Dark Oxidation of Unsaturated Lipids by the Photoxidized 8-Methoxypsoralen

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8-Methoxypsoralen (8-MOP) in ethanol, acetone, benzene, or CCl_4 is photoxidized under UV-irradiation (320–400 nm). Photoxidized 8-MOP (O_2-8-MOP) is stable in the organic solvents, but it is destructed in water or in liposome suspension. The destruction rate constants are 0.04 s^{-1} in water and 0.004 s^{-1} in liposome membranes as estimated by the kinetics of the chemiluminescence accompanying the destruction. In course of O_2-8-MOP destruction the residues of phospholipid unsaturated fatty acids are oxidized. Generation of the singlet oxygen ($^1\Delta_g$) by excited 8-MOP is observed neither in acetone, nor in ethanol. Quantum yield of $^1\Delta_g$ formation in CCl_4 is less than 3%. A pattern is proposed for 8-MOP-sensitized oxidation of unsaturated lipids proceeding without direct attack of lipids by singlet oxygen.

Psoralens combined with UV-irradiation (PUVA, 320–400 nm) are used for treatment of psoriasis and other skin diseases [1]. The photobiological effects of psoralens are believed to be based on the photoaddition reactions of psoralens to nucleic acids which do not require oxygen [1–3]. However, alongside with nonoxidative reactions some photoxidative ones are known to be sensitized by psoralens. The role of those in therapy is not quite clear. 8-Methoxypsoralen (8-MOP), for example, photoxidizes tryptophan [4, 5], unsaturated lipids [6], dioxyphenylalanine into melanine [7, 8]; 8-MOP also photoxidizes and inactivates lysozyme [9], and *E. coli* ribosomes [10]. The photoxidation was almost always supposed to proceed with participation of the singlet oxygen ($^1O_2$). Molecules of 8-MOP are known to generate the triplet excited state (after absorbing light quanta) with the energy of 262 kJ/mol [11]. The quantum yield of triplet generation in water is 0.14, that in benzene is 0.011 [12]. Lifetimes of the 8-MOP triplet state at + 20 °C in the solvents described vary in the range of 1.8 [9]–1.1 μs [12]. All these data suggest that 8-MOP is able to generate $^1O_2$. It should be noted that the energy of the 8-MOP triplet state is sufficient for generation of both known excited singlet states of oxygen designated $^1\Sigma_{g+}$ (157 kJ/mol, 762 nm) and $^1\Delta_g$ (95 kJ/mol, 1270 nm). It is the $^1\Delta_g$ state which is the most important in photoxidation reactions.

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However, the data on generation of the $^1\Delta_g$ by 8-MOP are obtained with indirect methods and thus, may be interpreted differently. For example, the rate of photosensitized 8-MOP oxidation was higher in D_2O than in H_2O [8, 9, 10]; the photoxidation was inhibited by the quenchers of $^1O_2$ – 1,4-diazabicyclo-[2,2,2]-octane [8], β-carotene [8], NaN_3 and some other [7, 10]. In the presence of air 8-MOP was observed to photosensitize nitroxide radicals formation of 2,2,6,6-tetramethylpiperidine [13].

Now there is no direct evidence for the ability of 8-MOP to generate $^1\Delta_g$ or $^1\Sigma_{g+}$. On the other hand, some data have been obtained on the capacity of 8-MOP to participate in the photoxidation reaction without direct attack of the substrate by singlet oxygen. Under UV-irradiation psoralen [14–16] and 8-MOP are oxidized. The photoxidized psoralens are more toxic than the original ones. They are capable of covalent binding with proteins in the dark [17]. They also can inactivate potato tyrozinase and succinatdehydrogenase [15], and depress the respiration of brain and liver homogenates [16].

The purposes of the present paper were: (i) to investigate the capability of 8-MOP of $^1\Delta_g$ generation by means of controlling photosensitized luminescence of oxygen from the $^1\Delta_g$ state. Such luminescence has been recently observed in solutions of various sensitizers [18–20]. (ii) to elucidate if 8-MOP is able to photosensitize lipids oxidation by some mechanism different from the direct attack of lipids by $^1O_2$. In this case 8-MOP is oxidized at first, and then, the photoproduct oxidizes lipids in the dark.

It is very important for successive PUVA-therapy to know the molecular mechanisms of the photoxidative reactions of psoralens. These reactions may be significant in the induction of PUVA-erythema and in the changes of mechanoelectric skin properties, as it follows from the experiments showing inhibition of the effects mentioned by the well-known antioxidant α-tocopherol [21].

Materials and Methods

Synthetic dipalmitoylphosphatidylcholine (DPPC) (M. M. Shemyakin Institute of Biorganic Chemistry) was used. 8-MOP was kindly supplied by Prof. G. Rodighiero (the University of Padua, Italy).

Phospholipid bulk fraction was isolated from egg yolk lecithin by the method [22] on the day of experiment. Liposomes of phospholipids (PL) (4 mg/ml) were prepared in a phosphate buffer (0.05 mol/l, pH 7.4) and three times exposed to freezethawing for comminution and to obtain a more stable suspension [23]. DPPC suspension (5 mg/ml) in phosphate buffer was obtained by means of heating to 40 °C and subsequent cooling to room temperature.

High pressure quartz mercury lamp SVD-120A was used as light source. Light was passed through a cutt-off filter BS-10 (< 350 nm), through a water filter, and focused with a quartz lens on a quartz cuvette. The suspension was irradiated for 30 min, dose at 365 nm being 47 J/cm² (actinometer Osram UVA/B Messgerät, Typ 78). 8-MOP solution in ethanol, or acetone, or benzene, or CCl_4 (10^{-3} mol/l) was placed in a quartz vacuum cuvette, cooled in liquid nitrogen, and vacuumized with forevacuum pump. Then, the vacuum tap was turned down, the cuvette was heated to room temperature and refrozen. The vacuumizing procedure was repeated three times. After air evacuation the solution was heated to room temperature and either irradiated in vacuum, or irradiated in the presence of oxygen, or, in the third case, remained unirradiated. During the irradiation the solutions were stirred with a magnetic stirrer.

Chemiluminescence was measured with the apparatus described [24]. Irradiated or unirradiated 8-MOP (0.04 ml) in the organic solvent was added to 4 ml of lipid suspension (4 mg/ml) with vigorous stirring; 0.04 ml of the solvent were added to the control samples. The chemiluminescence initiated by 8-MOP preparation was measured (Fig. 1). In 3–5 min (Table 1) 1 ml $FeSO_4$ solution (final concentration 2×10^{-3} mol/l) was added. In some cases a flash of chemiluminescence occurred as a response to introduction of Fe^{2+} ions, its intensity ($J_{Fe^{2+}}$) in our experiments was proportional to square concentration of lipid peroxides present in the suspension [25]. $J_{Fe^{2+}}$ was counted with the value of the background chemiluminescence J_b (in the absence of Fe^{2+}) considered equal to zero (Fig. 1):

$$J_{Fe^{2+}} = J_{max} - J_b .$$

$J_{Fe^{2+}}$ in suspension of PL incubated with photoxidized 8-MOP i.e. O_2-8-MOP (Table I, sample 2) was considered 100% for all our experiments.

Products of lipid peroxidation which react with 2-thiobarbituric acid (TBA) were estimated. The procedure was as follows: 1.25 ml PL liposome suspension (4 mg/ml) and 1 ml of 0.5% aqueous solution of TBA and 2 ml of 25% acetic acid were mixed (Fig. 2, Table II). Or 2 ml PL liposome suspension (2 mg/ml) and 2 ml 0.5% TBA with 1 ml of 10% trichloracetic acid were mixed (Fig. 3). The mixture was incubated 20 min at 60 °C (and also at 80 °C and 100 °C only in Fig. 3); cooled to room temperature and then absorption spectra in the range of 400–600 nm were immediately measured on a SF-14 spectrophotometer supplied with integrating sphere, optical path was 0.5 cm; the control sample contained neither 8-MOP, nor phospholipids.

Photosensitized by 8-MOP luminescence of O_2 from $^1\Delta_g$ state was measured in CCl_4, or acetone, or D_2O solutions using the apparatus with photomultipliers [19, 20].

Results and Discussion

Generation of $^1\Delta_g$ by 8-MOP was investigated in CCl_4, acetone, and D_2O by registering the 8-MOP-sensitized luminescence of $^1\Delta_g$ in these solvents. Only in CCl_4 luminescence was observed which was extremely weak, its quantum yield being at least 20 times lower than that when the luminescence is sensitized by anthracene. Since the quantum yield of the anthracene intersystem crossing is 0.7 [26], then that of $^1\Delta_g$ generation by 8-MOP in CCl_4 is lower than 3 per cent.

Chemiluminescence of O_2-8-MOP. In Fig. 1 (curve 4) it is seen that addition of O_2-8-MOP ethanol solu-

tion to water (phosphate buffer) is accompanied by appearance of chemiluminescence which decays by kinetics of the first order. The rate constant of the decay was 0.04 s^{-1}. The addition of O_2-8-MOP to liposome suspension from PL was accompanied by more intensive chemiluminescence (Fig. 1, curve 1) whose decay may be presented as a sum of two exponents of the first order with the constants 0.04 s^{-1} and 0.004 s^{-1}. 8-MOP irradiated in vacuum or unirradiated initiated by far the less intensive chemiluminescence when added to liposomes (Fig. 1, curves 2 and 3), and still less intensive chemiluminescence when added to the buffer without PL (data are not presented).

Similar results have been obtained with 8-MOP irradiated in acetone, or CCl_4, or benzene; while photoxidation of 8-MOP in water caused no effects presented in Fig. 1. O_2-8-MOP could be kept more than a month in all the organic solvents used without changes in the ability to initiate chemiluminescence when added to water or liposome suspension. Apparently, chemiluminescence appears as a result of destruction of O_2-8-MOP in water with formation of electronically excited products. The fast stage of chemiluminescence decay (0.04 s^{-1}) must correspond to the destruction going in the water phase. In the presence of liposomes a part of O_2-8-MOP is readily bound to liposome membranes. It may be assumed that the slow stage of chemiluminescence decay (0.004 s^{-1}) is the result of water-induced destruction of O_2-8-MOP bound to the membranes. Thus, O_2-8-MOP is stable in the organic solvents and is readily destructed in water.

Chemiluminescent determination of lipid hydroperoxides. Introduction of Fe^{2+} ions into suspension of lipids containing hydroperoxides is known to induce a flash of chemiluminescence, whose intensity ($J_{Fe^{2+}}$) is proportional to the square concentration of hydroperoxides [25]. This phenomenon was used for determination of lipid hydroperoxides. In Fig. 1 the kinetics of chemiluminescence after introduction of Fe^{2+} ions into the system is shown with dotted lines. Fe^{2+}-induced chemiluminescence flash occurred in suspension of egg PL with O_2-8-MOP. In the absence of PL in the phosphate buffer no flash was observed; on the contrary, Fe^{2+} ions quenched chemiluminescence accompanying O_2-8-MOP destruction. The values of $J_{Fe^{2+}}$ registered in different systems are summarized in Table I. In the experiment 1 UV-irradiation of liposomes from egg PL

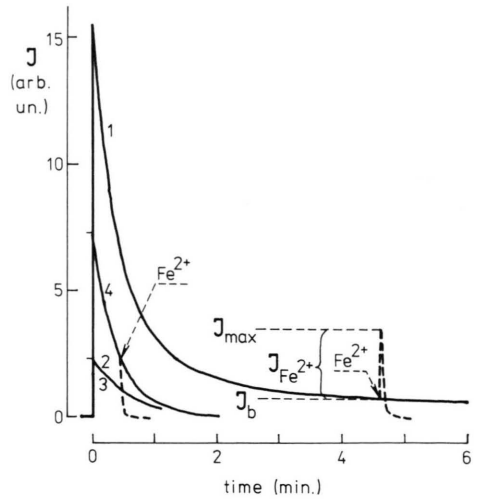

Fig. 1. Kinetics of chemiluminescence at the introduction of different samples of 8-MOP (in ethanol) into liposome suspension (1, 2, 3), or in phosphate buffer (4). 1, 4 – O_2-8-MOP, 2 – 8-MOP irradiated in vacuum, 3 – 8-MOP, final concentration in all cases was 10^{-5} mol/l. Liposomes are from egg phospholipids (4 mg/ml). Dash lines show the kinetic changes at addition of Fe^{2+}. $J_{Fe^{2+}}$ is intensity of chemiluminescence flash initiated by Fe^{2+} ions, 2×10^{-3} mol/l. J_b and J_{max} are chemiluminescence intensities before addition of Fe^{2+}, and the maximal intensity in the presence of Fe^{2+}. Abscissae: time after addition of sample of 8-MOP (min.). Ordinates: J chemiluminescence intensity (arb. units).

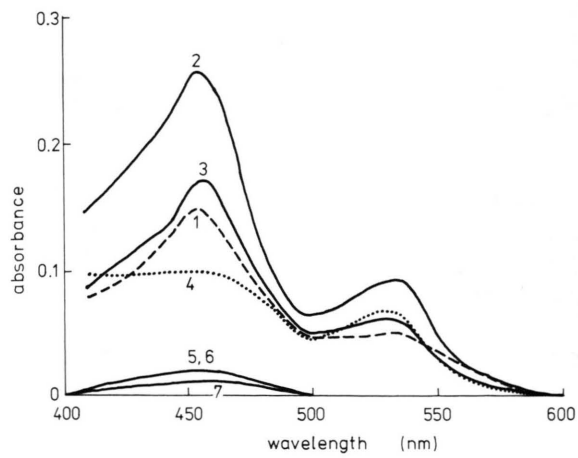

Fig. 2. Absorption spectra of liposomes from egg phospholipids treated by ethanol solutions of different 8-MOP samples after reaction with 2-thiobarbituric acid. Added to phospholipids: 1 – 1% ethanol, 2 – O_2-8-MOP, 3 – 8-MOP, 4 – 8-MOP UV-irradiated in vacuum; 5, 6, 7 – spectra of TBA products of 8-MOP, O_2-8-MOP, and 8-MOP irradiated in vacuum, respectively.

Table I. Lipid peroxidation in liposome suspension induced by different 8-MOP preparations.

Nos.	Experiment	TBA-reaction		Chemiluminescence
		$A_{454} \pm s$	$A_{533} \pm s$	$J_{Fe^{2+}} \pm s$
1.	8-MOP + PL + UV	–	–	178 ± 14 (6)
2.	O_2-8-MOP + PL	0.257 ± 0.007 (3)	0.094 ± 0.005 (3)	100 (17)
3.	8-MOP + PL	0.170 ± 0.009 (3)	0.062 ± 0.004 (3)	50 ± 3 (17)
4.	(8-MOP + UV, vacuum) + PL	0.099 ± 0.006 (3)	0.068 ± 0.002 (3)	37 ± 4 (3)
5.	PL	0.148 ± 0.010 (3)	0.050 ± 0.003 (3)	< 3 (17)
6.	PL + UV	–	–	37 ± 4 (3)
7.	DPPC	–	–	< 3 (2)
8.	8-MOP + DPPC	–	–	< 3 (2)
9.	O_2-8-MOP + DPPC	–	–	< 3 (2)

Comments: 8-MOP, O_2-8-MOP, (8-MOP + UV, vacuum) are ethanol solutions of 8-methoxypsoralen unirradiated, UV-irradiated in the presence of air, and UV-irradiated in vacuum, respectively. PL and DPPC are liposomes of egg yolk phospholipids (4 mg/ml) and dipalmitoylphosphatidylcholine suspension (5 mg/ml) in phosphate buffer, respectively. A_{454} and A_{533} are absorbance values at 454 nm and 533 nm. Figures in brackets designate number of experiments.

was carried out in the presence of 8-MOP. In experiment 2 photoxidized 8-MOP is added to PL. In experiments 3 and 4 unirradiated 8-MOP or 8-MOP irradiated in vacuum is added, respectively. In experiment 5 neither 8-MOP nor UV-irradiation is applied to PL, in 6 PL are exposed to UV-irradiation only without 8-MOP. A comparison of experiments 1 and 6 shows that 8-MOP photosensitizes lipid oxidation, this confirms the results of our previous paper [6]. It follows from experiments 2–5 that O_2-8-MOP leads to the oxidation of PL, *i.e.*, it exerts an effect similar to the combined action of 8-MOP and UV-irradiation. It should be noted that conditions of the photochemical reactions of 8-MOP in tests 1 and 2 are different – in the first case under UV-treatment 8-MOP is contained in water suspension of liposomes, in the second it is in ethanol solution. Therefore, one may compare these two tests only qualitatively.

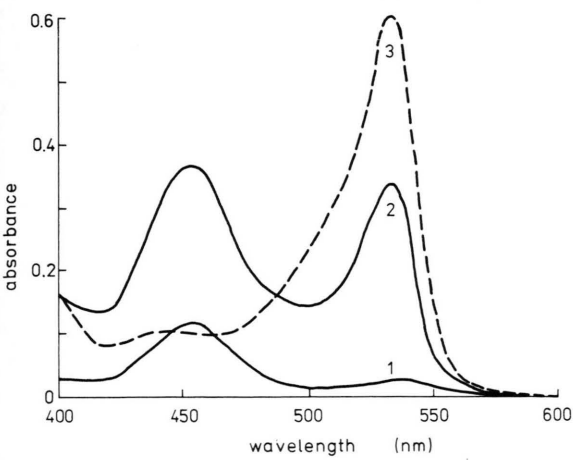

Fig. 3. Absorption spectra of TBA products of liposomes from egg phospholipids. Incubation time with TBA was 20 min at: 1 – 60 °C, 2 – 80 °C, 3 – 100 °C.

Unirradiated or irradiated in vacuum 8-MOP led to 2 or 2.5 times less intensive flashes ($J_{Fe^{2+}}$) than photoxidized 8-MOP. When liposomes from saturated lipids (DPPC) were used no chemiluminescence flash was observed (experiments 7–9).

Formation of TBA-products. In the absorption spectrum of PL liposome suspension after TBA-reaction two maxima appeared (Fig. 2, curve 1). The maximum near 533 nm is believed to belong to the TBA interaction with malonic aldehyde at heating [27]. The maximum near 454 nm appears when TBA is heated to 60–80 °C with various long-chain aldehydes. This chromophore is destructed at boiling [28]. In our experiments the maximum at 454 nm as well as in [28], was pronounced at 60 and 80 °C and disappeared at boiling (Fig. 3). Perhaps, in our experiments its formation involves long-chain aldehydes which are the products of oxidation of PL unsaturated fatty acids residues.

The experiments summarized in Table I confirm the chemiluminescence data. It is seen that photoxidized 8-MOP leads to formation of the biggest amount of TBA products. The proxidant effect of unirradiated 8-MOP or of 8-MOP irradiated in vacuum is considerably less pronounced. It is peculiar that 8-MOP irradiated in vacuum leads to a

partial destruction of long-chain aldehydes (A_{454} decreases). A small proxidant effect of unirradiated 8-MOP may be accounted for by the impurity of the oxidized 8-MOP.

Analysing the data obtained in the present paper one may propose the following scheme of 8-MOP-photosensitized oxidation of unsaturated lipids without direct action of the singlet oxygen on the lipids: at first, under UV-irradiation photoxidation of 8-MOP occurs. At the second stage in the dark the residues of PL unsaturated fatty acids are oxidized by O_2-8-MOP. In course of the second stage hydroperoxides, malonic aldehyde and other aldehydes are formed.

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