O_2^--dependent lipid peroxidation does not affect the molecular order in hepatoma microsomes

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Microsomal membranes from rat liver and from the fast-growing Morris hepatoma 3942A have been peroxidized to different extents and the order parameter of the membranes measured by fluorescence depolarization of the probe 1,6-diphenyl-1,3,5-hexatriene. The data have been analysed by applying a mathematical approach that takes into account simultaneously static and dynamic fluorescence parameters. It appears that tumour membranes are more ordered than the control and their order parameter does not increase with greater exposure to the action of O_2^- radicals in contrast to liver membranes. The fatty acid composition of the membrane lipids has been studied under different experimental conditions and correlated to the behaviour of the physical parameter.

(Morris hepatoma 3924A) *Microsome* *Lipid peroxidation* *Lipid order* *Fluorescence*

1. INTRODUCTION

It is well-documented that the cytotoxicity of free radicals is mainly due to their action on DNA, proteins and membrane lipids [1]. The basic process by which oxy- and related radicals cause membrane damage is lipid peroxidation, which results in the formation of lipid peroxide, lipid alcohol and aldehyde byproducts [2].

Changes in chemical composition of fatty acids lead to alterations in the structural organization and functional properties of the membrane [3]. In particular, such membrane functional damage has been linked to changes in molecular order and fluidity of the bilayer [4–6]. These physical properties of the membrane can be studied by the use of ESR [7] and fluorescence spectroscopy [8–10].

It has been reported that tumour membranes are characterized by changes in lipid constituents and membrane lipid fluidity [11]. We have observed that microsomal and plasma membranes isolated from hepatomas with different degrees of differentiation exhibit an increase in the order of the lipid domain and a decrease in fluidity strictly related to the nature and content of phospholipids [10,12,13]. In particular, the more undifferentiated and rapidly growing were the tumours, the lower was their lipid content, degree of fatty acid unsaturation, lipid/cholesterol ratio and lipid/protein ratio. Similar alterations of chemical and physical properties in erythrocyte ghosts and normal microsomal membranes have been reported as being caused by in vitro lipoperoxidation [5,6,14].

It has been postulated that transformed cells may undergo in vivo peroxidation damage due to the loss of anti-oxidant protective enzymes [15]. The aim of this paper is to investigate the correlation between the peroxidizability of hepatoma microsomal membranes and the changes in molecular order of the lipid bilayer.

We chose the most deviated tumour, Morris hepatoma 3924A, as being most representative of

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the possible answer of tumours to in vitro peroxidation and carried out fluorescence depolarization studies of 1,6-diphenyl-1,3,5-hexatriene (DPH) to measure the physical parameters.

2. MATERIALS AND METHODS

Morris hepatoma 3924A was propagated in inbred rats of the ACI/T strain and utilized for membrane preparation after 3–4 weeks of growth. Normal male rats (150–200 g) of the same strain were used as controls. Microsomal membranes were isolated as in [16] and proteins estimated by the biuret method [17].

Lipid peroxidation was measured at 25°C as malondialdehyde formation, by the thiobarbituric acid assay, and as lipid hydroperoxide formation by iodometric assay [2]. Membranes (0.2 mg protein/ml) were suspended in 0.15 M KCl, 50 mM Tris-HCl (pH 7.5) saturated with oxygen, containing 1 mM ADP, 0.05 mM $FeCl_3$ and 0.33 mM xanthine. The peroxidation reaction was initiated by 50 μg/ml xanthine oxidase (Sigma, type I) and 0.5-ml samples withdrawn after 5 and 10 min incubation of the suspension in a Dubnoff metabolic shaker under oxygen tension. Microsomes 'peroxidized' for 5 and 10 min were obtained by stopping peroxidation at the time indicated by the addition of 1.3 μM bovine superoxide dismutase (Sigma) and rapid cooling to 0°C of the flask containing the suspension. These membranes, spun down by centrifugation at 105000 $\times$ g for 60 min, were utilized for lipid analysis and fluorescence measurements.

Lipids were extracted from isolated membranes by the method of Folch et al. [18]. Phospholipid phosphorus was determined by the method of Bartlett [19] modified by digestion with 70% $HClO_4$, according to Marinetti [20]. For analysis of fatty acids by gas-liquid chromatography, methyl esters were prepared by inter-esterification of lipid samples for 4 h at 100°C with 5% anhydrous HCl/CH_3OH, then extracted with light petroleum and examined with a gas chromatograph. The samples were injected into a poly(ethylene glycol) succinate column operating at 180°C. The column was calibrated with standard methyl esters of the different fatty acids.

DPH was obtained from Aldrich-Europe (Beerse, Belgium) and purified by sublimation. Acetone and all organic solvents were of fluorimetric grade. All other chemicals were of reagent grade quality.

Fluorescence polarization measurements of DPH were carried out as described in [10], using a probe concentration of 5×10^{-7} M and a probe/phospholipid ratio between 1:150 and 1:300. Steady-state polarization anisotropy was measured as in [10]. Measurements of fluorescence lifetime were carried out as in [14]. Fluorescence decay curves were deconvoluted using the non-linear least-squares fit–fast Fourier transform method developed in [21]. The fluorescence intensities were assumed to decay via a biexponential, i.e.

$$F(t) = C_1\exp - (t/\tau_1) + C_2\exp - (t/\tau_2) \quad (1)$$

Fits were also performed using a monoexponential decay. However, when comparing the fitted and experimental decays, poor χ^2 and residue distribution were found.

3. RESULTS AND DISCUSSION

In table 1 we report lipid peroxidation expressed as formation of malondialdehyde and lipid hydroperoxides with time in microsomal membranes from rat liver and from hepatoma 3924A. Clearly, peroxidation is rather fast and reaches high values in rat liver membranes whereas it is severely depressed in the tumour.

The dependence of the static fluorescence anisotropy of DPH on temperature in the two membranes studied at different times of peroxidation is reported in fig.1. In both cases the parameter increases markedly with peroxidation time. It is well-known that in several previous studies this has been interpreted as indicating a decrease in membrane 'fluidity' [11]. Such an interpretation is of course incorrect, since r_s contains both static and dynamic components, i.e. it is a function of the order parameter, $\langle P_2 \rangle$, of the rotational correlation time of the probe, Φ, and of its fluorescence lifetime τ_F [22,23]. In order to calculate $\langle P_2 \rangle$ and Φ measurements of τ_F are required.

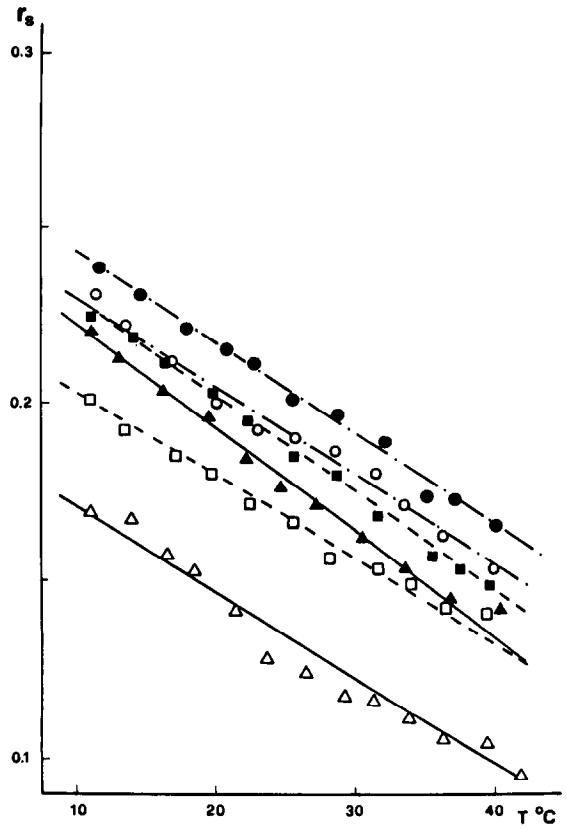
The τ_F values of DPH measured under our experimental conditions are reported in fig.2. In some experiments fittings with poor χ^2 and residue distribution were found. As a consequence, only

Table 1

Relationship between formation of malondialdehyde and lipid hydroperoxides and changes in the double-bond index induced by peroxidation in rat liver and hepatoma 3924A microsomes

Microsomes	Double-bond index	Malondialdehyde (nmol/mg protein)	Lipid hydroperoxides (nmol/mg protein)
Rat liver (ACI/T)			
Control	102	–	–
Peroxidized, 5 min	73	35.8 ± 4.2 (8)	88.5 ± 17.2 (6)
Peroxidized, 10 min	55	68.1 ± 5.9 (8)	201.2 ± 26.8 (6)
Hepatoma 3924A			
Control	32	–	–
Peroxidized, 5 min	38	7.1 ± 0.4 (7)	41.9 ± 5.5 (7)
Peroxidized, 10 min	27	13.1 ± 1.1 (7)	53.4 ± 5.4 (7)

The double-bond index was calculated as the sum of the values obtained by multiplying the percentage of the unsaturated fatty acid by the number of double bonds in that fatty acid. Values are expressed as means ± SE (number of observations)



those fits with χ^2 values not too far from unity ($\chi^2 < 2$) have been considered. It is possible that the scattering of the τ_F values is due to the presence of a residual fluorescence in peroxidized membranes amounting to about 20% of the fluorescence intensity of DPH. Such fluorescence, due to end products of lipid peroxidation, has been reported previously and has also been used to determine the degree of peroxidation of biological membranes [24]. To solve the distortions caused by the near coincidence of λ_{ex} and λ_{em} of this fluorescence with that of DPH, we have subtracted, during the deconvolution process, channel by channel from the histogram of the sample with DPH a blank monitored for the same time period (typically 1000 s). The reason why DPH shows two lifetimes in normal membranes is not cleary understood. Similar behaviour has been noted by Brand and coworkers [25] in model membranes. A possible explanation could be the localization of DPH both in the core of the lipid bilayer (long lifetime) and in a more polar environment near the polar heads of

Fig.1. Dependence of the static anisotropy on temperature of microsomal membranes. Rat liver microsomes: not peroxidized (△), after 5 min (□) and 10 min (○) peroxidation. Hepatoma 3924A microsomes: not peroxidized (▲), after 5 min (■) and 10 min (●) peroxidation.

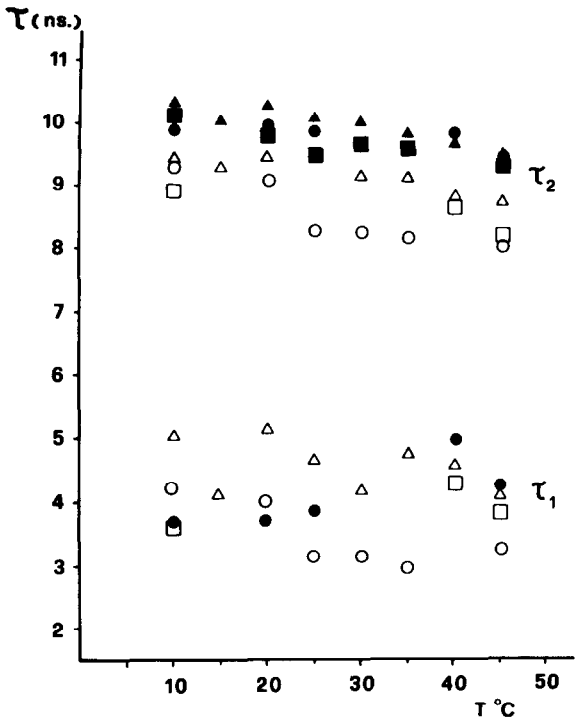


Fig.2. Dependence of DPH lifetime on temperature after incorporation into microsomal membranes. Symbols as in fig.1. The values in the range 3–6 ns are relative to lifetime τ_1 and those between 8 and 11 ns to lifetime τ_2.

the phospholipids (short lifetime), as in organic solvents. Conversely, in tumour cells DPH consistently shows a single lifetime, independent of temperature, which is longer than that in normal membranes. It can be assumed that the higher 'molecular order' in tumour membranes, discussed below, segregates DPH in the lipid core. In peroxidized membranes, the probe lifetime changes are not easily explained due to the complexity of the environment experienced by the probe.

Fig.3 reports the dependence of the order parameter, $\langle P_2 \rangle$, of DPH on temperature in microsomal membranes of rat liver and hepatoma at different degrees of peroxidation. $\langle P_2 \rangle$ has been calculated as reported in [12]. The order parameter varies significantly with the time of peroxidation in normal membranes, while it is nearly constant in the transformed membranes. It is important to note that $\langle P_2 \rangle$ reflects the order of the lipid region where DPH is located. Experiments carried out with the spin probes, 5-, 12- and 16-doxylstearic

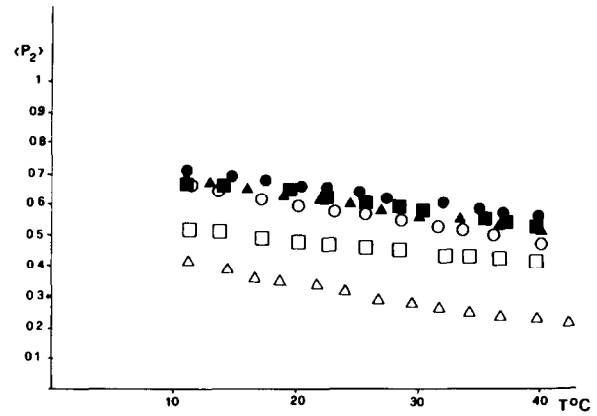


Fig.3. Plot of the order parameter of DPH vs temperature. Symbols as in fig.1.

acid, located at different depths of the bilayer [26] in model [7] and microsomal membranes [14], showed that peroxidation resulted in an increase in the order parameter of the bilayer, dependent on the peroxidation time, temperature and location of the probe within the bilayer. Of the 3 probes, 12-doxylstearate was the most sensitive to changes in membrane organization, monitoring the higher degree of order caused by peroxidation. This is the bilayer region where DPH should be located. However, it should be pointed out that such comparisons should be made with great caution, since the observable determined is the order parameter of the probe; having such probes with quite different steric properties, they can mimic their molecular environment in quite a different way. The order parameters calculated from fluorescence depolarization data are in good agreement with those available in the literature [6]. However, the increase or decrease in these parameters, as well as in r_s values, has been interpreted as indicating changes in fluidity. It has to be stressed that the order parameter is a purely static, structural property of the system, giving no direct information on the fluidity of the bilayer, which is a dynamic property. In fact, comparison of figs 1 and 3 shows that r_s and $\langle P_2 \rangle$ behave differently with temperature, the difference being due to the fact that the dynamic component, Φ, also contributes to the former.

In order to measure the correlation time, viz. the local fluidity, time-dependent polarization studies are now being performed.

The increase in molecular order of normal membranes upon peroxidation and the consistently high value of the parameter found in tumour membranes correlate well with the alterations in chemical nature of the fatty acids (see table 1). Indeed, the double-bond index is severely reduced as a result of exposure of the normal membranes to O_2^- radicals. In hepatoma 3924A, on the other hand, the double-bond index is already lower for control membranes than for the most peroxidized rat liver membranes and does not change greatly upon peroxidation. The insensitivity of this parameter to peroxidation is certainly attributable to the very low content of unsaturated fatty acid chains in this kind of tumour membrane. In fact, fig.4 shows that in rat liver microsomes peroxidation causes an increase in saturated fatty acid content, a very slight increase in monoenoic and a marked decrease in polyenoic acyl residues. In tumour membranes an initial lower content of saturated fatty acids is also followed by an increase after 5 min peroxidation and stays constant upon further exposure to the oxidizing agent. However, the most noticeable feature is the initial very low content in polyenoic fatty acids, which does not undergo variations with peroxidation. The monoenoic fatty acids show a slight increase as previously noted for normal membranes.

The fatty acid composition and molecular order of the lipid found for tumour membranes, when compared to normal membranes, seem to be indicative of a membrane that behaves globally as one which has already undergone peroxidative damage in vivo. This can be partially explained by the decrease in the protecting effect of cytosolic

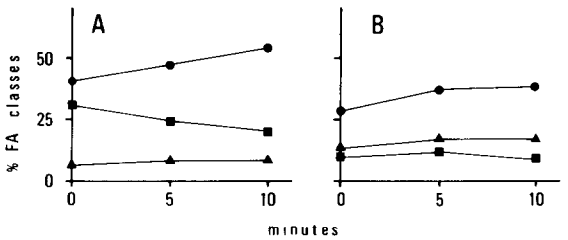


Fig.4. Changes in percentage (w/w) of saturated (●), monounsaturated (▲) and polyunsaturated (■) fatty acids (FA) induced by 5 and 10 min peroxidation in rat liver (A) and hepatoma 3924A (B) microsomes. The fatty acids reported are 16:0, 18:0, 18:1, 18:2 and 20:4.

superoxide dismutase and glutathione peroxidase previously reported [15,27]. However, to prove further the hypothesis that tumour membranes undergo oxy radical-induced damage in vivo, measurements of lipid peroxidation end products within the membranes are needed and are now in progress.

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