Plant Physiol. (1993) 102: 213–218

Modification of Phospholipid Catabolism in Microsomal Membranes of γ-Irradiated Cauliflower (*Brassica oleracea* L.)[1]

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Acceleration of membrane deterioration has been observed recently during storage of γ-irradiated cauliflower (*Brassica oleracea* L., Botrytis group). In the present study, the activity of microsome-associated lipolytic enzymes was investigated in cauliflower florets exposed to 0 or 4 kilograys of γ radiation and stored for 8 d at 13°C. Radiolabeled breakdown products obtained from the metabolism of (16:0/18:2*)-phosphatidylcholine and (16:0/16:0)-phosphatidyl-[*N*-methyl-^{3}H]choline by microsomal membranes indicated that phospholipase D (EC 3.1.4.4), phosphatidic acid phosphatase (EC 3.1.3.4), and lipolytic acyl hydrolase were associated with the membranes. The rate of phosphatidylcholine catabolism by the membranes increased slowly in control cauliflower during storage. γ irradiation caused an immediate rise in phosphatidylcholine catabolism that remained higher than that of the controls during subsequent storage. Collectively, the data suggest that enhancement of membrane lipolytic activity results from free-radical-induced stress. Rapid increase of the membrane-associated phospholipase D activity may be a key event leading to accelerated membrane deterioration following γ irradiation.

Ionizing energy can be used as a postharvest treatment to delay ripening or senescence of plant foods. However, the response and tolerance of produce to γ radiation vary considerably. Undesirable side effects, e.g. softening or enzymic browning of tissues, are frequent (Kader, 1986), and the mechanisms underlying these physiological effects remain poorly understood (Urbain, 1986). γ irradiation accelerated the degradation of membrane phospholipids in cauliflower (*Brassica oleracea* L.) florets during subsequent storage (Voisine et al., 1991). Membrane deterioration was suggested to be induced by chemical deesterification of phospholipids by free radicals generated during irradiation, which in turn triggered further enzymic breakdown of membrane lipids during storage.

Progressive decline in membrane phospholipid content is a common characteristic of membrane deterioration in senescing plant tissues, e.g. bean leaves (McKersie et al., 1978), carnation (Thompson et al., 1982), and rose petals (Borochov et al., 1978). Decreased synthesis of phospholipids, increased phospholipid catabolism, or both have been proposed to be responsible for the process (Suttle and Kende, 1980; Borochov et al., 1982). Phospholipase D, PA phosphatase, and lipolytic acyl hydrolase were associated with microsomal membranes of carnation flowers (Paliyath et al., 1987; Brown et al., 1990), bean cotyledons (Paliyath and Thompson, 1987), and tomato fruits (Todd et al., 1990). However, declining microsomal phospholipid content in senescing carnation flowers was not correlated with increased membrane-associated phospholipase activity (Brown et al., 1991). Degradation of exogenous radiolabeled phospholipids by microsomal membranes was not affected in *Tradescantia* petals (Suttle and Kende, 1980) or in carnation flowers during senescence (Paliyath et al., 1987).

Voisine et al. (1991) suggested enzyme involvement in membrane degradation after irradiation. To better understand the mechanisms responsible for the accelerated membrane degradation, the present study identified the lipolytic enzymes associated with cauliflower membranes and determined their activity after γ irradiation. Free radicals produced by the radiation rapidly stimulated membrane lipolytic activity.

MATERIALS AND METHODS

Irradiation and Storage

Cauliflower heads (*Brassica oleracea* L., Botrytis group) were obtained from a local market. Florets were washed in cold 0.1% hypochlorite (w/v), rinsed, and held overnight in 2-L plastic baskets at 1°C under a water-saturated air flow. The florets were irradiated at ambient temperature in a cobalt-60 γ irradiator (Gammacell 220; Atomic Energy of

[1] This work was supported in part by a grant from the Natural Sciences and Engineering Research Council of Canada to C.W. R.V. was supported by a graduate fellowship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche, Quebec. Contribution No. 280 from the Food Research and Development Center, Agriculture Canada Saint-Hyacinthe, and No. 455 from the Research Station, Agriculture Canada, Sainte-Foy. Publication No. 76 from the Centre de Recherches en Horticulture, Université Laval.

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Abbreviations: DG, diacylglycerol; FFA, free fatty acids; kGy, kilogray; OP, oxidation products; PA, phosphatidic acid; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acids.

Canada Ltd., Kanata, Ontario, Canada) at a dose of 4 kGy. Unirradiated controls were held at ambient temperature during the treatment. Control and irradiated florets were stored in the dark at 13°C under a water-saturated air flow. Analyses were carried out within 60 min after irradiation (day 0) and after 4 and 8 d of storage.

Isolation of Membranes

Cauliflower inflorescence, approximately 22 g, was homogenized with an Ultra-Turrax apparatus (Janke and Kunkel GmbH, Hohenstaufen, Germany) in 30 mL of ice-cold extraction buffer (50 mM Mops-KOH, pH 7.2, 0.6% insoluble PVP [w/v], 10 mM EGTA, and 7% [w/v] Suc). The homogenate was filtered through a single layer of Miracloth and centrifuged at 10,000*g* for 30 min. The supernatant was centrifuged at 141,000*g* for 60 min. The pellet was resuspended in wash buffer (50 mM Mops-KOH, pH 7.2, 2 mM EGTA, and 7% Suc) to prevent adhesion of cytosolic contaminants, and again centrifuged at 141,000*g* for 60 min. The resulting pellet was used as a source of microsomal membranes. Some microsomal pellets were partly solubilized by resuspension in wash buffer containing 0.35% (w/v) Triton X-100, agitated for 20 min on ice, and centrifuged at 141,000*g* for 60 min. All the microsomal pellets were finally resuspended in 1 mL of a buffer containing 0.2 mM EGTA, 150 mM KCl, 1 mM $MgCl_2$, and 50 mM Hepes, pH 7.0. Protein was measured by the method of Bradford (1976) using BSA as standard.

Phospholipid Degradation

Phospholipid catabolism was measured by a method adapted from Todd et al. (1990) using labeled (16:0/18:2*)-PC (L-3-phosphatidylcholine, 1-palmitoyl-2-[1-^{14}C]linoleoyl; 2.02 GBq/mmol, Amersham) as substrate. The reaction mixture contained 3.7 kBq of radiolabeled substrate, 40 μL of membrane suspension (75–150 mg of protein), 0.2 mM EGTA, 0.250 mM $CaCl_2$, 150 mM KCl, 1 mM $MgCl_2$, 0.1% of Triton X-100 (w/v), and 50 mM Hepes, pH 7.0, in a final volume of 500 μL. The reaction was carried out for 60 min at 30°C and stopped by addition of 100 μL of 4 N HCl. Lipids were extracted by the procedure of Bligh and Dyer (1959). A 700-mL sample of the chloroform phase was evaporated under nitrogen, dissolved in 20 μL of chloroform:methanol (2:1, v/v), and spotted on a 250-μm silica gel G plate (Analtech, Newark, DE). Authentic standards (PC, PA, FFA, DG), and ether-extracted FFA peroxides obtained from peroxidation of linoleic acid by soybean lipoxygenase (Brown et al., 1990) were run on parallel lanes. The plates were developed halfway in chloroform:acetic acid:methanol:water (70:25:5:2, v/v; Todd et al., 1990), dried under nitrogen, and fully developed in hexane:diethyl ether:acetic acid (70:30:1, v/v). After drying under nitrogen, the lipids were briefly visualized with iodine vapor and identified by comparison with the standards. Iodine was evaporated under nitrogen and the plates were autoradiographed for 6 d. The radioactive spots were scraped into 8 mL of scintillation cocktail (Ready Protein, Beckman) and assayed.

Phospholipase D, lipolytic acyl hydrolase, and phospholipase C activities were assessed by measuring the release of radiolabeled choline, glycerophosphorylcholine, and phosphorylcholine from (16:0/16:0)-PC* (L-3-phosphatidyl [*N*-methyl-^{3}H]choline, 1,2-dipalmitoyl; 3.0 TBq/mmol, Amersham) incubated with the membranes at 30°C. One hundred microliters of membrane suspension (185–375 mg of protein) was mixed with an assay buffer containing 37 kBq of (16:0/16:0)-PC* to obtain a final volume of 800 μL and a final reaction mixture similar to that described above. The reaction was stopped after 60 min with 200 μL of 4 N HCl. A 500-μL sample from the aqueous phase obtained after extraction (Bligh and Dyer, 1959) was mixed with 10 mL of scintillation cocktail (Ready Protein, Beckman) and assayed. The remaining aqueous fraction was dried under nitrogen, resuspended in 1 mL of deionized water, and neutralized by adding NaOH. The proportions of labeled choline, phosphorylcholine, and glycerophosphorylcholine in the aqueous fraction were evaluated after separation on an anion-exchange column (Paliyath et al., 1987).

RESULTS

Cauliflower Microsomal Enzymes Metabolizing Phospholipids

Four labeled breakdown products, PA, DG, FFA, and OP, were detected following the incubation of microsomal membranes with (16:0/18:2*)-PC. PA, liberated by the action of phospholipase D, was the major breakdown product and progressively accumulated during the 60-min incubation (Fig. 1). DG, which can be produced from metabolism of PA by PA phosphatase and from PC by phospholipase C, increased during the first 40 min of incubation and remained at 0.8% of the total radioactivity (Fig. 1). FFA, produced by lipolytic acyl hydrolase, represented 1% of the total radioactivity after 10 min and remained at that level. OP accumulated progres-

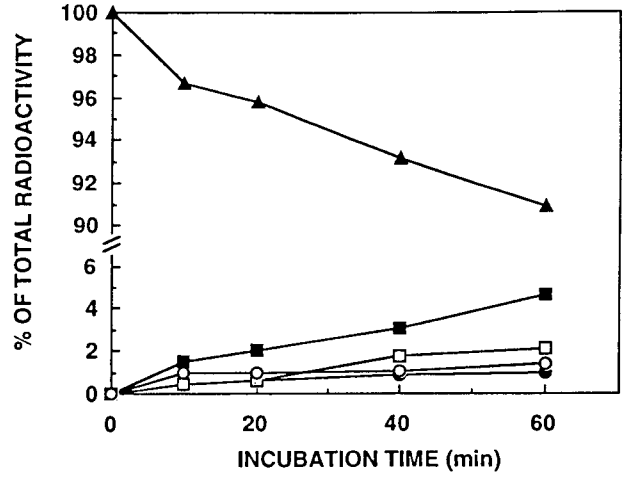

Figure 1. Time course for the metabolism of radiolabeled (16:0/18:2*)-PC by microsomal membranes from cauliflower. Results are from one of three experiments showing the same trend and are expressed as percent of total radioactivity in scraped TLC bands. ■, PA; □, OP; ●, DG; O, FFA; ▲, PC.

sively above the level of FFA and DG and reached 2% of the total radioactivity after 60 min.

Radiolabeled water-soluble metabolites accumulated during incubation of (16:0/16:0)-PC* with microsomal membranes (Fig. 2). Labeled phosphorylcholine and glycerophosphorylcholine were not detected in the aqueous fraction and only labeled choline was liberated from (16:0/16:0)-PC* (not shown). Therefore, the water-soluble radioactivity was associated with phospholipase D activity.

It is noteworthy that OP migrated on the TLC plate below the peroxidized FFA used as reference (not shown), suggesting that OP resulted from further rapid, probably enzymic, degradation of fatty acid hydroperoxides. OP were not formed by incubation of (16:0/18:2*)-PC with heat-inactivated membranes. Addition of 2 mM propyl gallate, a lipoxygenase inhibitor, reduced the production of OP by 80%. Further indication of a rapid enzymic conversion of peroxidized FFA to OP was obtained by promoting the liberation of labeled linoleic acid from (16:0/18:2*)-PC during membrane incubation. The addition of exogenous phospholipase A_2 to incubated membranes led to increased PC degradation, large OP accumulation, and a slight enhancement of FFA labeling. No FFA hydroperoxides were produced (not shown). Addition of exogenous soybean lipoxygenase to the incubated membranes yielded fatty acid hydroperoxides, but many of the other labeled metabolites were different from those observed after incubation of the membranes without added lipoxygenase.

Phospholipid Catabolism by Membranes from Control and Irradiated Cauliflower

The rate of degradation of (16:0/18:2*)-PC by microsomal membranes was accelerated after storage of control cauliflower florets (Fig. 3), despite some loss of membrane protein (Fig. 4). Degradation of PC increased by 50% over 8 d, from 8.7% at day 0 to 13.1% at day 8. The liberation of choline from (16:0/16:0)-PC* by phospholipase D was increased by 35% within the same time (Fig. 5).

The enhanced catabolism of (16:0/18:2*)-PC after storage yielded only a slight increase in labeled PA but a large accumulation of OP (Fig. 6A). Production of FFA remained constant, whereas DG levels slightly decreased. The contribution of the intermediate metabolites PA, DG, and FFA to the total radioactivity of the breakdown products was reduced from 75 to 57% after 8 d of storage. Further catabolism of PC fatty acids to OP was apparently facilitated, since OP contribution to the pool of breakdown products increased from 25 to 43% after storage.

Although membranes extracted from florets immediately after γ irradiation had 20% less protein than those from nonirradiated controls (Fig. 4), they metabolized PC on day

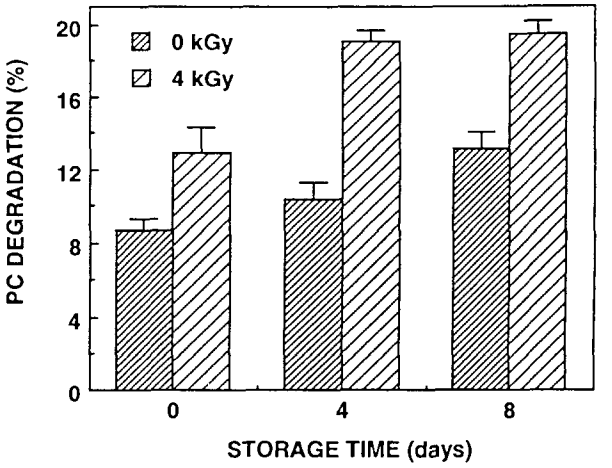

Figure 3. Degradation of radiolabeled (16:0/18:2*)-PC by microsomal membranes from control and irradiated cauliflower during storage at 13°C. The reaction was allowed to proceed for 60 min. Means ± SE for three experiments.

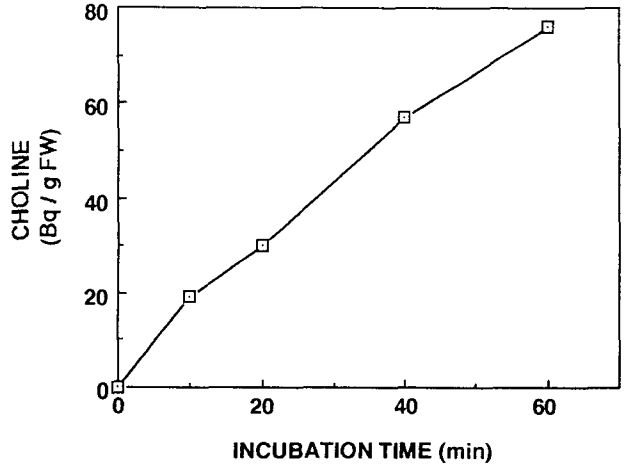

Figure 2. Time course for the release of radiolabeled water-soluble products from (16:0/16:0)-PC* by microsomal membranes from cauliflower. Results are from one of three experiments showing the same trend.

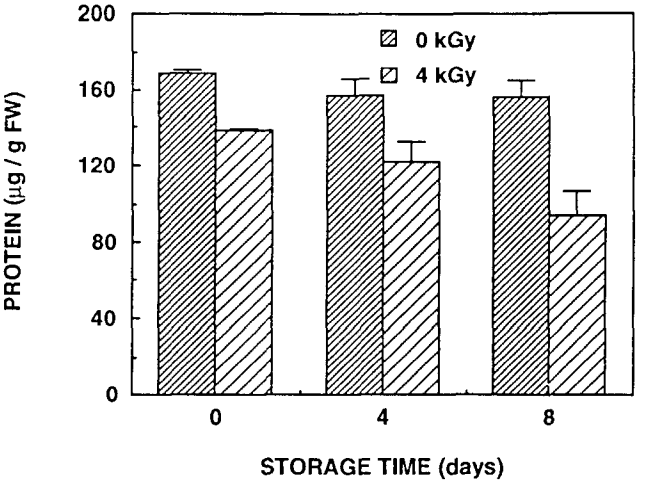

Figure 4. Protein content of microsomal membranes from control and irradiated cauliflower during storage at 13°C. Means ± SE for three separate experiments.

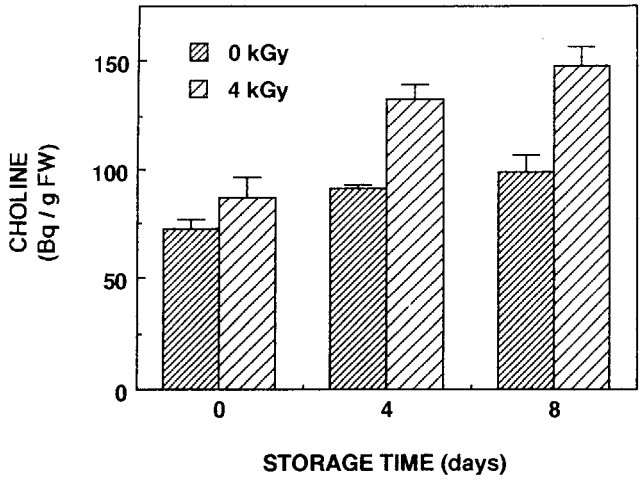

Figure 5. Liberation of radiolabeled water-soluble products from (16:0/16:0)-PC* by microsomal membranes from control and irradiated cauliflower during storage at 13°C. Only choline was detected in the aqueous fraction. The reaction was allowed to proceed for 60 min. Means ± SE for three separate experiments.

0 at a level comparable to that of membranes from control cauliflower stored for 8 d (Fig. 3). During the 8 d of storage, continued loss of protein occurred and reached approximately 40% of the initial membrane protein content of the control. Notwithstanding this extensive protein loss, the rate of PC catabolism was further increased after storage of irradiated florets and reached 19% over the 60-min incubation after 4 d, twice the initial value of the control. Concomitantly, release of labeled choline by microsomal phospholipase D was increased immediately after irradiation of the florets and was further increased after 4 or 8 d of storage (Fig. 5).

The profile of the breakdown products provided additional evidence of the modification of PC catabolism in irradiated florets (Fig. 6B). In freshly irradiated tissue, the profile was similar to that of control florets stored for 8 d. After 4 d of storage, more PA, FFA, and OP and less DG were produced. Thereafter, PA increased and OP slightly decreased.

Association of Lipolytic Enzymes with Microsomal Membranes

In the above experiments, microsomal membranes were routinely washed to remove cytosolic contaminants. To estimate the degree of association of the lipolytic enzymes with the membranes, some microsomal pellets were partly solubilized with Triton X-100, 0.35% (w/v) (Paliyath and Thompson, 1987; Todd et al., 1990). The treatment depleted protein by 53 and 58% in membranes from control and irradiated florets stored for 4 d, respectively (not shown). However, the membranes retained the ability to metabolize PC into PA, DG, FFA, and OP (Fig. 7). The production of PA from PC by the detergent-treated membranes increased 2.5- and 2.2-fold on a protein basis for control and irradiated florets, respectively. Total activity in the membranes increased by 17% or decreased by 8% in the membranes, for control and irradiated florets, respectively, after treatment with Triton X-100, confirming the resistance of the enzyme to solubilization. The accumulation of PA with little OP production indicated that metabolism of PA was considerably reduced. The specific activity of phospholipase D from Triton X-100-treated membranes, measured from the liberation of labeled choline, increased by 24 and 8% for control and irradiated florets, respectively (not shown). These data correspond closely to the amount of PC degraded (i.e. the sum of its degradation products: PA + DG + FFA + OP) per mg of protein, 30 and 9% for control and irradiated tissue, respectively. PC catabolism by phospholipase D, whether measured from the release of PA or free choline, still remained higher in detergent-treated microsomes from irradiated florets than in those from controls.

DISCUSSION

The breakdown products yielded from the degradation of exogenous PC by cauliflower microsomal membranes show the involvement of phospholipase D, PA phosphatase, and lipolytic acyl hydrolase. The same enzymes were previously reported in microsomal membranes from bean cotyledons,

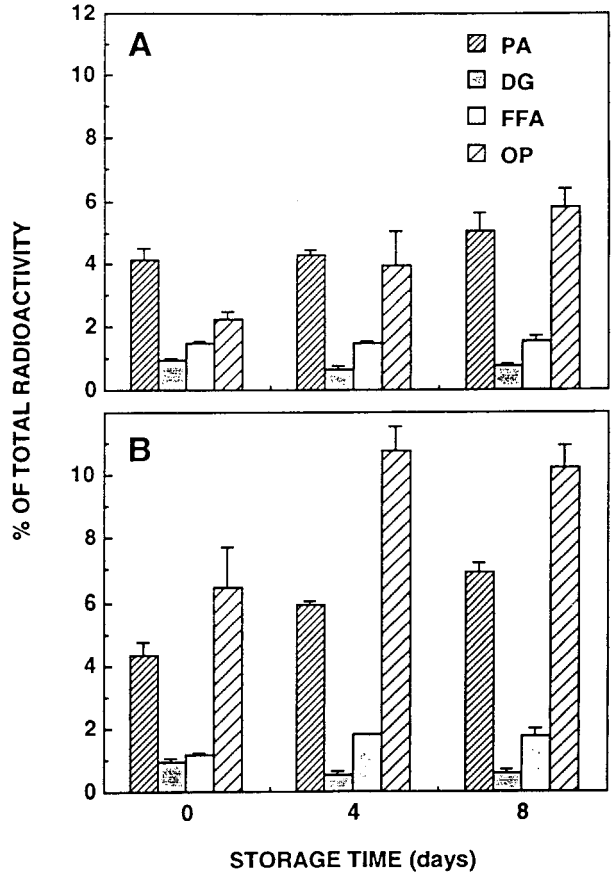

Figure 6. Radiolabeled products derived from the catabolism of (16:0/18:2*)-PC by microsomal membranes from control (A) and 4 kGy-irradiated cauliflower (B) stored at 13°C. The reaction was allowed to proceed for 60 min. Means ± SE for three separate experiments. Results are expressed as percent of total radioactivity in scraped TLC bands.

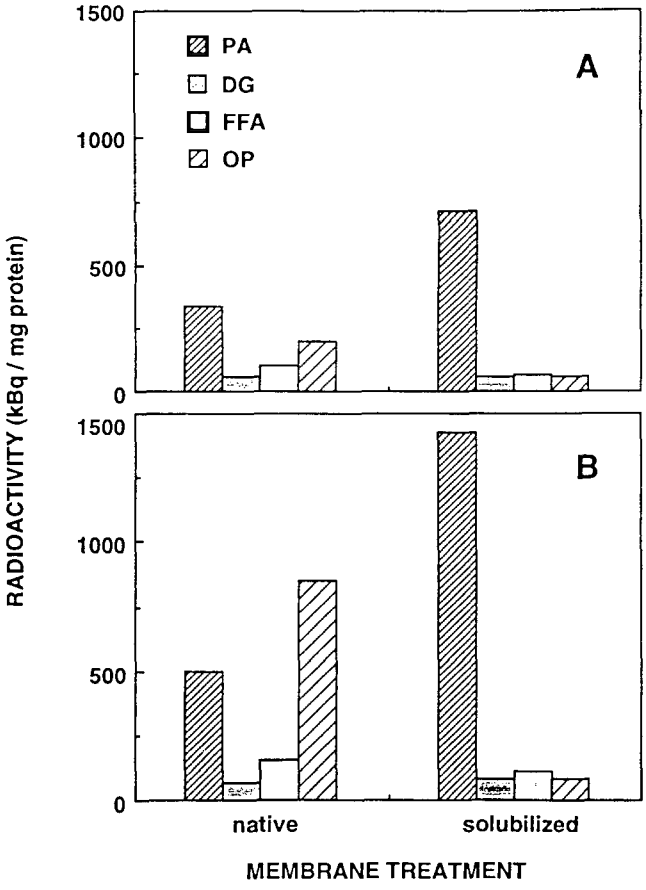

Figure 7. Effect of membrane partial solubilization with Triton X-100 on the metabolization of (16:0/18:2*)-PC. Membranes were from control (A) and 4 kGy-irradiated cauliflower (B), stored at 13 °C for 4 d. The reaction was allowed to proceed for 60 min. Results are from one of two experiments showing the same trend.

carnation flowers, tomato fruits, and broccoli (Paliyath and Thompson, 1987; Paliyath et al., 1987; Todd et al., 1990; Deschene et al., 1991), whereas lipolytic acyl hydrolase activity was detected in a particulate fraction from cauliflower (Wardale and Galliard, 1977).

The enzymes were associated with the microsomal membranes since they were not removed by washing and since their activities could still be detected after partial solubilization of the membranes with Triton X-100. On a specific activity basis, the enhanced PC catabolism by solubilized membranes probably resulted from the enrichment in phospholipase D activity. Phospholipase D was apparently more intimately bound to the membranes than PA phosphatase and lipolytic acyl hydrolase activities because PA accumulated at the expense of the other breakdown products. Partial membrane solubilization with Triton X-100 led to enrichment of phospholipase D and PA phosphatase activities in bean membranes, but it also led to reduced PC catabolism in carnation membranes (Paliyath and Thompson, 1987; Brown et al., 1990). The absence of OP accumulation in solubilized cauliflower membranes might be interpreted as reduced lipoxygenase activity. However, as partly solubilized membranes were shown to be enriched in lipoxygenase activity (Voisine et al., unpublished data), the absence of OP probably reflects reduced lipolytic acyl hydrolase activity and liberation of FFA, the substrate of lipoxygenase.

Lack of even traces of phosphorylcholine among the water-soluble products generated from PC by cauliflower microsomal membranes makes involvement of phospholipase C unlikely, which is in agreement with observations on membranes of carnation petals (Paliyath et al., 1987) and bean cotyledons (Paliyath and Thompson, 1987). The involvement of a nonspecific phosphatase hydrolyzing phosphorylcholine also seems unlikely: phosphorylcholine is metabolically inert in higher plant cells unless it is used for synthesis of PC, and it resists hydrolysis by nonspecific phosphatases (Bligny et al., 1989). PC was not a substrate for lipolytic acyl hydrolase because glycerophosphorylcholine was not detected in the water-soluble fraction. FFA were most likely produced by deesterification of PA or DG. The latter is a better substrate for lipolytic acyl hydrolase than phospholipids (Galliard, 1980). Collectively, the above results show the importance of phospholipase D as initiator of PC degradation, since PA must be generated first from PC before further catabolism can ensue.

The limitation to the use of (16:0/16:0)-PC*, the only available PC molecular species labeled in the choline moiety, is recognized. This species is normally absent from plant microsomal membranes and may be metabolized in a different way than the endogenous species. Todd et al. (1990) used the same substrate to identify water-soluble breakdown products of PC in tomato fruit. The same group (Brown et al., 1990) showed that unsaturated species of PC were more rapidly degraded than saturated (16:0/16:0)-PC. It is, therefore, more than likely that, had naturally occurring less-saturated species been used as substrates, the changes observed during senescence and after γ irradiation would have been similar, although probably enhanced.

Metabolism of PUFA was probably initiated by lipoxygenase (Voisine et al., 1991). The activity was detectable in cauliflower microsomes and increased during storage of control and irradiated cauliflower (Voisine et al., unpublished data). Rapid conversion of the resulting hydroperoxides into OP different from FFA hydroperoxides was probably catalyzed by enzymes like lyase, isomerase, and cyclase (Vick and Zimmerman, 1987). Recently, membrane-associated enzymes were reported to catalyze the epoxidation of PUFA by transfer of oxygen from hydroperoxides to PUFA (Blée and Schuber, 1990; Hamberg and Hamberg, 1990). The precise nature of the OP is currently under investigation. Production of OP resulting from further metabolism of hydroperoxides was also reported for microsomal membranes of tomato fruit (Todd et al., 1990).

The increased capability of microsomal membranes to degrade labeled PC after storage of control florets suggests that a progressive rise in lipolytic activity accounted for the enhanced loss of phospholipids previously observed in stored florets (Voisine et al., 1991). Increased lipolytic activity was associated with reduced phospholipid content in membranes from senescent rose petals (Borochov et al., 1982). In contrast, enhanced phospholipid catabolism was not associated with increased phospholipase activity in *Tradescantia* petals (Suttle

and Kende, 1980) or in microsomal membranes from senescent carnation petals (Paliyath et al., 1987). Changes in the molecular organization of the floret microsomal lipid bilayer may have facilitated accessibility of exogenous PC to the membrane-associated lipolytic enzymes. However, membrane rigidification was observed in senescing carnation petals (Mayak et al., 1983; Fobel et al., 1987) without enhanced catabolism of exogenous PC (Paliyath et al., 1987). Increasing membrane-associated phospholipase activity, which may be due to an increased level of lipase or to physical changes in the membrane, is probably involved in the degradation of microsomal membranes during storage of cauliflower.

The greater proportion of FFA, mainly composed of saturated and monounsaturated fatty acids, in membranes extracted from freshly irradiated florets was attributed to deesterification of phospholipids by free radicals generated during γ irradiation (Voisine et al., 1991). Activation of lipolytic enzymes also contributed to the increased FFA content. Phospholipase D activity increased immediately after irradiation, PA and DG were rapidly metabolized, and higher levels of OP indicated enhanced liberation of PUFA available for lipoxygenase. The lack of specificity of lipolytic acyl hydrolase with respect to the fatty acid position on the phospholipid, and the degradation of free PUFA by lipoxygenase may explain the accumulation of monounsaturated and saturated fatty acids in the membranes. γ irradiation did not alter the pattern of PC metabolism associated with senescence of control florets, but it strongly accelerated the process. The mechanism underlying the activation of phospholipases in irradiated florets remains unresolved. Activation of phospholipases by calcium during the assay is excluded because calcium was chelated by EGTA during membrane extractions and because calcium concentration was controlled during the assays. Membrane destabilization after γ irradiation may have stimulated phospholipase activity.

In conclusion, membrane-associated phospholipase D activation may be a key event leading to accelerated membrane deterioration in irradiated tissue. Once initiated, phospholipid degradation by phospholipase D leads to further lipid catabolism by phosphatidic acid phosphatase and acyl hydrolase, oxidation of PUFA by lipoxygenase into hydroperoxides, rapid conversion of the latter into OP, and accumulation of saturated FFA. These phospholipid breakdown products most likely perturb the bilayer, and thus may increase its susceptibility to phospholipase attack and irreversibly accelerate the deterioration process.

Received November 2, 1992; accepted February 11, 1993.
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