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Possible involvement of the lipid-peroxidation product 4-hydroxynonenal in the formation of fluorescent chromolipids

Hermann ESTERBAUER,* Ernst KOLLER,* Regina G. SLEE† and Johan F. KOSTER†

*Institute of Biochemistry, University of Graz, Schubertstraße 1, A-8010 Graz, Austria, and †Department of Biochemistry I, Medical Faculty, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

The effects of the lipid-peroxidation product 4-hydroxynonenal on the formation of fluorescent chromolipids from microsomes, mitochondria and phospholipids were studied. Incubation of freshly prepared rat liver microsomes or mitochondria with 4-hydroxynonenal results in a slow formation of a fluorophore with an excitation maximum at 360 nm and an emission maximum at 430 nm. The rate and extent of the development of the 430 nm fluorescence can be significantly enhanced by ADP-iron (Fe^{3+}). With microsomes, yet not with mitochondria, NADPH has a catalytic effect similar to that of ADP-iron. Fluorescent chromolipids with maximum excitation and emission at 360/430 nm are also formed during the NADPH-linked ADP-iron-stimulated lipid peroxidation. Phosphatidylethanolamine and phosphatidylserine react with 4-hydroxynonenal revealing a fluorophore with the same spectral characteristics as that obtained in the microsomal and mitochondrial system. The findings suggest that the fluorescent chromolipids formed by lipid peroxidation are not derived from malonaldehyde, but are formed from 4-hydroxynonenal or similar reactive aldehydes via a NADPH and/or ADP-iron-catalysed reaction with phosphatidylethanolamine and phosphatidylserine contained in the membrane.

INTRODUCTION

It is known that lipid peroxidation in biological samples leads to the formation of fluorescent chromolipids (Dillard & Tappel, 1971; Tappel, 1978; Koster & Slee, 1980; Koster *et al.*, 1982). Because of the high sensitivity, fluorescence measurements were widely used as a parameter of lipid peroxidation *in vitro* and *in vivo* (Tappel, 1980; Tsuchida *et al.*, 1985). It was suggested that the reaction of malonaldehyde, formed in the peroxidation process, with amino groups in phosphatidylethanolamine or proteins leads to the formation of fluorescent Schiff's bases with the 1-amino-3-iminopropene structure (Tappel, 1980). Recently other investigators proposed dihydropyridine derivatives as the molecular species responsible for the fluorescence (Kikugawa *et al.*, 1981*a*, *b*). Fluorescent compounds with similar spectral characteristics were also detected in lipid extracts of age pigments (Tappel, 1980).

During ADP-iron (Fe^{3+})- or haloalkane-stimulated lipid peroxidation of microsomes, a great diversity of aldehydes other than malonaldehyde are also formed (Esterbauer, 1982; Esterbauer *et al.*, 1982; Poli *et al.*, 1985). Among them, 4-hydroxynonenal has received particular attention, since it is formed in rather large quantities and is highly reactive towards proteins and other biomolecules (Schauenstein *et al.*, 1977; Benedetti *et al.*, 1980, 1981; Jürgens *et al.*, 1986). The involvement of aldehydes other than malonaldehyde in the formation of fluorescent chromolipids during lipid peroxidation has not yet been investigated. We report here that 4-hydroxynonenal reacts with model phospholipids revealing products possessing fluorescence properties similar to those found in the lipid extract of peroxidized microsomes and mitochondria.

MATERIALS AND METHODS

Experiments with microsomes and mitochondria

Rat liver microsomes were isolated from the liver homogenate (in 0.25 M-sucrose) by differential centrifugation. The pellet obtained after 30 min centrifugation at 200000 *g* was washed with, and finally taken up in, 0.15 M-KCl/5 mM-Tris/maleate, pH 7.4. For liver mitochondria the liver was homogenized in 0.15 mM-Tris/maleate buffer, pH 7.4. The homogenate was centrifuged for 5 min at 600 *g* and the mitochondria were obtained by centrifuging for 10 min at 5000 *g*.

For the formation of fluorescent chromolipids the microsomes or mitochondria (for concentrations, see the legends to Figs. 1, 2 and 3) were incubated in 0.1 M-Tris/HCl, pH 7.5 at 37 °C, up to 120 min in the absence and presence of 4-hydroxynonenal (0.77–1.54 mM). 4-Hydroxynonenal was prepared by a chemical synthesis previously described (Esterbauer & Weger, 1967). The incubation system was supplemented with either NADPH (0.4 mM) or ADP-iron (10 mM/0.37 mM) or NADPH plus ADP-iron. At the indicated times, 0.5 ml samples were withdrawn, mixed with 1.875 ml of chloroform/methanol (1:2, v/v) and centrifuged. After centrifugation, 0.625 ml of chloroform and 0.625 ml of water were added to the supernatant. A 1 ml sample was taken from the chloroform layer and mixed with 0.1 ml of methanol. The fluorescence intensity of this solution was measured at an excitation wavelength of 360 nm and an emission wavelength of 430 nm by using quinine sulphate (0.1 μg/ml) in 0.05 M-H_2SO_4 as standard.

Experiments with model phospholipids

L-α-Phosphatidylethanolamine type VII, L-α-phosphatidylcholine type III-L, L-α-phosphatidylinositol and L-α-phosphatidyl-L-serine were purchased from Sigma. To study the reaction with 4-hydroxynonenal, 0.1 ml of a solution of the phospholipids in chloroform (10 mg/ml) was added to 0.95 ml of 0.1 M-Tris/HCl, pH 7.4, and the chloroform was removed on a Rotavapor. The resulting sample was sonified for 2 min and mixed with 50 μl of 4-hydroxynonenal (20 mM) dissolved in water. The mixture was incubated at 37 °C in an open vial. Samples (0.1 ml) were analysed for fluorescence exactly as described above for microsomes or mitochondria. The consumption of 4-hydroxynonenal by the phospholipids was measured as previously described (Lang *et al.*, 1985).

RESULTS

Incubation of rat liver microsomes with NADPH and ADP-iron led to a rapid development of chloroform/methanol-extractable fluorescent material (= chromolipids) with an excitation maximum at 360 nm and an emission maximum at 430 nm (Table 1; Fig. 1*a*). In the absence of either NADPH or ADP-iron the rate and extent of the formation of the chromolipids was slow (Fig. 1). The time course of the development of the 430 nm fluorescence was more or less parallel with the extent of lipid peroxidation as measured by the evolution of malonaldehyde or 4-hydroxynonenal (Koster *et al.*, 1986; Esterbauer *et al.*, 1986). To investigate whether 4-hydroxynonenal is involved in the formation of the chromolipids, microsomes were also incubated with this aldehyde. A weak concentration- and time-dependent increase in the chromolipids occurred when the microsomes were incubated only with the aldehyde (Fig. 1*b*). Supplementation with either NADPH, ADP-iron or NADPH + ADP-iron resulted in a strong enhancement of the 4-hydroxynonenal-mediated 430 nm fluorescence (Fig. 1*a*). Boiled microsomes (5 min, 100 °C) did not form the fluorescent material with the aldehyde when incubated in the presence of NADPH, but still did if

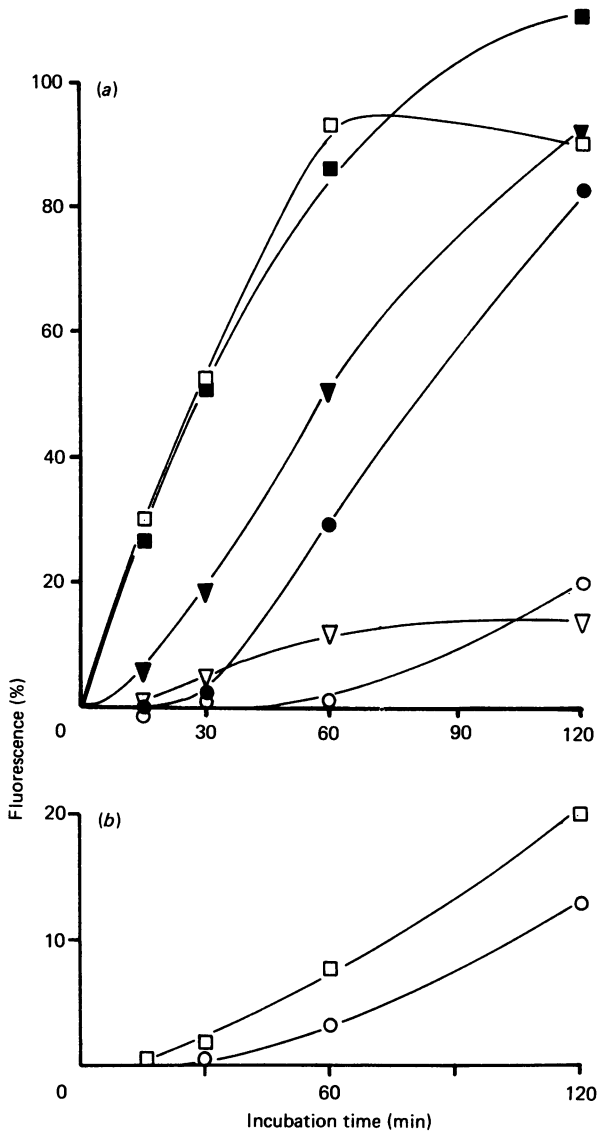

Fig. 1. Time-dependency of the formation of fluorescent chromolipids in microsomes in the presence of ADP-iron, NADPH or 4-hydroxynonenal

(*a*) ●, ○, ADP-iron; ▼, ▽, NADPH; ■, □, ADP-iron plus NADPH. Open symbols indicate the absence, and closed symbols the prescence, of 4-hydroxynonenal (0.77 mM). (*b*) ○, 0.77 mM-4-hydroxynonenal; □, 1.54 mM-4-hydroxynonenal in the absence of ADP-iron and NADPH. The amount of microsomes added was 0.89 mg of protein/ml.

Table 1. Effect of NADPH, ADP-iron and 4-hydroxynonenal (HNE) on the formation of fluorescent products by microsomes and mitochondria

Microsomes (0.9 mg/ml) or mitochondria (4.4 mg/ml) were incubated in the presence of the indicated supplements for 120 min and the fluorescence of the chloroform/methanol-extractable lipid material was measured relative to a 0.1 μg/ml quinine standard (= 100%); the HNE concentration was 0.77 mM. The values are means ± S.D. from three experiments.

	Fluorescence (%)	
Supplements	Microsomes	Mitochondria
NADPH	14±2	8±2
ADP-iron	18±2	8±3
NADPH + ADP-iron	100±5	65±3
HNE	12±2	8±2
NADPH + HNE	90±3	18±3
ADP-iron + HNE	85±2	50±4
NADPH + ADP-iron + HNE	105±5	105±6

ADP-iron was present. Butylated hydroxytoluene (100 μg/ml) or thiourea (30 mM) prevented the 4-hydroxynonenal-dependent generation of chromolipids.

Similar to microsomes, mitochondria exposed to NADPH and ADP-iron also produced chromolipids with excitation at 360 nm/emission at 430 nm, and only a weak fluorescence developed when either NADPH or ADP-iron was omitted (Table 1; Fig. 2*a*). Like the situation with microsomes, incubation of mitochondria with 4-hydroxynonenal alone led to a small concentration- and time-dependent increase of the fluorescent chromolipids (Fig. 2*b*), which was, however, significantly enhanced when the system was supplemented with NADPH + ADP-iron or ADP-iron. Unlike the situation

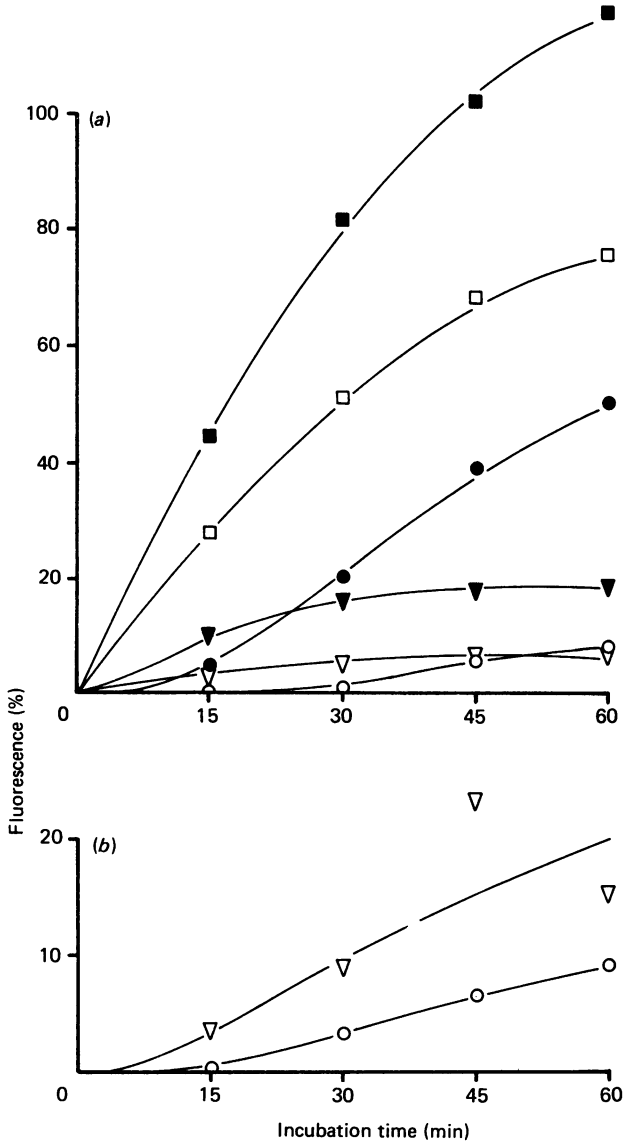

Fig. 2. Time-dependency of the formation of fluorescent chromolipids in mitochondria in the presence of NADPH, ADP-iron or 4-hydroxynonenal

(*a*) ●, ○, ADP-iron; ▼, ▽, NADPH; ■, □, ADP-iron plus NADPH. Open symbols indicate the absence, and closed symbols the presence, of 4-hydroxynonenal (0.77 mM). (*b*) ○, 0.77 mM-4-hydroxynonenal; ▽, 1.54 mM-4-hydroxynonenal in the absence of ADP-iron and NADPH. The amount of mitochondria added was 4.37 mg/ml.

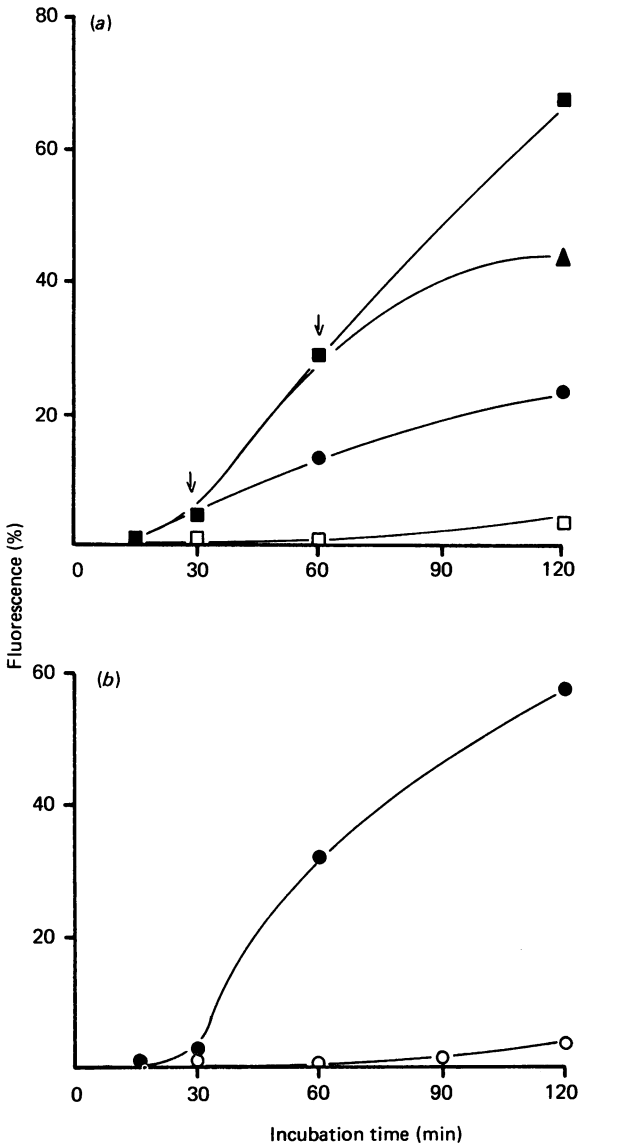

Fig. 3. Effect of desferral on the ADP-iron-enhanced formation of fluorescent chromolipids by 4-hydroxynonenal in microsomes and mitochondria

The arrows indicate the time of addition of desferral (1.5 mM) (*a*) Microsomes: ■, control; □, desferral added at zero time; ●, ▲, desferral added 30 min (●) and at 60 min (▲) after starting the reaction. (*b*) Mitochondria: ●, control; ○, desferral added at zero time. Amounts added: microsomes, 0.73 mg/ml; mitochondria, 3.38 mg/ml; 4-hydroxynonenal, 0.75 mM.

with microsomes, the combination NADPH + 4-hydroxynonenal did not stimulate chromolipid formation (Figs. 1*a* and 2*a*). The iron chelator desferral added simultaneously with ADP-iron prevented the formation of chromolipids by 4-hydroxynonenal in both microsomes and mitochondria (Fig. 3). If desferral was added 30 or 60 min after the addition of ADP-iron, the subsequent production of chromolipids was decreased significantly.

To study the possible involvement of 4-hydroxynonenal in the formation of the fluorescent chromolipids, phospholipid liposomes were incubated with the aldehyde (Fig. 4). Phosphatidylethanolamine was most reactive and consumed the aldehyde in a time-dependent process with a constant rate over the 90 min incubation period examined. Somewhat less reactive was phosphatidylserine, whereas phosphatidylinositol reacted only slowly and phosphatidylcholine reacted not at all with 4-hydroxynonenal. The reaction of 4-hydroxynonenal with phosphatidylethanolamine and phosphatidylserine led to the formation of fluorescent chromolipids with the same fluorescence characteristics (excitation 360 nm/emission 430 nm) as generated by the microsomes or mitochondria. Phosphatidylinositol showed a weak intrinsic fluorescence, most likely due to traces of impurities or degradation products; the fluorescence, however, did not increase upon incubation with 4-hydroxynonenal; similarly, phosphatidylcholine did not give any fluorescent material with 4-hydroxynonenal. Fig. 5 shows

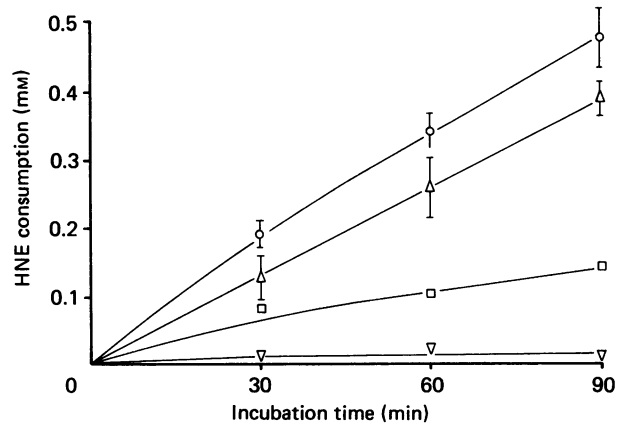

Fig. 4. Time course of the consumption of 4-hydroxynonenal (HNE) by phospholipids

Phosphatidylethanolamine (○), phosphatidylserine (△), phosphatidylinositol (□) and phosphatidylcholine (▽) were incubated with 1 mM-4-hydroxynonenal in 0.1 M-Tris/HCl buffer, pH 7.4, at 37 °C. The phospholipid concentration was 1 mg/ml.

that the fluorescence intensity of phosphatidylserine and phosphatidylethanolamine increases proportionally with the degree of modification by 4-hydroxynonenal, i.e. mol of aldehyde bound per mol of phospholipid. On the basis of the same degree of modification the fluorescence intensity of phosphatidylserine was, however, clearly higher than that of phosphatidylethanolamine. Incubation of phosphatidylethanolamine and phosphatidylserine in the absence of 4-hydroxynonenal also led to an increase of the 430 nm fluorescence, most likely through autoxidation. This spontaneous fluorescence was taken into account in the construction of the curve shown in Fig. 5. ADP-iron (10 mM/0.3 mM) did not accelerate the

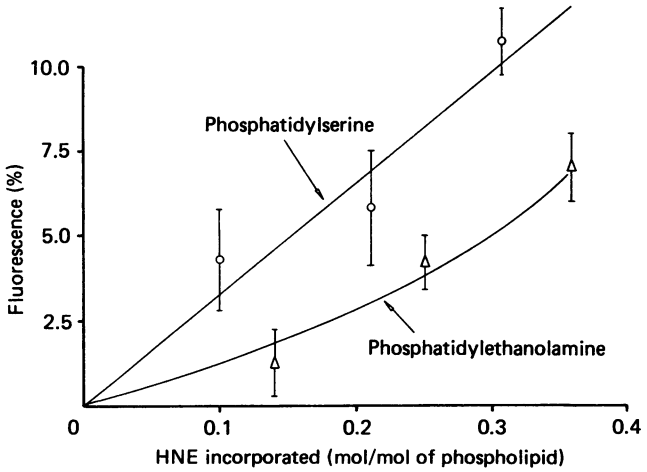

Fig. 5. Relationship between fluorescence intensity of phospholipids and the degree of modification by 4-hydroxynonenal (HNE)

Phosphatidylserine (○) and phosphatidylethanolamine (△) were incubated with 4-hydroxynonenal as described in Fig. 4; at different times the fluorescence intensity (excitation 360 nm/emission 430 nm) and the consumed aldehyde were measured.

4-hydroxynonenal consumption by the four phospholipids and had no effect on the rate and extent of the formation of the 430 nm-fluorescent material.

DISCUSSION

Lipid peroxidation in biological membranes leads to the formation of a number of aldehydic degradation products such as malonaldehyde, 4-hydroxynonenal, n-alkanals and 2-alkenals (Dillard & Tappel, 1971; Esterbauer *et al.*, 1982; Poli *et al.*, 1985). It is believed that the increase in fluorescence in the lipid extract of peroxidized biological samples is due to modification of phospholipids by malonaldehyde (Tappel, 1980). Model studies have shown that phosphatidylethanolamine reacts with this aldehyde to a form of fluorescent product (Bidlack & Tappel, 1973). This product, however, has spectral characteristics (excitation 400 nm, emission 475 nm) that are not at all similar to the fluorescence characteristics of lipids extracted from peroxidized microsomes that show excitation at 350–360 nm and emission at 430 nm (Dillard & Tappel, 1971; Koster & Slee, 1980). Moreover, it was reported (Dillard & Tappel, 1973) that, by reaction of peroxidizing arachidonic or docosahexaenoic acid with synthetic dipalmitoyl phosphatidylethanolamine, fluorophores with excitation at 360 nm and emission at 430–440 nm are formed. The fluorescence intensity in this model system increased linearly with the thiobarbituric acid-reactive material, and it was therefore assumed that malonaldehyde is involved in the formation of the fluorophore. The spectral difference between the malonaldehyde-phosphatidylethanolamine fluorophore and the fluorophore contained in peroxidized microsomes cannot be neglected and rather indicates that substances other than malonaldehyde are responsible. In agreement with previous reports (Dillard & Tappel, 1971; Koster & Slee, 1980) we found that microsomes respond towards oxidative stimuli with a time-dependent formation of fluorescent chromolipids with excitation at 360 nm and emission at 430 nm. Mitochondria also produce significant amounts of these fluorochromes when exposed to NADPH and ADP-iron. This suggests that the formation of this chromophore during lipid peroxidation is a general phenomenon independent of the type of system studied.

It was shown that 4-hydroxynonenal is formed during lipid-peroxidation processes in rather large amounts (Benedetti *et al.*, 1980; Esterbauer *et al.*, 1982, 1986; Poli *et al.*, 1985) and that this aldehyde is highly reactive towards thiol groups (Schauenstein *et al.*, 1977) and other nucleophilic species such as lysine residues in low-density lipoprotein (Jürgens *et al.*, 1986). 4-Hydroxynonenal is most reactive to phosphatidylethanolamine and phosphatidylserine, and the reaction leads to the formation of a fluorescent chromophore identical in the emission maximum (430 nm) and excitation maximum (360 nm) with the fluorophore formed in peroxidizing microsomes and mitochondria. Moreover, the fluorescence intensity is linearly related to the number of 4-hydroxynonenal molecules that had reacted with the phospholipids. This suggests that the fluorescent lipid material formed during lipid peroxidation processes is generated by interaction of 4-hydroxynonenal or similar aldehydes with phosphatidylethanolamine and phosphatidylserine contained in the membrane. With our present

knowledge it is not possible to propose structures for the products formed. The formation of the ultimate fluorescent compound can occur in uncatalysed systems as shown by the model experiments with phospholipids (Fig. 5) as well as in incubations of microsomes and mitochondria with 4-hydroxynonenal alone (Figs. 1*b* and 2*b*). In microsomes, NADPH exerts a strong catalytic activity in the formation of chromolipids by 4-hydroxynonenal, and this NADPH-linked activity is most likely enzymic, since it is completely abolished by heat inactivation. Mitochondria obviously are low in this activity, since NADPH leads only to a weak stimulation. The enhancement of the formation of fluorescent chromolipids by ADP-iron is probably not coupled with an enzyme activity, since boiled and fresh microsomes gave more or less the same fluorescence intensity upon exposure to ADP-iron and 4-hydroxynonenal. Nevertheless, ADP-iron is only effective in the biological systems (microsomes, mitochondria) and ineffective with the phospholipid liposomes. The reasons for this may be manyfold (different accessibility for the complexed iron, traces of preformed hydroperoxides in the organelles, catalytic effects of proteins, among others), but at present we have no plausible explanation for this phenomenon.

The biological significance of the present findings relies in the more precise characterization of the possible origin of the fluorescent lipid chromophores formed during lipid peroxidation. Our findings strongly suggest that the fluorophors are not formed from malonaldehyde but from 4-hydroxynonenal or similar aldehydes in an NADPH- and ADP-iron-catalysed reaction with phosphatidylethanolamine and phosphatidylserine.

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