine the importance of lipid peroxidation in food [2] and biological systems, and in various situations involving the formation of chemical adducts with MDA.

Previous studies [1–6] led to explanations implying a chemical bound formation. This study shows that a natural lipid peroxidation product such as MDA can interact by itself with synthetic membranes, with no evidence of chemical bound formation with phospholipids. However, the existence of the latter mechanism cannot be refuted, particularly for high molar ratios where a gel formation is observed.

Many molecules interact exclusively with the polar headgroup of the membrane. In several cases, destructuring or fluidizing properties are found, and a detergent effect is found at high drug concentrations [19–21]. Conversely, MDA shows quite different properties: while a similar disordering effect is also found at low MDA:membrane ratios, high MDA concentrations lead to the formation of a new phase. Indeed, the very stable gel formed is quite different from a single detergent effect. This could be consistent with chemical bound formation, i.e. between the aldehyde groups of MDA and the lipid molecules. TLC studies [22] were performed on extracts of UVC-irradiated liposomes. While breakdown products of lipids were found after a 24 hr irradiation, no lipid–MDA adduct could be identified clearly. Furthermore, the MDA molecule could still be identified and dosed after irradiation using the reaction with added thiobarbituric acid [2]. However, no direct argument for a bound formation was found in our NMR experiments, either in SUV or in solution, since no new resonances were detected after MDA addition, except those of MDA itself. Thus, in the absence of any evidence for chemical bound formation, the hypothesis of a non-specific interaction can be proposed.

This study shows a quite similar effect of MDA on model membranes and on erythrocytes ghosts, except in the latter case that larger amounts of drug are required. This can be attributed to the cohesion enhancement that other membrane components, i.e. the proteins and the cytoskeleton, provide.

The ratios of MDA we used, even though not physiological, are generally met in biophysical studies of drug–membrane interactions, particularly when lipid mixtures are used [21, 23, 24]. Furthermore, some pathological situations give rise to high local concentrations of MDA. This is the case for inflammatory diseases or after irradiation where self-amplified lipid peroxidation processes arise. Thus, after the first radiobiological effect (i.e. the formation of free radicals) successive lipid molecules are peroxidized (up to 12 molecules). As supposed by Guille *et al.* [25], a high concentration of MDA in a membrane can lead to, besides the formation of adducts with proteins, the MDA–lipid interaction [26]. Thus, our results show that a peroxidation product such as MDA interacts with membranes and may contribute to additional membrane damage.

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