PROTECTION OF CHLOROPHYLL BY PHOSPHOLIPIDS FROM PHOTOOXIDATION

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SUMMARY: Rapid photooxidation of chlorophyll in chloroform was shown to be partly inhibited by various biologically significant compounds including β-carotene, xanthophyll and several synthetic and natural phospholipids. Protection from bleaching by phospholipids was most evident for the phosphatidyl cholines and was less for the phosphatidyl ethanolamines. Protection was independent of the chain length and unsaturation of the esterified fatty acids and depended primarily on the nature of the polar head group of the phospholipids.

The irreversible photooxidation (bleaching) of chlorophyll has been known for many years. Bleaching has been studied in organic solvents (1,2,3), quantasomes (4), chloroplasts (5), and recently in liposomes (6). The lability of chlorophyll to photooxidation has been demonstrated to be very dependent on the environment that the chlorophyll is in; the order of chlorophyll lability being: in vivo < quantasomes < liposomes < organic solvents (6). Stanier (7,8,9) and others (10) have shown that the irreversible photooxidation of chlorophyll could be slowed greatly by the presence of carotenoids both in vivo and in vitro. The role of carotenoids as protecting agents for the photooxidation of chlorophyll has been proposed for all photosynthetic organisms from bacteria (8) and algae (9) to higher plants (10).

Undoubtedly part of the reason chlorophyll is more stable in vivo than in organic solvents is due to the presence of carotenoids properly arranged in the membrane. However, it seemed possible that other lipid-soluble components might also be playing an active role in protecting chlorophyll from oxidative photobleaching. It was the purpose of this study to investigate the effect of various phospholipids and other lipid-soluble biological materials on the photooxidation of chlorophyll.

MATERIALS AND METHODS: Chlorophyll was extracted from fresh spinach by the method previously described (11). The amount of chlorophyll was determined as reported by (Mackinney (12)). Most of the phospholipids were purchased from Sigma Chemical Co., St. Louis, MO (L-α-lecithin, type II-s from soy beans;

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L-α-phosphatidyl choline, type IX-E from egg yolk; L-α-phosphatidyl ethanolamine, type II from bovine brain; brain extract, from bovine brain; L-α-phosphatidyl choline, type III-E from egg yolk; and the synthetic phospholipids D,L-α- phosphatidyl choline dipalmitoyl; L-α-phosphatidyl choline dioleoyl; L-α-phosphatidyl choline distearoyl; and L-α-phosphatidyl choline dimyristoyl). D,L-α-cephalin was purchased from Nutritional Biochemicals, Cleveland, OH; vegetable lecithin from Eastman Organic, Rochester, NY; and asolectin (purified by the method of Kagawa and Racker (13)), Associated Concentrates, Woodside, NY. Of the organic molecules tested for their ability to prevent the photooxidation of chlorophyll; K & K Laboratories, Plainview, NY supplied the glyceryl monooleate, glyceryl dioleate, glyceryl monoricinoleate, glyceryl monostearate, glyceryl distearate and cholesterol; Nutritional Biochemicals, the phytol and xanthophyll and Eastman, the 2,5 ditert.-butyl hydroquinone, tert-butyl hydroquinone and squalene. β-Carotene (ICN Pharamacuticals, Cleveland, OH), D,L-α-tocopherol (Sigma) and Benzoquinone (Aldrich, purified by sublimation) were also purchased.

Stock solutions (7.85 mM) of the organic materials to be tested were made fresh in spectral grade chloroform (Fischer Scientific Co., Fairlawn, NJ). The chlorophyll stock contained 17.5 mg of chlorophyll per 10 ml of chloroform. In the dark, various quantities of the organic stock was mixed with 0.7 ml of the chlorophyll stock and this was diluted to 6.0 ml with chloroform. The samples initially all had an absorbance of 1.0. The samples were placed in Bausch and Lomb Spectronic 20 Colorimeter Test Tubes and were illuminated through water heat filters with a 500 Watt Sylvania CZX-DAB Projection Lamp at 35 cm and 2 Sylvania F4T5/D Florescent Lamps at 20 cm. Each experiment was illuminated adjacent to a control tube containing only chlorophyll in chloroform. The samples were illuminated for one minute time intervals after which the absorbance at 663 nM was measured. The initial rate of photodestruction of chlorophyll was determined and this was plotted against the amount of organic material in solution.

RESULTS: The rate of photooxidation of chlorophyll in chloroform was measured spectrophotometrically. The initial rate of chlorophyll degradation (determined from the average of over 100 identical experiments) was 12.3% of the initial chlorophyll oxidized per minute. In these experiment, this amounted to 0.15 mg of chlorophyll oxidized/minute. Several quantities of various biologically significant organic materials were added to the chlorophyll solution and the rate of photodegradation was measured compared to the control experiment (with no added material). Photooxidation of identical chlorophyll solutions was measured in the presence of 0, 0.079, 0.393, 0.785, 1.57, 4.71 and 15.7 μ moles of added material. These mixtures gave material/chlorophyll mole ratios of 0, 0.06, 0.28, 0.58, 1.15, 3.44 and 11.46, respectively. Several compounds were shown to have no effect on the rate of photooxidation of chlorophyll even at the highest concentrations used (15.7μ moles/6.0 ml of illuminated chlorophyll solution with a material/chlorophyll ratio of 11.46). These ineffective compounds included glyceryl monooleate, glyceryl dioleate, glyceryl monoricinoleate,

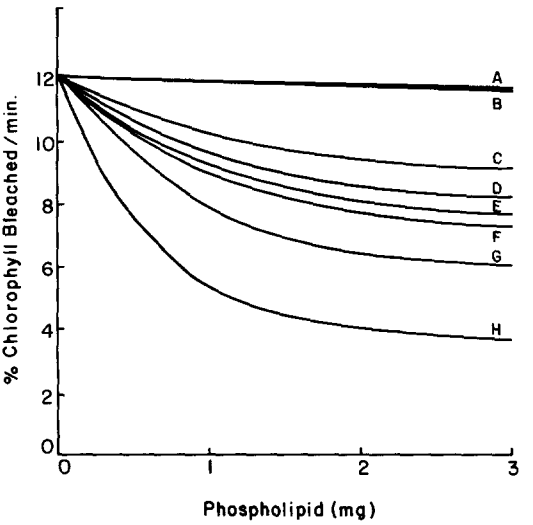

Figure 1. Effect of phospholipids extracted from biological sources on preventing the photooxidation of chlorophyll. A) D,L-α-cephalin, B) brain extract, from bovine brain, C) L-α-phosphatidyl ethanolamine, type II from bovine brain, D) L-α-lecithin, from soy beans, E) asolectin, F) lecithin, vegetable, G) L-α-phosphatidyl choline, type IX-E from egg yolks, and H) L-α-phosphatidyl choline, type III-E from egg yolks.

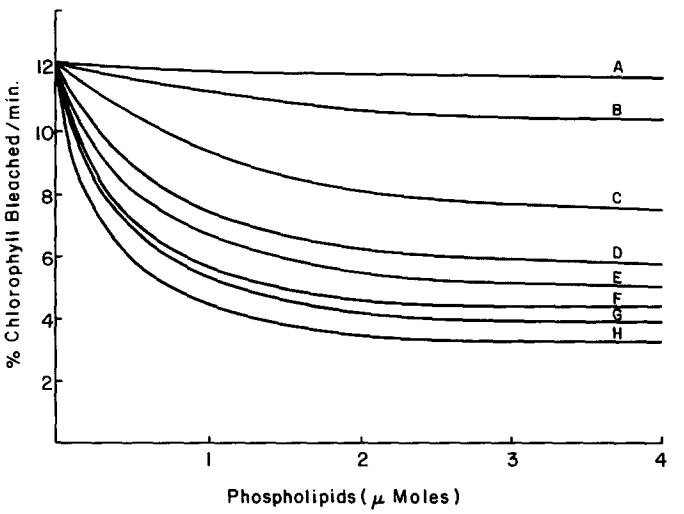

Figure 2. Effect of phospholipids, both synthetic and natural, on preventing the oxidation of chlorophyll. A) D, L-α-cephalin, B) L-α-phosphatidyl ethanolamine, type II from bovine brain, C) asolectin, D) L-α-phosphatidyl choline, type IX-E from egg yolks, E) L-α-phosphatidyl choline dimyristoyl, F) L-α-phosphatidyl choline distearyl, G) L-α-phosphatidyl choline dioleoyl, and H) D,L-α-phosphatidyl choline dipalmitoyl.

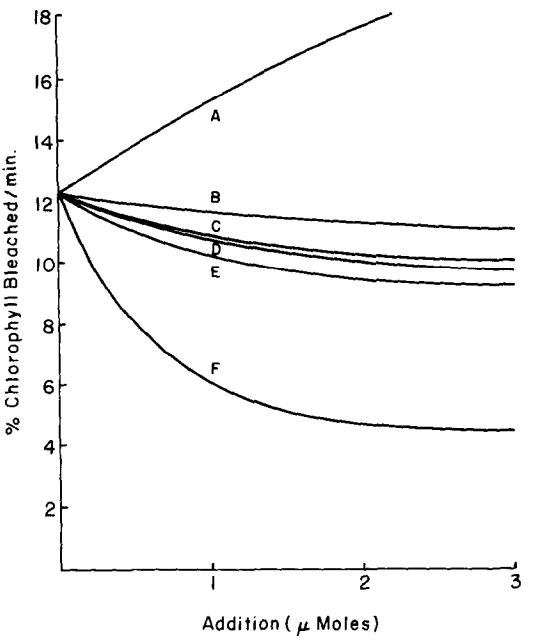

Figure 3. Effect of various agents on the preventing the photooxidation of chlorophyll. A) acetic acid, B) α-tocopherol, C) benzoquinone, D) pyridine, E) xanthophyll, and F) β-carotene.

glyceryl monostearate, glyceryl distearate, phytol, squalene and cholesterol. The rate of photodestruction of chlorophyll was shown to be slightly decreased (less than 10%) by 15.7μ moles of hydroquinone, mono-tert.-butyl hydroquinone and ditert.-butyl hydroquinone. Several compounds were shown to greatly inhibit the rate of photooxidation, even in the lower concentration ranges tested. These compounds included various phospholipids extracted from biological materials and often used in planar bimolecular lipid membrane and liposome studies (Figure 1) as well as synthetic phospholipids (Figure 2) and some other important lipid-soluble components (Figure 3).

DISCUSSION: Chlorophyll was shown to readily photooxidize in aerobic chloroform solution. As had been shown by Stanier (7,8,9) and Anderson (10) β-carotene in small quantities substantially reduces the rate of degradation of chlorophyll (Figure 3). However, other biological compounds were shown to be as

effective as β-carotene in protecting chlorophyll from the ravages of light and air. All the phospholipids tested provided chlorophyll with some protection. For effectivness in protection of chlorophyll from photooxidation, the following sequence of phospholipids extracted from biological materials was obtained (Figure 1): L-α-phosphatidyl choline (H), type III-E from egg > L-α-phosphatidyl choline (G), type IX-E from eggs > (vegetable) lecithin (F) > asolectin (E) > L-α-lecithin (D), from soy beans type II-s > L-α-phosphatidyl ethanolamine, type II from bovine brain (C) > brain extract, from bovine brain (B) > D,L-α-cephalin (A). The phosphatidyl cholines, (G) and (H), were best at protection, while the compounds high in phosphatidyl ethanolamine, (A), (B), and (C), were the poorest. The three vegetable lecithin extracts, (D), (E), and (F), which have approximately equivilant amounts of phosphatidyl choline and phosphatidyl ethanolamine (14), were intermediate. Since glyceryl monooleate, glyceryl dioleate, glyceryl monoricinoleate, glyceryl monostearate and glyceryl distearate were totally ineffective in preventing the photooxidation of chlorophyll, it appears that the polar head group is a necessary requirement for protection. From the results reported in Figures 1 and 2, phosphatidyl cholines are considerably more effective than phosphatidyl ethanolamines. Degradation rates in the presence of the four synthetic phospholipids; L-α-phosphatidyl choline distearoyl, D,L,-α-phosphatidyl choline dipalmitoyl, L-α-phosphatidyl choline dimyristoyl and L-α-phosphatidyl choline dioleoyl, demonstrated that chain length and unsaturation of the esterified fatty acids was not as significant in contributing to the protecting properties as was the nature of the polar head group. The position and arrangement of the porphrin ring of the chlrophyll molecule in bilayer lipid membranes have been considered (11,15). Recently, Oettmeier et al. (16) have shown that in D, L-α-dipalmitoyl phosphatidyl choline-chlorophyll lipisomes, the porphyrin ring sits in the polar head region of the membrane. Therefore, in the membrane proper, chlorophyll is in an environment (adjacent to the polar head groups of phospholipids) ideally suited for maximum protection from photooxidation. Perhaps the location of the porphyrin ring of chlorophyll

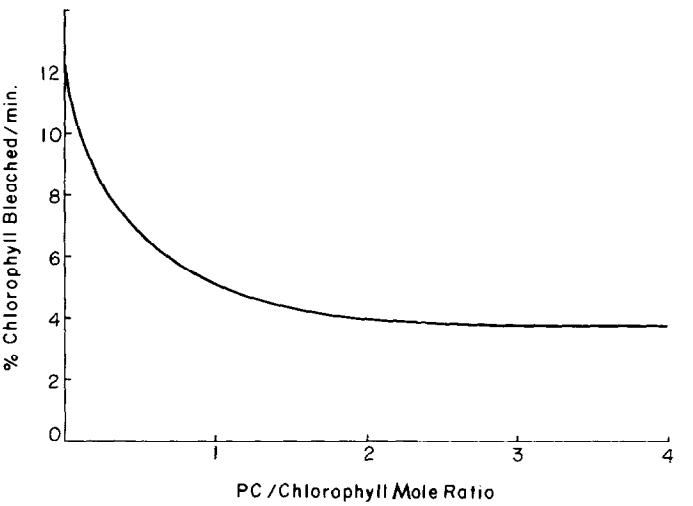

Figure 4. The aerobic photodegradation of chlorophyll as a function of the L-α-phosphatidyl choline, type III-E/chlorophyll mole ratio.

in the lipid bilayer is partly responsible for the marked increase in stability of chlorophyll toward photooxidation in chloroplasts and quantasomes as opposed to in free solution.

Other compounds were also added to the solutions to test for their ability to protect chlorophyll from photooxidation. These results are presented in Figure 3. Chlorophyll was very unstable and degradation was rapid when photooxidized in the presence of acetic acid. Acid lability (resulting in pheophytins) has been known for a long time. Some protection was afforded by the base-pyridine, the antioxidant-α-tocopherol and benzoquinone (a compound known to prevent the photooxidation of bacteriochlorophyll (2,6)). Xanthophyll was also shown to signiciantly slow the rate of photobleaching, however, it was only about one fifth as effective as the classic protecting agent, β-carotene (Figure 3).

Recently several papers have appeared on incorporation of chlorophyll into liposomes in an attempt to model the thylakoid membrane (15-18). Chlorophyll was demonstrated to be much more resistant to photooxidation in liposomes than

in free solution (6). This increased stability could be understood in terms of the protection role of phospholipids to the photooxidation of chlorophyll as outlined here. In Figure 4 the rate of degrdation of chlorophyll in chloroform is plotted against the L-α-phosphatidyl choline, type III-E/chlorophyll ratio. In free solution phosphatidyl choline reaches its maximum protecting efficiency by the time the phosphatidyl choline/chlorophyll mole ratio reaches about 1. Additional phosphatidyl choline does not result in a substantial further increase in protection. Chlorophyll-containing liposomes can not accommodate more than about one molecule of chlorophyll per 30 molecules of phospholipid. Therefore, in liposomes, the phospholipid/chlorophyll ratio is well into the maximum protection range for chlorophyll photooxidation.

The great increase in stability toward photooxidation of chlorophyll in vivo as opposed to that in free solution appears to be the result of proper orientation of chlorophyll in the phospholipid bilayer as well as the immediate presence of such hyrophobic molecules as β-carotene and xanthophyll. Additional stability might be provided be incorporation of appropriate lipoproteins as well as water soluble proteins. Such studies are currently in progress.

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