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Absence of Lipid Oxidation during Accelerated Aging of Soybean Seeds[1]

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DAVID A. PRIESTLEY AND A. CARL LEOPOLD
Boyce Thompson Institute, Ithaca, New York 14853

ABSTRACT

When seeds of soybean were subjected to accelerated aging, the amount of total lipid which was extracted from the whole seed increased with "age," whereas the extractable phospholipid decreased slightly. This small decline primarily reflected changes in the amounts of phosphatidylcholine and phosphatidylethanolamine. The levels of unsaturated fatty acids in the whole seed and in the seed axis showed no decline during aging. Similarly, the fatty acids in a polar lipid extract from the whole seed showed little change in unsaturation. These results suggest that oxidation of seed lipids may be unrelated to the process of seed aging.

The aging of dried seeds in storage is thought to be accompanied by changes in membranes (8, 24) and nucleic acids (15). Alterations in the membranes of aged seeds are considered to lead to a greatly enhanced leakage of solutes during seed imbibition (2, 13). This may be indicative of an inability to re-form coherent membranes during rehydration of the seed, resulting in loss of vigor and lack of germination. Changes in dry or rehydrated membranes of aged seeds have been noted at the ultrastructural level (6). Lesions in nucleic acids may also be important in deterioration of seed (15), although probably less significant during the first minutes of imbibition when protein and nucleic acid synthesis is probably minimal.

Several workers have argued that the alterations to seed membranes during aging may result from peroxidation (10, 24). Autooxidation of polyunsaturated fatty acids leads to free radical formation and possible peroxidative damage to lipids, proteins, and nucleic acids. Peroxidation of membrane lipids gives rise to increased permeability of artificial bilayers (23) and a decrease in membrane fluidity (5). Spencer *et al.* (20) noted an increased level of oxygenated fatty acids in seeds of *Cichorium* and *Crepis* during long term storage, although these workers supplied no germination data. Most studies have been performed using systems of accelerated aging, in which the symptoms of natural aging are induced over a relatively short time span by exposing the seeds to conditions of high temperature and humidity (4). Koostra and Harrington (10) applied TLC to lipids extracted from cucumber seed and suggested that any phosphorus-containing spot which remained immobile in their solvent system was probably a peroxidized phospholipid; applying this principle, they indicated that peroxidation increased severalfold during aging, peroxidized phospholipids representing over 20% of the total phospholipids in seeds rendered nongerminable. UV spectroscopy of seed fatty acids of *Protea compacta* by Van Staden *et al.* (22) gave no indication of increased peroxidation during storage, although this form of analysis can be considered only semiquantitative. Harman and Mattick (7) studied accelerated aging of pea seeds and found that the decrease in germination rate was paralleled by a pronounced decline in linoleic (C18:2) and linolenic (C18:3) acids whereas the saturated and monoenoic fatty acids remained unchanged. They suggested that this decline in dienoic and trienoic fatty acids is consistent with oxidation of unsaturated lipids and free radical formation. Conditions of high electrical potential which may bring about a reduction of free radicals during seed aging have been reported to prevent deterioration of seeds (12) and lead to a decrease in leakage of solutes during imbibition. Our own study was conducted on seeds of soybean and was directed toward a quantitative analysis of fatty acids and phospholipids during accelerated aging.

MATERIALS AND METHODS

Soybean seeds (*Glycine max* [L.] Merr. cv. Chippewa 64) produced in 1975 by the Minnesota Seeds Foundation were subjected to accelerated aging by incubation for periods up to 5 days in a water bath maintained at 40 C and close to 100% RH. The seeds increased in weight during the aging process due to water uptake from the atmosphere but were air-dried to their original weight before use. Germination assays were performed on 49 or 50 seeds per treatment. The seeds were rolled in paper toweling which was kept moist by capillary action. Radicle emergence was measured after 3 days of growth in darkness at room temperature. Seeds with radicles longer than 10 mm were considered to have germinated. Growth rate was measured as the mean length of all emerged radicles.

Whole seeds were ground for 30 s using a Waring coffee mill. Seed axes were prepared by splitting them from the dry seed using a razor blade and then grinding them in a mortar. Solvent extracts of the particles from whole seeds were made using chloroform-methanol (2:1, v/v) containing 0.005% (w/v) butylated hydroxytoluene to minimize oxidation during extraction. Extraction was performed under argon at 24 C for 30 min using 20 ml solvent/5 g seed tissue (36–38 seeds). The solution was filtered through Whatman GF/A glass microfiber paper and partitioned once against 0.2 volumes of 0.9% (w/v) NaCl and twice against 0.5 volumes of methanol-water (1:1, v/v) containing 0.9% (w/v) NaCl. Total lipid yield was estimated by drying the solution under argon to constant weight. Phospholipids in the total lipid extract were estimated by the procedure of Raheja *et al.* (14). Polar lipid fractions were prepared by precipitation of 100 μl of total lipids using 5 ml acetone. After incubation at 4 C for 1 h, polar lipids were pelleted by centrifugation at 1,000*g* for 2 min.

TLC was performed using precoated Silica Gel G plates (200 × 200 mm) (Applied Sciences Labs) with a layer thickness of 250 μm. Total lipid samples were separated by three-solvent two-dimensional chromatography (1). A sample of 10 μl of soybean oil was used for each plate. The plates were fully developed in the first dimension using chloroform, dried, and then fully developed

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again in the same direction using chloroform-methanol-7 M NH_4OH (65:30:4, v/v/v). The separation was completed using chloroform-methanol-acetic acid-water (170:25:25:4, v/v/v/v) in the second dimension. Spots were visualized using a spray of 1% (w/v) iodine in methanol. Phospholipids were identified using a molybdenum blue spray reagent, PI[2] and PG using Schiff's reagent, PC using Dragendorff's reagent, and PE using a ninhydrin stain. Authentic standards were employed to assist identification. Phospholipids were removed from the plate into recovery tubes, eluted, and quantified (14).

Esterification of fatty acids was performed using two different methods. In one procedure extracts of total or polar lipids were incubated in 5 ml of methanol-benzene-sulfuric acid (100:5:5, v/v/v) for 2 h at 80 C. The methyl esters were extracted into *n*-hexane for injection into the gas chromatograph. In the alternative procedure seed lipids were subjected to direct methanolysis without prior solvent extraction (7). Freshly ground seed material (100–500 mg) was placed in a flask fitted with a condenser and boiled with 4 ml of 0.5 M KOH in methanol for 5 min. Four ml of 14% (w/v) BF_3 in methanol was added through the condenser and the sample was boiled for an additional 2 min. It was then rapidly cooled and 5 ml of saturated NaCl solution was added. The methyl esters were extracted by partition into *n*-hexane. Heneicosanoic acid (C21:0) was used as internal standard for all samples. Fatty acid methyl esters were analyzed using a Hewlett-Packard 5730A gas chromatograph equipped with a flame ionization detector and coupled to an electronic integrator. Separations were performed on a 180-cm glass column (internal diameter 4 mm) packed with 3% (w/w) Silar-5CP on 100/120 mesh Gas-chrom Q (Applied Science Labs). A temperature of 200 C was employed with N_2 (15 ml min^{-1}) as carrier.

RESULTS

Accelerated aging treatments caused a decline in both vigor and germinability of soybean seeds (Fig. 1). The total lipid extracted from each seed increased slightly with "age" (Fig. 2a). In contrast, the extractable phospholipid declined (Fig. 2b). In unaged seeds the phospholipids represented approximately 5% of the total lipid present. Analysis of the phospholipids by TLC (Fig. 3) suggested that the decline in extractable phospholipids with "age" was principally a loss of PC and PE. Amounts of PA, PG, and a spot tentatively identified as DPG increased slightly during the aging treatment. At least one minor phospholipid was not identified; this chromatographed under, or immediately adjacent to, the triglyceride area and could not be routinely removed from the plate. Identifiable phospholipids recovered following TLC accounted for about 94% of the total phospholipid extracted from unaged seeds, declining to 88% in samples from seeds aged for 5 days. No phospholipid was encountered at the origin at any stage.

The fatty acids present in the total lipid extract from whole seeds are shown in Figure 4a. Fatty acid levels remained unchanged during the aging treatment, with no indication of a decline in unsaturated forms. When the fatty acids of particles from the whole seed were esterified directly using methanol-BF_3, an almost identical result was obtained (Fig. 4b). The acetone-insoluble polar lipid fraction from whole seeds (primarily phospholipids [11]) similarly showed little change (Fig. 5a). The total fatty acids of the seed axis (which represents about 4% of the weight of the dry seed) displayed no change during aging (Fig. 5b) although they were more unsaturated than the fatty acids present in the remainder of the seed.

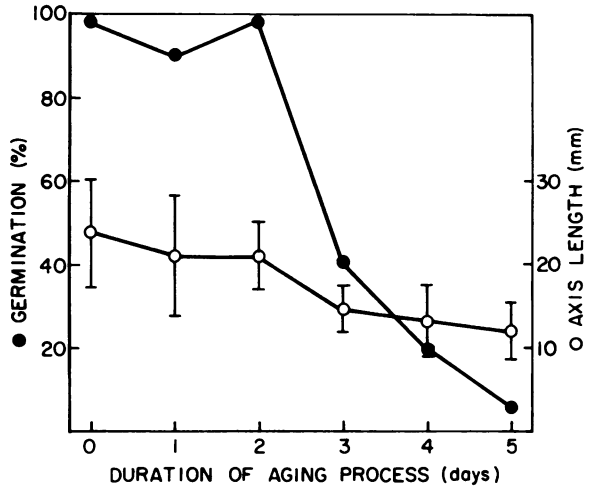

FIG. 1. Percentage germination and axis length of artificially aged soybeans. Data on axis growth represent mean length of axes (± SD) from germinated seeds only.

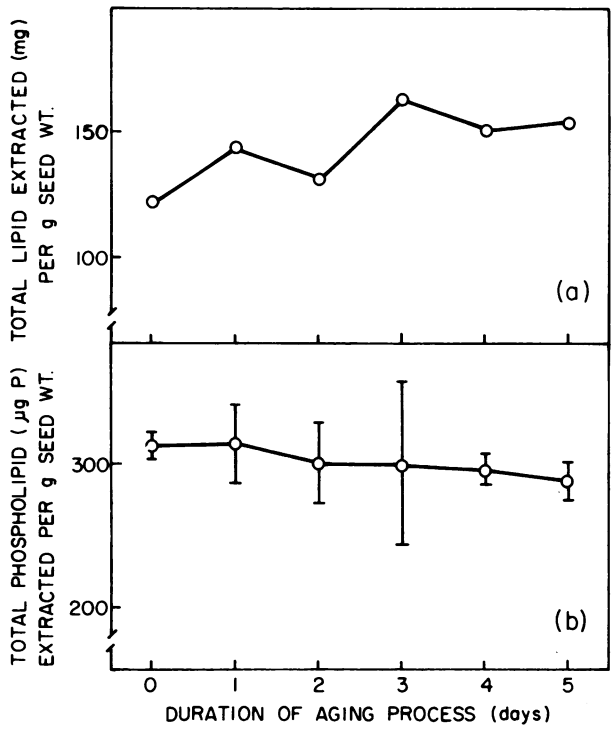

FIG. 2. Total lipid (a) and phospholipid (b) extracted from whole soybean seeds aged artificially. Each point represents a single observation (a) or mean ± SD of three observations (b) made on the same seed extracts.

DISCUSSION

These experiments show that the accelerated aging of soybean seeds to an extent which reduced vigor and essentially eliminated germination caused small changes in the phospholipid components of the seed but did not alter the extent of oxidation of the fatty acids.

Soybean seeds contain at least two enzymes capable of altering lipids during extraction. Lipoxygenase (EC 1.13.11.12) catalyzes the formation of peroxidized lipids from unsaturated fatty acids by the addition of O_2. Assays of soybean lipoxygenase preparations using an O_2 electrode showed that the enzyme is immediately and completely inactivated upon contact with chloroform-meth-

[2] Abbreviations: DPG: diphosphatidylglycerol; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol.

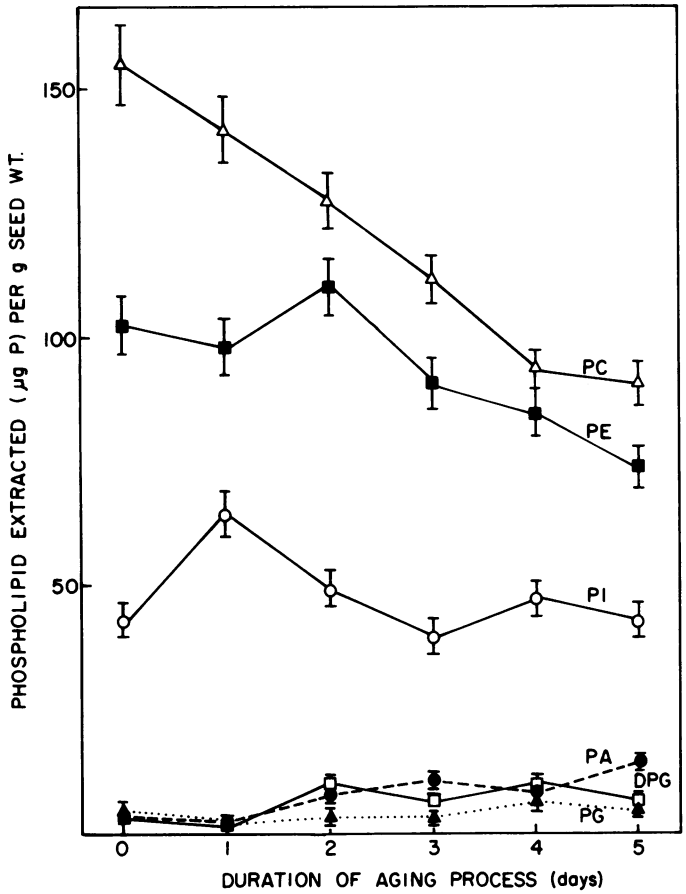

FIG. 3. Phospholipid classes extracted from whole soybean seeds aged artificially. Each point represents the mean ± SD of three observations made on the same seed extracts.

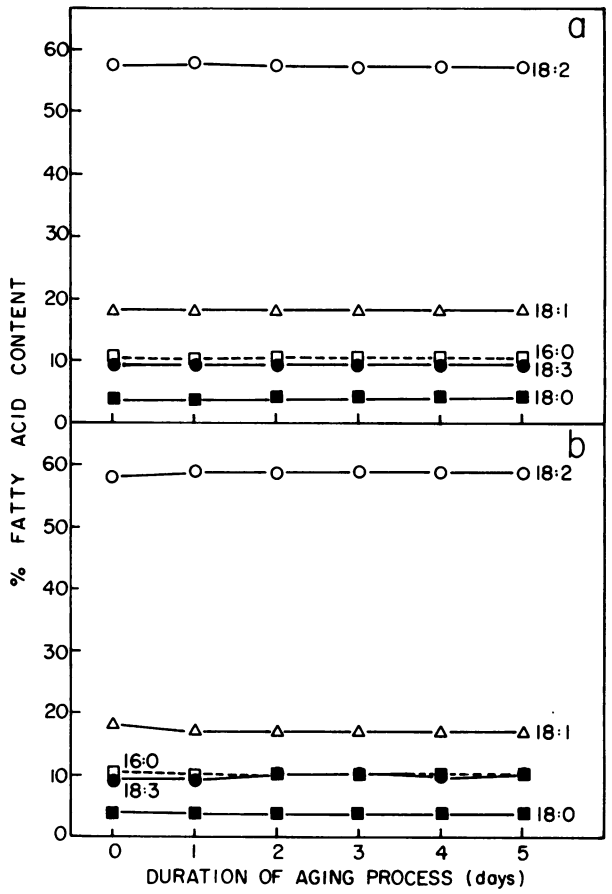

FIG. 4. Percentage fatty acid content of whole soybean seeds. (a): Esterification of total lipid fraction produced by solvent extraction; methanol-benzene-sulfuric acid procedure. (b): Direct esterification of dry seed particles without prior solvent extraction; BF_3-methanol procedure. Results given are for one representative experiment.

anol (2:1, v/v) used for extracting the seed lipids (Priestley and Leopold, unpublished data). Phospholipase D (EC 3.1.4.4), which promotes the hydrolysis of several phospholipids, also occurs in soybean seeds but this activity is probably insignificant when dry seeds are extracted into chloroform-methanol (16). It is unlikely that the lipid extracts used for the present study were grossly modified by enzymic activity. Furthermore, the fatty acids of the lipids present in chloroform-methanol solvent extracts from seeds were almost identical to fatty acids prepared directly from powdered dry seed by the methanol-BF_3 procedure. This suggests that the process of solvent extraction did not result in the preferential selection of particular fatty acids.

The phospholipid components encountered in unaged seeds in the present study appear to be similar to those of mature seeds of other cultivars of this species (25). Two minor phospholipids whose presence has been reported in mature soybean seeds (21, 25) could not be identified with confidence. Although some shifts in the proportion of phospholipid classes occurred during aging, no evidence for phospholipids remaining immobile at the origin of the TLC plate was obtained. If this feature is indeed indicative of peroxidation (10), the evidence from soybean would suggest that age-induced peroxidation of the phospholipid fraction was minimal. The marked decline in linoleic and linolenic acids of aging seeds reported by Harman and Mattick (7) did not occur in our experiments either in the whole seed or in the axis of soybean. The duration of accelerated aging in their experiments was 10 weeks, however. Five days was sufficient to age soybean seeds to a nongerminable condition for the present work. It is possible that soybeans subjected to several weeks of accelerated aging might also show pronounced oxidation of fatty acids, although we have been unable to investigate this due to fungal contamination of seed batches aged for periods longer than 1 week. Our results from soybean suggest that if changes in fatty acids play a major role in seed deterioration, they must occur at a level far below those previously considered significant.

The reasons for the slight decline in extractable phospholipid with seed "age" are obscure. Koostra and Harrington (10) reported a 3-fold drop in polar lipids of cucumber seeds during 4 weeks of accelerated aging but no change in the same fraction when naturally aged material was analyzed. Sedenko (17) recorded a 2-fold drop in phospholipid content of nonviable maize seed stored at a water content of 14.9% for 9 years when compared to viable seed of the same batch stored with a moisture content of 9%. Chapman and Robertson (1) have given data for soybeans stored at 35 C and 85% RH but supplied no germination data. They encountered a doubling of phospholipid content within 10 days of storage which they suggested arose from the onset of seed germination under conditions of high humidity. Under the accelerated aging treatment which we employed soybean seeds increased their water content to approximately 30% (over 5 days) by uptake from the atmosphere. This exceeds the water levels at which membranes have been postulated to re-form into coherent bilayers (19) but is still about 20% below the moisture content required for successful germination of soybeans (9). It is possible that hydrolytic enzymes may be functional under conditions of accelerated aging at a stage when cellular organization is still incomplete. Simon (18) has

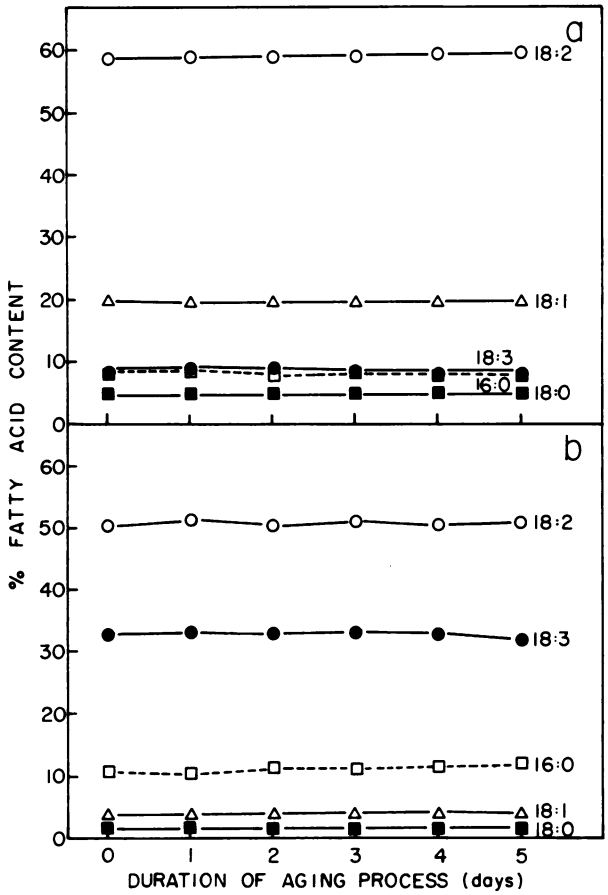

FIG. 5. Percentage fatty acid content. (a): Esterification of polar lipid fraction of whole seeds produced by solvent extraction and precipitation; methanol-benzene-sulfuric acid procedure. (b): Direct esterification of seed axis particles without prior solvent extraction; BF_3-methanol procedure. Results given are for one representative experiment.

speculated that a decrease in phospholipid levels and a consequent decline in membrane integrity are key features in the physiologically unrelated phenomenon of organ senescence. Seeds which are held dormant in a fully imbibed state are thought to possess the ability for membrane repair (3, 24) but full hydration and complete cellular organization may be prerequisites for this process.

The deteriorative changes which are capable of causing aging and death of seeds are probably manifold. The most important cause of deterioration may vary according to species or the form of storage employed and we cannot exclude the possibility that certain forms of degradation may be more significant during accelerated aging than under natural conditions. It is evident from our data that artificial aging of soybean is accomplished with minimal changes in the degree of unsaturation of seed fatty acids.

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