BENZOPHENONE-SENSITIZED PEROXIDATION IN COMPRESSED LIPID MONOLAYERS AT AIR–WATER INTERFACE

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The paper reports on a study of radical-type lipid peroxidation of biomembrane lipid constituents in compressed monolayers, with incorporated lipoidic benzophenone photosensitizers. Their triplets abstract allylic and double-allylic hydrogen atoms from hydrophobic moieties of the lipid molecules. The results confirmed the H abstraction occurrence (and thus the initiation of the peroxidizing chain mechanism), and absence of lipid peroxide formation at the same time. The reason is in the "cage effect": highly restricted spatial area of compressed lipid monolayers limits mobility of the formed radicals (lipid and ketyl radicals) and leads to their recombination, preventing the propagation step of the chain mechanism. With certain approximation, one may conclude that these results have clear implication for real biomembranes: their structure is one of the main factors in prevention of spreading of the chain reaction, and lipid peroxide formation.

Keywords: Free radical; Lipid peroxidation; Photosensitizers; Benzophenone; Monolayers; Photolysis; Peroxides.

The lipid peroxidation phenomenon, implying oxidative destruction of polyunsaturated moieties[1], may be ascribed in large part to the presence of double bonds in the hydrocarbon parts, and the adjacent allylic and doubly allylic sites at which hydrogen abstraction (thus lipid radical formation) may be facilitated[2,3].

While the chain peroxidation effects have been studied extensively *via* autooxidation[4], quantitative characterization of the degradation requires controlled initiation of the H abstraction from allylic and double-allylic sites. Several radiation-chemistry studies carried out using $OH^{\bullet}$ radicals as the H abstraction agents suffered from non-selectivity as to the site of attack in complex environments[5–7]. On the other hand, use of benzophenone (BZP), already well known as a very efficient initiator of polymerization processes[8,9], permits very selective abstraction from allylic and double-allylic sites by its triplet (^{3}BZP) and appeared to be a promising approach for further quantitative chain peroxidation studies[10]. Benzophenone is a

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typical Type I photosensitizer, reacting directly with a lipid to generate reactive lipid radicals[11,12]. The H abstraction by longer-lived triplet states of aromatic ketones is a well-known reaction in organic photochemistry[13].

There are two possible approaches to study of the mechanisms of photosensitized lipid peroxidation in biological membranes. The first one deals with experiments *in vivo*. A complexity of the processes involved appears to be a limiting factor for such approach. The second one includes experiments on model membranes, with various degrees of molecular organization (micelles, compressed monolayers, vesicles), providing better control of the chain process inside. The latter approach has been used in our reports[10,14,15]. To get basic kinetic data, deprived of any molecular organization influence, a series of measurements studying reactions of BZP with unsaturated lipid fatty acids has been done in benzene solution[10]. The same reactions were also studied in micelles of sodium dodecyl sulfate[14] (SDS) and linoleic acid[15] (LA) to be able to estimate spatial, molecular organization effects, by comparing two sets of kinetic data from two media. This report is a step further, since it describes the BZP-sensitized peroxidation in compressed lipid monolayers at the air–water interface – in a medium which is more organized and mimics the real biomembranes.

EXPERIMENTAL

Synthesis of Benzophenone Derivatives

11-[4-(4-Heptylbenzoyl)phenyl]undecanoic acid (BHUA) has been synthesized according to the described procedure[14]. The second BZP derivative, diphenyl 1-*O*-hexadecyl-2-*O*-{5-[4-(4-heptylbenzoyl)phenyl]pentanoyl}-*sn*-glycerol 3-phosphate (DBP) has been synthesized according to the recepee for the synthesis of very similar compounds[16,17]. The Structures of BHUA and DBP are shown in Fig. 1.

A lipid used to form monolayers, 1,2-di-*O*-linoleoyl-3-*sn*-phosphatidylcholine (1,2-DLPC) was purchased from Avanty Polar Lipids (Birmingham, AL).

Photolysis Experiments on Monolayers

The photolysis experiments on lipid monolayers with incorporated BZP derivatives as the photosensitizers have been done on a specially constructed home-made experimental set-up. A Teflon trough 15 cm × 54 cm × 3 mm (depth) was filled with water. A still rod was fixed parallel to and above the longer trough edge. It serves as a support for two parallel Teflon barriers immersed 2–2.5 mm in the water subphase. The barriers, in parallel position to the trough shorter edge, glide along the rod, approaching to each other and compressing the lipid monolayer already created on the water surface. The monolayer compression speed, in the range 0.016–1.6 cm/min, has been controlled by four-phase Airpax stepping motor model K82954-MS (North American Phillips Controls Corp.). To prevent the heating during the operation, the motor was cooled by water flowing through the copper tubing coiled

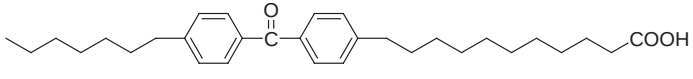
around its cylindrical surface. A small box with the scales of Cahn 2000 electrobalance was fixed at the top of the experimental set-up. At the left arm of the scales, a nichrome wire was hooked, ending with a 0.95 cm square filter paper, immersed 1–2 mm into the water subphase, straight into the center of the trough. It serves for registration of changes of the surface pressure resulting from the monolayer compression. The right arm was fixed to the Cahn electrobalance which transforms stretching of the balance arms (caused by the surface pressure changes) into millivolts. The calibration was done prior to the experiments. The electrobalance is connected to a Hewlett–Packard chart recorder, model 745A.

After the lipid (1,2-DLPC) monolayer formation and the compression start, surface pressure (π) changes have been registered on a chart (the *Y*-axis in mN/m), together with molecular packing (σ) changes expressed in $Å^2$ per molecule (the *X*-axis). The σ values were calculated easily, knowing the exact volume and the concentration of the 1,2-DLPC solution (used for the monolayer formation), as well as the trough dimensions. The π–σ isotherm is shown in Fig. 4a.

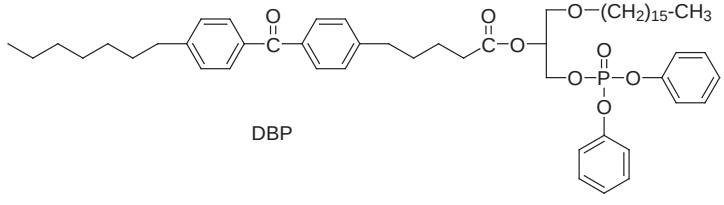
The monolayer samples were mixtures of the lipid (1,2-DLPC) and the photosensitizer (BHUA, DBP). The lipid/sensitizer ratios used in the experiments were 4 : 1 and 6 : 1. Usually, the aliquots of (5–50) · 10^{-6} dm^3 were used (in chloroform), with an approximate total (lipid + sensitizer) concentration of 1.5 mmol dm^{-3}.

Low-pressure mercury lamps (manufactured by the Southern New England Ultraviolet Co., Hamden, CN) were used in the photolysis experiments. The lamps were packed into two separated sets, fixed about 15 cm above the water level on a solid adjustable rack bearer. Each lamp housing contained 10 individually water-jacketed lamps, spaced 3.7 cm apart and positioned beyond the whole monolayer area (each set covering approximately half of the area). To prevent the lamp heating and possible film destruction, a distilled water was circulated *via* the tubing coiled around the lamps. This provided temperature control within ±0.5 °C.

Before the photolysis experiments, the lamps were turned on and thrmostatted for about 10 min. The emission, directed toward the film, was blocked until the start of the experiment. The lamp emission profile with negligible intensities beyond 300 nm prevents de-



BHUA



DBP

FIG. 1
Structure of lipoidic benzophenone derivatives: BHUA and DBP

struction of possibly formed peroxide dienes structures, with absorption maximum at 234 mn (ref.[18]).

Since the surface pressure measurement is highly dependent on temperature[19,20], the whole trough and the lamp apparatus were placed on a solid rectangular block and housed inside a 92 × 69 × 61 cm acrylic glass glove box. Since particular experiments needed a special atmosphere inside the acrylic glass box (N_2, O_2, air), gas channels for the box interior have been provided.

Post-Photolysis Procedure

At the end of the photolysis, the monolayers were aspirated with a Pasteur pipette using strong vacuum into a specially designed cylindrical flask. A few cm^3 of the water phase have usually been aspirated, too (despite the effort to avoid it or to diminish the quantity). The collected water phase was then evaporated under vacuum in a small round-bottom flask. A volume of 1 cm^3 of CH_3CN was then added to dissolve the monolayer post-photolytical material. The solution was analyzed by HPLC. The water subphase was periodically analyzed by this procedure to detect a possible (undesirable) presence of the monolayer post-photolytical material inside. About 30% of the monolayer post-photolytical material have been lost during the manipulation by before the HPLC analysis. This value was estimated by a comparative method, based on the detection of 1,2-DLPC peroxides photosynthesized in benzene, and then used together with the rest of non-oxidized 1,2-DLPC for the monolayers formation.

HPLC Detection

μ-Bondapak C-18 column 8 mm × 10 cm and 90% aqueous MeOH as the mobile phase were used for the HPLC analysis (Waters Associates, Milford, MA) of the monolayer post-photolytical material, by analogy with the report of Crawford and coworkers[21]. The total concentration of the analyte was in the 10^{-5} mol dm^{-3} range. The peroxides were detected at 234 nm, at the maximal absorbance (A_{max}) of the formed diene structures[18]. The photosensitizers were detected in the 250–270 nm wavelength range.

RESULTS AND DISCUSSION

Before the photolysis experiments on monolayers, a “blank” experiment with LA instead of 1,2-DLPC was done, *i.e.*, LA was used as the lipid substrate in solution and micelles[10,14,15]. LA and 1,2-DLPC do not differ significantly (1,2-DLPC may be represented by two LA “branches” together with a polar phosphate head (Fig. 2)). Still, the presence of the polar head (totally irrelevant for the peroxidation process) might cause steric restriction for the initiation process, especially in the monolayer. The 1,2-DLPC peroxidation with BHUA as a sensitizer, was done in benzene by continuous photolysis. The result is shown in Fig. 3. Figure 3a shows the HPLC chromatogram before photolysis (the photosensitizer peak only). Figure 3b shows the HPLC chromatogram after 2-min UV-irradiation: the two peroxide peaks and the

photosensitizer peak in the middle can be seen on the same absorbance scale (to make comparison easier). The two peroxide peaks indicate probably an incomplete, one-branch peroxidation, otherwise one peak, only should be expected.

1,2-Di-*O*-linoleoyl-3-*sn*-phosphatidylcholine (1,2-DLPC) was used as a lipid substrate spread in the form of a monolayer at the air–water interface. The two chosen BZP sensitizers were BHUA and DBP. The choice of the lipid and the sensitizers was not accidental. 1,2-DLPC is naturally present in certain types of biomembranes. The two double bonds in the two hydrophobic LA branches should permit high reactivity with the incorporated photosensitizers, mainly an easy H abstraction. On the other hand, BHUA and in particular DBP (which is a benzophenone lipide, Fig. 1) should permit a better incorporation inside the 1,2-DLPC monolayer, and the best possible positioning of the BZP chromophore relative to the main H abstraction targets: the two double-allylic and four allylic H atoms. The assumed optimum position of the BZP chromophore inside the compressed 1,2-DLPC monolayer is shown in Fig. 2.

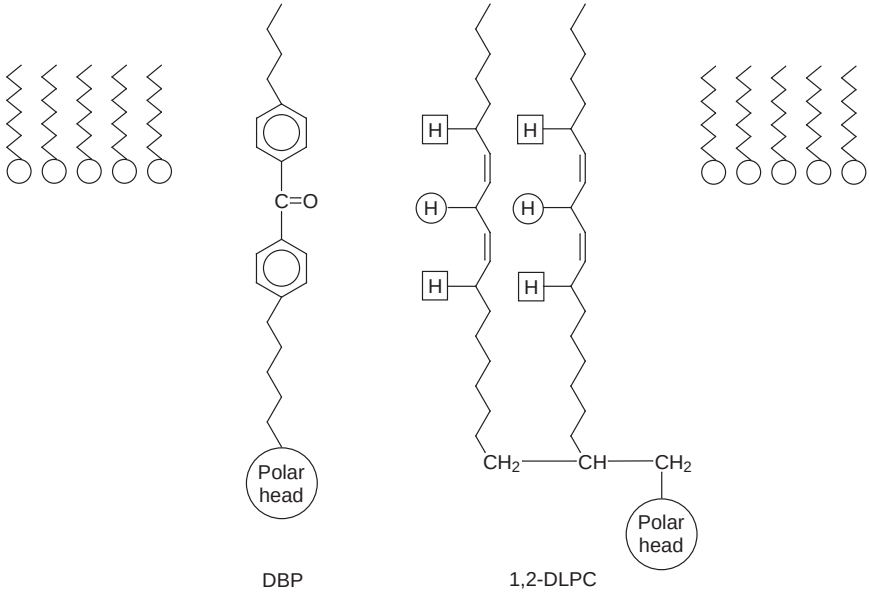


FIG. 2
An anticipated optimum position of carbonyl chromophore of benzophenone derivatives (BHUA and DBP) in compressed 1,2-DLPC monolayers relative to main potential H abstraction targets: allylic and double-allylic H atoms

The idea of incorporating the photosensitizing chromophore into a lipid monolayer has been already exploited. Bohorquez and Patterson[22] used lipophilic pyrene for monolayer study by monitoring the probe excimer fluorescence. More recently and more relevant to this report, Maziere and coworkers[23] used 1,6-diphenyl-1,3,5-hexatriene (DPH)-labeled lipids as a potential tool to study lipid peroxidation in monolayer films. DPH was previously known as a fluorescent probe for monitoring lipoprotein peroxidation[24]. However, while the DPH fluorescence served as a probe to follow lipid peroxidation caused by another agent[23], in this report, the DBP initiates the peroxidation process.

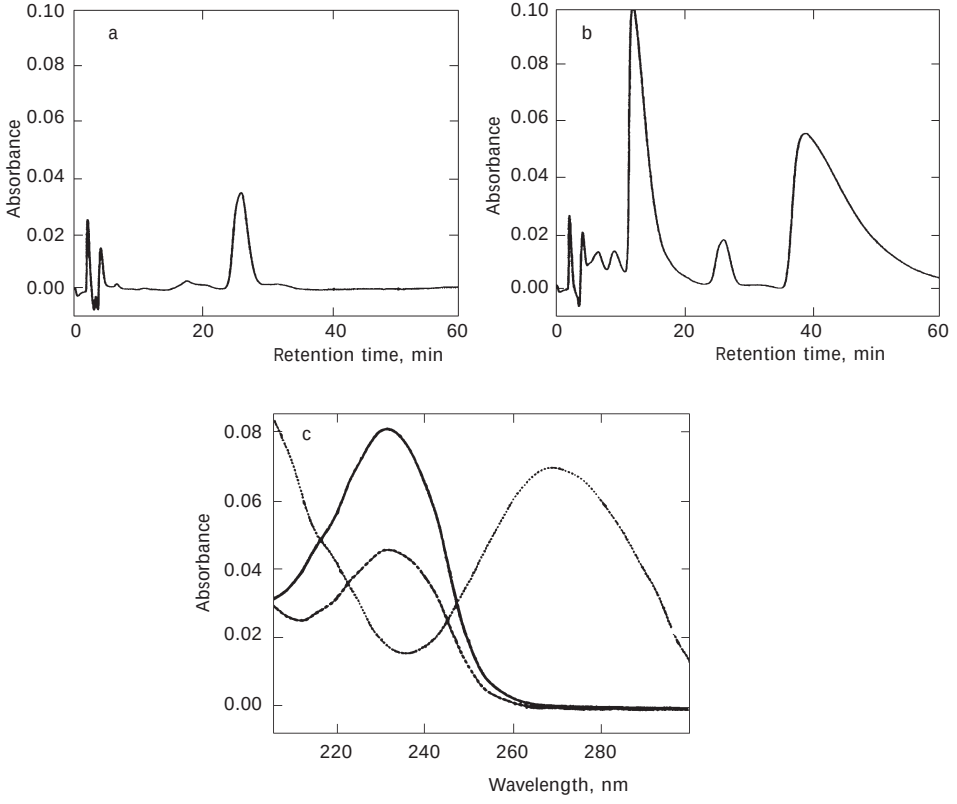


FIG. 3

a HPLC chromatogram of a mixture of 1.26 mmol dm^{-3} 1,2-DLPC with 0.1 mmol dm^{-3} BHUA in aerated benzene, before photolysis; b HPLC chromatogram of the same mixture, after 2-min UV-irradiation (the same absorbance scale); c spectra of pure compounds: 1,2-DLPC peroxides (———) and (- - - -), BHUA (· · · ·)

Since the photosensitized peroxidation of 1,2-DLPC with BHUA is evidently possible (Fig. 3), the implication for possibility of its occurrence in the compressed monolayers (submitted to the oxygen-containg atmosphere) is the following. If the 1,2-DLPC peroxides do not appear in HPLC chromatograms (under the same separation and detection conditions as those in Fig. 3), the reason could be: (i) the prevented or at least significantly elliminated initiation caused by steric limitations in the compressed monolayer "cage" or (ii) recombination of the formed radical pairs due to the "cage effect", logically expected to be even more pronounced compared with SDS micelles[14].

The photolysis experiments were done with the lipid (1,2-DLPC)-to-photosensitizer (BHUA, DBP) ratios of 4 : 1 and 6 : 1, in a broad range of surface pressures (π) (5–30 mN/m), and irradiation time intervals (1–10 min). The ratios were not accidentally chosen. At these two ratios, the monolayers showed a good stability before irradiation in the whole π range. A good stability of a non-irradiated "blank" monolayer is necessary, since otherwise it is not possible to attribute the appearance of possible (photolytically induced) changes exclusively to the lipid-sensitizer reaction, but also other factors have to be considered. Furthermore, the ratios (4 : 1 and 6 : 1) prevented the triplet self-quenching (or "fusion"), which has already been detected in solution[25], and which may be expected also in the compressed monolayer "cage".

An indirect evidence for the (lipid/sensitizer) reaction is given in Fig. 4, showing the π–σ isotherm of the 1,2-DLPC/BHUA (4 : 1) monolayer, Fig. 4a before, and 4b after the photolysis, in a 10-min period in a nitrogen atmosphere. The isotherm itself has a shape typical of unsaturated compounds, where the double bond presence prevents sharp phase changes during the monolayer compression. A simple visual comparison of the isotherms given in Fig. 4a and 4b proves an increase in the surface pressure during the photolysis, performed at a constant σ value (the compressing Teflon barriers in a fixed position). But, more solid evidence of the occurrence of the lipid/sensitizer reaction is given in Fig. 5, showing a decrease in BHUA concentration in the monolayer 1,2-DLPC/BHUA (6 : 1) during the photolysis. This is a reliable evidence of ^{3}BHUA H abstraction from 1,2-DLPC, since no other types of reactions of BHUA with 1,2-DLPC come into account in the monolayer (and also in general) which would lead to a BHUA concentration decrease (Fig. 5), *i.e.* which would cause its disappearance as a chemical species. The physical quenching of ^{3}BHUA by interaction with the double bonds of 1,2-DLPC does not transform ^{3}BHUA into the corresponding ketyl radical[26].

So, not surprisingly, the HPLC chromatogram recorded after the photolysis in the oxygen atmosphere did not exhibit traces of the 1,2-DLPC peroxides (not shown). That was something to be expected, based on the results obtained in SDS micelles, where only 7–14% of the formed radical pairs escaped the recombination inside the micellar cage[14]. The effect of the

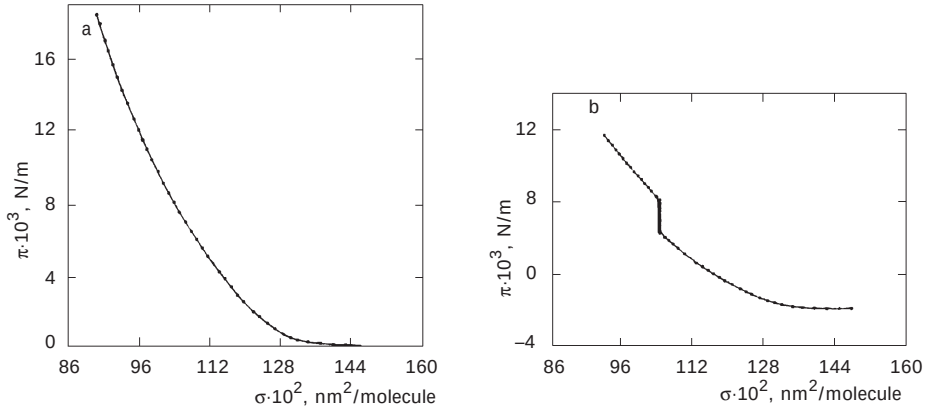


FIG. 4

π–σ isotherm of the 1,2-DLPC/BHUA (4 : 1) monolayer before (a), and after 10-min photolysis (b). The compressing Teflon barriers were in a fixed position (constant σ value) during the photolysis. The π values are expressed in mN/m, and σ values show the surface area (in nm^2) per molecule. The experiment was done in nitrogen atmosphere

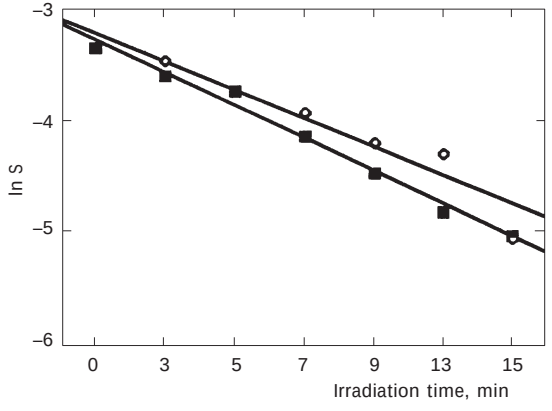


FIG. 5

Kinetics of BHUA reduction in 1,2-DLPC/BHUA (6 : 1) monolayers. The full squares indicate actinometer (BHUA in EtOH), and the open suares integrated area of BHUA peaks at 270 nm

radical-pair recombination is certainly even more pronounced in the 1,2-DLPC compressed monolayer "cage" since the "escape event" is even less probable. Clearly, the last statement does not exclude, in an absolute sense, the possibility of the lipid peroxide production in the investigated monolayers.

Certainly, one must not forget that the peroxide detection is limited by the HPLC conditions established for the "blank" (BHUA and l,2-DLPC in benzene). So, a limited peroxide production cannot be excluded. To be able to quantify the detection limit, the following procedure has been performed. 1,2-DLPC peroxides liave been sythesized in benzene with BHUA, ("blank" experiment). After benzene evaporation, $CHCl_3$ was added and the peroxide concentration was adjusted to 1.3 mmol dm^{-3} (the concentration used for the monolayer formation). By spreading few aliquots in the $(5–50) \cdot 10^{-3}$ cm^3 range from the bulk solution at the water surface, a set of monolayers has been formed. Then the post-photolytical procedure (described in Experimental) has been used. This time the 1,2-DLPC peroxides were detected by HPLC (not shown): one peak only was found, with the retention time close to the first peak in Fig. 3b.

The integrated areas of the peaks represent the *X*-axis values. The second (*Y*-axis) set of data came from the same ($(5–50) \cdot 10^{-3}$ cm^3) aliquots. $CHCl_3$ was evaporated and the peroxides were then dissolved in 1 cm^3 of CH_3CN (the same amount of the same solvent used for the final dissolution of the post-photolytical monolayer material). The sample absorbances were then measured on a UV-VIS spectrophotometer at 234 nm (the A_{max} value) to get the *Y*-axis values (it is important to emphasize that the measured values reflect mostly the peroxide absorbance, and to a negligible extent, the concentration of non-oxidized 1,2-DLPC molecules).

The calibration plot is given in Fig. 6. From the least, but still detectable peak in the HPLC chromatogram (the lower detection limit), and the integrated area value, the corresponding absorbance has been determined, and the concentration of 1,2-DLPC peroxides was calculated. The last number is then expressed as the percentage calculated relative to the known concentration of the non-oxidized 1,2-DLPC. The final result of 1–3% is the detection limit. If the post-photolytic peroxide concentration does not exceed 1–3% of the total lipid (1,2-DLPC) monolayer material, it will not be detected under given HPLC conditions. The small percentage value is a very, strong proof supporting the basic conclusion about the predominant "cage" effect influence on the peroxidation process inhibition. It is reasonable to conclude that the percentage of the photolytically induced 1,2-DLPC peroxides in monolayers is probably less than 1%: the presence of 1–3% lipid

peroxides in freshly isolated or synthesized non-oxidized lipid material is considered as normal, due to autooxidation.

Finally, an additional proof supporting the absence of a significant peroxidation extent in compressed lipid monolayers came from the experiments made at a constant surface pressure. This time, the photolytically induced change was expressed in the area per molecule (in nm^2). The

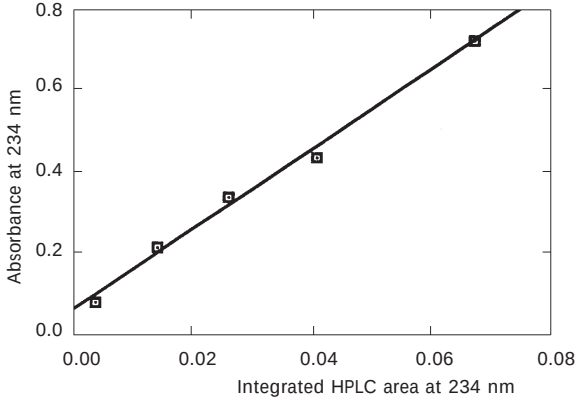


FIG.6
Calibration diagram for determination of minimum 1,2-DLPC peroxides concentrations (*i.e.* the detection limit) under the chosen HPLC conditions

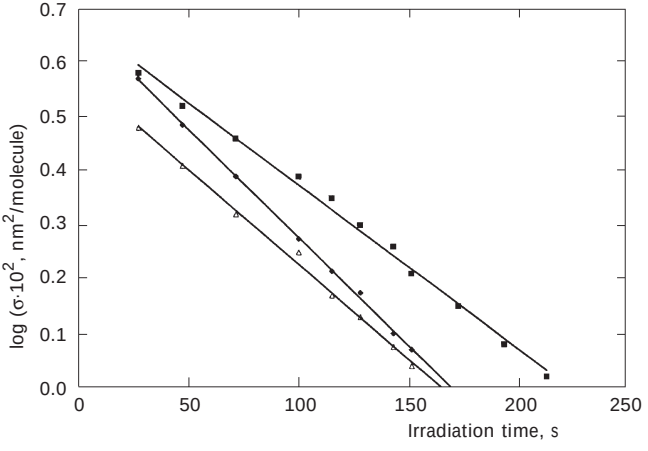


FIG. 7
Plots of time changes of molecular packing in 1,2-DLPC/DBP (6 : 1) monolayers during photolysis (in nm^2 per molecule) under various surface pressures. π values: ◆ 5, ■ 15, Δ 25 · 10^{-3} N/m

calculated value is based on the knowledge of all necessary data: the area framed by the compressing Teflon barriers, concentrations as well as the volumes of the aliquots used for the monolayers formation. The log plots representing temporal changes in the molecular packing of the monolayers 1,2-DLPC/DBP (6 : 1) during the photolysis have been shown in Fig. 7 for three different surface pressures.

The two clear facts can be seen from the plots. First, the ^{3}DBP–1,2-DLPC reaction is of the pseudo-first order, reflecting the significantly smaller triplet sensitizer concentration (compared to its counterpart, *i.e.* the lipid). The reaction rate depends on the dynamics of the triplet disappearance only. Second, the molecular packing changes during the photolysis (calculated from the *Y*-axis in Fig. 7), do not exceed 5 Å^2 per molecule ($5 \cdot 10^{-2}$ nm^2 per molecule), for all the experimental surface pressures (5, 15, and 25 mN/m). For the lipid/sensitizer, ratio of 6 : 1 used in the experiments, the maximal change is about 30 Å^2 ($30 \cdot 10^{-2}$ nm^2 per molecule). The last value clearly and additionally proves the significant peroxidation absence in the compressed 1,2-DLPC monolayers. Otherwise, the number would be much higher.

CONCLUSIONS

1. Production of peroxides in monolayers of 1,2-DLPC with the incorporated lipoid photosensitizers has not been detected (up to 1–3% relative to the total amount of the lipid used for formation of the monolayers). The result has been obtained from experiments made with increasing surface pressures. It is not because the photochemical reaction inside the monolayers does not occur. A clear decrease in the photosensitizer concentration with increasing irradiation time has been found. The H abstraction inside the 1,2-DLPC monolayer (by ^{3}BHUA and ^{3}DBP) certainly occurs, but 1,2-DLPC peroxides have not been detected because of the predominant radical pair recombination inside the "cage" of the compressed monolayer, in which their mobility is extremely restricted. An additional proof of this statement consists in proportional small changes of the surface area per molecule in the investigated monolayers. The change would be certainly bigger if the lipid peroxidation chain reaction significantly occurred.

2. Though compressed monolayers are only artificial models of biomembranes, the basic conclusion concerning the possibility of the lipid peroxidation occurrence inside the monolayer can be applied to biomembranes, with certain approximation. At least one of the factors which prevent propagation of the lipid peroxidation chain mechanism inside

biomembranes consists their structure. It clearly appears that biomembranes have some kind of self-protective mechanism against the peroxidation reaction, consisting in the radical pair recombination due to the "cage effect".

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