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Effect of Aluminium Ions on Liposomal Membranes as Detected by Laurdan Fluorescence

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We report here an investigation of the influence of aluminium on iron-induced peroxidation in brain model membranes. Laurdan fluorescence emission spectra and generalised polarisation measurements have been used to investigate how ferrous and aluminium ions can affect the phase components of phospholipid membranes. An increase in the generalised polarisation of oxidised liposomes with respect to controls has been observed, which reveals the presence of a less polar environment surrounding the probe that changes the properties of the bilayer.

Aluminium has been shown to facilitate iron-mediated oxidation as detected from emission fluorescence spectra. However, no quantitative influence has been calculated relative to general polarisation and derived phase state determinations. The structural influence of aluminium on membranes may therefore be less significantly marked than initially expected.

Keywords: Aluminium, membranes, oxidation, Laurdan, fluorescence, spectra, generalised polarisation

INTRODUCTION

Membrane degeneration resulting from oxygen radical-mediated lipid peroxidation is associated with a variety of pathological events.[1] Iron is known to act as an effective catalyst in this process. In particular, iron is considered as the most probable agent responsible for lipid peroxidative damage in the brain.[2,3]

It is well documented that oxidative damage caused by free radicals is at the origin of changes in the central nervous system that are associated with aging.[4] In this context, it has been suggested that Alzheimer's disease (AD) may represent a specific brain vulnerability to age-related oxidation.[5] Free radicals are effectively involved in the neurodegeneration of AD[4]—oxidative

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stress being associated with several carbonyl-related modifications characteristic of the disease[6,7]—and a possible disruption of brain iron homeostasis in AD has been evoked.[8] More specifically, it has been demonstrated that iron-catalysed free radical attack can induce aggregation of the β-amyloid peptide (βA4) which is considered the major neuropathological hallmark of the disease,[2,9] and that βA4 neurotoxicity is both related to the aggregation process[10] and mediated by free radicals.[4]

Aluminium also has been attributed a possible contributory role in the pathogenesis of Alzheimer's disease[11–13] a controversial hypothesis that recent epidemiological studies rather tend to confirm.[14] Interestingly, aluminium equally promotes βA4 aggregation in solution[15,16] through conformational changes due to Al^{3+} binding to the peptide.[17–20] Moreover, aluminium has been shown to stimulate iron-induced lipid peroxidation in mouse brain homogenates[21] via changes in the arrangement of membrane lipids.[22] The prooxidant effect of the Al^{3+} ion is correlated with its capacity to promote liposome aggregation, permeability and fusion, as well as fatty acids chain packing.[23] In a recent study on this effect,[24] aluminium facilitation of Fe-mediated lipid peroxidation has been shown to be dependent on substrate, pH, and Al and Fe concentrations, with bovine brain-derived phosphatidylserine being the most susceptible substrate among the lipids tested.[24] The dependence of iron-catalysed lipid peroxidation on the surface charges of membranes has lately been confirmed.[25]

Finally, it has also been shown in a recent work from our group that aluminium interactions with citrate—a strong ligand of Al^{3+} and Fe^{3+} whose concentration in the cerebrospinal fluid is double that in blood plasma—can aggravate Fe(II)-induced peroxidation in brain model membranes.[26] The reason for this effect is the competition of Al^{3+} ions with Fe^{3+} ions normally bound to citrate, whose release tends to favour the oxidation process.

With a view to obtain more information on the mechanism through which iron-mediated lipid peroxidation is facilitated by aluminium, the present work reports an investigation of the effect of Al^{3+} ions on the structural organisation of phospholipids submitted to oxidation using Laurdan fluorescence emission spectroscopy.[27] Advantage is taken of the fluorescence properties of the Laurdan molecule to be sensitive to the polarity of the microenvironment where it is located. It is shown that the fluorescence properties of Laurdan are effectively sensitive to the presence of Fe^{2+} (and Al^{3+}) ions on liposomes.

MATERIALS AND METHODS

Liposome Preparation

Phospholipid unilamellar liposomes composed of L-α-phosphatidylcholine and L-α-phosphatidylserine from brain (60:40, molar ratio) were prepared by evaporating a chloroform solution of phospholipids (Sigma Chemical Co., St. Louis, MO, USA) under a nitrogen stream, resuspending the dried film in 100 mM acetate buffered solution (pH 5.5), vortexing for 1 min, incubating the samples at 45°C for 10 minutes. Then, the mixture was sonicated three times 15 minutes as described by Oteiza.[23] Final phospholipid concentration was 0.5 mg/ml.

Evaluation of Lipid Peroxidation

For these experiments, liposomes were agitated for 90 min in a shaking incubator at 37°C with metal salts (25 μM Fe^{2+} or 25 μM Fe^{2+} and 100 μM Al^{3+} as their sulfates) in the presence of 50 μM citrate.

Lipid peroxidation was evaluated at the end of the incubation as 2-thiobarbituric acid-reactive substances (TBARS) production as described by Oteiza.[23] Incubations were stopped by the addition of 0.1 ml of 4% (w/v) butylated hydroxytoluene in ethanol. Sodium dodecyl sulfate

(0.25 ml of 3% (w/v)) was added to the incubation mixtures (0.5 ml) and, after mixing, 0.5ml of 1% (w/v) 2-thiobarbituric acid and 0.5ml of 25% (v/v) HCl were added. Samples were vortexed and heated for 15 min at 95°C, and TBARS were extracted in 2.5 ml of butan-1-ol. After centrifugation at 1000 g for 10 min, the butanolic phase was measured at 535 nm. The malondialdehyde standard was made from 1.1.3.3-tetramethoxypropane in HCl.

Fluorescence Measurements: Labelling with Laurdan

For fluorescence experiments, incubations were stopped by congelation in dry ice. The fluorescent probe 2-dimethylamino-6-lauroylnaphtalen (Laurdan) (Molecular Probes, Eugene, OR, USA) was stored in ethanol at the concentration of 10^{-3}M and stored at −40°C.[28] Labelling of liposomes with the probe was performed by incubating control or oxidised liposomes[28] with a labelling buffer freshly prepared by adding an aliquot of the stock solution in saline buffer. The final concentration of the probe used was 10^{-6}M, with the ratio of probe to liposome being 1:1000. As the fluorescence intensity of Laurdan is negligible in aqueous buffer, the increase in fluorescence intensity was used to follow the incorporation of the probe into liposome lipids. The fluorescence spectra of Laurdan were carried out by means of a Perkin-Elmer spectrofluorimeter using 340 nm and 390 nm as excitation wavelengths. (Phospholipids in gel phase absorb in the 390 nm region whereas phospholipids in liquid-crystalline phase absorb in the 340 nm region.)[29] Samples were incubated for 30 min, then transferred into the cuvette and allowed to equilibrate at 20°C or 37°C in the cell holder compartment of the fluorimeter for 10 min prior measurement.

The generalised polarisation (GP) value of Laurdan was calculated as defined by Parasassi *et al.*:[30] $GP = (I_{435} - I_{490})/(I_{435} + I_{490})$, where I_{435} and I_{490} are the emission intensities at 435 and 490 nm, respectively. Generalised polarisation measurements of Laurdan fluorescence were determined in control liposomes and in liposomes oxidised by the addition of ferrous salts or ferrous and aluminium salts. Moreover the fractional intensity of the gel phase was quantified as described by Parasassi *et al.*,[27] the GP values being used to assess the two phases (gel and liquid-crystalline) in membranes following the equation:

$$GP = x.\ GP_g + (1-x)\ GP_l \qquad (1)$$

where x is the fractional intensity of the gel phase. Experiments have been carried out in duplicate.

RESULTS AND DISCUSSION

Formation of TBARS

The prooxidant effect of aluminium on phosphatidylcholine:phosphatidylserine, 60:40, liposomes *in vitro* has been confirmed by comparing the effect of iron-induced peroxidation in the absence and presence of aluminium (Table I). Clearly, aluminium stimulates iron-induced free radical formation as reported previously.[26,31–33] (It was checked that Al had no prooxidant effect by itself.)

Excitation and Emission Spectra of Laurdan Liposomes

The fluorescence intensity of the probe in buffer in the absence of liposomes was confirmed to be negligible. Following the addition of liposomes

TABLE I Iron-induced peroxidation of phosphatidylcholine:phosphatidylserine, 60:40, liposomes in the absence and presence of Al

Untreated liposomes	0.8 ± 0.6
Fe-treated liposomes	10.9 ± 3.1
Fe/Al-treated liposomes	18.8 ± 3.9

Concentrations are Fe^{2+}: 25μM; Al^{3+}: 100μM (Al_2SO_4: 50μM); citrate: 50μM. Results are expressed as μM of MDA. Mean ± SD of 6 separate experiments.

(control or treated), there was an increase in the fluorescence intensity and, after a few minutes at 20°C, a well structured emission spectrum appeared, which was characterised by a maximum at about 440 nm (Figure 1).

Normalised excitation and emission spectra of Laurdan incorporated in control liposomes (phosphatidylcholine/phosphatidylserine, 60:40) and in oxidised liposomes are shown in Figures 1 and 2. In the excitation spectrum relative to untreated liposomes, the intensity of the peak at 350 nm is higher than that of the peak at 380 nm (Figure 1A). This result can be expected from the composition of the liposomes used in this study in which unsaturated fatty acids in PC and PS represent 43.2% of total fatty acids. Indeed, previous studies have shown that the Laurdan excitation spectrum in the liquid-crystalline phase is characterised by a decrease in intensity at longer wavelengths with respect to the excitation spectrum observed in the gel phase. These peculiar features of Laurdan excitation spectra have been widely reported in model membranes and have been used to study phase coexistence and interconversion in mixed phospholipid vesicles.[27]

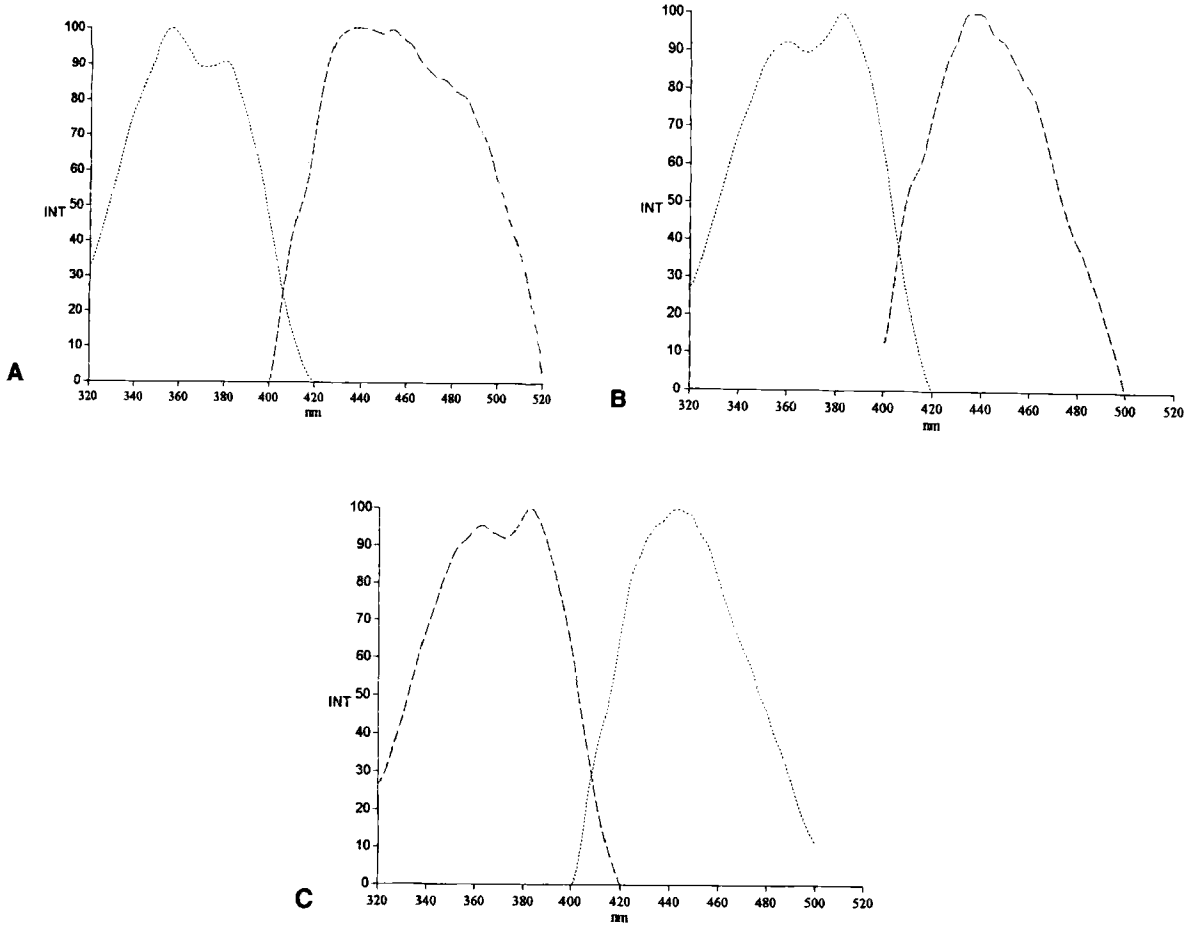

FIGURE 1 Normalised excitation and emission spectra of Laurdan incorporated in control liposomes **(A)** and in liposomes oxidised in the presence of Fe^{2+} **(B)** or Fe^{2+} plus Al^{3+} **(C)** at 20°C (λ_{exc} = 340 nm). (INT is given for fluorescence intensity.)

The position of the maximum emission of Laurdan in untreated liposomes was found near 440 nm with a shoulder at about 480 nm at 20°C (Figure 1A), the position of maximum emission being centered at 480 nm at 37°C (Figure 2A). This red-shifted position at 37°C indicates an increase in polarity with respect to 20°C.

In oxidised liposomes, the Laurdan excitation spectra show a decrease in intensity of the blue band at 350 nm and a parallel increase in the intensity of the peak at 380 nm with respect to untreated liposomes (Figures 1B and 1C). These results indicate the occurrence of modifications in the physical properties of the microenvironment of the probe.

Modifications are also observed in the emission spectrum relative to oxidised liposomes with the disappearance of the shoulder at 480 nm (Figures 1B and 1C). This result is characteristic of a lower polarity in oxidised liposomes with respect to untreated liposomes. This is in part due to the disappearance of unsaturated fatty acids in the membrane consecutive to oxidative stress (data not shown). Small variations in emission intensity are also observed to be induced by the presence of aluminium: the maximum recorded near 440 nm

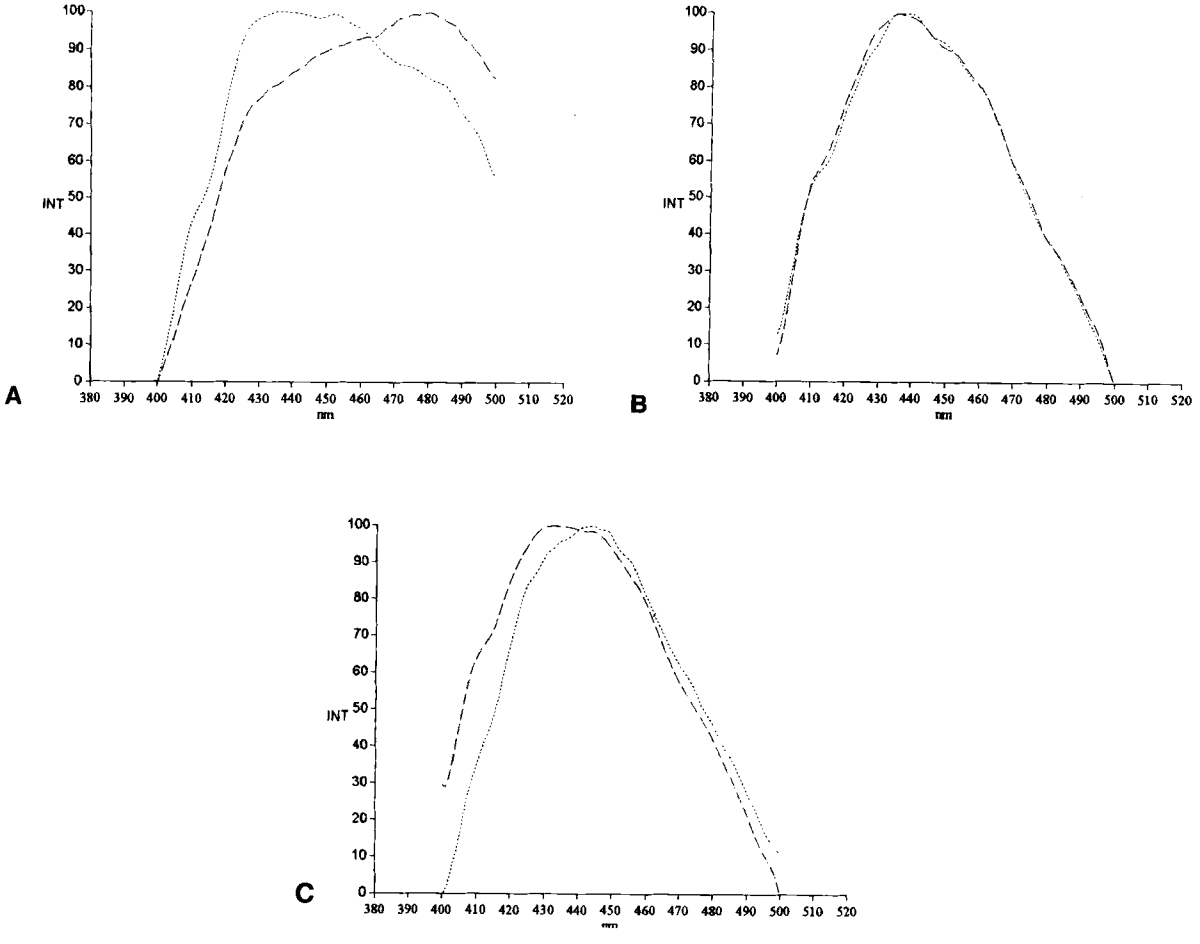

FIGURE 2 Temperature-related modifications of the position of the maximum emission of Laurdan in untreated liposomes **(A)** and in liposomes oxidised by Fe^{2+} **(B)** and by Fe^{2+} plus Al^{3+} **(C)** (λ_{exc} = 340 nm; ..... at 20°C; ------ at 37°C). (INT is given for fluorescence intensity.)

in Figure 1B is shifted to the red by some 10 nm in Figure 1C—the rest of the spectrum remaining qualitatively identical—which is the sign of an aggravation of oxidation.

Moreover, no temperature-related modifications of the position of the maximum has been observed in liposomes oxidised by ferrous ions (Figure 2B), but a small difference appears to be due to the presence of aluminium (Figure 2C): the order of maximum intensities observed for control liposomes is slightly reversed, this being also indicative of a more important oxidation.

TABLE II Generalised Polarisation (GP) of Laurdan incorporated in untreated liposomes and oxidized liposomes

A—Excitation wavelength 340nm		
Temperature	20°C	37°C
Untreated liposomes	0.066	−0.037
Fe-treated liposomes	0.229	0.210
Fe/Al-treated liposomes	0.234	0.247
B—Excitation wavelength 390nm		
Temperature	20°C	37°C
Untreated liposomes	0.054	−0.078
Fe-treated liposomes	0.241	0.200
Fe/Al-treated liposomes	0.219	0.220

Generalised Polarisation (GP) and Values of the Fractional Intensity of the Gel Phase in Untreated and in Oxidised Liposomes

The value of GP calculated in untreated liposomes was 0.066 at 20°C, decreasing to −0.037 at 37°C. The changes observed in the spectra and these GP values suggest that temperature-related modifications of the phase state occur in the lipid environment of the probe in untreated liposomes, which is in line with the findings of previous studies in model membranes.[29]

The aforementioned modifications of the spectral properties of Laurdan in oxidised liposomes are associated with a significant increase in the GP value of liposomes oxidised either in the presence of Fe^{2+} or Fe^{2+} and Al^{3+} (Table II). In addition, the effects of oxidation on GP are positively correlated with the extent of oxidation ($r = +0.87$, $p < 0.02$).

In a previous study, characteristic GP values in phospholipid vesicles have been determined to be about 0.6 (GP_g) and −0.2 (GP_l) for the gel and the liquid-crystalline phase, respectively, and independent of the phospholipid composition and the pH.[27] Combining these previous data with the GP values obtained in the present study the two phases (gel and liquid-crystalline) in control liposomes and in liposomes oxidised by Fe^{2+} and Fe^{2+} with Al^{3+} have been quantified (Figure 3).

For both wavelengths reported in Figure 3, the gel phase in untreated liposomes at 20°C is about double that observed at 37°C (33 and 31.75% with respect to 20 and 15%). Also, the fractional intensity of the gel phase relative to oxidised liposomes is about double that of untreated liposomes at 20°C and triple at 37°C. This reflects the fact that temperature-related modifications of spectral properties of Laurdan are observed only in control, untreated liposomes, whereas there are no temperature-related modifications of phase state in oxidised liposomes. In total, oxidised liposomes display similar values of the position of the maximum emission, of GP and of the fractional intensity of the gel phase at 20°C and 37°C.

In contrast with the GP value of control liposomes which is logically negative at 37°C due to the high proportion of unsaturated fatty acids in the phospholipid, the GP value observed for oxidised liposomes (Fe^{2+} and Fe^{2+}/Al^{3+}) is relatively high. Thus, an increase in GP values is observed when liposomes are submitted to oxidative stress (ferrous ions and ferrous ions plus aluminium ions). Laurdan is an amphiphilic molecule that has been reported to be incorporated at the hydrophilic-hydrophobic interface of model membranes[34] and used to study modifications induced by ozonation. Its spectral sensitivity to the phospholipid phase state has been demonstrated in model membranes.[35] The present study shows that ferrous (and aluminium) ions induce modifications of the polarity in the microenvironment of the probe, as revealed by

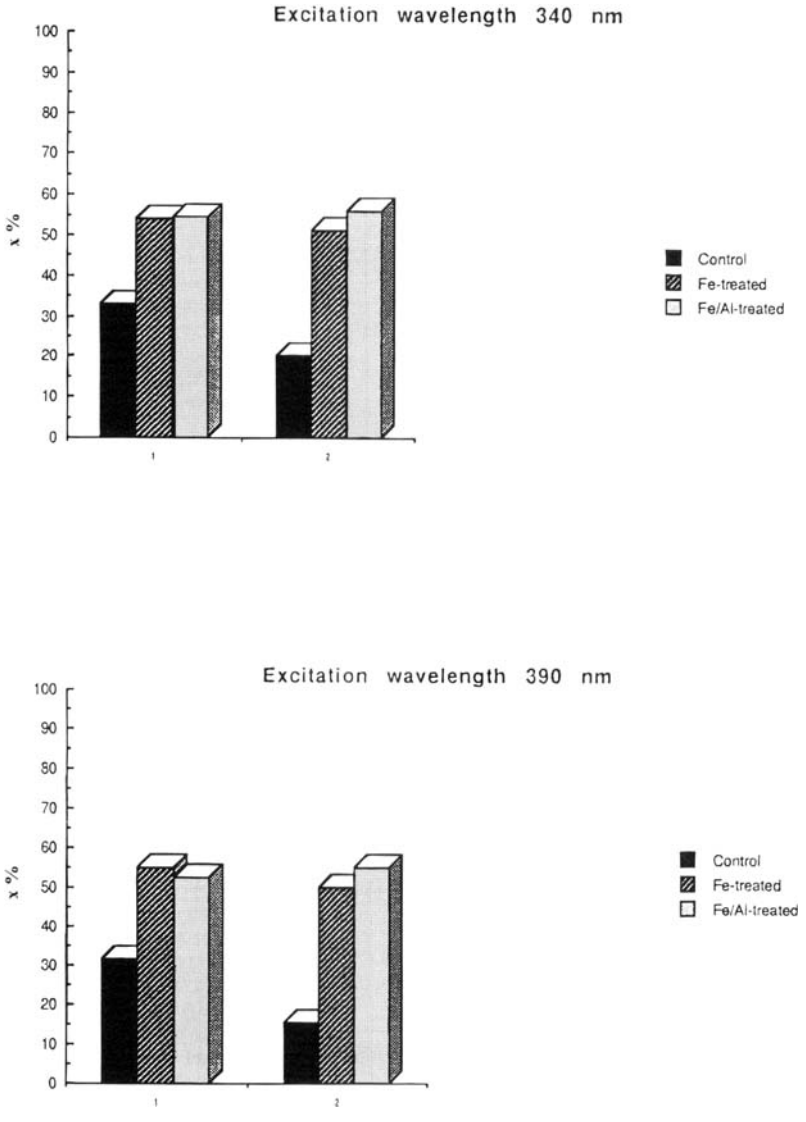

FIGURE 3 Fractional intensity of gel phase (x %) of untreated and oxidised liposomes, at 20°C (1) and 37°C (2).

the significant increase in the GP value with respect to control liposomes.

In a previous ESR study by Oteiza,[23] aluminium was shown to increase the packing of membrane lipids in the presence of Fe^{2+} by facilitating the propagation of oxidation, even though the order parameter and the hyperfine interaction constant did not vary with the Al concentration. In line with this, Mc Lean and Hagaman[36] have advanced the hypothesis that an increase in the packing density of the acyl chains of lipids in liposomes promotes lipid peroxidation and favors phase separation.

The present results tend to corroborate these previous findings in that the presence of Al^{3+} ions has been shown to induce slight variations in emission fluorescence spectra, indicating aggravated oxidation. However, no significant influence of aluminium has been observed on GP values, nor on derived phase state determina-

tions. Dynamic fluorescence studies involving time-resolved experiments will be necessary to further elucidate the specific effect of Al^{3+} ions on membrane structures. These are currently in progress in our laboratories.

References

[1] Minotti, G., Mordente, A. and Cavaliere, A. F. (1995). Metal ions, free radicals and disease. In *Handbook of Metal-ligand Interactions in Biological Fluids: Bioinorganic Medicine* Vol. 2 (G. Berthon, ed.) Marcel Dekker, New York, pp. 962–975.

[2] Dyrks, T., Dyrks, E., Hartmann, T., Masters, C. and Beyreuther, K. (1992). Amyloidogenicity of βA4 and βA4-bearing amyloid protein precursor fragments by metal-catalyzed oxidation. *Journal of Biological Chemistry*, **267**, 18210–18217.

[3] Arai, H., Kogure, K., Sugioka, K. and Nakano, M. (1987). Importance of two iron-reducing systems in lipid peroxidation of rat brain: implications for oxygen toxicity in the central nervous system. *Biochemistry International*, **14**, 714–749.

[4] Behl, C., Davis, J. B., Lesley, R. and Schubert, D. (1994). Hydrogen peroxide mediates amyloid β protein toxicity. *Cell*, **77**, 817–827.

[5] Smith, C. D., Carney, J. M., Starke-Reed, P. E., Oliver, C. N., Stadtman, E. R., Floyd, R. A. and Markesbery, W. R. (1991). Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proceedings of National Academy of Sciences USA*, **88**, 10540–10543.

[6] Smith, M. A., Sayre, L. M., Monnier, V. M. and Perry, G. (1995). Radical Ageing in Alzheimer's disease. *Trends Neurosciences*, **18**, 172–176.

[7] Smith, M. A. and Perry, G. (1995). Free radical damage, iron and Alzheimer's disease. *Journal of Neurological Sciences*, **134**, 92–94.

[8] Connor, J. R., Menzies, S. L., St. Martin, S. M. and Mufson, E. J. (1992). A histochemical study of iron, transferrin, and ferritin in Alzheimer's diseased brains. *Journal of Neuroscience Research*, **31**, 75–83.

[9] Dyrks, T., Dyrks, E., Masters, C. L. and Beyreuther, K. (1993). Amyloidogenicity of rodent and human βA4 sequences. *FEBS Letters*, **324**, 231–236.

[10] Pike, C. J., Walencewicz, A. J., Glabe, C. G. and Cotman, C. D. W. (1991). *In vitro* aging of β-amyloid protein causes peptide aggregation and neurotoxicity. *Brain Research*, **562**, 311–314.

[11] Mera, S. L. (1991). Aluminum, amyloid, and Alzheimer's disease. *Medical Laboratory Science*, **48**, 283–295.

[12] Forbes, W. F., Gentleman, J. F. and Maxwell, C. J. (1995). Concerning the role of aluminum in causing dementia. *Experimental Gerontology*, **30**, 23–32.

[13] Mc Lachlan, D. R., Lukiw, W. J. and Kruck, T. P. A. (1995). Aluminum and Alzheimer's disease. In *Handbook of Metal-Ligand Interactions in Biological Fluids: Bioinorganic Medicine* Vol. 2 (G. Berthon, ed.), Marcel Dekker, New York, pp. 935–944.

[14] Jacqmin-Gadda, H., Commenges, D., Letenneur, L. and Dartigues, J. F. (1996). Silica and aluminum in drinking water and cognitive impairment in the elderly. *Epidemiology*, **7**, 281–285.

[15] Kawahara, M., Muramoto, K., Kobayashi, K., Mori, H. and Kuroda, Y. (1994). Aluminum promotes the aggregation of Alzheimer's amyloid β in vitro. *Biochemical Biophysical Research Communications*, **198**, 531–535.

[16] Mantyh, P. W., Ghilardi, J. R., Rogers, S., De Master, E., Allen, C. J., Stimson, E. R. and Maggio, J. E. (1993). Aluminum, iron, and zinc promote aggregation of physiological concentrations of β-amyloid peptide. *Journal of Neurochemistry*, **61**, 1171–1174.

[17] Exley, C., Price, N. C., Kelly, S. M. and Birchall, J. D. (1993). An interaction of β-amyloid with aluminum in vitro. *FEBS Letters*, **324**, 293–295.

[18] Fasman, G. D. and Moore, C. D. (1994). The solubilization of model Alzheimer tangles: reversing the β-sheet conformation induced by aluminum with silicates. *Proceedings of National Academy of Sciences USA*, **91**, 11232–11235.

[19] Fasman, G. D., Perczel, A. and Moore, C. D. (1995). Solubilization of β-amyloid-(1–42)-peptide: reversing the β-sheet conformation induced by aluminum with silicates. *Proceedings of National Academy of Sciences USA*, **92**, 369–371.

[20] Vyas, S. B. and Duffy, L. K. (1995). Stabilization of secondary structure of Alzheimer β-protein by aluminum (III) ions and D-Asp substitutions. *Biochemical Biophysical Research Communications*, **206**, 718–723.

[21] Fraga, C. G., Oteiza, P. I., Golub, M. S., Gershwin, M. E. and Keen, C. L. (1990). Effects of aluminum on brain lipid peroxidation. *Toxicology Letters*, **51**, 213–219.

[22] Oteiza, P. I., Fraga, C. G. and Keen, C. L. (1993). Aluminum has both oxidant and antioxidant effects in mouse brain membranes. *Archives of Biochemistry and Biophysics*, **300**, 517–521.

[23] Oteiza, P. I. (1994). A mechanism for the stimulatory effect of aluminum on iron-induced lipid peroxidation. *Archives of Biochemistry and Biophysics*, **308**, 374–379.

[24] Xie, C. X. and Yokel, R. A. (1996). Aluminum facilitation of iron-mediated lipid peroxidation is dependent on substrate, pH, and aluminum and iron concentrations. *Archives of Biochemistry and Biophysics*, **327**, 222–226.

[25] Tampo, Y. and Yonaha, M. (1996). Effects of membrane charges and hydroperoxides on Fe(II)-supported lipid peroxidation in liposomes. *Lipids*, **31**, 1029–1038.

[26] Berthon, G. and Dousset, N. (1996). Al(III)-citrate interactions aggravate Fe(II)-induced peroxidation of brain model membranes. Potential implications for the pathogenesis of Alzheimer's disease. *Redox Reports*, **2**, 412–413.

[27] Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R. M. and Gratton, E. (1991). Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of Laurdan Fluorescence. *Biophysical Journal*, **60**, 179–180.

[28] Dousset, N., Ferretti, G., Taus, M., Valdiguié, P. and Curatola, G. (1994). Fluorescence analysis of lipoprotein peroxidation. In *Methods in Enzymology* Vol. 233 (L. Packer, ed.), Academic Press, London and New York, pp. 459–469.

[29] Parasassi, T., Di Stefano, M., Loiero, M., Ravagnan, G. and Gratton, E. (1994). Influence of cholesterol on phospholipid bilayers phase domains as detected by Laurdan fluorescence. *Biophysical Journal*, **66**, 120–132.

[30] Parasassi, T., De Stasio, G., D'Ubaldo, A. and Gratton, E. (1990). Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophysical Journal*, **57**, 1179–1186.

[31] Ohtawa, M., Seko, M. and Takayama, F. (1983). Effect of aluminum ingestion on lipid peroxidation in rats. *Chem. Pharm. Bull. Tokyo*, **31**, 1415–1418.

[32] Gutteridge, J. M. C., Quinlan, G., Clark, I. and Halliwell, B. (1985). Aluminum salts accelerate peroxidation of membrane lipids stimulated by iron salts. *Biochim. Biophys. Acta*, **835**, 441–447.

[33] Quinlan, G. J., Halliwell, B., Moorhouse, C. P. and Gutteridge, J. M. C. (1988). Action of lead(II) and aluminum(III) ions on iron-stimulated lipid peroxidation in liposomes, erythrocytes and rat liver microsomal fractions. *Biochim. Biophys. Acta*, **944**, 196–200.

[34] Salgo, M. G., Cueto, R. and Pryor, W. A. (1995). Effect of lipid ozonation products on liposomal membranes detected by Laurdan fluorescence. *Free Radical Biology and Medicine*, **19**, 609–616.

[35] Parasassi, T., Conti, F. and Gratton, E. (1986). Time-resolved fluorescence emission spectra of Laurdan in phospholipid vesicles by multifrequency phase and modulation fluorometry. *Cellular Molecular Biology*, **32**, 103–108.

[36] Mc Lean, L. R. and Hagaman, K. A. (1992). Effect of lipid physical state on the rate of peroxidation of liposomes. *Free Radical Biology and Medicine*, **12**, 113–119.