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Pyrene derivatives as markers of transbilayer effect of lipid peroxidation on neuronal membranes

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Two different pyrene derivatives, namely 12-(1-pyrene)dodecanoic acid (P_{12}-FA) and *N*-(12-(1-pyrene)dodecanoyl)-galactosylsphingosine I^3-sulfate (P_{12}-CS) have been used to follow lipid peroxidation both in model and natural membranes. The malondialdehyde (MDA) production in small unilamellar vesicles of dipalmitoylphosphatidylcholine/arachidonic acid (80:20, molar ratio), symmetrically labelled with both probes determined a progressive decrease of pyrene fluorescence due to an involvement of pyrene in the peroxidative reaction. Nervous membranes are particularly sensitive to lipid oxidation which differentially acts on the two layers of the membrane determining a greater rigidity of the exofacial one. Thus, we consider the possibility to asymmetrically introduce the pyrene ring, as P_{12}-FA or P_{12}-CS, in synaptosomes for monitoring lipid peroxidation in each layer of the membrane. The amount of the two probes incorporated in the membrane was 20 ± 3 and 10 ± 2 nmol/mg of protein for P_{12}-FA and P_{12}-CS, respectively. P_{12}-FA was symmetrically distributed in the two layers, whereas 95% of P_{12}-CS was incorporated in the exofacial layer of the membrane as determined by TNBS measurements. The decrease in fluorescence of synaptosome associated pyrene was, in the early stages of lipid peroxidation, greater for P_{12}-CS than for P_{12}-FA labelled membranes, indicating a greater susceptibility of the exofacial layer to iron-induced peroxidation.

Introduction

Literature data strongly support the importance of an asymmetric structure in membrane functions [1–4]. Asymmetry in membrane structure is determined by two possible non homogeneous distributions of the bilayer components of the membrane: (i) in the plane (lateral asymmetry); (ii) normal to the plane (transbilayer or vertical asymmetry). Both asymmetric features of the bilayer structure may be of significance to membrane functions [1–7].

Let us look at the latter case and consider the outer and inner leaflets of the bilayer, asymmetric both in lipid [2,5] and protein [8] composition as well as in fluidity [9,10], as distinct domains.

Moreover, the asymmetry is not only related to intrinsic properties and functions of the bilayer but also to such exogenous events as the in vivo or in vitro administration of anesthetic drugs [11,12], which can differentially affect the outer or inner layer of the membrane.

Peroxidation, which enhances the transbilayer movements of phospholipids in mammalian cell membranes [13], differentially acts on neuronal membranes increasing the rigidity of the outer layer and causing minor changes in the inner one [14].

It should, therefore, be of interest to follow the lipid peroxidation in each layer of the membrane. A lipid-soluble fluorescent structure susceptible to radical attack could be used for this purpose, the decrease in fluorescence being the measure of lipid peroxidation. In this study we tested the possibility of asymmetrically incorporating into synaptosomal membranes an aromatic structure, such as pyrene fatty acid in its free form or covalently linked to sulfogalactosylsphingosine, to provide a sensitive probe for the monitoring of lipid peroxidation in each of the two layers.

Materials and Methods

Analytical grade chemicals, distilled solvents and doubly distilled water were used. Dipalmitoylphospha-

Abbreviations: AA, arachidonic acid; DPPC, dipalmitoylphosphatidylcholine; MDA, malondialdehyde; P_{12}-FA, 12-(1-pyrene)dodecanoic acid; P_{12}-CS, *N*-(12-(1-pyrene)dodecanoyl)galactosylsphingosine I^3-sulfate; TNBS, trinitrobenzenesulfonic acid; TBA, thiobarbituric acid.

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tidylcholine (DPPC), arachidonic acid (AA), trinitrobenzenesulfonic acid (TNBS) and fatty acid free bovine serum albumin were purchased from Sigma Chemicals Co. 12-(1-Pyrene)dodecanoic acid (P_{12}-FA) from Molecular Probes, Inc. (Junction City, OR, U.S.A.). This last was purified on a C_{18} reverse phase HPLC column [15]. *N*-(12-(1-pyrene)dodecanoyl)galactosylsphingosine I^3-sulfate (P_{12}-CS) was prepared from galactosylsphingosine I^3-sulfate following the procedure of Marchesini et al. [16].

Preparation of small unilamellar vesicles

Small unilamellar vesicles containing different lipid mixtures were prepared in 10 mM Tris-HCl, 154 mM NaCl (pH 7.4) by sonication of multilamellar vesicles [171. The phospholipid content was determined according to Bartlett [18]. Liposomes were extracted with 20 vol. of chloroform/methanol (1 : 1, v : v) for evaluating absorption and emission spectra of pyrene lipids before and after iron-ascorbate induced lipid peroxidation.

Membrane preparation and characterization

Sprague-Dawley male rats (100–125 g) were obtained from Charles River Co. Synaptosomes were prepared by using the procedure of Whittaker and Barker [19] with minor modifications [20]. Synaptosomal fractions were enriched 3.5–4 fold over the total homogenate in Na^+, K^+-ATPase (EC 3.6.1.3) activity assayed according to Morgan et al. [21].

Protein determination

The protein content was determined according to Lowry [22] using bovine serum albumin as standard.

Synaptosome labelling with 12-(1-pyrene)dodecanoic acid and N-(12-(1-pyrene)dodecanoyl)galactosylsphingosine I^3-sulfate

Suitable amounts of 12-(1-pyrene)dodecanoic acid (P_{12}-FA) or *N*-(12-1-pyrene)dodecanoyl)galactosylsphingosine I^3-sulfate (P_{12}-CS) in chloroform/methanol (2 : 1, v/v), were evaporated under a nitrogen stream, further dried under vacuum for 30 min and resuspended by vigorous vortexing in dimethylsulfoxide (DMSO) to 4 mM final concentration. 10 nmol of the respective probes, dissolved in DMSO, were injected in 2 ml of 1 mM Tris-HCl, 154 mM NaCl, 0.1 mM EDTA (pH 7.4), then 300 μg of synaptosomal proteins resuspended in the same buffer were added and the mixture incubated at 37 °C for different times. The kinetics of incorporation of P_{12}-FA or P_{12}-CS in synaptosomes was followed by monitoring the increase of pyrene fluorescence emission at 379 nm. After incubation the membranes were centrifuged 10 min at 86 500 × *g* at 4 °C and resuspended in 1 mM Tris-HCl, 154 mM NaCl, 0.1 mM EDTA (pH 7.4). The respective total amounts of P_{12}-FA and P_{12}-CS incorporated into synaptosomes were determined by extracting labelled membranes with 20 volumes of chloroform/methanol (1 : 1, v : v) and reading the fluorescence in the lipid extract using P_{12}-FA and P_{12}-CS standard solutions for the calibration curves.

Fluorescence determination of the transbilayer distribution of 12-(1-pyrene)dodecanoic acid and N-(12-(1-pyrene)dodecanoyl)galactosylsphingosine I^3-sulfate

0.2 ml of P_{12}-FA labelled synaptosomes (300 μg protein) were mixed with 70 μl of 10% and (w/v) fatty acid free bovine serum albumin solution in 1 mM Tris-HCl, 154 mM NaCl, 0.1 mM EDTA (pH 7.4). The mixture was incubated 1 min on ice [23] and centrifuged 10 min at 86 500 × *g* at 4 °C. The respective amounts of P_{12}-FA in the pellet and supernatant were determined, as described above, by reading the fluorescence in the lipid extract.

The P_{12}-CS transbilayer distribution in synaptosomal membranes was determined by labelling synaptosomes with trinitrobenzenesulfonic acid (TNBS), according to Schroeder [24]. P_{12}-CS labelled synaptosomes were resuspended at 4 °C in one volume of 120 mM $NaHCO_3$, 11 mM glucose, 50 mM NaCl (pH 8.5), then one volume of the same buffer containing 0.8 mM TNBS was added and the synaptosomes were incubated for 60 min at 4 °C and 37 °C, respectively. The reaction was stopped by adding ethanolamine dissolved in the same buffer, corresponding to 2 mM final concentration. Samples were then centrifuged 10 min at 86 500 × *g* and the pellets resuspended in 1 mM Tris-HCl, 154 mM NaCl, 0.1 mM EDTA (pH 7.4) for fluorescence determinations.

Peroxidation of small unilamellar vesicles and synaptosomes

Peroxidation of small unilamellar vesicles was induced by adding $FeSO_4$ (50 μM) and ascorbate (500 μM) to liposomes resuspended in 10 mM Tris-HCl, 154 mM NaCl (pH 7.4) according to Cervato et al. [25]. At different times the reaction was stopped by adding 0.1 ml of 5 mM EDTA to 0.5 ml of the liposomal suspension.

Peroxidation of the control and labelled synaptosomes was induced by adding $FeSO_4$ (84 μM) and ascorbic acid (400 μM) to 0.4 mg of synaptosomal protein resuspended in 3.2 ml 1 mM Tris-HCl, 154 mM NaCl, 0.1 mM EDTA (pH 7.4) [20]. At different times 0.5 ml of the mixture were withdrawn and the peroxidative reaction stopped by adding 0.1 ml of 5 mM EDTA.

Peroxidation was measured as thiobarbituric acid (TBA) reactive products formed, according to the method of Buege et al. [26] and using 1,1,3,3-tetraethoxypropane for the calibration curve. A molar absorption coefficient $\epsilon_{535} = 1.54 \cdot 10^5$ mol^{-1} cm^{-1} was obtained.

When peroxidation was monitored by determining the decrease in pyrene fluorescence, the absorption and emission spectra were recorded at different times. The fluorescence decrease was expressed as F/F_0, where F_0 represents the fluorescence intensity at 379 nm at zero time of peroxidation and F is the residual fluorescence at 379 nm for a given peroxidation time.

Fluorescence measurements

All fluorescence measurements were carried out in a Jasco 770 spectrophotofluorimeter equipped with a cuvette holder with the temperature maintained by a Haake G D3 thermostatic circulating bath. The temperature was monitored with a Subline PT 100 Digital thermometer.

Absorption and emission spectra of pyrene derivatives were determined in the range 300–360 nm and 360–550 nm, respectively.

Results and Discussion

The sensitivity of the pyrene ring to iron-ascorbate induced peroxidation was first evaluated using small unilamellar vesicles composed by a satured phospholipid DPPC, a peroxidable substrate arachidonic acid (AA), which contained 0.1 mol% hydroperoxides as contaminant, and 1 mol% of the two respective fluorescent probes symmetrically distributed in both layers of liposomes (Fig. 1). The iron-ascorbate induced peroxidation, monitored as malondialdehyde (MDA) formed, determines a progressive decrease of pyrene fluorescence using both labelled fatty acid and sulfatide. With both probes F/F_0 reaches a limit value of 0.4, indicating that not all the probe present in the vesicle is involved in the peroxidative reaction. When peroxidation was induced in the same model system lacking arachidonic acid, no TBA-reactive products nor any decrease in fluorescence were observed, indicating that iron-ascorbate alone does not modify the fluorescence emission of the pyrene ring.

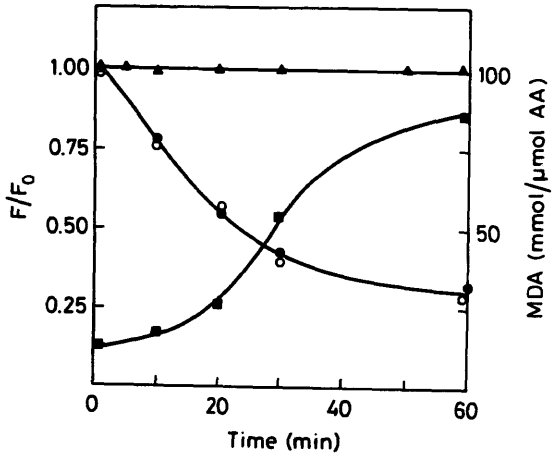

Fig. 1. Time-dependent increase of TBA reactive products (■———■) and fluorescence decrease, expressed as F/F_0, of P_{12}-FA (●———●) or P_{12}-CS (○———○) consequent to iron-ascorbate induced peroxidation of DPPC/AA (80:20 molar ratio) small unilamellar vesicles, containing 1 mol% of P_{12}-FA or P_{12}-CS; time-dependent fluorescence of P_{12}-FA (▲———▲) after iron-ascorbate treatment of the same samples lacking arachidonic acid.

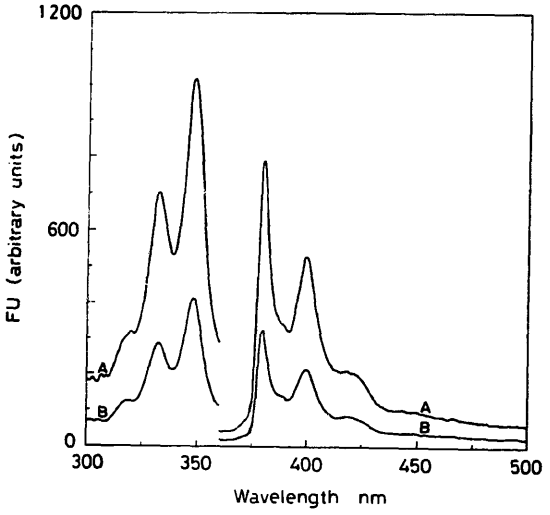

Fig. 2. Absorption and emission spectra of P_{12}-FA in liposome lipid extracts before (curves A) and after 30 min peroxidation (curves B). Spectra were recorded in chloroform/methanol (1:1, v/v).

We previously reported [25] that, in these model systems, iron-ascorbate induced peroxidation seems to proceed through the Fe^{2+}-mediated breakdown of contaminant arachidonic acid hydroperoxides to lipid alkoxyl (LO•) or lipid peroxyl (LOO•) radicals which are known to be involved in the propagation of lipid peroxidation [27,28]. Thus the observed decrease in fluorescence could be due to an involvement of pyrene in the radical reactions, which propagate peroxidation, or to a reduction of the fluorescence quantum yield due to end products of lipid peroxidation. The first hypothesis is strongly supported by two facts: (i) in the early stages of lipid peroxidation the decrease in fluorescence is greater than the increase in TBA-reactive products (Fig. 1); (ii) the absorption spectra of pyrene recorded on the lipid extracts after peroxidation (Fig. 2) showed a reduction of the same magnitude of the monitored fluorescence decrease, indicating that the molecular structure of the pyrene ring is modified during peroxidation.

The validity of pyrene lipids as markers of lipid peroxidation in model systems prompted us to consider the use of pyrene derivatives to follow lipid peroxidation in natural membranes. In particular we tested the possibility that these probes, if incorporated asymmetrically in the exofacial or cytoplasmatic leaflet of synaptosomal membranes, could provide a sensitive marker for monitoring lipid peroxidation in each layer.

The spectroscopic characteristics of pyrene have been used to monitor the incorporation of P_{12}-FA and P_{12}-CS

monomers into synaptosomal membranes. In fact, when DMSO solutions of both probes were injected in buffer to a final concentration $5 \cdot 10^{-6}$ M, the emission spectra mainly showed an excimeric peak at 470 nm, characteristic of pyrene aggregates, while the emission at 379 and 400 nm, characteristic of the monomeric form, was greatly reduced.

Thus the synaptosomal uptake of P_{12}-FA was determined spectrofluorimetrically by following the time-dependent increase of fluorescence at 379 nm after addition of 300 μg protein to $5 \cdot 10^{-6}$ M dispersion of P_{12}-FA (Fig. 3A). The emission spectra recorded at different times of incubation (Fig. 3B) showed an increase in monomeric fluorescence, with a concomitant decrease in the excimeric fluorescence, indicating that a transfer of monomers between P_{12}-FA aggregates and synaptosomal membranes occurs. The incorporation of P_{12}-FA reached a plateau value in 20 min. After 30 min of incubation the amount of P_{12}-FA incorporated in the membrane was 20 ± 3 nmol/mg protein, as calculated from the fluorescence of synaptosomal lipid extracts.

Also in the case of P_{12}-CS the synaptosomal uptake was determined following the time-dependent increase of fluorescence at 379 nm (Fig. 4), together with the modifications of the emission spectrum which showed the same characteristics described for P_{12}-FA. The kinetic of incorporation of P_{12}-CS in synaptosomes was similar to that of P_{12}-FA. Membrane associated sulfatide increase linearly in the first minutes of incubation reaching a plateau value after approx. 10 min. The amount of P_{12}-CS incorporated in synaptosomes after 10 min was 10 ± 2 nmol/mg protein.

The emission spectra obtained with intact membranes after incorporation of P_{12}-FA and P_{12}-CS (Figs. 5 and 6, respectively) clearly indicate that both fluorescent probes were present as monomers in the bilayer. The P_{12}-FA and P_{12}-CS incorporated in the membrane, respectively, represent about 2 and 1 mol% and of the

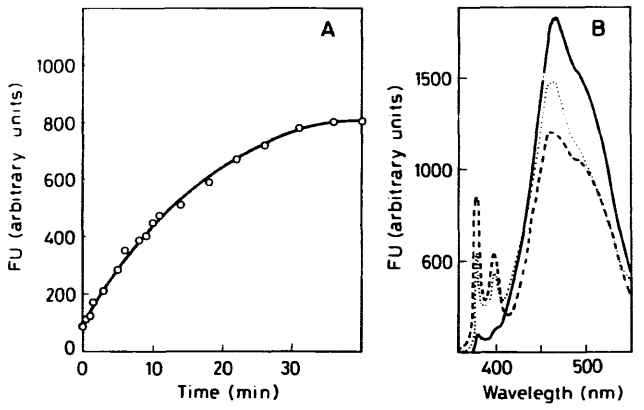

Fig. 3. (Panel A) Kinetics of P_{12}-FA incorporation into synaptosomes as measured by increase in its fluorescence at 379 nm. (Panel B) Emission spectra of P_{12}-FA at different times of incubation with synaptosomes. ———, time 0; ·····, 15 min; — — —, 30 min.

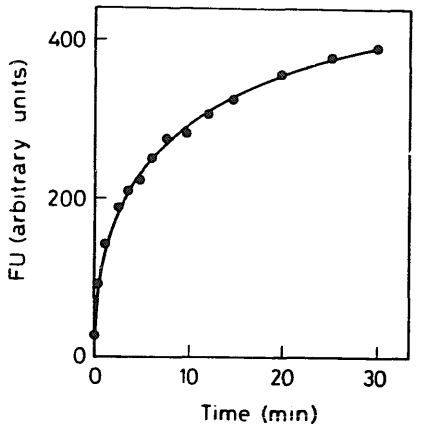

Fig. 4. Kinetics of P_{12}-CS incorporation into synaptosomes as measured by increase in its fluorescence at 379 nm.

total synaptosomal lipids [29]. It is in fact very important that only small amounts of probe are incorporated in the membrane to ensure that it does not perturb the structure and the organization of the bilayer.

Furthermore, the exogenous fluorescent lipids incorporated should have a different distribution between the two layers forming the membrane. Fatty acids, which have fast flip-flop movements in the bilayer [30], should in fact be symmetrically distributed in both the membrane layers whereas sulfatide which is naturally present almost exclusively in the outer layer of the membrane [31] and for which no transbilayer movements were determined in model membranes [32], should be present only in the exofacial leaflet of synaptosomes.

To test the transbilayer distribution of P_{12}-FA across the synaptosomal membrane, labelled synaptosomes were incubated with bovine serum albumin (fatty acid free) which can remove only the fatty acids present in the outer layer [23,33]. The method was described for the selective removal of paramagnetic fatty acids from the outer layer of an erythrocyte membrane without modifying the stability of the membrane and the shape of the cell. Spectra reported in Fig. 5 showed that the non extractable fraction of label, i.e. the fraction of label that accumulates in the inner layer, was about 50% of the initial membrane associated fluorescence, thus indicating a symmetric distribution of the pyrene fatty acid across the membrane. Moreover, the incubation of labelled synaptosomes with increasing albumin concentrations did not modify the amount of P_{12}-FA removed by the albumin.

A similar procedure was attempted to determine the P_{12}-CS transbilayer distribution, but less than 9% fluorescent label was extracted by the albumin even after increasing the albumin concentration 4-fold. It is very difficult to believe, however, that exogenously added monomers of sulfatide can selectively reach the inner layer of the membrane and thus assuming a distribution opposite that of the natural glycolipid. Therefore these

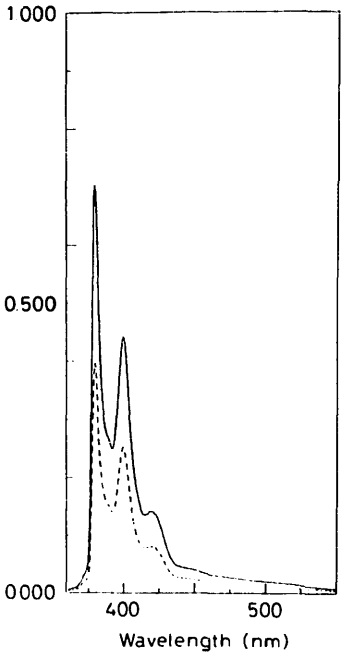

Fig. 5. Emission spectra of P_{12}-FA incorporated in synaptosomes before (———) and after (— — —) treatment with fatty acid free albumin 1 min at 4°C.

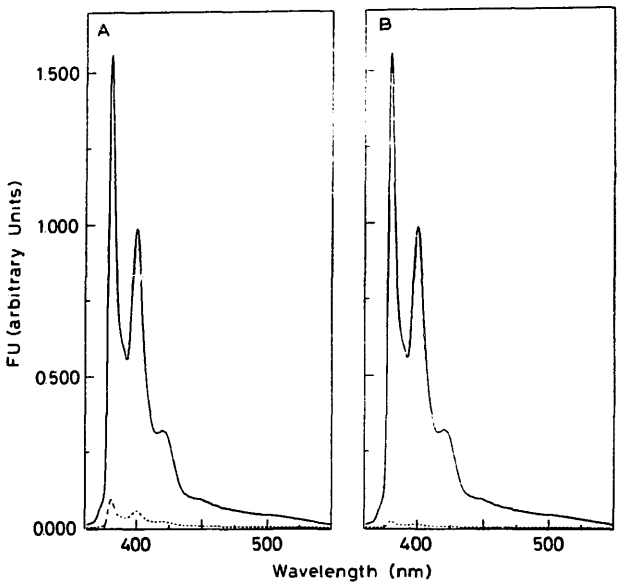

Fig. 6. Quenching of fluorescence emission of P_{12}-CS by trinitrophenyl groups in synaptosomal membranes. Emission spectra before (———) and after (— — —) TNBS treatment. Trinitrophenylation was done at 4°C (panel A) and at 37°C (panel B).

data seem to indicate that albumin is not able to remove sulfatide incorporated in the membrane, probably because of the more complex structure of the glycolipid characterized by a saccharide polar moiety and a hydrophobic portion constituted by two acyl chains. For this purpose it should be noted that the selective removal of lysophospholipids (one acyl chain and a simpler polar head) needs more drastic conditions than those required for the removal of fatty acids [23].

To evaluate the transbilayer distribution of P_{12}-CS across the synaptosomal membrane we took a different approach based on the quenching of pyrene fluorescence by a chemical quenching agent [34], namely trinitrobenzenesulfonic acid (TNBS).

Fluorescence quenching by energy transfer occurs with greater efficiency when the absorption band of the acceptor coincides with that of fluorophore emission. This is the case of trinitrophenylated amino groups that have an absorption band at 400 nm, thus presenting a large overlap with the emission band of pyrene (379 and 400 nm) (Fig. 6). According to the Forster theory [35] the energy transfer from fluorescent molecules to acceptor dyes depends on the distance between the donor and acceptor molecules and only those donor molecules satisfying this distance can be bleached by an energy transfer mechanism. Thus quenching, after treating only the outside of the membrane with TNBS, involves only the fluorescent molecules located in the exofacial leaflet of the synaptosomes, whereas trinitrophenylation of the amino groups of both the layers will result in the quenching of all the fluorescent molecules present in the bilayer. Incubation of P_{12}-CS labelled synaptosomes with TNBS under penetrating conditions (37°C) resulted in a 99% fluorescence quenching (Fig. 6B); also trinitrophenylation of the only exofacial amino groups, obtained by incubating labelled synaptosomes with TNBS under non penetrating conditions (4°C), resulted in an almost complete pyrene quenching. In fact in these conditions 95% and of the P_{12}-CS fluorescence was quenched (Fig. 6A), thus demonstrating that almost all

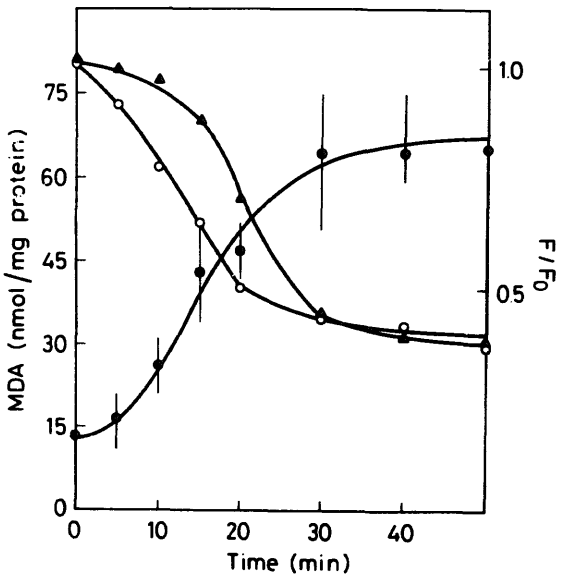

Fig. 7. Time dependent decrease of P_{12}-FA (▲———▲) and P_{12}-CS (○———○) fluorescence consequent to iron-ascorbate lipid peroxidation of labelled synaptosomes. Time-dependent increase in TBA reactive products (●———●) in the same samples was also reported.

the sulfatide molecules are asymmetrically distributed on the outer layer of the plasma membrane.

The validity of energy tranfer applications for selective bleaching is based on the hypothesis that the quenching will be of a non trivial nature, i.e. it must be due to dipole-dipole coupling and just not be the result of a simple reabsorption of fluorescence emission, thus it is important to demonstrate that non trivial quenching by energy transfer occurs. The spectra reported confirm this because all parts of the emission bands are equally quenched.

TNBS treatment of P_{12}-FA labelled synaptosomes (data not shown) confirm the symmetrical distribution obtained with selective removal of fatty acids with albumin.

Synaptosomes labelled only in the outer layer with P_{12}-CS, and those labelled in both layers with P_{12}-FA, were then subjected to iron-ascorbate lipid peroxidation (Fig. 7).

In both cases the peroxidative stress causes a decrease in fluorescence intensity (Fig. 7). During the peroxidative process, moreover, also the absorption peaks of the pyrene decrease (data not shown). This indicates that the decrease in fluorescence is due to a modification of the polyene structure of pyrene to non fluorescent compounds rather than to an environment dependent modification of the fluorescence quantum yield.

In Fig. 7 the time-dependent decrease in fluorescence expressed as F/F_0 for both P_{12}-CS and P_{12}-FA labelled synaptosomes was measured in comparison with time-dependent production of MDA. In the initial stage of peroxidation the decrease of pyrene-sulfatide fluorescence is greater than that of the pyrene fatty acid. The time necessary to obtain 50% over the maximum fluorescence reduction ($t_{1/2}$ox) is 15 and 21 min for P_{12}-CS and P_{12}-FA, respectively. The delay in P_{12}-FA fluorescence decrease indicates that, at the early stages of the peroxidative reaction, the percentage of P_{12}-FA involved in the peroxidation is lower than that of P_{12}-CS, suggesting a greater susceptibility of the outer layer to iron-ascorbate induced peroxidation. This could provide a demonstration that iron-ascorbate induced peroxidation develops preferentially in the exofacial leaflet rather than in the cytoplasmatic one. As the reaction proceeds the decrease of fluorescence becomes similar for both probes probably as a consequence of a loss of membrane integrity which renders the cytoplasmatic layer equally sensitive to lipid peroxidation. These differences in reactivity of the two layers to lipid peroxidation could give, in part, a possible explanation for the loss in fluidity gradient observed after a peroxidative damage [14] and during aging [14].

Furthemore the possibility of synthesizing different lipids carrying a 'peroxidation sensitive fluorescent probe' such as pyrene, together with the capacity to incorporate these probes selectively in different regions of natural membranes, could provide a useful tool for a 'topological' study of peroxidation in the membrane.

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