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# Lipid Peroxidation Associated with Accelerated Aging of Soybean Axes[1]

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## ABSTRACT

**Soybean seeds age rapidly during storage at high temperature and high relative humidity. The axes of such aged seeds contain high levels of malondialdehyde, a product of the peroxidation of unsaturated fatty acids. The levels of linoleic (18:2) and linolenic (18:3) acids in a polar lipid (phospholipid) fraction decrease during aging and more dramatically during postaging deterioration. None of these changes occurred in seeds that have been stored at high temperature but low relative humidity. No superoxide dismutase activity was detected in any nonimbibed seed. In viable seeds, activity was detectable 1.5 hours after the onset of imbibition, but none was found in aged seeds up to 5 hours. It is suggested that aging leads to peroxidative changes to lipids and that these could contribute to loss of viability.**

Seeds deteriorate during periods of prolonged storage. This aging is manifested as a reduction in percentage germination, while those seeds that do germinate produce weak seedlings (4, 12). Recently, seed aging has been the subject of extensive research, but still it is unclear what the fundamental processes of aging are. Several different processes have been implicated (*e.g.* [9]), but there is little definitive evidence for any primary process of aging damage. Parrish and Leopold (14) suggested that aging in soybean seeds resulted from deteriorative changes in membranes, probably via peroxidation reactions involving unsaturated fatty acids. However, their data were largely circumstantial. More recently, Priestley and Leopold (15) have presented data to show that there is little or no change in the level of unsaturated fatty acids during aging of whole soybean seeds, and hence suggest that oxidation of lipids is unrelated to seed aging. Harman and Mattick (6) have demonstrated that aging of pea seeds results in a pronounced decline in dienoic and trienoic fatty acids, whereas saturated and monoenoic fatty acids remain unchanged. The results presented here using isolated axes of soybean also show that there is a loss of unsaturated fatty acids as a consequence of accelerated aging and an increase in a product of peroxidation.

## MATERIALS AND METHODS

**Plant Material.** Seeds of soybean (*Glycine max* [L.] Merr., cv. Pride X005) were obtained from the Agriculture Canada Experimental Research Station, Lethbridge, Alberta, Canada, and stored at 5 C until required.

**Aging Treatment.** Seeds were aged by storing them at 45 C (air temperature) above the water level in a covered water bath (14). As a control seeds were kept dry by storing them at 45 C, with desiccant silica gel. Excision of the axes occurred after aging.

**Germination Treatment.** Seeds of various aging conditions were dispensed into 15-cm Petri dishes lined with Whatman No. 1 filter paper and wetted with distilled $H_2O$. Germination was scored 48 h after imbibition.

**Electrolyte Leakage.** Imbibitional leakage was measured by excising two axes from the aged seeds and placing them in 5 ml deionized $H_2O$ for 20 min with occasional agitation. A further 5 ml deionized $H_2O$ were added and the conductivity of the solution was measured. The axes then were homogenized and the conductivity of the homogenate was measured. The percentage leakage was calculated:

$$\text{Percentage leakage} = \frac{\text{Conductivity of leachate}}{\text{Conductivity of homogenate}} \times 100$$

**Peroxidation Product Estimation.** Malondialdehyde (1,3-propandial) was measured by a colorimetric method (8). Two axes, excised from soybeans at various times after imbibition, were homogenized in 5 ml of distilled $H_2O$. An equal volume of 0.5% TBA[2] in 20% trichloroacetic acid solution was added and the sample was incubated at 95 C for 30 min. The reaction was stopped by putting the reaction tubes in an ice bucket. The samples then were centrifuged at 10,000*g* for 30 min. The supernatant was removed, $A$ was read at 532 nm, and the value for nonspecific absorption at 600 nm was read and subtracted from this. The amount of malondialdehyde present was calculated from the extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$ (10). It has been pointed out (3) that a number of organic compounds may interfere with the TBA assay for malondialdehyde. Although this problem is reduced by subtracting nonspecific absorption obtained in the assay it was deemed necessary to correlate these data with an analysis of the fatty acid precursor from which malondialdehyde is ultimately produced.

**Polar Lipid Fatty Acid Analysis.** The fatty acids of the polar lipids (largely phospholipids [17]) were assayed as follows: 10 axes were excised from imbibed seeds and homogenized in boiling propan-2-ol. The extract was boiled for 30 min and then centrifuged at 5,000*g* for 5 min. The supernatant was removed, the pellet was resuspended in chloroform-methanol (2:1), and recentrifuged. This was repeated; the organic phases were combined, taken to dryness, and redissolved in chloroform. The chloroform solution was partitioned twice against 200 mM aqueous sodium carbonate to remove any free acids, and then taken to dryness and redissolved in a small volume of 1% acetic acid in chloroform. The sample was loaded onto a short column (0.5 × 5 cm) of silica gel (type 1; Sigma Chemical Co.) which was premixed and washed with 1% acetic acid in chloroform. The column then was washed with 25 ml of the acetic acid-chloroform to remove the neutral lipids, and with 25 ml of methanol to remove the polar lipids. The

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[2] Abbreviation: TBA: 2-thiobarbituric acid.

methanol solution was taken to dryness under $N_2$ and 20 ml of 1 M KOH in water was added. This was incubated at 95 C for 30 min, was cooled, and an equal volume of distilled $H_2O$ was added. The aqueous solution was partitioned against diethyl ether, the organic phase being discarded. Although this may result in the loss of 5–10% fatty acids, it effectively removes most contaminants.

The aqueous solution then was taken to pH 2 with concentrated $H_2SO_4$ and extracted three times against diethyl ether; the aqueous phase was discarded, while the organic phases were combined and frozen to remove residual water. The solution was taken to dryness *in vacuo*, and the extract was methylated using diazomethane and dried once more. The extract was redissolved in 100 μl of methanol and assayed by GLC on a Varian 3700 gas chromatograph, using a stainless steel column (1.8m × 0.32 cm o.d.) packed with 10% SP 2330 on Supelcoport 100/120 mesh (Supelco Inc., Bellefonte, Pa.). The mode of detection was flame ionization, and a temperature program of 160 C for 1 min, 5 C $min^{-1}$ to 225 C was run.

**Superoxide Dismutase Assay.** Superoxide dismutase was assayed on the basis of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (2). Ten axes were excised and homogenized in cold 50 mM phosphate buffer (pH 7). The homogenate was centrifuged at 30,000*g* for 30 min and the supernatant obtained was the enzyme extract. A 3-ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM nitro blue tetrazolium, 2 μM riboflavin, 100 nM EDTA, and 0–200 μl of enzyme extract. Riboflavin was added last and the tubes were shaken and placed 30 cm below a light bank consisting of two 15-w fluorescent tubes. The reaction was started by switching on the light and it was allowed to run for 10 min, before being stopped by switching off the light, when the tubes were covered with a black cloth. *A* by the reaction mixture was read at 560 nm. A nonirradiated reaction mixture had an *A* of zero at 560 nm. The reaction mixture lacking enzyme developed the most color and this decreased with increasing volume of extract added. Log $A_{560}$ was plotted as a function of the volume of enzyme extract in the reaction mixture (5). The volume of enzyme extract producing 50% inhibition of the reaction was read from the resultant graph. One unit of superoxide dismutase activity was defined as that amount of enzyme which caused 50% inhibition of the initial rate of the reaction in the absence of enzyme (2).

## RESULTS

**Germination and Imbibitional Leakage.** Storage of soybean seeds at an elevated temperature and humidity has a profound effect upon both their ability to germinate and upon the extent of imbibitional leakage (Fig. 1). However, the elevated temperature without high humidity has no noticeable effect upon these parameters.

During storage at elevated temperatures and low humidity the water content of the seed declines from an initial 7.9% to 4.6% during the 1st day, and to 3.3% by day 7. At high humidity the water content increases to 18.7% on the 1st day, to 22.7% on the 2nd day, and to 35.1% by day 7.

**Malondialdehyde Content.** When triunsaturated fatty acids with double bonds 3 carbon atoms apart (linolenic [9,12,15-octadecatrienoic] acid) are subjected to peroxidation a product is malondialdehyde. With increasing high humidity aging of the seed, up to 4 days, more malondialdehyde is present during the first few hours of subsequent imbibition (Fig. 2). However, after 4 days the malondialdehyde content of the axes declines rapidly. On the other hand, the dry aged seeds show a pattern similar to that of the unaged seeds, an initially high content, which rapidly falls. On days 6 and 7, even this initially high level is reduced.

If this malondialdehyde is a product of the peroxidation of triunsaturated lipids, then the levels of the triunsaturated fatty acids in the polar lipid fraction also should decline during aging.

**Polar Lipid Fatty Acids.** During aging at high temperature and humidity the degree of saturation of the polar lipids is much increased (Table I). This effect is not due to an increase in the saturated acids stearic and palmitic because the levels of these acids also fall slightly during aging. The reduction in the degree of saturation is due to a large decrease in the content of linoleic and linolenic acids (Table I), particularly linolenic acid which drops from 57% of the total fatty acids in unaged seeds to 33% in 7-day-aged seeds.

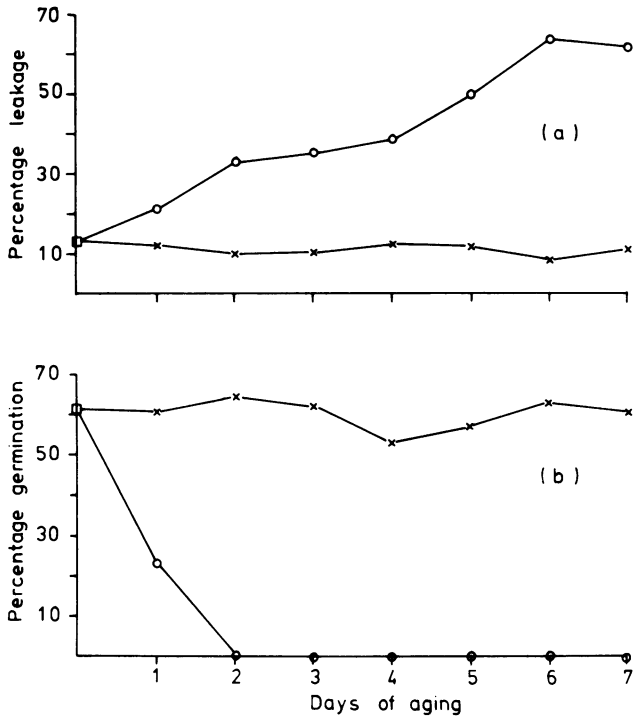


FIG. 1. a: Percentage leakage from axes in distilled $H_2O$ for 20 min. These axes were excised from seeds which had been aged for various times. □: Unaged seeds; ○: high-humidity-aged seeds; ×: low-humidity-aged seeds. b: Percentage germination of soybean seeds aged for various times: symbols as in (a).

In the dry-aged seeds the degree of saturation changes little during 6 days storage at 45 C.

**Superoxide Dismutase Content.** No superoxide dismutase is detectable in aged or unaged seeds during the first hour of imbibition (Fig. 3). Thereafter the dry-aged and the unaged seeds contain levels that increase and reach a maximum at 3.5 h from the start of imbibition. The levels then decline, but there still is detectable superoxide dismutase activity. There is no superoxide dismutase activity in seeds that have been aged for 7 days in an atmosphere of high RH.

## DISCUSSION

The effect on the seeds of storage at high temperatures and high RH follows the rule of thumb mentioned by Parrish and Leopold (14) inasmuch as an increase of 4 C over their storage temperature doubled the rate of aging damage, as expressed in germination reduction.

From the data presented in Figures 1 and 2 it appears likely that peroxidation of fatty acids occurs during and following aging. It seems best to divide the events that have been followed here into two phases—aging, which occurs during the first 2 days, and postaging deterioration, which occurs after the 2nd day and when seeds are aged and have lost their ability to germinate (Fig. 1). Peroxidation of unsaturated fatty acids occurs during aging at high RH, linolenic acid declining appreciably on the 1st day, and linoleic acid by the 2nd day. There after, both unsaturated fatty acids show extensive losses as the seeds deteriorate. These studies

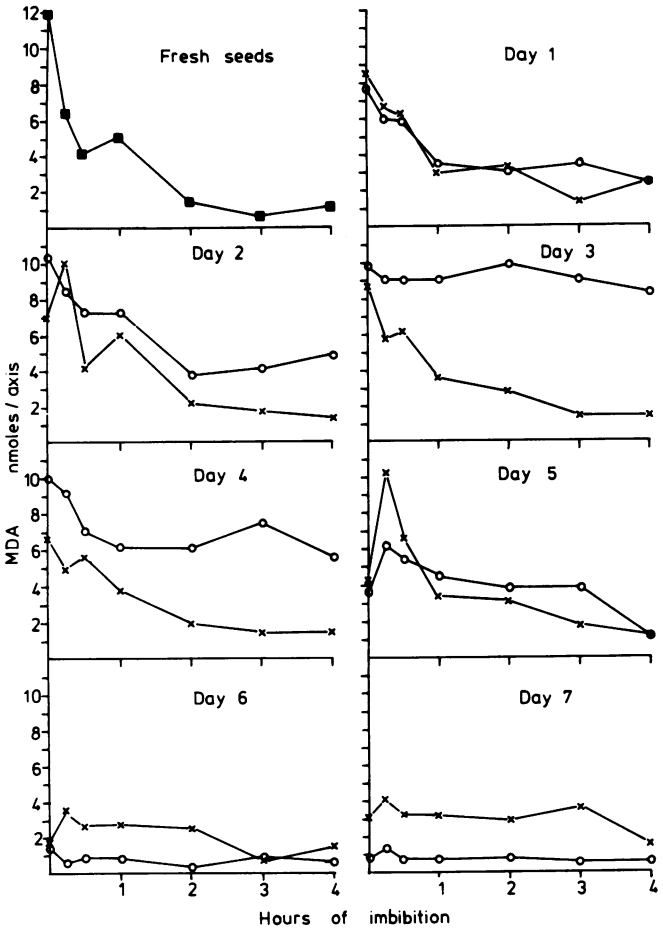


FIG. 2. Levels of malondialdehyde in axes of soybeans during the first few hours from the start of imbibition. Seeds were aged to various times (days 1–7) before imbibition. ■: Unaged (fresh) seeds; ○: high-humidity-aged seeds; ×: low-humidity-aged seeds.

Table I. *Content of Fatty Acids in the Polar Lipid Fraction, during Aging at High and Low Humidity*

| | Fatty Acid Content | | | | |
|---|---|---|---|---|---|
| | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
| | *% of total* | | | | |
| Unaged seeds | 16.6 | 3.1 | 3.8 | 57.3 | 19.3 |
| Days of high humidity aging | | | | | |
| 1 | 20.6 | 4.2 | 3.5 | 56.4 | 15.4 |
| 2 | 22.6 | 5.9 | 5.0 | 52.4 | 14.0 |
| 3 | 32.1 | 7.1 | 9.6 | 40.5 | 10.7 |
| 4 | 42.1 | 8.1 | 16.6 | 28.8 | 4.4 |
| 6 | 39.6 | 13.0 | 7.0 | 33.9 | 6.5 |
| Days of low humidity aging | | | | | |
| 2 | 19.6 | 3.1 | 2.1 | 60.2 | 14.9 |
| 4 | 19.4 | 3.0 | 2.4 | 58.1 | 17.0 |
| 6 | 21.1 | 3.0 | 2.2 | 58.8 | 14.9 |

agree with the work on peas by Harman and Mattick (6) but disagree, in part, with the studies of Priestley and Leopold (15).

For this study the axes of soybeans were used, rather than cotyledons or whole seeds (14, 15). This is because the cotyledons are a source of stored lipids, and it was deemed undesirable to study polar lipid changes and lipid peroxidation in a tissue that contained considerable quantities of nonmembrane lipids. Priestley and Leopold showed no changes in unsaturated fatty acids in whole soybean seeds (15), but state that they obtained similar results with isolated axes. Harman and Mattick (6) showed changes both in axes and in intact seeds.

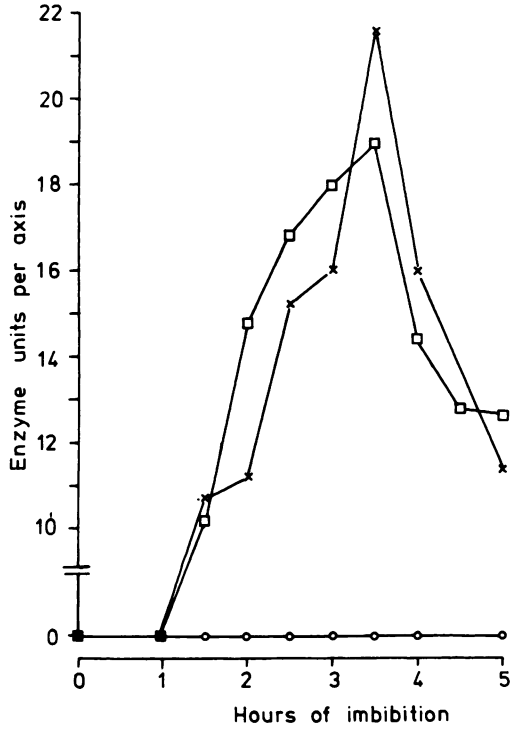


FIG. 3. Superoxide dismutase content of soybean seeds during the first 5 hours of imbibition. □: Unaged seeds; ○: seeds aged at high humidity for 7 days; ×: seeds aged at low humidity for 7 days.

On the 2nd day of aging at high RH and temperature there is an increase in malondialdehyde upon imbibition of the axes (Fig. 2). This suggests that lipid peroxidation is occurring. After prolonged storage under these conditions the levels of malondialdehyde in the aged seeds declines, presumably because it is oxidized further (3, 10), and also because the level of the unsaturated fatty acid precursor (linolenic acid) is reduced (Table I). Malondialdehyde is water-soluble, and if the membranes have deteriorated it is likely that any malondialdehyde in the axis will be leached into the medium during and following imbibition. The levels of malondialdehyde in the medium of high humidity and high temperature-aged seeds are higher than those of the dry-aged seeds (data not presented).

It has long been suggested that lipid peroxidation (or autoxidation) is a primary cause of seed deterioration (*e.g.* 9). However, as shown in Figure 2, dry aged seeds that retain their viability also contain malondialdehyde. The level of this product is reduced after 6–7 days dry aging. This might be because during dry aging the water content of the seeds drops below that of the unaged seeds. Thus, the free radical chain reactions (which are implicated as the source of peroxidation damage [3, 11, 13]) do not occur readily. It has been suggested (1, 7) that the major peroxidation damage occurs during storage, but that this is expressed only upon imbibition. In this study it is apparent that some changes occur during aging, but that they occur most obviously in deteriorating aged seeds.

It seems unlikely that superoxide dismutase has any role to play in the aging itself, since even viable (unaged and dry aged) seeds contain no detectable superoxide dismutase activity until 90 min after imbibition has started. It may play a key role in restricting

peroxidation damage after this time. Since the high humidity-aged axes are unable to synthesize (or activate) the superoxide dismutase after imbibition, the free radical chain reactions will be uncontrolled and the aging damage will be compounded. The increase in superoxide dismutase may represent a method of recovery from the deleterious effects of dry storage (19): a recovery that is not possible for excessively aged seeds.

The biochemical consequences of membrane deterioration are profound. Apart from the obvious loss of solute control, the highly ordered system of membrane-associated enzymes is dispersed. This results, among other effects, in the build-up of the products of glycolysis, since the Kreb's cycle enzymes are membrane associated. The build-up of acetaldehyde and ethanol has been reported in many tissues in which the membrane phospholipids have been altered (16, 18).

The changes in malondialdehyde and in the degree of polar lipid saturation show a reasonable correlation with loss of viability during accelerated aging. The enzyme superoxide dismutase is not detectable in nonimbibed unaged seeds and it seems unlikely that this enzyme plays a role in aging *per se*. The most important factor seems to be water content during aging since the presence of this water increases peroxidation damage.

There is no obvious reason why our results should be different from those of Priestley and Leopold (15). Several factors could contribute: different seed variety, more severe aging conditions, different polar lipid extraction procedures. Nevertheless, we have shown that some peroxidation of lipids (mostly phospholipids [17], which are important constituents of membranes) occurs during accelerated aging of soybean axes. We must agree with Priestley and Leopold (15) that the changes associated with aging are probably many and variable, and whether the changes that we observe are of primary significance in aging remains to be determined. Certainly any oxidation of lipids and loss of membrane integrity must be a contributing factor. What is evident is that the accelerated deterioration that occurs following loss of viability leaves no chance for the aging process to be reversed.

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## LITERATURE CITED

1. ABDUL-BAKI AA, JD ANDERSON 1972 Physiological and biochemical deterioration of seeds. *In* TT Kozlowski, ed, Seed Biology, Vol II. Academic Press, New York, pp 283–315
2. BEAUCHAMP C, I FRIDOVICH 1971 Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276–287
3. BEUGE JA, SD AUST 1978 Microsomal lipid peroxidation. Methods Enzymol 52: 302–310
4. BYRD HW, JC DELOUCHE 1971 Deterioration of soybean seeds in storage. Proc Assoc Official Seed Anal 61: 41–57
5. FRIDOVICH I 1975 Superoxide dismutases. Annu Rev Biochem 44: 147–159
6. HARMAN GE, LR MATTICK 1976 Association of lipid oxidation with seed ageing and death. Nature 260: 323–324
7. HARRINGTON JF 1972 Seed storage and longevity. *In* TT Kozlowski, ed, Seed Biology, Vol III. Academic Press, New York, pp 145–245
8. HEATH RL, L PARKER 1968 Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125: 189–198
9. KOOSTRA PT, JF HARRINGTON 1968 Biochemical effects of age on membranal lipids of *Cucumis sativus* L. seed. 15th Int Seed Testing Congr, New Zealand, pp 1–12
10. KWON T-W, DB MENZEL, HS OLCOTT 1965 Reactivity of malondialdehyde with food constituents. J Food Sci 30: 808–813
11. LEA CH 1962 The oxidative deterioration of food lipids. *In* HW Shultz, ed, Lipids and Their Oxidation. Avi Publishing Company, Westport, Conn, pp 3–29
12. MCDONALD MB 1976 A review and evaluation of seed vigor tests. Proc Assoc Official Seed Anal 65: 109–139
13. PAMMENTER NW, JH ADAMSON, P BERJAK 1974 Viability of stored seed: extension by cathodic protection. Science 186: 1123–1124
14. PARRISH DJ, AC LEOPOLD 1978 On the mechanism of aging in soybean seeds. Plant Physiol 61: 365–369
15. PRIESTLEY DA, AC LEOPOLD 1979 Absence of lipid oxidation during accelerated aging of soybean seeds. Plant Physiol 63: 726–729
16. RAISON JK 1973 Temperature-induced changes in membrane lipids and their influence on metabolic regulation. Symp Soc Exp Biol 27: 485–512
17. SINGH H, OS PRIVETT 1970 Studies on the glycolipids and phospholipids of immature soybeans. Lipids 5: 692–697
18. SMAGULA JM, WJ BRAMLAGE 1977 Measurement of acetaldehyde in senescing apple fruits. J. Am Soc Hort Sci 102: 318–320
19. VILLIERS TA, DJ EDGCUMBE 1975 On the cause of seed deterioration in dry storage. Seed Sci Technol 3: 761–764