An ultrastructural study of the localization of lipoxygenase by immunogold labelling and mobilization of food reserves in soybean seeds and seedlings

Youngsun Song
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AN ULTRASTRUCTURAL STUDY OF THE LOCALIZATION OF LIPOXYGENASE BY IMMUNOGOLD LABELLING AND MOBILIZATION OF FOOD RESERVES IN SOYBEAN SEEDS AND SEEDLINGS

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An ultrastructural study of the localization of lipoxygenase by immunogold labelling and mobilization of food reserves in soybean seeds and seedlings

by

Youngsun Song

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW</td>
<td>4</td>
</tr>
<tr>
<td>Lipoxygenase in Relation to Foods</td>
<td>4</td>
</tr>
<tr>
<td>Purification of Lipoxygenase Isozymes</td>
<td>10</td>
</tr>
<tr>
<td>Distribution of Lipoxygenase</td>
<td>14</td>
</tr>
<tr>
<td>Physiological Function of Lipoxygenase</td>
<td>16</td>
</tr>
<tr>
<td>Definition of Germination</td>
<td>18</td>
</tr>
<tr>
<td>Structure of Soybean Seeds</td>
<td>19</td>
</tr>
<tr>
<td>Mobilization of Food Reserves during Germination and Seedling Growth</td>
<td>20</td>
</tr>
<tr>
<td>Proteins</td>
<td>20</td>
</tr>
<tr>
<td>Lipids</td>
<td>24</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>27</td>
</tr>
<tr>
<td>Patterns of reserve mobilization in germinating seeds</td>
<td>28</td>
</tr>
<tr>
<td>Ultrastructural Changes during Germination and Seedling Growth</td>
<td>29</td>
</tr>
<tr>
<td>Plastids</td>
<td>30</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>30</td>
</tr>
<tr>
<td>Endoplasmic reticulum (ER)</td>
<td>31</td>
</tr>
<tr>
<td>Glyoxysomes</td>
<td>33</td>
</tr>
<tr>
<td>Immunogold Labelling in Electron Microscopy</td>
<td>34</td>
</tr>
<tr>
<td>OBJECTIVES</td>
<td>38</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>39</td>
</tr>
<tr>
<td>Plant Materials</td>
<td>39</td>
</tr>
<tr>
<td>Preparation of Acetone Powder from Soybean Seeds</td>
<td>39</td>
</tr>
<tr>
<td>Preparation of Chromatography Columns</td>
<td>39</td>
</tr>
<tr>
<td>Sephadex G-100 column</td>
<td>39</td>
</tr>
<tr>
<td>DEAE-sephadex A-50 column</td>
<td>40</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Purification of Lipoxygenase Isozymes</td>
<td>41</td>
</tr>
<tr>
<td>Protein Determination</td>
<td>43</td>
</tr>
<tr>
<td>Lipoxygenase Assay</td>
<td>44</td>
</tr>
<tr>
<td>Polyacrylamide Gel Electrophoresis (PAGE)</td>
<td>45</td>
</tr>
<tr>
<td>Antibody Production</td>
<td>47</td>
</tr>
<tr>
<td>Seed Germination</td>
<td>49</td>
</tr>
<tr>
<td>Tissue Processing</td>
<td>49</td>
</tr>
<tr>
<td>Ultrastructural study</td>
<td>49</td>
</tr>
<tr>
<td>Immunocytochemical study</td>
<td>50</td>
</tr>
<tr>
<td>Microscopy</td>
<td>51</td>
</tr>
<tr>
<td>Ultrastructural study</td>
<td>51</td>
</tr>
<tr>
<td>Immunocytochemical study</td>
<td>51</td>
</tr>
<tr>
<td>Localization of Lipoxygenase by Physical and Biochemical Techniques</td>
<td>54</td>
</tr>
<tr>
<td>Tissue extraction</td>
<td>54</td>
</tr>
<tr>
<td>Differential centrifugation</td>
<td>54</td>
</tr>
<tr>
<td>Sucrose density gradient fractionation</td>
<td>55</td>
</tr>
<tr>
<td>Marker enzyme assays</td>
<td>55</td>
</tr>
<tr>
<td>RESULTS</td>
<td>57</td>
</tr>
<tr>
<td>Purification of Lipoxygenase Isozymes</td>
<td>57</td>
</tr>
<tr>
<td>Morphology and Lipoxygenase Activity Changes during Germination and Seedling Growth</td>
<td>59</td>
</tr>
<tr>
<td>Mobilization of Food Reserves during Germination and Seedling Growth</td>
<td>60</td>
</tr>
<tr>
<td>Proteins</td>
<td>60</td>
</tr>
<tr>
<td>Lipids</td>
<td>64</td>
</tr>
<tr>
<td>Starch</td>
<td>66</td>
</tr>
<tr>
<td>Ultrastructural Changes during Germination and Seedling Growth</td>
<td>66</td>
</tr>
<tr>
<td>Localization of Lipoxygenase</td>
<td>72</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>----</td>
</tr>
<tr>
<td>Differential centrifugation</td>
<td>72</td>
</tr>
<tr>
<td>Sucrose density gradient fractionation</td>
<td>73</td>
</tr>
<tr>
<td>Immunogold labelling</td>
<td>73</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>DISCUSSION</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purification of Lipoxygenase Isozymes</td>
<td>75</td>
</tr>
<tr>
<td>Mobilization of Food Reserves and Ultrastructural Changes during Germination and Seedling Growth</td>
<td>78</td>
</tr>
<tr>
<td>Localization of Lipoxygenase-1 and -2</td>
<td>88</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CONCLUSIONS</th>
<th>93</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>BIBLIOGRAPHY</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>116</td>
</tr>
<tr>
<td>APPENDIX: FIGURES</td>
<td>117</td>
</tr>
</tbody>
</table>
INTRODUCTION

Lipoxygenase (E.C.1.13.11.12; linoleate: oxygen oxidoreductase) is a dioxygenase containing nonheme iron. It catalyzes the oxidation of polyunsaturated fatty acids having a cis,cis 1,4-pentadiene system to produce conjugated hydroperoxydiene derivatives through the insertion of molecular oxygen into the substrate. These primary products are converted into secondary products, such as alcohols, aldehydes, ketones, and epoxy compounds by enzymatic and nonenzymatlc processes, which are responsible for the development of off-flavors during the processing of certain plant food stuffs, particularly soybeans (Mustakas et al., 1969; Kon et al., 1970; Ashraf and Snyder, 1981).

Seeds of soybean (Glycine max [L.] Merr.) contain significant amounts of lipoxygenase-1 and -2 (1.4 and 2.8 mg/g dry weight, respectively) (Slappendel, 1983; refer to Vernooy-Gerritsen et al., 1984). The properties of lipoxygenase and the methods for its purification have been studied extensively, and the existence of multiple lipoxygenases in soybeans is also well-established (Christopher et al., 1972; Yamamoto et al., 1970; Verhue and Francke, 1972). However, the distribution and physiological function of lipoxygenase in plants are far from clear.

For the better understanding of action, distribution and physiological function of lipoxygenase in plants, the study of the exact cellular and subcellular localization of lipoxygenase has been emphasized. Most of the early work on the localization of lipoxygenase was done by using differential centrifugation and density gradient fractionation.
Unfortunately, evidence for the subcellular localization of lipoxygenase is not equally helpful. Douillard and Bergeron (1981) found that lipoxygenase activity was distributed in chloroplast stroma of young pea shoots, and Haydar and Hadziyev (1973) reported that lipoxygenase in pea seeds and seedlings was not compartmentalized in any particulate fraction. In pea root, lipoxygenase was localized in the lysosomal fractions (Wardale and Galliard, 1977).

In potato tuber and soybean, lipoxygenase was present in the particle-free supernatant fraction (Wardale and Galliard, 1975). It was claimed that the activity of lipoxygenase would not sediment, possibly because of the destructive action of lipolytic acyl hydrolase on subcellular membranes during isolation.

To determine whether soybean lipoxygenase activity, present in the supernatant of density gradient fractions, originates from lysed cytoplasmic organelles or from the cytosol, Vernooy-Gerritsen et al. (1984) introduced a very specific immunocytochemical technique for the localization of lipoxygenase-1 and -2. Their results by indirect labelling with protein A-colloidal gold complexes showed that both lipoxygenase-1 and -2 were mainly localized in the cytoplasm of storage parenchyma cells of soybean cotyledons.

In this study, immunocytochemical, physical, and biochemical techniques were incorporated to investigate the subcellular distribution of lipoxygenase in soybean cotyledons. For immunocytochemical study, antibodies directed against lipoxygenase-1 and -2 were raised from goats. Antibodies adsorbed onto gold particles (immunogold complexes) were used
for direct labelling of lipoxygenase-1 and -2 in the cotyledons of germinating soybean seeds and seedlings. The immunocytochemical results will be discussed and compared to these results obtained from differential centrifugation and sucrose density gradient fractionation.

Furthermore, the mobilization of food reserves in soybean cotyledons, such as proteins, lipids, and starch, was studied by using light microscopy and transmission electron microscopy. This study will present a general description of fine structural changes which occur during germination and seedling growth. These structural changes will be discussed as far as possible with reference to the metabolism of cotyledons and with the results from localization of lipoxygenase-1 and -2 in cotyledons of soybean seeds and seedlings.
LITERATURE REVIEW

Lipoxygenase in Relation to Foods

The beneficial role of lipoxygenase in the baking industry has been known for a long time. In 1934, Haas and Bohn described in a patent the use of ground soybeans as a bleaching agent for wheat flour during white bread making. They speculated that an enzyme in soybeans caused bleaching of wheat flour pigments. The bleaching occurred as a coupled oxidation during the peroxidation of unsaturated fatty acids by this enzyme (Sumner and Sumner, 1940). Since that discovery, enzyme active soy flour has been added as a bleaching agent in white bread at a level of approximately 1% (flour weight basis) in Canada, the United States, and England (Pringle, 1974; Wood, 1967).

Subsequent research implicated lipoxygenase in the oxidation of the dough protein gluten, which appeared to be associated with the improvement of the rheological and baking properties of dough (Wood, 1967; Fischer, 1985; Pringle, 1974; Frazier et al., 1973). Daniels et al. (1971) suggested that oxidized lipid intermediates are able to act on the hydrophobic sites of lipid-binding proteins, resulting in oxidation of sulfhydryl groups. Later, Frazier et al. (1973) provided further evidence for the mechanism when they noticed that addition of purified soybean lipoxygenase resulted in a marked increase in the release of bound lipids during dough development in air. This phenomenon did not occur after heat treatment or with a nitrogen atmosphere during dough-mixing. Their later study found the rheological effect of lipoxygenase to occur only in the presence of oxidizable, polyunsaturated, free-lipids. They also observed
this improvement was achieved only if the polyunsaturated lipid oxidation
occurred in dough during mixing where oxygen, polyunsaturated fatty acids,
and lipoxygenase were all present (Frazier et al., 1977).

The oxidation of gluten proteins, probably promoted by free-radical
transfer from lipid to protein, seems to be effective, provided the
contacts between lipid and protein are sufficiently frequent (Nicholas and
Drapron, 1983).

Besides the rheological effects, several researchers have supported the
hypothesis that oxidation coupled by lipoxygenase improves bread loaf

Apart from the beneficial effects of lipoxygenase in the baking
industry, this enzyme is responsible for the destruction of essential fatty
acids and fat-soluble vitamins (Garssen et al., 1971; Grossman et al.,
1969; Ben-Aziz et al., 1971) and oxidative damage to proteins, enzymes, and
amino acids (Roubal and Tappel, 1966), as well as the loss of color (Matsuo
et al., 1970; Irvine, 1956; Buckle and Edwards, 1970; Holden, 1965; Cohen
et al., 1984) and off-flavor production in many foods (Mattick and Hand,
1969; Fujimaki et al., 1965; Rackis et al., 1979; Sessa et al., 1969; Cowan
et al., 1970).

Lipoxygenase, which was named "carotene oxidase" initially (Sumner
and Sumner, 1940), is known to co-oxidize carotene and chlorophyll. Pigment
destruction during spaghetti processing, where the loss of color is
undesirable, was observed (Matsuo et al., 1970; Irvine, 1955). Wheat flour
lipoxygenase seemed to be involved in oxidation of β-carotene, producing
β-carotene epoxide and conjugated polyene ketones.
The blanching of chlorophyll-containing vegetables has been universally recognized as an important factor in the retention of color (Buckle and Edwards, 1970). Many workers have shown that the inactivation of lipoxygenase prevents color changes associated with the conversion of chlorophyll to pheophytin (Holden, 1965; Buckle and Edwards, 1970; Cohen et al., 1984). Holden (1965) conducted an extensive investigation to explain the relationship between lipoxygenase activity and chlorophyll bleaching in legume seeds. She found that the bleaching activity of soybean extract clearly bore some relation to lipoxygenase activity but did not parallel it exactly. The pH optimum was different for bleaching and for peroxidation, which can be explained by the existence of isozymes (Kies, 1947; Koch et al., 1958; Christopher et al., 1970; Yamamoto et al., 1970).

The nature and mechanism of the pigment bleaching reaction of lipoxygenase has not been established yet. Some researchers suggested that an unstable intermediate fatty acid peroxide compound (Arens et al., 1973) and singlet oxygen (Pistorius, 1974) might be responsible for the destruction of the pigment.

The production of off-flavors during processing is a major factor that limits the use of many vegetable proteins in foods, especially soy protein (Wolf, 1975; Wilkens et al., 1967; Eldridge et al., 1963; Ashraf and Snyder, 1981; Mattick and Hand, 1969). Haas and Bohn (1934) mentioned that addition of raw soy flour at levels above 2% contributed an undesirable beany flavor in bread. This fact has been confirmed by many workers who attempted to add soy flour at levels above that required for bleaching in
order to increase protein level as well as to correct the lysine deficiency in wheat flour (Finney et al., 1950; Ofelt et al., 1952).

Evidence indicates that lipoxygenase plays a major role in the development of beany and bitter flavors in soybean products under certain processing conditions (Wilkens et al., 1967; Mustakas et al., 1969; Kon et al., 1970; Ashraf and Snyder, 1981). Several volatile components, such as ethyl vinyl ketone, cis-3-hexenal, trans-2-hexenal, n-hexanal, have been identified as a major contributor to the beany odor and flavor (Mattick and Hand, 1969; Fujimaki et al., 1965; Wolf, 1975; Sessa et al., 1969). Recently, Matoba et al. (1985) reported that lipoxygenase-2 is responsible for the generation of n-hexanal in soybean homogenate.

In other foods, the many short chain aldehydes produced from polyunsaturated fatty acids contribute to desirable flavor: tomato (Stone et al., 1975; Jadhav et al., 1972; Galliard and Matthew, 1977), green tea Yamashish et al., 1966), and black tea (Saikyo and Takeo, 1972). But at high concentrations, these aldehydes elicit rancid flavors (Kazeniac and Hall, 1970).

To improve the flavor of soybean products, numerous efforts have been made to inactivate lipoxygenase. The processed soy protein industry has improved the flavor of soy flours by carefully controlled moist heat treatment referred to as toasting. This process eliminates much of the flavors found in raw soy flours and, in turn, develops a nutty or toasted flavor and darkens the product (Wolf, 1975).

Several researchers could eliminate lipoxygenase-generated off-flavor development in soymilk by grinding the unsoaked, dehulled soybeans with
boiling water (Wilkens et al., 1967; Mustakas et al., 1969) or by soaking soybeans in 15% ethanol at 50°C for 6 hours (Borhan and Snyder, 1979; Ashraf and Snyder, 1981).

Rice et al. (1981) found that steam heat treatment of soybeans prior to oil extraction was beneficial to the quality of both oil and flakes. Concomitant protein insolubilization which limits usefulness of the resulting product could be minimized by the existence of a critical initial moisture range of 19-20g H₂O/100g solids (Wapinski, 1977) and by the addition of carbonates to the hydration water during heat treatment (Brown et al., 1982).

Adjustment of pH also appeared to be an effective method for inactivating lipoxygenase, particularly in combination with a heat treatment (Kon et al., 1970; Ashraf and Snyder, 1981).

Numerous researchers reported the ability of antioxidants, such as norhydroguaiaretic acid (NDGA), propyl gallate, α-tocopherol, butylated hydroxyanisole (BHA), hydroquinone, to inhibit lipoxygenase (Tappel et al., 1953; Rhee and Watts, 1966; Yasumoto et al., 1970; Al-Obaidy and Siddiqi, 1981b). Furthermore, sulfhydryl compounds and metal binding agents (Al-Obaidy and Siddiqi, 1981b), mannitol (Morrison et al., 1982), saturated monohydric alcohols (Mitsuda et al., 1967b), quercetin (Takahama, 1985), acetylenic compounds (Downing et al., 1970), and erucic acid and tannin (St. Angelo et al., 1979) showed an inhibitory effect on the lipoxygenase activity.

Considerable effort has been made on improving soybean quality by producing low linolenic acid or low lipoxygenase strains through plant
breeding (Brim et al., 1968; Hammond et al., 1972; Chapman et al., 1976; Hammond and Fehr, 1975; Hammond and Fehr, 1983; Hildebrand and Hymomitz, 1981, 1982; Matoba et al., 1985). Treatment of soybean seeds with X-rays and ethyl methylsulfonate has been successful in increasing the incidence of low-linolenic acid soybean strains (Hammond and Fehr, 1975).

There is disagreement on the effect on the fatty acid composition and oil content of seeds. Brim et al. (1968) proposed that oil content and fatty acid composition of seeds were determined primarily by the genotype of the maternal parent, whereas Chapman et al. (1976) proposed that those were affected by environment. However, the lipoxygenase reduction work revealed that the level of lipoxygenase activity in soybeans appeared to be genetically controlled and not influenced by the environment (Hammond et al., 1972; Chapman et al., 1976). Currently, a few groups have successfully released new soybean germplasm lines which do not contain isozymes of lipoxygenase (Matoba et al., 1985; Kinney and Baumgardt, 1986).

Developing biotechnological methods, such as the use of molecular probes and mutator genes, will likely accelerate the bioengineering of the soybean seeds into a more acceptable composition. Compared to the classical breeding approach of repeated backcrossing, time and effort can be saved and entirely new lines with enhanced composition and desirable biochemical properties can become reasonable targets (Moshy, 1985).
Purification of Lipoxygenase Isozymes

Lipoxygenase was first purified in 1947 from soybeans by Theorell et al. (1947a, 1947b). They obtained a purified enzyme by a combination of ammonium sulfate fractionation and electrophoresis. Since then, a number of groups have worked on the purification of lipoxygenase, primarily from soybeans (Mitsuda et al., 1967a; Allen, 1968; Catsimpoolas, 1969; Stevens et al., 1970; Grossman et al., 1972b; Allen et al., 1977; Finazzi-Agrò et al., 1973). Other plant sources from which lipoxygenase was purified were peas (Hale et al., 1969; Arens et al., 1973; Haydar and Hadziyev, 1973; Yoon and Klein, 1979; Reynolds and Klein, 1982), alfalfa (Grossman et al., 1974), potato tubers (Pinsky et al., 1973; Galliard and Phillips, 1971), cauliflower (O'Reilly et al., 1969), wheat (Wallace and Wheeler, 1975), apple (Kim and Grosch, 1979), broad bean (Ai-Obaidy and Siddiqi, 1981a), and eggplant (Grossman et al., 1972a). Recently, several researchers purified lipoxygenase from haricot seed (Kermasha and Metche, 1986), immature English peas (Chen and Whitaker, 1986), and germinating sunflower seeds (Leoni et al., 1985).

Mostly, lipoxygenase has been purified by ammonium sulfate fractionation, gel filtration, and ion-exchange chromatography (Eriksson and Svensson, 1970; Stevens et al., 1970; Yoon and Klein, 1979; Reynolds and Klein, 1982). These techniques are reliable, although they are time-consuming and tedious (Eskin et al., 1977). A few nonconventional techniques of purification were also used. Using an isoelectric focusing technique, Catsimpoolas (1969) isolated soybean lipoxygenase with a 230-fold purification. Affinity chromatography, which separates the enzyme
based on the affinity of the enzyme for the substrate, also has been incorporated into the procedure of lipoxygenase isolation (Grossman et al., 1972b; Allen et al., 1977; Vernooy-Gerritsen et al., 1982), and Grossman et al. (1972b) achieved the separation by passing the crude extract through a linoleyl agarose column. Allen et al. (1977) evaluated a number of aminohexyl derivatives of unsaturated fatty acids as materials for the affinity chromatography of soybean and pea lipoxygenase. Chromatography on the linoleate derivatives was very satisfactory, and docosa-4,7,10,13,16,19-hexenoic acid with the highest degree of unsaturation, which is commercially available as lipoxygenase substrate, was the most satisfactory ligand used in their work.

They also discussed relative importance of the type of bonding involved in enzyme-substrate binding. Indeed, they suggested that the major specific substrate-binding force is not a hydrophobic interaction with the saturated hydrocarbon moiety of the substrate. Although saturated alcohols up to heptanol bond hydrophobically to the lipoxygenase, their effectiveness as inhibitors of the reaction has been reported to be minimal (Mitsuda et al., 1967b).

Vernooy-Gerritsen et al. (1982) separated lipoxygenase-2 from traces of lipoxygenase-1 by affinity purification over a column with lipoxygenase-1 antibodies coupled to sepharose 4B. When they checked the purity of isozymes by using Ouchterlony double diffusion test, no fused lines were detected.

The earliest indication of the existence of lipoxygenase isozymes came from Kies (1947), who discovered that a partially purified preparation of
soybean lipoxygenase lost its carotene bleaching activity on two minutes exposure to 70°C without a corresponding loss of peroxidation activity. From these findings, a dual enzyme system was proposed: a "carotene oxidizing factor" and a "peroxidation factor," suggesting the presence of more than one lipoxygenase.

Another indication of the existence of isozymes resulted from pH optimum studies. Employing soybean meal as the enzyme source, Smith (1948) observed a pH optimum of 6.5 for lipoxygenase. However, Holman (1947) obtained a pH optimum of 9.0 for crystalline soybean lipoxygenase. At this juncture, the problem remained until Koch et al. (1958) succeeded in fractionating a soybean extract into two entities with lipoxygenase activity. These fractions differed in optimum pH and substrate preference. One utilized linoleic acid as a substrate within a pH range of 8 to 9. The other was more active on trilinolein and methyl linoleate and showed a broad optimum pH in the range of 5 to 6.

Several researchers confirmed these results (Christopher et al., 1970; Yamamoto et al., 1970; Verhue and Francke, 1972). Christopher et al. (1970) isolated and purified an isozyme (lipoxygenase-2), which differed from the well-known crystalline lipoxygenase of Theorell (lipoxygenase-1). Lipoxygenase-2 showed an optimum activity at pH 6.5, while lipoxygenase-1 showed an optimum activity at pH 9.0. For the substrate specificity, they observed the same results as Koch et al. (1958). On gel electrophoresis, lipoxygenase-2 revealed one protein band (R_f = 0.25) which was distinct from the lipoxygenase-1 with an R_f of 0.34. Later the same group of researchers isolated two additional isozymes from soybeans and named them
lipoxygenase 3a and 3b (Christopher et al., 1972). These two isozymes differed from the two previously purified isozymes by their elution profiles from DEAE-sephadex, isoelectric points, pH profiles, and the effect of calcium ion activity. In comparison with lipoxygenase-1 and -2, lipoxygenase 3a and 3b were not very active and showed no distinct optimum pH. Lipoxygenase 3a and 3b were alike in all aspects except their chromatographic behaviors on columns of hydroxyapatite and CM-sephadex. The mixture can be treated as a single enzyme (Axelrod et al., 1981).

Yamamoto et al. (1970) isolated two isozymes from soybeans, which were named as lipoxygenase a and b. They found that lipoxygenase b activity was dependent on calcium ion, and the catalytic nature of lipoxygenase a was similar to that of Theorell lipoxygenase (lipoxygenase-1).

Verhue and Francke (1972) also separated two fractions of lipoxygenase from soybean extracts on ion-exchange resins. One showed preferential activity for the free acid, the other for the methyl ester. They suggested that this substrate specificity between the "acid" and "ester" lipoxygenase was due to a different way of binding the substrate, since the "ester" lipoxygenase was capable of catalyzing oxygenation of linoleic acid at neutral pHs but was inactive at pH 9.0, when the fatty acid was completely dissociated.

Later, Roza and Francke (1973) examined the product specificity of purified "acid" and "ester" lipoxygenase from soybeans. "Acid" lipoxygenase yielded exclusively the 13-hydroperoxide at pH 9.0 and 0°C, while at pH 9.0 and room temperature, 9-isomers were also found. At pH 7.0 and room temperature, yields of 13- and 9-isomers were in the ratio of about 1:1.
The "ester" enzyme was characterized by a preferential oxygenation at the ends of the unsaturated system, both in free fatty acids and methyl esters.

**Distribution of Lipoxygenase**

Until the mid-1960s, lipoxygenase activity was generally thought to be restricted to seeds of leguminous plants and certain cereals (Tappel, 1963). It is now clear that lipoxygenase is widely distributed in higher plants and in various plant organs. Extensive lists of plants known to contain lipoxygenase have appeared in numerous reviews (Axelrod, 1974; Eskin et al., 1977; Galliard and Chan, 1980), and its activity in a wide range of plant tissues has been measured (Rhee and Watts, 1966; Pinsky et al., 1971; Galliard and Matthew, 1973).

Apart from the general occurrence of lipoxygenase in legume seeds, distribution of lipoxygenase among the tissues of a plant varies. In *Brassica*, activity is high in florets but low in leaves and fruits (Pinsky et al., 1971); in watermelon seedlings, activity in cotyledons is 50-fold higher than in the roots or hypocotyl (Vick and Zimmerman, 1976). The extractable lipoxygenase was found mainly in pea cotyledon, and the concentration of enzyme was somewhat higher in the inner than in the outer part of cotyledon (Eriksson, 1967).

Furthermore, lipoxygenase also has been found in animal tissues. Nugteren (1975) reported arachidonate lipoxygenase in blood platelets. German and Kinsella (1985) suggested that endogenous skin lipoxygenase from trout might contribute a significant source of initiating radicals leading to subsequent lipid peroxidation in fish tissue. Whether the mammalian
enzyme is identical to plant lipoxygenase has not been determined. However, Bild et al. (1978) reported that lipoxygenase-2 from soybeans catalyzed the oxygenation of arachidonic acid to form significant amounts of an eicosanoid product \textit{in vitro}.

For the better understanding of lipoxygenase action and physiological function in plants, the study of the exact cellular and subcellular localization of lipoxygenase has been emphasized (Galliard and Chan, 1980; Vernooy-Gerritsen et al., 1984). Most of the early work on the localization of lipoxygenase was done by using differential centrifugation and density gradient fractionation. Unfortunately, evidence on the subcellular localization of lipoxygenase is not equally helpful.

Douillard and Bergeron found that lipoxygenase activity was distributed in the chloroplast lamellae of young wheat (1979) and in chloroplast stroma of young pea shoots (1981). High lipoxygenase activity was found in the intact proplasts and vacuoles from both peel and flesh tissue of cucumber (Wardale and Lambert, 1980).

Haydar and Hadziyev (1973) found that lipoxygenase in pea seeds and seedlings was not compartmentalized in any particulate fraction. In pea root, lipoxygenase was localized in the lysosomal fractions, whereas in \textit{Brassica florets} (cauliflower) lipoxygenase was present in a heavy body with a density similar to plastids (Wardale and Galliard, 1977). Grossman et al. (1972c) detected the greatest lipoxygenase activity in alfalfa from mitochondria, followed by chloroplasts and then microsomes.

In potato tuber and soybean, lipoxygenase was present in the particle-free supernatant fraction (Wardale and Galliard, 1975; Vernooy-Gerritsen
et al., 1983a, 1984). It was claimed that the activities of lipoxygenase would not sediment, possibly because of the destructive action of lipolytic acyl hydrolase on subcellular membranes during isolation (Wardale and Galliard, 1975).

To determine whether soybean lipoxygenase activity, present in the supernatant of density gradient fractions, originates from lysed cytoplasmic organelles or from the cytosol, a very specific immunocytochemical technique has been introduced by Vernooy-Gerritsen et al. (1984). Their results showed that lipoxygenase-1 and -2 were localized in the cytoplasm of storage parenchyma cells and in aberrant types of protein bodies occurring in hypodermis and vascular bundle cells. No association has been found with protein bodies in storage parenchyma cells or lipid bodies, mitochondria, and other organelles in any type of cell.

Physiological Function of Lipoxygenase

Despite the widespread distribution of lipoxygenase and increasing concern in the reactions catalyzed by this enzyme, there is not yet complete understanding for the physiological role of lipoxygenase. Several possible functions have been suggested (Gallaird et al., 1968; Lieberman and Mapson, 1964; Hitchcock and Nichols, 1971; Galliard and Chan, 1980), but none has emerged as generally applicable.

It has long been known that damage to plant tissues can result in the formation of large amounts of a substance which induces cell proliferation, which was identified as traumatic acid (Hitchcock and Nichols, 1971). Lipoxygenase activity is commonly exhibited in wound tissues (Veldink
et al., 1977), and relevant observations include the increase in lipoxygenase activity in response to wounding and the formation of a "wound hormone" via a presumed lipoxygenase-mediated process. Hitchcock and Nichols (1971) proposed that traumatic acid was formed from linoleic or linolenic acid hydroperoxide. The 12-oxo-10-\textit{trans}-dodecenoic acid was reported as the active component of traumatin by Zimmerman and Coudron (1979). Perhaps the strongest circumstantial evidence for a physiological function of lipoxygenase is in the area of wound response of plants (Galliard and Chan, 1980; Galliard, 1978).

The involvement of linoleic acid and lipoxygenase in ethylene production in plants is controversial (Galliard et al., 1968; Lieberman and Mapson, 1964). Although methionine is generally recognized as the precursor of ethylene (Mapson et al., 1970; Mapson and Wardale, 1968), this role is still not fully resolved. Recently, Lynch et al. (1985) suggested that lipoxygenase-generated hydroperoxides account for the nonphysiological features of ethylene formation from 1-aminocyclopropane-1-carboxylic acid (ACC) by the microsomal membranes of carnation flowers. They also indicated that this \textit{in vitro} conversion of ACC to ethylene is not reflective of the reaction mediated by the native \textit{in situ} ethylene-forming enzyme.

Another possibility remains that ethane may be derived from lipoxygenase-mediated reactions in disrupted tissues. Elstner and Konze (1976) demonstrated that, in damaged leaf tissue, ethane production is proportional to the number of damaged cells. This suggested that ethane production was a function of disrupted cells (Galliard, 1978).
An earlier suggestion that lipoxygenase is involved in cutin formation (Mazliak, 1968) has not been substantiated. Nevertheless, there are indications from several sources that lipoxygenase could well be involved both in the microbial and insect interactions with plants (Galliard and Chan, 1980) and the production of volatile compounds in the biosynthetic process of unsaturated and oxygenated fatty acids (Veldink et al., 1977; Galliard and Chan, 1980; Galliard, 1978).

Recently, possible significant function of lipoxygenase during germination was proposed by Vernooy-Gerritsen et al. (1983a, 1984). If there is random distribution of lipoxygenase in the cytoplasm of storage parenchyma cells, the general function might lie in the dioxygenation of fatty acids to make their transport from lipid bodies to glyoxysomes.

Definition of Germination

Germination is essentially a resumption of embryo growth after uptake of water or imbibition (Esau, 1977). During imbibition, the water content of seeds rises quickly at first, then more slowly (Parrish and Leopold, 1977). As a consequence of imbibition, the quiescent tissue becomes metabolically active. Enzymes already present are activated (Trelease et al., 1971; Muto and Beevers, 1974; Vick and Zimmerman, 1976), and new proteins are synthesized (Yomo and Srinivasen, 1973; Juliano and Varner, 1969) for maintaining the metabolism of the active tissue. Cell extension and cell division are initiated (Smith and Flinn, 1967), and ultrastructural changes are prominent (Öpik, 1966; Bain and Mercer, 1966;
Treffry et al., 1967; Webster and Leopold, 1977; Vernooy-Gerritsen et al., 1984). Further, the mobilization of food reserves in storage tissue and the embryo, itself, become active to provide nutrients before the embryo can become a self-supporting seedling (Webster and Leopold, 1977; Treffry et al., 1967; Kermode et al., 1985; Taylor et al., 1985).

Recently, Bewley and Black (1985a) define germination as follows: "It begins with the start of elongation of the seed (imbibition) and ends with the start of elongation by the embryonic axis, usually the radicle."

According to them, germination, therefore, does not include seedling growth which commences when germination finishes. They, as physiologists, do not encourage the inclusion of the mobilization of food reserves as a germination event, since the mobilization mostly happens during seedling growth (Smith, 1974a; Öpik, 1966; Kermode et al., 1985). Nevertheless, a definition of germination including seedling growth has been widely used (Smith, 1974a; Öpik, 1966; Treffry et al., 1967; Smith and Flinn, 1967; Vernooy-Gerritsen et al., 1984).

Structure of Soybean Seeds

In germinating soybean seeds, the embryo is composed of the embryonic axis and two cotyledons. The axis incorporates the embryonic root (radicle), the hypocotyl to which the cotyledons are attached and the shoot apex with the first true leaves (plumule). In seeds without endosperm, like soybeans, the cotyledons are the site of reserve nutrient storage and account for almost 90% of the seed mass (Figure 1).
Cotyledons of soybean comprise an epidermis, a distinct hypodermis, storage parenchyma, and vascular bundles. Vascular bundles, surrounded by a parenchymatous bundle sheath, are situated along the boundary between adaxial and abaxial parenchyma. The cotyledon has an extensive system of intercellular spaces, which can be observed as triangles at every cell wall junction of the parenchyma, when it is viewed as transverse sections (Figure 2-5).

**Mobilization of Food Reserves during Germination and Seedling Growth**

**Proteins**

The storage proteins of seed are found primarily in subcellular organelles called protein bodies (Herman et al., 1981; Ashton, 1976; Lott, 1981). Other terms such as protein storage vacuoles (Herman et al., 1981), and aleurone grains (Khokhlova, 1971; Bewley and Black, 1985b) are also used.

Protein bodies are spherical or oval shaped in section (Lott, 1981; Citharel and Citharel, 1985; Weber and Neumann, 1980) and bound by a single limiting membrane (Lott, 1981; Vernooy-Gerritsen et al., 1984; Pernollet, 1978). They vary in size from one plant to another and from one tissue to another (Citharel and Citharel, 1985). Diameters have been reported from 0.1 and 25 μm with the mean diameter in soybean being 2-8 μm (Lott and Buttrose, 1977; Tombs, 1967; Vernooy-Gerritsen et al., 1984).

Evidence for the localization of storage proteins within protein bodies came from biochemical and histochemical investigations. It has been demonstrated by differential staining that protein bodies contain almost exclusively proteins (Smith, 1974a; Harris and Chrispeels, 1975). Proteins
obtained from isolated protein bodies were shown on the basis of critical studies by density gradient centrifugation to be identical with the respective storage proteins of the seeds (Tombs, 1967), gel electrophoresis (Burgess and Miflin, 1985; Citharel and Citharel, 1985; Kermode et al., 1985; Taylor et al., 1985), amino acid composition (Taylor et al., 1985), and immunocytochemical technique (Craig et al., 1980).

In protein bodies that lack any structure, storage proteins (7s vicilin- and 11s legumin-type) and albumins might be uniformly distributed throughout the entire organelles (Weber and Neumann, 1980; Craig et al., 1980). Though, in most species, the portion of albumin is much smaller (Weber et al., 1978), the most prominent feature of albumin fraction is their enzymatic activity. Several acid hydrolases such as protease (St. Angelo et al., 1968; Ory, 1972) or phosphatase (Matile, 1968) were observed as constituents of protein bodies. According to several researchers, protein bodies also contain hydrolytic enzymes specific against substrate outside of these organelles, such as lipases, gluco-sidases, and amylases (Matile, 1968; Tronier and Ory, 1970). For this reason, Matile (1968) considered protein bodies as lytic compartments of the plant cell, similar to lysosomes of animal cells. This suggestion was confirmed by electron microscopic evidence showing that protein bodies often contain cell organelles during their degradation (Herman et al., 1981).

The highly structured protein bodies have one or more subunits embedded within the finely granular proteinaceous matrix. Rost (1972) has used the types of subunits to categorize protein bodies into three groups:
Group 1, protein bodies without subunits; Group 2, protein bodies with globoid subunits; Group 3, protein bodies with globoid and crystalloid subunits. It had been thought that soybean seeds did not possess any inclusions (Tombs, 1967). Later, some protein bodies were observed with a few large globoid inclusions in mesophyll cells (Lott and Buttrose, 1977; Lott, 1981).

The globoids consist of storage phosphate compounds. The major compound is phytin, an insoluble calcium magnesium salt of inositol hexaphosphoric acid (Lott and Buttrose, 1977; Lott, 1981; Ashton, 1976). The globoid is very brittle; therefore, it is not penetrated well by fixatives and resins and tends to shatter when cut. This occurrence leaves a hole in the section or small fragments of globoid distributed around the globoid area (Ashton, 1976). These technical difficulties have been described in detail elsewhere (Lott and Buttrose, 1977; Lott, 1980). An alternative method, freeze-etching which minimizes fixation artifacts, was introduced by Lott and Buttrose (1977) and Lott (1980).

Considerable new information on the element composition of the protein bodies was derived from energy dispersive X-ray microanalysis (EDX) which allows in situ studies of elements with atomic number over 11 (Lott and Buttrose, 1977; Lott, 1981). A predominance of P and K in protein bodies and globoids was present, whereas, Ca and Mg were present in lower amounts or only in traces.

Lipids have been reported to be present in protein bodies (Tombs, 1967; Pernollet, 1978); however, these may be contaminants except for the membrane phospholipids (Tombs, 1967). Other minor biochemical components
include RNA (Tombs, 1967; Pernollet, 1978; Yokoyama et al., 1972), oxalic acid, and carbohydrates (excluding starch) (Pernollet, 1978).

Several ultrastructural studies on the fate of the protein body during seed germination have been reported (Treffry et al., 1967; Õpik, 1966; Pernollet, 1978; Taylor et al., 1985; Vernooy-Gerritsen et al., 1984). The pattern of protein body degradation most commonly reported is a swelling of the organelles, followed by internal degradation of protein and, finally, fusion into larger vacuoles or to protein masses (Õpik, 1966; Bain and Mercer, 1966; Smith and Flinn, 1967; Webster and Leopold, 1977; Treffry et al., 1967; Pernollet, 1978). Recently, Taylor et al. (1985) observed a different mode of protein body degradation in germinating sorghum, where protein bodies did not swell and fuse and were degraded predominantly from the protein body boundary membrane inward.

In germinating soybean cotyledons, protein bodies become more granular or flocculent. Eventually, these become irregular and sometimes coalesce into a single mass (Tombs, 1967; Vernooy-Gerritsen et al., 1984), giving rise to the central vacuole, while the integrity of their membrane is maintained (Pernollet, 1978; Webster and Leopold, 1977). Ashton (1976) speculated that a flocculent appearance of protein bodies in electron micrographs indicates endogenous proteolytic activities within these organelles. However, discrepancies exist between ultrastructural and biochemical changes of protein body contents in germinating Vicia faba seeds, where the protein body becomes flocculent in the electron micrographs without any detectable storage protein degradation (Lichtenfeld et al., 1979). Similarly, a flocculent appearance of the protein body
contents was demonstrated by Mollenhauer and Totten (1971) as a consequence of the fixation procedure for seeds. Vernooy-Gerritsen et al. (1984) observed flocculent protein bodies only in the vascular bundle and hypodermis cells but not in the storage parenchyma cells of germinating soybeans.

During germination, the contents of free amino acids are increased due to breakdown of storage protein by proteinase (Harris and Chrispeels, 1975). The majority of liberated amino acids are translocated to the growing axis, and some of these amino acids are reutilized in protein synthesis in situ or further metabolized to yield energy (Ashton, 1976; Bewley and Black, 1985b). Amino acids, such as valine, phenylalanine, proline, and arginine, which are not gluconeogenic, were found to be only slowly metabolized by the cotyledon and were recovered unchanged in the seedling axis (Durzan and Chalupa, 1968).

### Lipids

The triglyceride reserves of soybean cotyledons are in the form of discrete subcellular organelles, the lipid bodies. A variety of names have been used for these storage organelles: spherosomes (Sorokin, 1967; Jacks et al., 1967; Yatsu and Jacks, 1972), oil bodies (Smith, 1947b), oleosomes (Bergfeld et al., 1978), lipid vesicles (Mollenhauer and Totten, 1971), and lipid bodies (Wanner et al., 1981).

Lipid bodies, which stain uniformly gray (Smith, 1974b; Horner and Arnott, 1966), are spherical and appear to be bound by a half-unit membrane (Yatsu and Jacks, 1972; Jacks et al., 1967; Wanner and Theimer, 1978).
They vary in size from one plant to another. Diameters have been reported from 0.1 to 2.0 μm (Sorokin, 1967; Smith, 1974b; Jacks et al., 1967; Vernooy-Gerritsen et al., 1984).

Observations on lipid bodies have suggested that these are consumed for cell metabolism during germination and seedling growth (Wanner and Theimer, 1978; Mollenhauer and Totten, 1971). Mobilization of lipid reserves during germination results in their gradual disappearance (McAlister and Krober, 1951; Treffry et al., 1967). Lipid bodies become frequently irregular in outline and often larger (Webster and Leopold, 1977). In the germinating soybeans, mobilization of the stored lipid begins on about the third day after imbibition (Vernooy-Gerritsen et al., 1984), and lipid bodies are not seen in cytoplasm after 10 days, but they become very frequent in the plastids (Treffry et al., 1967). Horner and Arnott (1966) reported that lipid is the last of original seed reserves to be utilized completely in germinating Yucca seeds.

Several biochemical analyses (Yoshida, 1984; Holman, 1948) on the lipid contents in the germinating soybeans showed an agreement with the results of ultrastructural studies (Treffry et al., 1967; Webster and Leopold, 1977; Vernooy-Gerritsen et al., 1984). Yoshida (1984) also reported that 85% of triacylglycerols had been consumed at day 12 of seedling growth and triacylglycerols with polyunsaturated fatty acids were hydrolyzed slightly faster than other species.

Lipid bodies are randomly distributed through the cytoplasm between the protein bodies, in a single layer around protein bodies, and linearly on the inner surface of the plasma membrane (Webster and Leopold, 1977; Allen
et al., 1982; Treffry et al., 1967; Vernooy-Gerritsen et al., 1984). Hexagonal arrangement of lipid bodies around protein bodies was also reported by Allen et al. (1982). Lipid bodies, occasionally, are closely associated with mitochondria and endoplasmic reticulum (Stein and Stein, 1968) and glyoxysomes (Bergfeld et al., 1978; Wanner and Theimer, 1978; Trelease et al., 1971) during germination.

Several electron microscopic studies showed that, in intermediate stages of lipid depletion, lipid bodies became invaginated with cytoplasmic material and became surrounded by a membrane with a thick lipid layer (Wanner and Theimer, 1978; Bergfeld et al., 1978). Wanner and Theimer (1978) further suggested that the appendices of lipid bodies represent the lipase carrying membranes, and the glyoxysomes may receive their substrates from the appendices or any area of lipid body surface.

Different modes of lipid degradation during germination have been reported. Mollenhauer and Totten (1971) observed the transformation of composite lipid vesicles into flattened saccules in bean cotyledon, and Horner and Arnott (1966) observed a fine reticulation of gray oil material in germinated Yucca seeds, followed by an increase in its size, breakdown of meshwork, leaving a lipid body vacuole that collapses in cells.

The breakdown of cotyledon lipid involves three discrete subcellular organelles: lipid bodies, mitochondria, and glyoxysomes. Briefly, these three organelles function biochemically as follows: (1) lipolysis in the lipid bodies to give fatty acids and glycerol occurs; (2) oxidation of fatty acids and synthesis of succinate in the glyoxysomes, which takes
place via the glyoxylate cycle; and (3) conversion of succinate to oxaloacetate in the mitochondria. The oxaloacetate is processed further in the cytoplasm, ultimately producing sucrose (Ching, 1973).

Since ß-oxidation also occurs in glyoxysomes and acetyl CoA is produced and consumed in the same organelle (Hutton and Stumpf, 1969), this biochemical processing has led to the concept that mitochondria and glyoxysomes cooperate closely in the conversion of succinate to malate and that succinate is transported from glyoxysomes to the mitochondrion for further conversion to oxaloacetate through citric acid cycle. The oxaloacetate released into the cytoplasm is converted to hexose by reversal of the glycolytic pathway. Some workers reported that gluconeogenesis might also occur within the plastids, an organelle from which a small amount of glycolic enzymes have been recovered (Thomas and Rees, 1972).

The major products of gluconeogenesis are sucrose and stachyose, which are presumably transported from the cotyledons to the growing axis (Bewley and Black, 1978a; Abrahamsen and Sudia, 1966) and starch, a transient reserve material which is utilized later in germination (Adams et al., 1980). Furthermore, conversion to amino acids, nucleotides, proteins, and lipids might occur to sustain the metabolism of persistent cotyledons (Thomas and Rees, 1972; Sinha and Cossins, 1965).

**Carbohydrates**

Respiration during initial water uptake, germination, and early growth requires a supply of readily-available substrates other than that derived from hydrolysis of the major storage reserves, since these only become
available after embryo growth commenced (Bewley and Black, 1978b). Sucrose, stachyose, and raffinose are good substrates for respiration (Abrahamsen and Sudia, 1966). In soybean, both raffinose and stachyose levels decline by about 50% over the first 48 hours after imbibition starts. Sucrose which is more abundant than the other two oligosaccharides only declines between the second and fourth day after imbibition starts (East et al., 1972).

Free fructose and glucose accumulate within seeds during sucrose, raffinose, and stachyose breakdown; however, no accumulation of galactose has ever been observed (Pazur et al., 1962). This fact is an indication of its rapid utilization, perhaps by incorporation into cell wall polysaccharides or into galactolipids of the newly forming organelles in the cells of the developing seedling (Bewley and Black, 1978a).

During germination of several legume species, amylase activity increases, and starch is depleted (Opik, 1966; Smith, 1974a), but soybeans apparently synthesize starch during imbibition and germination (Webster and Leopold, 1977; Treffry et al., 1967; Vernooy-Gerritsen et al., 1984) as a result of gluconeogenesis from storage lipid (Adams et al., 1980). These starch grains are accumulated as a transient reserve material, which is utilized later in seedling development.

Patterns of reserve mobilization in germinating seeds

Few extensive studies have been made on the breakdown of all the reserves from a single storage tissue (Opik, 1966; Smith, 1974a; Smith and Flinn, 1967; Vernooy-Gerritsen et al., 1983a, 1984). During the
germination of field pea seeds, mobilization of storage reserves begins at the abaxial side of the cotyledons and proceeds towards the adaxial side, which is at the center of the seeds (Smith and Flinn, 1967). The cotyledons of French beans show a different pattern of reserve mobilization. Öpik (1966) found that degradation begins in the cells farthest from the epidermis and the vascular bundles. These results were later confirmed by the report of Smith (1974a). In soybean cotyledons, mobilization begins at abaxial side of cotyledon (Vernooy-Gerritsen et al., 1983a) and around vascular bundles (Smith, D. L., 1974, refer to Bewley and Black, 1978b).

Ultrastructural Changes during Germination and Seedling Growth

The initial transformation of the dry embryo into an actively growing organism is associated with characteristic ultrastructural changes: progressive loss of reserve materials such as proteins, lipids, and carbohydrates (Horner and Arnott, 1965, 1966; Abrahamsen and Sudia, 1966; Ashton, 1976; Bergfeld et al., 1978; Adams et al., 1980; Taylor et al., 1985); an enhancement of the definition of cytoplasmic membranes that were rather obscure in the dry state (Webster and Leopold, 1977); appearance of certain organelles and membrane systems that seemed to be absent in the dormant seeds (Treffry et al., 1967; Harris and Chrispeels, 1980; Webster and Leopold, 1977; Wanner and Theimer, 1978; Horner and Arnott, 1966); and finally, a start of cell division and differentiation (Srivastava and Paulson, 1968). In this review, ultrastructural changes of several organelles which play important roles in the mobilization of food reserves
during germination, such as plastids, mitochondria, glyoxysomes, and endoplasmic reticulum, will be discussed.

**Plastids**

In the resting seeds, plastids lack internal membranes and contain shreds of a crystalline lattice formation, which may represent protein or part of a pro-lamellar body, osmiophilic globules, ribosomes, and electron-transparent regions, which may contain DNA (Webster and Leopold, 1977). There have been controversial reports about the existence of plastids with starch grains in resting cells of soybean cotyledons (Treffry et al., 1967; Webster and Leopold, 1977). Since the contents of starch in soybean are only 1% (Wilson et al., 1978) and a great number of protein bodies and lipid bodies fill each cell, it might be difficult to recognize any organelles (Horner and Arnott, 1966).

Plastids of cells of imbibed tissue contain large, conspicuous starch grains in a dense stroma (Treffry et al., 1967; Webster and Leopold, 1977; Vernooy-Gerristen et al., 1984). These grains are a result of gluconeogenesis (Adams et al., 1980). Like plastids of dry tissue, those of imbibed cells are irregular in size and shape (Webster and Leopold, 1977). After 10 days of germination, plastid degeneration has commenced. Swollen plastids containing few large grana are observed (Treffry et al., 1967).

**Mitochondria**

Mitochondria are not observed in resting cells (Övik, 1966), perhaps due to the dense array of lipid bodies obscuring other structures. They are frequently seen after imbibition (Treffry et al., 1967; Bain and
Mitochondria of imbibed cells are round or oval (Treffry et al., 1967). The outer membrane appears intact, and the inner is invaginated to form an elaborate substructure of numerous, narrow cristae (Treffry et al., 1967; Webster and Leopold, 1977).

The origin of mitochondria in imbibed cells is not known. It has been suggested that they could have arisen from pre-existent mitochondria that were not resolvable during dormancy; or from "ghosts" of mitochondria which have lost their enzyme machinery as cells became dormant (pro-mitochondria); or, finally, they may have arisen de novo as the cytoplasm became hydrated (Bain and Mercer, 1966).

In some cells of vascular bundles, mitochondria darken and their cristae swell at the late stages of germination (Öpik, 1966), possibly due to increased permeability of their membranes (Bain and Mercer, 1966). A thin band of cytoplasm and a plastid membrane surrounding the mitochondria are also observed (Treffry et al., 1967). In the cells where plastids are disrupted, vesicles lacking internal structures are seen. Treffry et al. (1967) identified these as degenerate mitochondria. This observed loss of mitochondrial structure parallels other existing biochemical data for germinating seeds (Young et al., 1960; Cherry, 1963).

Endoplasmic reticulum (ER)

It has been reported that ER with ribosomes were not seen in resting cells (Treffry et al., 1967; Öpik, 1966; Bain and Mercer, 1966). Webster and Leopold (1977) found tenuous, thread-like strands of membrane
occasionally lying parallel to lipid bodies and mitochondria in resting cells of soybean cotyledon, but they were not sure about the existence of ER. Recently, Harris and Chrispeels (1980) reported that the ER occurs in two forms: a cisternal form with associated ribosomes which can be seen at all stages from imbibition to cotyledon senescence and a tubular form which initially was associated with the ribosomes.

Most researchers reported that ER and ribosomes were frequently present after three days of germination (Bain and Mercer, 1966; Ópik, 1966; Treffry et al., 1967; Webster and Leopold, 1977), coinciding with gradually increasing water content and with the disappearance of lipid bodies from the cytoplasm (Bain and Mercer, 1966). Harris and Chrispeels (1980) also observed that cisternal ER was most commonly seen at the cell periphery close to the plasma membrane, and tubular ER was found abundantly between the protein bodies. After 24 hours of imbibition, short ER cisternae were often seen in close association with and wrapping around lipid bodies. Furthermore, their morphometry study showed the reduction in the total volume occupied by ER during germination and seedling growth, suggesting a preferential loss of tubular ER before protein mobilization.

The role of ER during germination has been investigated by several researchers. Bain and Mercer (1966) proposed that ER may have assisted the intracellular transport of substances, since the vascular tissue was not extensive in the cotyledon at the early stages of germination. Van Der Wilden et al. (1980) also reported that ER was involved in the transport of ribonuclease and proteinase to the protein bodies. Involvement of ER in
the biogenesis of glyoxysomes has been postulated by several researchers (Lord et al., 1973; Bowden and Lord, 1975, 1976).

The current view is that after imbibition, the plastids present in dry seeds begin to synthesize free fatty acids, mainly palmitic and oleic acids. These fatty acids are transported to the ER, where they are converted to membrane phospholipids by enzymes associated with the ER (Bowden and Lord, 1975). Membrane proteins are inserted into ER, and then by vesiculation glyoxysomes are formed (Bewley and Black, 1985b; Lord et al., 1973).

Glyoxysomes

The size and composition of these organelles varies little with species or tissue of fatty seedlings (Bewley and Black, 1978a). They have equilibrium density of 1.25g/cm$^3$ and are surrounded by a single unit membrane (Wanner and Theimer, 1978).

There is usually a rapid and more or less synchronous development of glyoxysomal enzymes (Gerhardt and Beevers, 1970; Huang and Beevers, 1974) from essentially zero in the dry seeds, and there is a corresponding increase in numbers of glyoxysomes during germination (Gerhardt and Beevers, 1970; Kagawa and Beevers, 1975). Electron micrographs clearly showed this increase in the glyoxysomal population (Vigil, 1970). When degradation of fat reserves is occurring most actively, glyoxysomes are often found closely associated with lipid bodies (Trelease et al., 1971; Wanner and Theimer, 1978).
During the early stages of germination when fat reserves are being consumed, glyoxysomes are functioning in the cotyledons, but later, leaf peroxisomes with their typical enzymes are present (Trelease et al., 1971). The changeover occurs normally when the cotyledons gain exposure to light (Beevers, 1979). It is not known whether glyoxysomes or peroxisomes are individual organelles or merely the same structure with different enzyme constituents (Beevers, 1971). However, it should be emphasized that this process is restricted to those fatty cotyledons that become (briefly) functional leaves. Glyoxysomes are never present in true leaves and leaf peroxisomes arise de novo (Beevers, 1979).

Immunolabelling in Electron Microscopy (EM)

There is a broad spectrum of application for markers in EM. They serve mainly as probes for the localization of cellular components (Vernooy-Gerritsen et al., 1984; Herman and Shannon, 1985; Doman and Trelease, 1985) for the labelling of intracellular transport processes (Craig et al., 1980; Herman and Shannon, 1984) and for visualizing affinity binding sites (Romano and Romano, 1977).

Feldherr and Marshall introduced colloidal gold particles as a trace for EM in 1962, but the first application of colloidal gold as a transmission electron microscope specific immunocytochemical marker was described by Faulk and Taylor (1971). Since then, immunogold labelling techniques have become increasingly popular, and their use has also been extended to scanning electron microscopy (Horisberger and Rosset, 1977) and light microscopy (Gu et al., 1981; DeMey et al., 1981). Furthermore,
multiple labelling experiments have been performed using gold particles of different sizes (Vernooy-Gerritsen et al., 1984; Geuze et al., 1981).

Preparation of colloidal gold is based on the reduction of a dilute solution of tetrachloroauric acid by sodium citrate (Frens, 1973). Different sizes of gold particles (5 to 150 nm) are prepared by controlling the amount of reducing agent. Gold particles carry a net negative charge which causes mutual repulsion and, as a consequence, stability of the colloid (Horisberger, 1984). The addition of electrolytes results in a compression of the ionic double layer surrounding the particles, resulting in coagulation of gold particles. However, coagulation can be prevented when a protective coat is added onto the particles by merely mixing the colloid with a solution of macromolecules (Romano et al., 1974; Vernooy-Gerritsen et al., 1984; Gu et al., 1981).

Colloidal gold binding to macromolecules, although poorly understood, is a simple matter of adsorption at the correct conditions of pH, reagent concentration, and ionic strength (Goodman et al., 1981; Romano and Romano, 1984). Much evidence indicates that macromolecules are adsorbed onto gold particles as a monolayer, presumably through a noncovalent binding process. They remain firmly attached and keep their bioactivities for months (Horisberger and Tacchini-Vonlanthen, 1983). Little change in their native state also was reported (Bauer et al., 1975), and multilamellar adsorption of protein to gold particles was proposed by Goodman et al. (1981), suggesting further investigation is needed as it bears directly on the stability of gold probes.
Successful attempts to label antibodies with colloidal gold particles ("direct" method) are far less numerous than for other macromolecules ("indirect" method) (Figure 6). The major problems with the production of stable immunoglobulin/gold conjugates for the "direct" method may be inherent to the nature of the antibodies themselves, since antibodies consist of many different immunoglobulins displaying a wide range of isoelectric points (Romano et al., 1974; Goodman et al., 1981).

DeMey et al. (1981) successfully introduced gold-labelled antibodies that are stable. Their stability was achieved by incubating the antibody solution with gold at the basic side of its isoelectric point and by using goat antibodies, since human and rabbit antibodies do not stabilize gold (Romano et al., 1974). The addition of polyethylene glycol 20K and bovine serum albumin as a stabilizing agent prevents further adsorption of protein to gold and blocks free binding sites still present at the gold surface, thereby, minimizing possible aggregation and enhancing probe stability (DeMey, 1983).

The basic schemes of the "direct" and "indirect" methods used for EM immunocytochemistry are shown in Figure 6. With the "direct" method, the labelled antibody binds directly to the antigen, whereas with the "indirect" method, the first antibody is visualized only indirectly by a labelled antibody directed against the first. In the "indirect" method, the attachment of a large marker molecule could lower the resolution but amplify the sensitivity of binding. In the "direct" method, it is vice versa (Plattner and Zingsheim, 1983).
The success of immunogold labelling is critically dependent on many factors, including appropriate fixation, dehydration and embedding, cellular permeability, probe concentration, and incubation time. A great deal of time and effort is necessary to determine optimal conditions of labelling; however, it is a technique that is versatile and very specific (Alexander et al., 1985; Goodman et al., 1981). Furthermore, the reliability of immunocytochemical data depends largely on the quality of the immunological tools used. Particularly, the requirement of highly specific antibodies has been stressed (Vernooy-Gerritsen et al., 1983b). Appropriate controls must be made to rule out unspecific adsorption artifacts (Vernooy-Gerritsen et al., 1984). These concerns have caused a few researchers to incorporate a pre-incubation step with buffered-glycine or -lysine solution before immunogold labelling to avoid nonspecific ionic binding of free aldehyde groups introduced during fixation (Doman and Trelease, 1985; Vernooy-Gerritsen et al, 1984).

In transmission electron microscopy, the localization of cellular components requires antigenic determinants undenatured and the ultrastructural morphology of the cell preserved during fixation. These two requirements tend to be mutually exclusive. However, the recently introduced low temperature embedding procedure has resulted in enhancement of structural preservation and immunocytochemical labelling (Roth et al., 1981; Herman and Shannon, 1985). Alternatively, cryoultramicrotomy allows optimal preservation of ultrastructure and immunoreactivity due to mild fixation and the possibility of avoiding dehydration steps (Vernooy-Gerritsen et al., 1984; Geuze et al., 1981).
OBJECTIVES

The objectives of this study are to:

1. Observe the changes of lipoxygenase-1 and -2 activities in germinating soybean seeds and seedlings;

2. Observe the mobilization of food reserves and ultrastructural changes in soybean seeds and seedlings by using light microscopy and transmission electron microscopy;

3. Examine the subcellular localization of lipoxygenase-1 and -2 in the cotyledons of soybean seeds and seedlings by (a) differential centrifugation, (b) sucrose density gradient fractionation, and (c) direct labelling with immunogold complexes;

4. Investigate any possible differences in subcellular distribution of lipoxygenase-1 and -2 in variety AmSoy and genetic mutant A5.
MATERIALS AND METHODS

Plant Materials

Soybean seeds (Glycine max [L.]. Merr. variety 1984 AmSoy and A5) were obtained from Dr. Fehr, Department of Agronomy, Iowa State University.

Preparation of Acetone Powder from Soybean Seeds

Forty grams of the soybean seeds were ground with dry ice in a blender for 7 min and blended for another 7 min with 200 ml of cold acetone containing dry ice. The slurry was filtered through Whatman No. 42 filter paper using a Büchner funnel. The residue on the filter paper was washed with five 50 ml aliquots of cold acetone, and the acetone was evaporated under a hood for 3 to 5 hrs. The residue on the filter paper was stored overnight in a desiccator. The powder was removed from the filter paper, pulverized in a mortar and pestle, and stored in a glass-stoppered bottle at -20°C until it was used.

Preparation of Chromatography Columns

Sephadex G-100 column

One hundred grams of Sephadex G-100 dextran gel (Sigma, St. Louis, MO) was hydrated in a boiling water bath for 5 hrs in approximately 2,500 ml of 50 mM sodium phosphate buffer (pH 6.8). Fine particles were removed by decanting excess buffer, adding fresh buffer, stirring, allowing the gel to settle, and again decanting the excess buffer. To prevent bubble formation in the gel bed during pouring, the gel slurry was degassed by aspiration at room temperature.
A Pharmacia K 50/100 chromatographic column (50 x 100 cm) fitted with a bottom flow adaptor was mounted in a cold room at 4°C and leveled. About 200 ml of the hydrating buffer (50 mM sodium phosphate buffer, pH 6.8) was poured into the column, and the column was filled with gel slurry. The gel particles were allowed to settle for 20 min. The eluent was drained to 3 to 5 cm of the surface of the packed region of the bed, and the outlet was closed. The upper flow adaptor was then installed while 3 to 5 cm of eluent remained above the surface of the bed.

The elution reservoir was connected to the column, and the bed was stabilized by allowing 2,000 ml of eluent to pass through the bed in descending eluent flow mode with approximately 30 cm hydrostatic pressure. After equilibration, a check for gel bed uniformity and a void volume determination were carried out simultaneously by chromatographing 5 ml of a 0.2% blue dextran 2000 solution. Between purifications, the column was reequilibrated with 2,000 ml of buffer. Sodium azide (0.01%) was added to eluent buffer to prevent microbial growth during storage.

**DEAE-Sephadex A-50 column**

Twenty grams of anion-exchange gel, DEAE-Sephadex A-50 (Sigma, St. Louis, MO) was stirred into 1,000 ml of deionized distilled water and hydrated for 2 hrs in a boiling water bath. The gel slurry was cooled and subjected to a washing cycle including: 1.0 M NaOH, water, 0.5 M NaCl, water, 1.0 M NaOH and water until excess alkali had been removed from the slurry. The entire process was carried out on a Büchner funnel. The filtrate was resuspended in 1,000 ml deionized distilled water and
refiltered. The process was repeated five times. Before packing the column, the pH of gel slurry was checked to insure all the alkali was removed and the slurry was degassed.

A Pharmacia K 26/70 chromatographic column (2.6 x 70 cm) was mounted in cold room at 4°C and leveled. About 40 ml of the starting buffer were poured into the column, and requisite gel slurry was added to the column. After the gel was allowed to settle for 20 min, eluent was drained from the gel bed until 2 cm remained above the bed surface. The bed was stabilized by allowing 1,000 ml of eluent to pass through the bed at 20 cm of hydrostatic pressure.

Purification of Lipoxygenase Isozymes

The purification procedure was a modification of the methods of Yoon and Klein (1979) and Axelrod et al. (1981). All steps in the procedure were performed at 4°C unless otherwise stated.

Sixty grams of defatted soybean powder was stirred for 2 hrs with 10 volumes of 50 mM sodium phosphate buffer (pH 6.8). The slurry was forced through two layers of cheesecloth and centrifuged at 16,000 g for 15 min in a Beckman model J-21 centrifuge (Palo Alto, CA). The precipitate was discarded, and the supernatant was brought to 25% saturation (144 g/l) with ammonium sulfate. The solution was allowed to stand for at least 1 hr and the precipitate was spun down at 16,000 g for 15 min. The supernatant was adjusted to pH 6.8 with 0.2 N NaOH and was brought to 60% saturation (230 g/l) with ammonium sulfate and allowed to stand for at least an hour. The resulting precipitate was collected by centrifugation at 16,000 g for 15 min. This was resuspended in 50 mM sodium phosphate buffer (pH 6.8) and
dialyzed overnight against two changes of 50 volumes each of 50 mM sodium phosphate buffer (pH 6.8).

Approximately 50 ml of the dialyzate (15 mg/ml) was applied to the Sephadex G-100 column. The gel bed was eluted by gravity flow with 1,500 ml of 50 mM sodium phosphate buffer (pH 6.8) at a rate of 50 to 75 ml per hour. Ten ml fractions were collected using a fraction collector.

Lipoxygenase activity and protein concentration were determined on alternate fractions from the columns. Those with lipoxygenase activity were pooled and concentrated by using a 70 ml S-series Stirred Cell (Nucleopore, Pleasanton, CA) under nitrogen pressure of 60 psi. The membrane cut-off molecular weight was 50,000. This concentrated enzyme solution was dialyzed against three changes of 50 volumes of 50 mM sodium phosphate buffer (pH 6.8).

Ten ml of enzyme solution (15 mg/ml) was loaded on a column of DEAE-Sephadex, which had been preequilibrated with 50 mM sodium phosphate buffer (pH 6.8). The enzyme was eluted with 2,000 ml of a linear gradient formed from 1,000 ml each of 50 and 220 mM sodium phosphate buffer (pH 6.8) at a flow rate of 20-25 ml/hr. Fractions (8 ml) were collected, and alternate fractions from the column were assayed for lipoxygenase activity and protein concentration.

Lipoxygenase-1 active fractions from DEAE-Sephadex column were collected, dialyzed against deionized distilled water for 6 hrs, and checked for purity by polyacrylamide gel electrophoresis (PAGE).

Fractions containing lipoxygenase-2 and -3 were pooled, and the solution was made up to 70% saturation by slow addition of ammonium sulfate
while maintaining the pH at 6.8 with 0.2 N NaOH. An additional 1 hr was allowed for complete precipitation of lipoxygenase-2 and -3. After centrifugation at 16,000 g for 15 min, the precipitate was dissolved in 25 ml of 50 mM sodium phosphate buffer (pH 6.8), and it was dialyzed against two changes of 50 volume each of 50 mM sodium phosphate buffer (pH 6.8).

The dialyzed protein solution was loaded on a second DEAE-Sephadex column. Elution was carried out with an increasing linear gradient formed from 750 ml each of 50 mM and 220 mM sodium phosphate buffer (pH 6.8). The flow rate was adjusted to 25 ml/hr, and fractions of 6 ml were collected.

Lipoxygenase-2 and -3 active fractions were pooled, dialyzed, and checked for purity by PAGE.

Protein Determination

Protein concentrations were determined by optical density at 280 nm and by the Lowry method (1951).

The absorbance of protein at 280 nm was used as an index of protein concentration in the column eluents. One hundred µl of the sample and 2.99 ml of buffer were pipetted into a cuvette, and optical density at 280 nm was measured with a Beckman DU model 2400 spectrophotometer (Palo Alto, CA) equipped with an Update (Madison, WI) for digital output.

The Lowry method, which is also known as the Folin-Ciocalteau method, was used to determine protein concentration of samples except those of column eluents. A standard curve was constructed by series of known concentrations of bovine serum albumin. The alkaline copper solution was made fresh daily by mixing 1 ml of 0.5% CuSO₄.5H₂O in 1% sodium tartrate
and 50 ml of 2% Na₂CO₃ in 0.1 N NaOH. Five ml of alkaline copper solution were added to samples containing unknown amounts of protein and immediately mixed. After 10 min, 0.5 ml of 1 N phenol reagent was added and thoroughly mixed in each tube. After an additional 30 min, the absorbance (λ = 600 nm) of the standard and sample tubes was read against a blank using a Beckman spectrophotometer. The protein concentration of the sample was calculated from the standard curve.

**Lipoxygenase Assay**

Lipoxygenase was determined polarographically using a YSI Oxygen Monitor Model 53 equipped with a Clark electrode (Yellow Springs Instruments, OH). Throughout purification, an aqueous linoleate substrate (Rackis et al., 1972) was used. A stock solution was prepared by adding 0.1 ml of linoleic acid (Sigma, St. Louis, MO) to 0.1 ml of tween 20 (Sigma, St. Louis, MO). Three-tenths ml of 0.1 N NaOH was then slowly added to obtain a clear solution, and the dispersion was brought to a final volume of 25 ml with deionized water. The stock substrate solution was diluted with 50 mM borax buffer (pH 9.0) and 50 mM sodium phosphate buffer (pH 6.8) for lipoxygenase-1 and -2 assay, respectively, giving a final concentration of 2.57 mM linoleic acid.

Appropriate substrates (2.99 to 2.95 ml) were pipetted into a reaction chamber and oxygenated for 30 sec. Enzyme solutions (10 to 50 μl) were added to the chamber, stirred with a magnetic stirrer, and the initial velocity of the enzyme reaction was recorded. Values for oxygen solubility in the reaction medium were obtained from Chappell (1964). One unit of
lipoxygenase activity corresponds to the consumption of 1 μM of oxygen per minute.

Polyacrylamide Gel Electrophoresis (PAGE)

Gel electrophoresis was performed to check the purity of lipoxygenase-1, -2, and -3 according to the methods of Davis (1964) and Guss et al. (1967). All the PAGE chemicals were obtained from Bio-Rad, Richmond, CA. Resolving gel systems were prepared as shown in Table 1. Immediately after mixing the reagents, gel tubes (0.5 cm inner diameter x 7.5 cm long) were filled. A layer of water was placed on top of the gel solution so that a flat and even surface would be obtained after polymerization.

Table 1. Reagents for acrylamide gel electrophoresis

<table>
<thead>
<tr>
<th>Stock solutions:</th>
<th>7% acrylamide, 0.18% N,N'-methylene-bisacrylamide (Bis), pH 9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) 1 N HCl, 48 ml</td>
<td></td>
</tr>
<tr>
<td>Trizma Base, 36.6 g</td>
<td></td>
</tr>
<tr>
<td>N,N,N',N'-tetramethylethylene-diamine (TEMED), 0.23 ml</td>
<td></td>
</tr>
<tr>
<td>H₂O to 100 ml</td>
<td></td>
</tr>
<tr>
<td>(b) Acrylamide, 28 g</td>
<td></td>
</tr>
<tr>
<td>Bis, 0.735 g</td>
<td></td>
</tr>
<tr>
<td>H₂O to 100 ml</td>
<td></td>
</tr>
<tr>
<td>(c) Ammonium persulfate, 0.14 g</td>
<td></td>
</tr>
<tr>
<td>H₂O to 100 ml</td>
<td></td>
</tr>
</tbody>
</table>

Working solution: 1 part (a), 2 parts (b), 4 parts (c), 1 part H₂O

Electrode buffer: Trizma base, 0.6 g |
Glycine, 2.88 g |
H₂O to 1,000 ml |
The gel tubes were inserted into a gel electrophoresis stem with the unfilled end of gel tubes up in a manner such that no air bubbles existed between the gel and the buffer in the lower reservoir.

A sample containing approximately 100 µg of protein was mixed with the same volume of 0.05% bromophenol blue (tracking dye) in 50% glycerol. The samples were then applied to the top of the gel, and buffer was carefully layered on top of each tube filling it completely with buffer solution. The upper compartment was filled with the same buffer.

The electrophoresis was performed at 3 to 5 mA per tube until the tracking dye was approximately 5 mm from the bottom of the gel.

For lipoxygenase specific staining, the gels were incubated in the substrate solution containing 2.57 mM linoleic acid in 50 mM tris-HCl buffer (pH 8.3) for approximately 30 min with frequent inversion to assure adequate aeration. The gels were then removed from the substrate solution, rinsed with distilled water, transferred to test tubes containing 0.02%, 3,3'-dimethoxybenzidine hydrochloride (Eastman Kodak Co., Rochester, NY), and stained 2 hrs at room temperature (DeLumen and Kazeniac, 1976). Reddish-brown bands were identified as the site of lipoxygenase migration. The distances of the dye and lipoxygenase band migration and the length of the gel were measured.

The protein staining was done by immersing the gels in 0.025% Coomassie blue R-250 in 5% methanol, 10% acetic acid for 10 hrs at room temperature. Finally, staining solution was decanted, and the gels were covered with the destaining solution consisting of 10% acetic acid and 5% methanol until discernable stained bands appeared.
Antibody Production

Antisera to lipoxygenase-1 and -2 were raised in young female goats by subcutaneous injections of the enzyme fractions (2 mg/ml), which were mixed with an equal volume of Freund's complete adjuvant (Difco Company, Detroit, MI). Animals were injected at two-week intervals until a suitable titer was obtained. Before injection, blood samples were taken and saved for the control experiment of immunolabelling in electron microscopy.

The blood sample was allowed to coagulate overnight. Removal of clotted material was obtained by centrifugation (13,600 g, 30 min) twice at room temperature.

The immunoglobulin G (Ig G) fractions of antisera were isolated by the precipitation of the sera with 0.15 M caprylic acid (Sigma, St. Louis, MO) according to the method of Steinbuch and Audran (1969). Caprylic acid (3.4 g) was added to the solution which contained 50 ml serum and 100 ml of 60 mM acetate buffer (pH 6.0) at room temperature to prevent gelation. After vigorous stirring during the dropwise addition of caprylic acid for 30 min, the serum was subjected to centrifugation (10,000 g) for 60 min. The supernatant was dialyzed against 50 mM sodium phosphate buffer (pH 7.4) with several changes. Specificity of antibody was analyzed by Ouchterlony double diffusion test (Ouchterlony, 1968).

A 70 ml S-series Stirred Cell (Nucleopore, Pleasanton, CA; M.W. cutoff 100,000) was used to concentrate the dialyzates of Ig G fractions, and affinity column chromatography was used to get more specific antibody fractions.
A CNBr-activated Sepharose column was set up according to the method of Vernooy-Gerritsen et al. (1983b); CNBr-activated Sepharose 4B (Sigma, St. Louis, MO) was swollen and washed with 1 mM HCl. Lipoxygenase-1 and -2 solutions (40 mg/g dry gel) in 0.1 M NaHCO\(_3\) (pH 8.5) containing 0.5 M NaCl were gently mixed with gel suspension for 15 hrs at 4°C. Unreacted active groups of the gel were blocked with 0.2 M glycine in 0.1 M NaHCO\(_3\) buffer (pH 8.0) containing 0.5 M NaCl. After five washings, alternatively with 0.1 M NaHCO\(_3\) (pH 8.3) and 0.1 M sodium acetate buffer (pH 4.0) each containing 0.5 M NaCl, the gel (2 ml) was applied onto the top of a column (1.6 x 30cm) packed with sephadex G-75 (15cm) which was equilibrated with 50 mM sodium phosphate buffer (pH 7.4) containing 0.09 M NaCl (PBS). The column was washed with 50 mM PBS (pH 7.4) with a flow rate of 15 ml/hr until absorbance at 280 nm of the eluent was zero.

Ig G fractions of sera directed against lipoxygenase-1 and -2 containing 50 mg Ig G (10 ml) were applied to the column with 10 intervals of 15 min. Unbound Ig G was removed by washing the column with 50 mM PBS (pH 7.4) until absorbance at 280 nm in the eluent was minimal. Bound Ig G was eluted with pulses of solutions of 2 M NH\(_4\)SCN (pH 7.2) and 3 M NH\(_4\)SCN (pH 7.2). Each pulse of 3 ml was followed by 50 mM PBS buffer (pH 7.4) until the A\(_{280}\) of the eluent was negligible. The eluted antibody was separated from the thiocyanate by the sephadex G-75 portion of the column. Lipoxygenase active fractions were collected, concentrated, and kept frozen at -20°C before use.
Seed Germination

Seeds of soybean were externally sterilized in 70% ethanol for 10 sec, rinsed with running tap water for 10 min, and soaked for 50 min in oxygen-flushed bi-distilled water. Seeds were then layered on wet filter paper on cotton wool in a plastic box with a lid. The box was placed in the dark at 28 ± 1°C.

Cotyledons of soybean seeds were selected randomly from 1 1/2 hrs through 10 days of germination. Time periods, where significant ultrastructural changes had been observed in preliminary experiments, were chosen.

Tissue Processing

Ultrastructural study

Cotyledons of germinated soybean seeds were placed in 4% glutaraldehyde-2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at room temperature. The tissue was cut with a razor blade into approximately 1mm³ blocks and placed in fresh buffered fixative overnight (12-24 hrs at 4°C.

Fixation was followed by three buffer rinses, 10 min each, and post-fixed in 1% osmium tetraoxide (OsO₄) for 4 hrs at room temperature using the 0.1 M sodium phosphate buffer (pH 7.4). The tissues were washed three times in buffer for 30 min followed by dehydration through a series of graded acetone solutions (30, 60, 90, to 100%). Specimens were infiltrated with Spurr® resin (Polysciences, Inc., Warrington, PA) according to
manufacturer's instruction for a "firm" block, cast in aluminum weighing pans, and placed in a 60°C oven overnight (12-24 hrs).

Immunocytochemical study

Tissues were processed according to the modification of the method of Herman and Shannon (1985). Cotyledons of germinated soybean seeds were placed in 2% glutaraldehyde-4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C. The tissues were then cut with a razor blade into approximately 1 mm$^3$ blocks and placed in fresh buffered fixative for 36 hrs. The tissues were rinsed in 0.1 M sodium phosphate buffer (pH 7.4) and dehydrated in dimethylformide (DMF; Aldrich Chemical Co., Milwaukee, WI) using 25%, 50%, 75%, and 90% (v/v) solutions for 1 hr each at 20°C. The dehydrated tissues were infiltrated with Lowicryl K 4M resin (Polysciences, Inc., Warrington, PA) containing 85% (W/W) monomer, 15% (W/W) crosslinker, and 0.5% (W/W) benzoin methyl ether. Infiltration was carried out at 20°C for 12 hrs in a 1:1 mixture of resin and 90% (V/V) DMF and then for two days in 100% resin. Aluminum foil was wrapped around the sample containers to minimize exposure to light.

Tissue samples were loaded into BEEM capsules (Ted Pella Co., Tustin, CA), filled with resin, and capped to preclude contact with air. The blocks were polymerized by illumination with longwave ultraviolet light for 24 hrs at 0°C followed by 24 hrs at 20°C. The blocks were stored in sealed containers to avoid hygroscopic softening.
Microscopy

Ultrastructural study

**Light microscopy (LM)** Thick sections were cut at 0.5-1.0 μm with glass knives on a Reichert Ultracut E ultramicrotome (Wien, Austria). Sections were collected with a stick applicator and placed on a water droplet on a glass microscope slide. The slide was placed on a hot plate (50-55°C) to remove section wrinkles and to adhere the section to the slide. Sections were stained with 1% toluidine blue in 1% borate buffer, washed, dried on warming tray, mounted in Permount, and dried on a warming tray for several days. Bright field observations were made on a Leitz Wetzlar Orthoplan Microscope (West Germany). Technical pan 2415 film was used in the attached Leitz Orthmat automatic camera.

**Transmission electron microscopy (TEM)** Ultrathin sections (silver and gray) of approximately 60-85 nm were cut with glass knives on a Reichert Ultracut E ultramicrotome, spread with chloroform fumes (Horner, 1976), and arranged for collection with an eyelash and collected on 200 and 300 mesh copper grids. Sections were stained with 20% aqueous or 5% methanolic uranyl acetate, followed by 2% lead citrate (Reynolds, 1963).

Observations were performed on Hitachi HU-11C-1 transmission electron microscope (Tokyo, Japan) at 50 KV accelerating voltage by using Dupont Cronar Ortho S Litho 3 1/4 x 4 inch sheet film.

Immunocytochemical study

**Gold preparation** Colloidal gold particles with an average diameter of 13-16 nm were obtained by reduction of tetrachloroauric acid (HAuCl₄;
Sigma, St. Louis, MO) with sodium citrate (Frens, 1973). One hundred ml
HAuCl₄ (0.01% by weight) solution were heated to boiling, and 2.5 ml of
sodium citrate (1%) were added, mixing rapidly. After 10 min of heating,
the solution turned red-orange, indicating the formation of mono-
disperse spherical particles (Frens, 1973).

Preparation of gold-labelled antibodies The procedure was based on
a modification of the methods of Geoghegan and Ackerman (1977) and
Horisberger et al. (1975). Purified goat Ig G directed against
lipoxygenase-1 and -2 was dialyzed against 2 mM borate buffer (pH 9.0).
The protein solution was centrifuged at 10,000 g for 1 hr at 4°C to remove
aggregates. The gold solution was adjusted to pH 9.0 with 0.2 M K₂CO₃.

The optimal amounts of protein needed to stabilize the gold solution
were determined according to the principals of Geoghegan and Ackerman
(1977). Twenty μg antibody/ml gold solution was optimal. This optimal
amount plus an extra 20% of antibody to insure gold stability was mixed
with the gold solution at pH 9.0. After 2 min stirring, bovine serum
albumin (BSA; Sigma, fraction V, 10% in 2 mM borax, pH 9.0) or polyethylene
glycol 20K (PEG 20K; Sigma, 10% in 2 mM borax, pH 9.0) was added to a final
centration of 1% or 0.1%, respectively. Unbound or loosely bound anti-
bodies, as well as insufficiently stabilized gold particles, were
eliminated by three sequential centrifugations at 14,000 g for 1 hr at 4°C.
The concentrated red pool at the bottom was resuspended in a volume equal
to the initial amount. The buffer for the resuspensions was 20 mM sodium
phosphate buffered-saline (pH 7.4) containing 1% BSA or 0.1% PEG 20K and
2 × 10⁻² M sodium azide.
The last red pool was resuspended in one-tenth of original volume. All diluted gold-labelled antibodies were centrifuged at low speed (250 g) just before use in order to remove any very sticky microaggregates.

**Immunolabelling procedure** All the immunolabelling procedures were performed at room temperature. Millipore-filtered bi-distilled water, which was degassed by boiling for 10 min, was used throughout all the procedures.

Grids with ultrathin section were incubated in 0.05 M glycine in 20 mM PBS (pH 7.4) for 20 min followed by three washings with PBS buffer for 30 min.

Immunogold complexes were diluted just before use until the color was faintly pink (Vernooy-Gerritsen et al., 1984). Immunocytochemical labelling was accomplished by incubating the grids in immunogold complex solution for 60 min. The sections were washed three times for 10 min each with 20 mM PBS (pH 7.4) and three times with degassed bi-distilled water.

As a control, other grids were incubated in pre-immune serum-gold complex solution prepared in the same manner as the immunogold complexes.

After drying in the air, the sections were stained in 5% aqueous uranyl acetate at 37°C for 20 min. The grids were rinsed in a stream of degassed bi-distilled water for 20 sec followed by air-drying in a dust-proof petri dish. The grids were examined and photographed using a Hitachi HU-11C-1 TEM at 50 kV accelerating voltage.
Soybean seeds were soaked in running water for 1 hr and germinated on vermiculite at 28 ± 1°C in the dark. All the chemicals for enzyme assays were obtained from Sigma Chemical Company, St. Louis, MO. All steps in the procedure were performed at 4°C unless otherwise stated.

**Tissue extraction**

The grinding medium contained 13% sucrose, 150 mM tricine buffer (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1% BSA. Germinated seeds were homogenized in a Waring Blender for 3 min. Homogenates were filtered through two layers of cheese cloth and centrifuged at 270 g for 10 min. The supernatant (S1) was saved and used for differential centrifugation and sucrose density gradient fractionation studies.

**Differential centrifugation**

Conditions of centrifugation for the separation of subcellular fractions (Grossman et al., 1972c) were as follows: whole and broken cells were removed by a 10 min centrifugation at 1,200 g. A chloroplast fraction (P1) was obtained by centrifugation at 4,000 g for 10 min, and an additional 10 min centrifugation at 8,000 g removed chloroplastic debris (P2). Mitochondria (P3) were obtained after 20 min at 15,000 g, followed by mitochondrial debris after 20 min at 25,000 g (P4). A final centrifugation at 105,000 g for 60 min yielded a microsomal fraction (P5) and supernatant cytosol (S2). Pellets were quickly frozen, washed with grinding buffer twice, and ground with a mortar and pestle for 3 min in 4 ml of grinding
buffer containing 0.1% Triton-X 100. The mixture was centrifuged at 20,000 g for 30 min and the pellets discarded. The supernatant was collected and assayed for protein content and lipoxygenase activity.

**Sucrose density gradient fractionation**

For the isolation of total organelle population, supernatant from tissue extraction was layered directly onto a sucrose gradient. The gradient consisted of a 3 ml cushion of 60% (W/W) sucrose, a 30 ml linear gradient from 13 to 60% (W/W) sucrose which was prepared with Hoefer gradient maker (San Francisco, CA), and 7 ml of sample in ultra-clear centrifuge tube (Beckman, Palo Alto, CA). All sucrose solutions contained 1 mM EDTA (pH 7.5). The gradients were centrifuged at 21,000 rpm for 4 hrs in a Beckman L5-65 preparative ultracentrifuge with a Spinco SW 27 rotor following which 1.0 ml fractions were collected from the top of the tube.

**Marker enzyme assays**

All enzymes were assayed spectrophotometrically. Sucrose concentrations were determined by using a Bausch and Lomb refractometer at room temperature.

Cytochrome oxidase, a marker enzyme of mitochondria, was assayed by the method of Wharton and Tzagoloff (1967) except that a trace amount of sodium hyposulfite was used to reduce the cytochrome C. Fifty mM sodium phosphate buffer (pH 7.1) and 1% reduced cytochrome C were incubated for 10 min at 38°C. Then enzyme solutions were added to the cuvette. The enzyme activity was determined by monitoring the decrease in absorbance at
550 nm, and the readings were taken against a blank cell containing all reagents plus 0.1 M potassium ferricyanide.

NADH dependent cytochrome C reductase was used as a marker enzyme for endoplasmic reticulum. The increase in absorbance at 550 nm was measured in a final volume of 3 ml containing 0.1 mM NADH, 0.1 mM oxidized cytochrome C, 0.2 M sodium phosphate buffer (pH 7.5), 0.1 M sodium azide, and enzyme solution. The reaction was run at 37°C, and the readings were taken against a blank cell containing the same amount of enzyme solution and all reagents except NADH (Bowles and Kauss, 1976).

Acid phosphatase activity, as a marker enzyme of the lysosomal fraction, was measured by monitoring the increase of absorbance at 420 nm. A standard curve was prepared by using a dilute solution of p-nitrophenol. The final concentration of the solution used was: 100 mM sodium acetate buffer (pH 5.6) and 5 mM p-nitrophenylphosphate and enzyme solution. After 10 min incubation at room temperature, the reaction was terminated by the addition of 0.02 N NaOH. Appropriate blank readings were made under the same condition without p-nitrophenolphosphate. The amounts of p-nitrophenol liberated from substrate were calculated by extrapolating the absorbance into the standard curve of p-nitrophenol.

Catalase activity, as a marker enzyme for the glyoxysomes, was measured at room temperature by monitoring the decrease of absorbance at 240 nm in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.5), 12.5 mM H₂O₂, and enzyme solution. The buffered H₂O₂ solutions were prepared fresh before use, and blank tests were done with H₂O₂-free water (Lück, 1965).
RESULTS

Purification of Lipoxygenase Isozymes

The purification of lipoxygenase isozymes from soybean seeds was accomplished by employing the traditional procedures for separation of proteins: ammonium sulfate fractionation, Sephadex G-100 gel filtration, and DEAE Sephadex A-50 ion exchange chromatography.

Specific activity and fold purification during purification of lipoxygenase from soybean seeds are summarized in Table 2. About a 19-fold purification for lipoxygenase-1 was obtained after DEAE Sephadex chromatography, while a 32-fold purification of lipoxygenase-2 was obtained after the rechromatography of the pooled fractions of lipoxygenase-2 and -3, resulting in a considerable loss of total enzyme activity. After ammonium sulfate fractionation, 39% of lipoxygenase-1 was recovered, while 81% of lipoxygenase-2 was recovered. There was not much lost of lipoxygenase-1 after gel filtration, whereas lipoxygenase-2 lost activity of about 37% compared to the ammonium sulfate precipitates. The total enzyme activity after DEAE-Sephadex remained 10% and 14%, respectively.

A typical elution pattern from a Sephadex G-100 column is shown in Figure 7. The activities at pH 9.0 and pH 6.8, optimum pH of lipoxygenase-1 and -2, respectively, were both eluted at the same fractions. The elution profile from DEAE Sephadex column is shown in Figure 8. Lipoxygenase-1 (Peak III) was sharply separated from lipoxygenase-2 and -3 (peak II and I, respectively), and appeared last, eluted at a concentration of 200-220 mM phosphate. The fractions of
Table 2. Summary of purification of lipoxygenase-1 and -2 from soybeans

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Total protein b (mg)</th>
<th>Total activity a (units)</th>
<th>Specific activity (unit/mg)</th>
<th>Fold purification</th>
<th>Recovery % LOX-1</th>
<th>LOX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LOX-1</td>
<td>LOX-2</td>
<td>LOX-1</td>
<td>LOX-2</td>
<td>LOX-1</td>
</tr>
<tr>
<td>Crude extract</td>
<td>6670.80</td>
<td>2847.60</td>
<td>8172.00</td>
<td>0.43</td>
<td>1.23</td>
<td>1.00</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 25-60% ppt</td>
<td>969.20</td>
<td>1110.85</td>
<td>6617.00</td>
<td>1.15</td>
<td>6.83</td>
<td>2.67</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>245.96</td>
<td>1075.00</td>
<td>4110.8</td>
<td>4.37</td>
<td>16.71</td>
<td>10.16</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>36.72</td>
<td>295.12</td>
<td>--</td>
<td>8.04</td>
<td>--</td>
<td>18.70</td>
</tr>
<tr>
<td>2nd DEAE-Sephadex</td>
<td>28.90</td>
<td>--</td>
<td>1130.50</td>
<td>--</td>
<td>39.11</td>
<td>--</td>
</tr>
</tbody>
</table>

aLipoxygenase activities are based on O₂ uptake using oxygen electrode.

bProtein values were determined by Lowry method (Lowry et al., 1951).
lipoxygenase-2 and -3 appeared to overlap. These fractions were pooled and then passed through the DEAE Sephadex column again. The elution profile from the second DEAE Sephadex column is shown in Figure 9, where lipoxygenase-2 appears immediately after lipoxygenase-3.

Employing the above procedures, it was possible to obtain preparations of lipoxygenase-1, -2, and -3 that appeared homogeneous on disc gel electrophoresis in tris-glycine buffer at pH 9.0. The respective $R_f$ values, employing lipoxygenase specific staining technique (DeLumen and Kazeniac, 1976), were 0.38, 0.29, and 0.33.

Morphology and Lipoxygenase Activity Changes during Germination and Seedling Growth

During germination of soybean seeds at 28 ± 1°C in the dark, the radicle emerged after day 1, and the hypocotyl began its elongation after the second day. Secondary roots appeared in four days, and cotyledons opened after day 6, revealing the expanding primary leaves.

Lipoxygenase-1 and -2/3 activities in AmSoy declined sharply after the second day, and after day 6, the activity of lipoxygenase decreased to a low level, the activity at pH 6.8 being somewhat higher than the activity at pH 9.0 (19 and 14% of maximal activity, respectively, Figure 10).

Lipoxygenase-1 and -2/3 activities in A₅ declined sharply after the third day, and after day 6, lipoxygenase-2/3 activity remained a little higher than the lipoxygenase-1 activity (23 and 22%, respectively) (Figure 11).

Protein content in AmSoy and A₅ during early stages of seedling growth sharply declined. Only 18% of proteins remained in AmSoy and A₅ after four
and five days of seedling growth, respectively. During late stages of seedling growth, the change of protein content was not prominent in both AmSoy and A₅ (Figures 10 and 11).

**Mobilization of Food Reserves during Germination and Seedling Growth**

The earliest stage at which the ultrastructure of the reserve tissues of soybean seeds was examined by electron microscopy was after 1 1/2 hr of germination. Attempts to examine the cotyledons in dry, ungerminated seeds were not very successful because of poor penetration of the fixative.

Two different varieties of soybean, AmSoy and A₅, did not show any significant differences in their ultrastructures. Therefore, for this study, *Glycine max* variety A₅ was used. Table 3 summarizes the mobilization of food reserves in the cotyledon which was observed from 1 1/2 hr to 10 days. Changes in each reserve material listed in Table 3 are discussed in more detail in the following sections.

**Proteins**

Storage protein bodies were present in all cells of the soybean cotyledons including vascular tissues at the beginning of germination. In the storage parenchyma cells, the protein bodies were large (diameter: 2-15 μm) and closely packed together around each nucleus and lipid bodies (Figures 2, 3, 13, and 17). The epidermis and hypodermis contained many small protein bodies, mostly up to 0.1-5 μm in diameter (Figures 2 and 17). Protein bodies of similar size also occurred in the cells of the vascular bundle (Figure 3).
Table 3. Mobilization of food reserves in soybean cotyledon during germination and seedling growth (1 1/2 hr ~ 10 days)

<table>
<thead>
<tr>
<th>Reserve materials</th>
<th>Types of cell</th>
<th>Periods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 1/2 hr</td>
</tr>
<tr>
<td>Protein (protein body)</td>
<td>Storage parenchyma (S.P.) numerous</td>
<td>(2-15 μm)</td>
</tr>
<tr>
<td></td>
<td>Epidermis (Ep1) (0.1-5 μm) numerous</td>
<td>degrading</td>
</tr>
<tr>
<td></td>
<td>Vascular bundle (V.B.) (0.1-5 μm) numerous</td>
<td>degrading</td>
</tr>
<tr>
<td>Lipid (lipid body)</td>
<td>S.P. numerous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epi numerous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V.B. numerous</td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>S.P. absent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epi absent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V.B. absent</td>
<td></td>
</tr>
<tr>
<td>Periods</td>
<td>3 day</td>
<td>6 day</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>irregular, larger</td>
<td>degrading</td>
<td>flocculent</td>
</tr>
<tr>
<td>vacuolating</td>
<td></td>
<td>vacuoles</td>
</tr>
<tr>
<td>flocculent</td>
<td>vacuolating</td>
<td>transparent vacuoles</td>
</tr>
<tr>
<td>less numerous</td>
<td>few</td>
<td></td>
</tr>
<tr>
<td>few</td>
<td>fewer</td>
<td>present</td>
</tr>
<tr>
<td>less numerous</td>
<td>few</td>
<td></td>
</tr>
<tr>
<td>numerous</td>
<td>degrading</td>
<td></td>
</tr>
<tr>
<td>few</td>
<td>degrading</td>
<td>absent</td>
</tr>
<tr>
<td>few</td>
<td>degrading</td>
<td></td>
</tr>
</tbody>
</table>
The shape of protein bodies was usually oval or round (Figures 2 and 3). During germination, the shape of protein bodies tended to vary due to changes induced by imbibition and pressure of adjacent protein bodies (Figures 39 and 40).

Each protein body was limited by a distinct single unit membrane (Figure 37) and had an internal structure that was usually homogeneous and without any inclusions (Figure 12). Sometimes, however, small globoids were found mainly in protein bodies in cotyledon parenchyma cells (Figures 13 and 14) and radicles (Figures 15 and 16). Sometimes protein bodies with aggregates of many small electron-transparent regions or individually scattered electron-transparent regions also were observed (Figure 31). Protein bodies in the same cell varied in their density of staining (Figure 24), supposedly due to different types of proteins being present.

As germination proceeds, changes in the internal structure of protein bodies were observed, as evidenced by an increase in electron-transparent areas (Figures 17 and 30) and a decrease in staining intensity (Figure 19), both of which suggest partial hydrolysis of proteins.

After nine hours of germination, protein bodies in epidermis stained less intensely than those in storage parenchyma cells (Figure 19). Internal degradation of protein bodies was observed in epidermis and hypodermis (Figure 17). However, protein bodies in the adaxial epidermis still showed intense staining (Figures 27 and 29).

Between two and six days, the protein bodies underwent several changes which differed within each cell and within different regions of the
cotyledons. In storage parenchyma cells, fusions of the protein bodies were often observed (Figures 35 and 36). Subsequently, larger and more irregularly shaped protein bodies appeared by day 3 (Figures 39 and 40). By day 6, these larger bodies showed a significant decrease in staining (Figures 47-50). In vascular bundles, each protein body matrix broke down in an irregular pattern, usually beginning in the center of the matrix (Figures 30, 32, and 34). The entire matrix of each protein body eventually became flocculent (Figure 33). The flocculent matrix was almost gone by day 6, leaving what have been termed as protein vacuoles (Figure 47). In epidermis, vacuolating protein bodies were observed at day 3 (Figures 42-44).

The cells of the storage tissue were mostly depleted of stainable storage protein, but the flocculent material remained visible (Figures 51-54) by day 7. In contrast, the flocculent material was absent both in the epidermal and vascular bundle protein bodies after days 3 and 6, respectively (Figures 42-44 and 47).

After day 9, the tonoplasts in epidermal cells appeared disrupted (Figure 73), which was the case in vascular bundles at day 10 (Figure 84). No tonoplast breakdown was observed in the storage tissue at this latter stage (Figures 75 and 76).

Lipids

Lipid bodies, along with protein bodies, constituted the most abundant organelles within soybean cotyledons. They were tightly packed in the
cytoplasm of cotyledon cells at the early stages of germination (Figures 13 and 14).

Lipid bodies, which stained uniformly gray, appeared to be bound by a thin electron dense lamella, which is called a half-unit membrane. Usually lipid bodies were round or oval (Figure 22), but frequently ones with irregular outlines were observed (Figure 45). The sizes of lipid bodies were smaller on the average than the protein bodies (diameter: 0.1-2.0 μm).

Lipid bodies closely associated with cisternal endoplasmic reticulum were commonly seen in storage parenchyma cells after 9 hours of germination (Figures 20-23). At day 2, lipid bodies were more scattered, and reduction in the number of lipid bodies per cell was observed (Figures 35 and 36). At day 3, significant reduction of lipid bodies was observed in the epidermis, where the degradation of protein bodies was completed (Figures 43, 44). The number of lipid bodies in storage parenchyma cells was significantly decreased at day 7, when protein body matrix became very flocculent (Figures 52 and 54). Lipid bodies showed an affinity for the inner side of plasma membrane (Figures 52 and 57).

In contrast to the starch grains and protein bodies, the lipid bodies were the last of the soybean seed reserves to be utilized completely. Frequently, a few undegraded lipid bodies are present in epidermal cells at day 9, where no starch grains and protein bodies are present and some cytoplasmic organelles have gone through their breakdown (Figures 73 and 74).
Starch

No starch grains were observed in any of the cotyledon cells at the early stage of germination (Figures 12-14). After 30 hrs, a few starch grains were observed in storage parenchyma plastids (Figure 31), whereas no starch grains were observed in epidermis and vascular bundles (Figures 32-34). At day 3, starch grains were abundant in storage parenchyma cells (Figures 39-41) and began to appear in the epidermis and vascular bundles (Figures 41 and 43). The number and size of starch grains within epidermal and vascular bundle cells were less than those observed in storage parenchyma cells. Between days 6 and 9, the reduction in the number and size of starch grains in cotyledons was evidently observed (Figures 56-58).

By day 9, starch grains disappeared completely in the epidermis (Figures 73 and 74), whereas few small starch grains were still found in storage parenchyma cells (Figure 5).

Ultrastructural Changes during Germination and Seedling Growth

Table 4 summarizes the ultrastructural changes in organelles of the soybean cotyledon which were observed from 1 1/2 hr to 10 days.

Mitochondria

At an early stage of germination (up to 9 hrs), mitochondria were only recognizable as membrane-bound organelles with no internal structure (Figures 12-14). Part of the outer membrane was distended so that it appeared singular, thin, and tenuous. The distended portion was electron-
<table>
<thead>
<tr>
<th>Organelles</th>
<th>Type of cell</th>
<th>1 1/2 hr</th>
<th>9 hr</th>
<th>30 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plastids</strong></td>
<td>Storage parenchyma (S.P.)</td>
<td>absent</td>
<td>present</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epidermis (Epi)</td>
<td>absent</td>
<td>present</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vascular bundle (V.B.)</td>
<td>absent</td>
<td>present</td>
<td></td>
</tr>
<tr>
<td><strong>Mitochondria</strong></td>
<td>S.P.</td>
<td>premature</td>
<td></td>
<td>few</td>
</tr>
<tr>
<td></td>
<td>Epi</td>
<td>premature</td>
<td></td>
<td>few</td>
</tr>
<tr>
<td></td>
<td>V.B.</td>
<td>premature</td>
<td></td>
<td>few</td>
</tr>
<tr>
<td><strong>ER</strong></td>
<td>S.P.</td>
<td>absent</td>
<td>numerous (short ER)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epi</td>
<td>absent</td>
<td>present</td>
<td>few</td>
</tr>
<tr>
<td></td>
<td>V.B.</td>
<td>absent</td>
<td>present</td>
<td>few</td>
</tr>
<tr>
<td><strong>Glyoxysomes</strong></td>
<td>S.P.</td>
<td>absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epi</td>
<td>absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>V.B.</td>
<td>absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 day</td>
<td>6 day</td>
<td>7 day</td>
<td>9 day</td>
</tr>
<tr>
<td>------------------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td><strong>common</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>common</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>common</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>numerous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>numerous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>numerous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>long &amp; short ER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>long ER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>frequently observed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>long ER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>short ER</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>few</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>few</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>few</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>present</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>absent</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
transparent or enclosed some granular material which was less electron
dense than that of the remainder of the organelle (Figure 12).

At 30 hrs, there were recognizable cristae in the mitochondria
(Figures 29 and 32). Mitochondria became more numerous, and the number of
cristae increased after day 3 (Figures 37 and 42-45), especially in
vascular bundles (Figures 71 and 72).

At day 3, in some cells of epidermis, mitochondria became dark, and
their cristae were swollen (Figure 44). The mitochondrial darkening and
swelling of cristae were increased in many cells of cotyledons between
days 6 and 10 (Figures 53 and 83). Furthermore, pleomorphic mitochondria,
ranging in shape from spherical to very elongated, were often observed
between days 3 and 10 (Figures 55, 61, 62, 64, and 78).

**Plastids**

At the beginning of early germination, no plastids were observed
(Figures 12-14). After 9 hrs of germination, few plastids were seen in all
cotyledon cells (Figures 18, 25, and 26). The membranes surrounding
plastids were well-defined, but internal lamellae were absent or poorly
represented (Figure 18). Each plastid had a granular matrix with a few
osmiophilic globules, ribosomes, and small electron-transparent regions
(Figures 25 and 26). No starch grains were observed (Figures 18, 25, and
26). Plastids were quite often irregularly shaped so that in sections they
often appeared to contain invaginations of cytoplasm with ribosomes and
lipid bodies (Figures 25 and 26).
After 30 hrs of germination, few plastids with starch grains were observed in storage parenchyma cells (Figure 31), whereas plastids with starch grains were seen in epidermal and vascular bundle cells at day 3 (Figures 41 and 43). Plastids containing large, conspicuous starch grains in a dense stroma were prominent in all cells of cotyledon between days 3 and 6 (Figures 41 and 52-54). At day 7, plastids in epidermal cells began to disintegrate (Figures 66 and 67) but contained prolamellar bodies and a few osmiophilic globules. Plastids in the vascular bundles and storage parenchyma cells at this stage remained intact and occasionally contained small starch grains (Figures 68 and 69).

**Glyoxysomes**

Glyoxysomes were absent at the early stages of germination. However, the cells of the cotyledons after three days of germination contained few glyoxysomes (Figures 37, 38, and 43). They were located primarily among and closely appressed to lipid bodies (Figures 37 and 38). The matrices of the glyoxysomes were uniformly dense.

Between days 3 and 7, glyoxysomes were commonly observed in all of the cells of the cotyledon and appeared to be less homogeneous (Figures 38, 62, and 65). Close association of glyoxysomes with the endoplasmic reticulum were frequently found (Figures 59-61, 81, and 82). At day 9, glyoxysomes were appressed to either lipid bodies or plastids (Figures 73, 79, and 81). The matrix of glyoxysomes was less flocculent than at day 7 (Figures 79-82), and crystalline inclusions were still observed in some of the glyoxysomes (Figure 81).
Throughout the period of seedling growth, glyoxysomes were much less numerous in vascular bundle cells than in storage parenchyma and epidermal cells.

Endoplasmic reticulum

Ribosomes were common in the cells at 1 1/2 hours germination, but ER with ribosomes attached were not seen (Figures 12-14). After 9 hrs of germination, short segments of ER cisternae were often seen in close association with and wrapping around spherosomes in storage parenchyma cells (Figures 20-23). In the adaxial epidermis, short ER segments wrapped around lipid bodies were present through 30 hrs of germination (Figure 29).

Short cisternal ER segments appeared more numerous after two days of germination (Figure 37) and were present in soybean cotyledons by day 10 (Figures 83 and 84). Relatively long ER cisternae and parallel arrays of ER along plasma membrane became prominent in the epidermal cells of three-day-old seedlings (Figures 43 and 44). Between days 5 and 8, these long cisternal ER segments were also commonly seen at the cell periphery close to the plasma membrane in storage parenchyma and vascular bundle cells (Figures 53 and 69).

At day 9, long cisternal ER segments formed concentric figures in epidermal cells (Figure 77). These figures are regarded as a degenerative change (Öpik, 1966).
Localization of Lipoxigenase

Differential centrifugation

After three days of seedling growth, most of the organelles are observed in the cotyledon tissues. This period was, therefore, chosen for the studies on subcellular localization of enzymes. Most of lipoxigenase-1 and -2/3 activities was present in the supernatant fraction after differential centrifugation of homogenates prepared from three-day-old seedlings; only 1.5% of the lipoxigenase activity remained in particulate fractions (Table 5). When this experiment was repeated using soybeans at early and late stages of seedling growth, little change was observed.

Table 5. Localization of lipoxigenase activity in fractions obtained from three-day-old seedlings of soybean by differential centrifugation

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Activity (unit/ml)(^a)</th>
<th>(%) in homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S_1)</td>
<td>118.70</td>
<td>164.80</td>
</tr>
<tr>
<td>(L_{OX-1})</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(L_{OX-2/3})</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>(P_1)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(L_{OX-1})</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(L_{OX-2/3})</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(P_2)</td>
<td>--</td>
<td>0.39</td>
</tr>
<tr>
<td>(L_{OX-1})</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>(L_{OX-2/3})</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>(P_3)</td>
<td>0.82</td>
<td>0.97</td>
</tr>
<tr>
<td>(L_{OX-1})</td>
<td>0.69</td>
<td>0.59</td>
</tr>
<tr>
<td>(L_{OX-2/3})</td>
<td>0.77</td>
<td>0.79</td>
</tr>
<tr>
<td>(P_4)</td>
<td>0.91</td>
<td>1.30</td>
</tr>
<tr>
<td>(L_{OX-1})</td>
<td>0.77</td>
<td>0.79</td>
</tr>
<tr>
<td>(L_{OX-2/3})</td>
<td>0.79</td>
<td>0.79</td>
</tr>
<tr>
<td>(S_2)</td>
<td>90.51</td>
<td>160.30</td>
</tr>
<tr>
<td>(L_{OX-1})</td>
<td>76.25</td>
<td>97.27</td>
</tr>
<tr>
<td>(L_{OX-2/3})</td>
<td>97.27</td>
<td>97.27</td>
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\(^a\)Lipoxigenase activity was measured by polarographic method.
Sucrose density gradient fractionation

The results of a sucrose linear gradient fractionation (three-day-old seedlings) showed that the lipoxygenase activity coincides with acid phosphatase at the densities of 1.19, 1.23, and 1.25 g/cm³ (Figure 85), even though most of lipoxygenase and acid phosphatase activities appeared in supernatant fractions. There was no indication that mitochondria contained any lipoxygenase activity, and it does not appear that glyoxysomes and ER contained any lipoxygenase activity either.

When this experiment was repeated by using one-day-old and five-day-old seedlings, no lipoxygenase activity appeared in any particulate fractions (Figures 86 and 87) as had been shown in the result from three-day-old seedlings (Figure 85). However, five-day-old seedlings showed small peaks of acid phosphatase activity at the high densities of 1.19 and 1.25 g/cm³ (Figure 87).

Immunogold labelling

Immunoelectron microscopy of ultrathin sections of soybean cotyledon showed that both lipoxygenase-1 and -2 were randomly distributed throughout the cytoplasm of storage parenchyma cells from day 1 up to day 7 (Figures 88-98). At day 7, few immunogold labels were found in cytoplasm (Figures 97 and 98). No gold label was found in either protein bodies, lipid bodies, mitochondria, or other organelles.

In the vascular bundle cells, both lipoxygenase-1 and -2 were observed in the cytoplasm at day 1 (Figures 99 and 100). After three days of seedling growth, lipoxygenase-1 and -2 appeared in cytoplasm and protein
bodies (Figures 102 and 103), while the control experiment incubated with nonimmune serum-gold complex did not show any gold label (Figure 101). Between days 5 and 7, immunogold labelling was only observed in the cytoplasm (Figures 104-107).

In epidermal cells, as in the storage parenchyma cells, both lipoxygenase-1 and -2 were found only in the cytoplasm. At day 1, immunogold labelling was very weak (Figures 108 and 109). Between days 3 and 5, more intense labelling was observed (Figures 110-113), and after seven days of seedling growth, few immunogold labels were found (Figures 114 and 115) in the cytoplasm. No immunogold labelling was observed in specific organelles.

In all cases, labelling was more intense after incubation with anti-lipoxygenase-2 Ig G-gold complexes than after incubation with anti-lipoxygenase-1 Ig G-gold complexes. No differences in subcellular distribution of isozymes of lipoxygenase were observed between variety AmSoy and genetic mutant A3.
DISCUSSION

Purification of Lipoxygenase Isozymes

For the purification and isolation of lipoxygenase, hexane-defatted soybean meal was used most commonly (Christopher et al., 1970, 1972; Yamamoto et al., 1970; Verhue and Francke, 1972). In this experiment, acetone-defatted soybean powder was subjected to the extraction of lipoxygenase isozymes with 50 mM sodium phosphate buffer (pH 6.8), which was useful for the isolation of both lipoxygenase-1 and -2 (Axelrod et al., 1981). They also reported that extraction of soybean meal at pH 4.5 offered the advantage of excluding large amounts of unwanted protein without any serious loss of lipoxygenase-1.

A few nonconventional techniques for the purification of lipoxygenase, such as isoelectric focusing and affinity chromatography, were reported to be very efficient and specific (Catsimpoolas, 1969; Grossman et al., 1972b). But these techniques suffer from the disadvantages that the one is one-step purification (Catsimpoolas, 1969), and the other does not separate the isozymes of lipoxygenase (Grossman et al., 1972b). For the separation of isozymes, traditional purification procedures have been reported to be successful (Christopher et al., 1970, 1972; Verhue and Francke, 1972; Yamamoto et al., 1970; Yoon and Klein, 1979) and very reliable (Eskin et al., 1977).

In this study, purification of soybean seed lipoxygenase isozymes was achieved satisfactorily by ammonium sulfate fractionation followed by Sephadex and DEAE-Sephadex column chromatography. The final preparation of
lipoxygenase-1 and -2 obtained was 19- and 32-fold purified, respectively, to the crude extract. But a considerable loss of the total enzyme activity occurred during purification. After ammonium sulfate fractionation, only 39% of the lipoxygenase-1 activity was recovered, while 81% of lipoxygenase-2 was recovered, which indicates that lipoxygenase-2 is more stable than lipoxygenase-1. The recovery of total isozyme activity after DEAE-Sephadex column remained between 10 and 14%, which has been reported by a few researchers (Haydar and Hadziyev, 1973; Christopher et al., 1972; Axelrod et al., 1981).

The elution profile from the DEAE-Sephadex column showed three peaks of lipoxygenase activity (Figure 9). This profile is similar to that of Christopher et al. (1972) who isolated a third enzyme of soybean lipoxygenase employing ammonium sulfate fractionation and DEAE-Sephadex chromatography. It is clear from this profile that at least three isozymes of lipoxygenase occur in soybean extracts. Later, Christopher (1972) could resolve lipoxygenase-3 into lipoxygenase-3a and -3b by incorporating a hydroxyapatite column. However, the mixture can be treated as a single enzyme (Axelrod et al., 1981), since the composition and behavior of these enzymes are somewhat alike (see Literature Review). Guss et al. (1967) and Verhue and Francke (1972) confirmed the presence of four isozymes of lipoxygenase in soybean using disc gel electrophoresis.

Lipoxygenase activity was measured by a polarographic method, based on the measurement of oxygen uptake during the reaction throughout this experiment. Turbidity of the reaction mixture originating either from crude extracts or poorly solubilized substrate did not present any...
difficulties in measuring the enzyme activity, since this method does not require an optically clear solution as does the spectrophotometric method (Grossman and Zakut, 1979). One disadvantage of this technique is that oxygen adsorption is not specific for lipoxygenase since oxygen uptake can be caused by other systems such as heme proteins (Axelrod et al., 1981). Therefore, controls were employed when the activity of crude extracts was tested in this assay. No significant activity by heme proteins was detected.

Linoleic acid, which is the best substrate for lipoxygenase, is not soluble below pH 7.0 but becomes increasingly soluble at higher pHs (Grossman and Zakut, 1979). The substrate solution prepared for lipoxygenase-2 and -3 assay at pH 6.8 was turbid. Poor solubility of linoleic acid was improved by the inclusion of an emulsifier (Tween 20), resulting in a soluble substrate over a rather wide pH range (Rackis et al., 1972). Allen (1969: refer to Yoon, 1978) also reported the successful use of a water-soluble substrate, potassium octadeca-9,12-dienyl sulphate or potassium linoleyl sulphate in kinetic studies, and pH profile determinations of lipoxygenase. However, no further studies with these substrates have been reported, and they are not commercially available yet.

Purified lipoxygenase-1, -2, and -3 were subjected to 7% polyacrylamide gel electrophoresis to check their purity. The proteins having lipoxygenase activity were localized by a specific staining technique based on the ability of enzymatically formed hydroperoxide to react with a dye, 3,3'-dimethoxybenzidine hydrochloride (DBH), producing a reddish-brown color (DeLumen and Kazeniac, 1976). This method is reported to be more
sensitive and to have less background staining from one described by Guss et al. (1967) who employed acidic potassium iodide on polyacrylamide gels impregnated with starch, which acts through the reaction of enzymatically produced hydroperoxide with acidic potassium iodide producing the elementary form of iodine. The iodine then reacts with starch in the gel to produce brownish violet bands as the site of lipoxygenase activity.

The respective Rf values of lipoxygenase-1, -2, and -3 in tris-glycine buffer (pH 9.0) were 0.38, 0.29, and 0.33. Axelrod et al. (1981) reported that the Rf values of lipoxygenase-1, -2, and -3, employing bromophenol blue as a standard in tris-glycine buffer (pH 9.5), were 0.30, 0.25, and 0.28, respectively. Under the same condition, Christopher et al. (1970) found lipoxygenase-1 and -2 localized at Rf values of 0.34 and 0.25, respectively. These differences could be ascribed to the different buffer systems employed.

Mobilization of Food Reserves and Ultrastructural Changes during Germination and Seedling Growth

The ultrastructure of dry seeds was difficult to preserve because there was little penetration of fixative due to the compactness of soybean cotyledon tissues. Cotyledons sampled after 1 1/2 hours of germination were taken as tissue representative of the beginning of germination. It is recognized that the possibility of some changes during the 12-hour fixation period cannot be excluded. However, most reserve mobilization and ultrastructural changes took place after three days of seedling growth. This 1 1/2 hour period, therefore, is arbitrarily designated as the beginning of germination.
The transformation of a dormant seed into an actively growing seedling was accompanied by marked changes in the fine structure of the cells. These changes include a progressive loss of storage reserve materials such as lipids, proteins, and carbohydrates and an increased number of subcellular structures such as mitochondria, plastids, glyoxysomes, and ER. In this discussion, the interrelationship between mobilization of storage reserves and appearance of subcellular organelles will be stressed.

The abundance of storage reserves, such as lipid and protein bodies, in the cotyledons of 1 1/2 hours of germination was striking. The protein bodies appeared very similar to those observed by Tombs (1967) with the exception of the existence of globoids. He reported that there were no inclusions in protein bodies. According to the classification of Rost (1972; see Literature Review), two types of protein bodies were observed in the cotyledon storage parenchyma cells, one with globoids and the other without any inclusions (Figures 12-14). This is in agreement with the results of Lott and Buttrose (1977) and Lott (1981).

Great size differences in protein bodies were observed; vascular bundle and epidermal cells have relatively small bodies, whereas storage parenchyma cells have very large ones. Similar observations were made by Lott et al. (1971) in squash cotyledons. Many agree that the protein bodies vary in size from one plant to another and from one tissue to another (Citharel and Citharel, 1985; Vernooy-Gerritsen et al., 1984; Smith, 1974a).
The variability in the degree of staining of protein bodies in a cell may reflect simply differences in composition or in protein concentration. This can be partly explained by the findings of Tombs (1967) who isolated two fractions of soybean protein bodies which have different densities of 1.29 and 1.30 g/cm$^3$. He also suggested that two fractions were mainly different in protein content (97.5 and 78.5%) and in the extent of nonproteinaceous materials such as phytic acids, RNA, and lipids.

A single limiting membrane was observed around each protein body prior to and during protein body degradation. The presence of a limiting membrane around each protein body in dormant seeds has been convincingly shown (Vernooy-Gerritsen et al., 1984; Lott, 1981; Pernollet, 1978). Evidence for the existence of intact membranes around protein bodies during protein body degradation is also accumulating (Webster and Leopold, 1977; Pernollet, 1978; Bain and Mercer, 1966). These observations suggest that the soluble products accumulate within the protein bodies and then leak through the membranes into the cytoplasm (Bain and Mercer, 1966) while the protein bodies are undergoing autolysis (Harris and Chrispeels, 1975).

The observed pattern of protein body degradation was more complex than those described for *Glycine* (Tombs, 1967; Treffry et al., 1967; Webster and Leopold, 1977; Vernooy-Gerritsen et al., 1984) or for other legumes, including *Pisum* (Smith and Flinn, 1967; Bain and Mercer, 1966), *Phaseolus* (Öpik, 1966; Smith, 1974a), and *Vigna* (Harris and Chrispeels, 1975). The pattern in the cells around the vascular bundles, where the proteins are degraded with little or no coalescence of bodies (Figure 30), is like that described for *Pisum sativum* (Bain and Mercer, 1966). Possible instances of
vacuolar fusion were encountered in these cells. The pattern in epidermis and storage parenchyma where the bodies swell, coalesce, and then are vacuolated was the one most frequently described (Öpik, 1966; Tombs, 1967; Webster and Leopold, 1977; Vernooy-Gerritsen et al., 1984). A similar pattern was found in seeds of Yucca schidigera where the protein bodies in the embryo coalesced before breaking down; however, the ones from the perisperm disappeared directly (Horner and Arnott, 1965).

A completely different pattern of protein body degradation was reported by Taylor et al. (1985) in germinating sorghum, where the protein bodies neither swelled nor formed a central vacuole. They were observed to undergo a progressive reduction in their size. Since the predominant storage protein in cereals is prolamin, which is hydrophobic and insoluble in water and salt solutions, protein bodies should not swell like the ones in germinating legumes which contain salt-soluble globulin proteins (Briarty et al., 1970). Furthermore, the lack of a protein body membrane would preclude vacuole formation (Taylor et al., 1985).

Flocculent protein bodies were observed in most of cotyledons between days 2 and 7. In the epidermis and vascular bundles, this type of protein body was observed earlier than in storage parenchyma cells due to earlier hydration and degradation. However, there have been contrasting reports on the occurrence of flocculent or granular protein bodies in germinating soybean seeds and seedlings. Treffry et al. (1967) observed a single vacuole with granular contents in some cells, and Vernooy-Gerritsen et al. (1984) observed flocculent protein bodies only in hypodermis and vascular bundles. These occurrences might be due to the internal degradation of
protein matrix (Ashton, 1976) by proteinase existing in protein bodies (St. Angelo et al., 1968; Ory, 1972), not as the result of fixation artifacts (Mollenhauer and Totten, 1971).

Some technical difficulties were met in monitoring the degradation of globoids in protein bodies. Since globoids are brittle, they are not penetrated well by fixatives and resins (Lott and Buttrose, 1977). As a result, they tend to shatter when cut (Ashton, 1976) and fall out of sections. This occurrence left a hole or small fragments of globoid around globoid area, as shown in Figure 13. Furthermore, globoids are easily displaced from the globoid area during microtoming and, thus, are found in other places (Figures 15 and 16).

As germination proceeds, a conspicuous electron-transparent region appeared around each globoid. Even though the existence of a globoid membrane is controversial (Lott et al., 1971; Khokhlova, 1971), it is thought that this electron-transparency arises from the swelling of the membrane (Khokhlova, 1971). After 30 hrs of germination, no globoids were observed, instead big holes occurred in the swollen protein bodies due to hydration. With these observations, it is not known whether the globoids dissolve as the matrix degrades or if they remain intact for some time. However, Khokhlova (1971) reported that globoids were the last material to be dissolved during protein body degradation in squash cotyledons because the phytin deposited in the globoids is the least soluble crystalloid (Ashton, 1976).

Another conspicuous change during germination and seedling growth was degradation of lipid bodies in the cytoplasm. At day 3, a significant
decrease of lipid bodies was observed. Concurrently starch grains and glyoxysomes appeared, and mitochondria became numerous. The number of cristae increased. Similar observations have been made by many researchers in germinating seeds (Öpik, 1966; Bain and Mercer, 1966; Webster and Leopold, 1977; Vernooy-Gerritsen et al., 1984). The close association of lipid bodies with glyoxysomes was often observed during seedling growth (between days 5 and 9). This relationship has been shown previously (Trelease et al., 1971; Vigil, 1970; Bergfeld et al., 1978; Wanner and Theimer, 1978).

It was difficult to follow the fate of lipid body degradation in this material. However, it seems apparent that the storage fats become hydrolyzed by lipase, and the fatty acids degraded after transfer into glyoxysomes through the glyoxylate cycle (Ching, 1973). The free fatty acids produced by the lipase should be distributed evenly over the entire lipid body surface (Yatsu and Jacks, 1972). As a result, the glyoxysomes responsible for the activation of free fatty acids and their conversion to succinate may obtain their substrate from the lipid body surface.

Wanner and Theimer (1978) proposed another mechanism for fatty acid utilization in oil seeds. They showed by electron microscopy that the half-unit membrane of each lipid body formed a handle-like appendix of lipid body which is the site of hydrolysis of triglycerides. Prior to any noticeable utilization of the storage fat, ribosomes were found to be attached to these handles. Bergfeld et al. (1978) confirmed the formation of a tripartite membrane, but they could not detect any obvious association of ribosomes with the collapsed part of lipid bodies.
The characteristic association observed between glyoxysomes and lipid bodies also reflects their mutual involvement in gluconeogenesis through the conversion of fatty acids to carbohydrates (Thomas and Bees, 1972; Adams et al., 1980). Biochemical measurements on glyoxylate cycle enzyme activity decline as fat degradation is completed (Trelease et al., 1971; Gerhardt and Beevers, 1970), while electron microscopic observation shows the disappearance of glyoxysomes (Trelease et al., 1971).

It is not known whether vacuoles are formed as a result of removal of storage fat from lipid bodies. However, the profiles of partly depleted lipid bodies were reported by Wanner and Theimer (1978) and Bergfeld et al. (1978). They indicated a further breakdown of the lipid body coat, since there was no striking accumulation of saccules in the cytoplasm during fat degradation (Bergfeld et al., 1978).

During germination and seedling growth of several legume species, starch is depleted (Juliano and Varner, 1969; Öpik, 1966; Smith and Flinn, 1967), but soybeans apparently accumulate starch grains in plastids as a result of glyconeogenesis (Adams et al., 1980). Biochemical data have shown that the reserve carbohydrates of soybean cotyledon consist primarily of low molecular weight oligosaccharides, particularly sucrose, stachyose, and raffinose (Abrahamsen and Sudia, 1966), and at maturity it contains approximately 1% of starch (Wilson et al., 1978). However, many electron microscopic observations confirm the accumulation of starch in soybean seedlings and other non-legume species as a transient reserve material (Webster and Leopold, 1977; Vernoov-Gerritsen et al., 1984; Treffry et al., 1967; Horner and Arnott, 1966).
The starch content rapidly declined at the late stage of seedling growth. Degrading and small starch grains were often found in the plastids at day 7 in storage parenchyma cells. A combined α-amylase and α-glucosidase system was reported to be responsible for starch utilization during the late stage of seedling growth (Adams et al., 1980).

The existence of ER in cotyledon cells became most prominent between days 3 and 5 when the most active reserve mobilization was observed. This observation confirms that reserve mobilization is accompanied by a proliferation of rough ER cisternae as described by Bain and Mercer (1966), Ópik (1966), and Harris and Chrispeels (1980). Furthermore, it agreed with analytical results showing that there was 10- to 20-fold increase in the activity of ER marker enzyme NADH cytochrome-C reductase during the first three to four days of castor bean endosperm seedling growth (Gilkes and Chrispeels, 1980).

Short segments of ER in association with lipid bodies were conspicuous after 9 hrs of germination. This profile also was observed by Harris and Chrispeels (1980), Stein and Stein (1968), and Bergfeld et al. (1978). The role of this association was not elucidated. However, Bain and Mercer (1966) presumed that fat material was used directly in the formation of the ER membrane system.

The role of ER in germinating seeds and seedlings is not completely understood; however, many researchers suggest that it plays an important role in the transport of the breakdown products of the storage reserves (Khokhlova, 1971; Bain and Mercer, 1966), ribonuclease and proteinase to the protein bodies (Van Der Wilden et al., 1980). A morphometric study by
Harris and Chripeels (1980) showed a 20 to 50% reduction in cell volume occupied by ER during seedling growth. This decline occurred first and was most pronounced in cells where reserve mobilization took place first, which may support the role of ER as a carrier of intermediate metabolites.

It has been suggested that ER is involved in the biogenesis of glyoxysomes by a process of invagination (Vigil, 1970; Lord et al., 1973; Bowden and Lord, 1976). A direct attachment between glyoxysome membrane and ER shown in Figures 59-61 and the similarity in polypeptide composition between ER and glyoxysomal membranes strongly support this suggestion (Bowden and Lord, 1975, 1976).

A tendency for the ER to become oriented in parallel lamellae along the cell periphery at late stage of germination, as observed in Figure 44, also seems to be characteristic of aging (Öpik, 1966).

Mitochondria at the early stage of germination (up to 9 hrs) showed little evidence of typical cristae and stroma, although they may be able to maintain certain oxidative functions (Mayer and Shain, 1974). The rapid development of numerous cristae and dense stroma after 30 hrs of germination may be related to the rapid initiation of respiration in soybean seeds (Parrish and Leopold, 1977).

At late stages of seedling growth (between days 5 and 9), mitochondria with dark matrices and swollen cristae were often observed in some cells. Bain and Mercer (1966) ascribed the swollen mitochondria to the increased permeability of cell and mitochondrial membrane. Öpik (1966) interpreted this as a degenerative change. She also found this occurred in the storage
tissue when hydrolysis became apparent but was delayed in the vascular bundles until the main period of translocation was over.

The general pattern of storage reserve mobilization in soybean cotyledons was found to be like that described by Smith (1974; refer to Bewley and Black, 1978a). The reserves disappeared first from the vascular bundles and epidermis (days 1 to 3). In the storage tissue, reserve mobilization began in adjacent cells around vascular bundles and epidermis and gradually progressed to the central storage tissue (days 3 to 7). At day 9, the epidermis completely lacked starch grains in plastids and protein bodies, but lipid bodies still were observed in all the cells of the cotyledons. It seems apparent from this observation that lipid is the last storage reserve to be utilized completely during soybean seedling growth. This fact has been shown also for Yucca seeds (Horner and Arnott, 1966).

A similar pattern of reserve mobilization was reported in Pisum (Smith and Flinn, 1967), where degradation began at the periphery of the cotyledons. Their results did not seem to show any correlation with the distance from the vascular bundles. Different zonation of reserve mobilization was reported in Phaseolus (Öpik, 1966; Smith, 1974a) and Vigna (Harris and Chrispeels, 1975) where reserve mobilization began in the cells farthest from the epidermis and from the vascular tissue; after four days of seedling growth, the central regions were vacuolated, while the cells around the vascular bundles and under the epidermis were still packed with reserves. Yomo and Taylor (1973) confirmed this observation by reporting
that protease activity was highest in the cells farthest from the vascular bundles in cotyledons of germinating *Phaseolus vulgaris*.

With this understanding of the basic patterns of hydrolysis of reserves, we may be better prepared to understand the complex metabolic controls which are associated with the breakdown and utilization of storage reserves.

**Localization of Lipoxygenase-1 and -2**

Previous work on the localization of lipoxygenase activity by differential centrifugation and density gradient fractionation from soybean seed homogenates had shown that 100% of lipoxygenase activity remained in the supernatant fraction (Vernooy-Gerritsen et al., 1983a; Wardale and Galliard, 1975). It was claimed that the activity of lipoxygenase could not be sedimented, possibly because of the destructive action of lipoxygenase on subcellular membrane, producing hydroperoxides which affect membrane structure.

Bovine serum albumin was included in the tissue extraction media to preserve the integrity of organelles (Haydar and Hadziyev, 1973). The MgCl₂ was excluded since Mg²⁺ ions are known to produce aggregation of organelles (Wardale and Galliard, 1977).

From the results in this study, it appears that confusion can easily arise if only one technique is employed in separating various organelles. The differential centrifugation study showed that about 1.5% of lipoxygenase-1 and -2/3 activities were localized in mitochondrial debris and microsomal fraction, while 98% of lipoxygenase-2/3 activity appeared in
supernatant fraction. It remains unclear at the moment whether this small activity is associated with mitochondria and microsomes or the result of contamination during centrifugation and subfractionation steps. The lipoxygenase activity in particulate fractions before and after the addition of 0.1% Triton-X 100 did not show any significant change, indicating lipoxygenase is not a membrane-bound enzyme, and the presence of lipoxygenase activity in mitochondrial and microsomal fractions may be a contaminant rather than being associated with the particles.

Subcellular organelles of a tissue preparation on sucrose density gradients are separated as determined mainly by their protein and lipid composition and, lastly, as influenced by their bound water content (Tolbert, 1974). After centrifugation, the gradient was first characterized by its sucrose concentration and lipoxygenase profile. Sucrose in each fraction was measured manually with a refractometer and converted to density at 20°C. The selection of a marker enzyme for each organelle depended upon organelle specificity and ease of assay. Catalase, NADH-dependent cytochrome c reductase, cytochrome oxidase, and acid phosphatase were used as marker enzymes for glyoxysomes, ER, mitochondria, and lysosomal fractions, respectively.

From the results of sucrose density gradient fractionation from three-day-old seedlings, lipoxygenase activity was coincident with acid phosphatase activity at the densities of 1.19, 1.23, 1.25 g/cm³. The appearance of the acid phosphatase activity at the high densities may be accounted for in several ways. Possibly, it is a trapping artifact, or it derives from protein bodies with different densities due to different
degrees of hydration and hydrolysis. However, it was not possible to observe these high density fractions by electron microscopy.

When the experiment was repeated by using one-day-old and five-day-old seedlings, no lipoxygenase activity was localized in any particulate fractions. However, five-day-old seedlings apparently showed two peaks of acid phosphatase activity at the densities of 1.19 and 1.25 g/cm$^3$, indicating that this enzyme is trapped or localized in protein bodies.

The NADH-cytochrome c reductase activity was present in the soluble fraction, and a peak at density of 1.09 g/cm$^3$, demonstrating that some leakage of marker enzyme from ER may have occurred. Cytochrome oxidase activity showed two broad peaks at the densities of 1.13 and 1.16 g/cm$^3$. The peak at lighter density might be due to broken mitochondria or the existence of nonspecific enzymes. The peak of catalase was somewhat symmetrical, which reflects the occurrence of homogeneous glyoxysomes present after three days of seedling growth. The electron microscopic observations at this stage confirmed the occurrence of fairly homogeneous glyoxysomes (Figures 37 and 43).

The results of immunoelectron microscopy showed that lipoxygenase-1 and -2 were localized in the cytoplasm of epidermal and storage parenchyma cells. In the vascular bundle cells, immunogold labelling was found in the cytoplasm and protein bodies of three-day-old seedlings. A similar observation was made by Vernooy-Gerritsen et al. (1984) who used an indirect labelling method with protein A-colloidal gold complexes. This finding also lends support to the interpretation of the results of sucrose
density gradient fractionation, where both lipoxygenase and acid phosphatase were localized in high densities of 1.19, 1.23, and 1.25 g/cm$^3$.

Evidence has been accumulating that the protein bodies are transformed into lysosomes in which their reserves are mobilized during germination and seedling growth. Cytochemical staining (Herman et al., 1981) and histochemical observation (Harris and Chrispeels, 1975) showed that the protein bodies in mung bean cotyledons were rich in acid phosphatase, a marker of lytic activity in cells. Biochemical analysis also revealed that the protein bodies contained protease, $\alpha$-glucosidase, phosphatase, and an esterase in pea seeds and mung beans (Matile, 1968; Harris and Chrispeels, 1975).

From all these results, it becomes quite evident that the particles with which lipoxygenase and acid phosphatase activity localized on a density gradient are protein bodies which have different internal matrices and which was shown in electron micrographs (Figures 39, 40, and 42-46).

The metabolic and physiological function of lipoxygenase in soybean cotyledons is still far from clear based on this observation. Lipid bodies were hypothesized for the lipoxygenase location, since enzyme and its substrate usually are associated with each other closely. From the observations of its distribution in cytoplasm of storage parenchyma cells, it is proposed that the function of lipoxygenase might lie in the dioxygenation of fatty acids to make their transport from lipid bodies to glyoxysomes possible (Vernooy-Gerritsen et al., 1984). The appearance of gold label in protein bodies of vascular bundle cells is considered as a stage in the digestion of lipoxygenase originating from the cytoplasm,
which may be explained by the finding that the protein bodies are the intracellular sites at which the digestion of cytoplasmic structure occurs (Herman et al., 1981; Harris and Chrispeels, 1975).
CONCLUSIONS

Conventional purification procedures including ammonium sulfate fractionation, Sephadex gel filtration, and DEAE Sephadex ion exchange chromatography successfully isolated three isozymes of lipoxygenase from acetone defatted raw soybean powder. On polyacrylamide gel electrophoresis at pH 9.0, employing lipoxygenase specific staining technique, lipoxygenase-1, -2, and -3 showed distinctive Rf values of 0.38, 0.29, and 0.33, respectively.

Though most lipoxygenase activity was recovered in the supernatant fraction, about 1.5% of lipoxygenase activity was localized in the mitochondrial debris and the microsomal fractions after differential centrifugation. About 1% of lipoxygenase activity was localized in protein bodies of three-day-old seedlings based on the sucrose density gradient separation. A direct immunogold labelling study showed that lipoxygenase-1 and -2 were localized in the cytoplasm of storage parenchyma and epidermal cells and in the protein bodies of vascular bundle cells. From these results, it seems apparent that lipoxygenase activity recovered in mitochondria and microsome is due to contamination and that confusion can easily arise if only one technique is employed in separating various organelles.

There was an attempt to draw the possible significance of lipoxygenase in the metabolism of its substrate and its possible function on the basis of its distribution and changes in lipoxygenase activity during germination and seedling growth. Electron microscopic observation showed that a
significant reduction of lipid bodies occurred after three days of seedling growth, when lipoxygenase activity declined sharply. Lipoxygenase activity decreased to a low level after day 6, when most reserve mobilization was completed. These results may reflect the possibility of its function being involved in mobilization of unsaturated fatty acids of storage lipids.

The observation of its distribution throughout the cytoplasm of storage tissue may reflect its function involving the dioxygenation of unsaturated fatty acids to make their transport from lipid bodies to glyoxysomes possible. The appearance of lipoxygenase activity in protein bodies of vascular bundle cells from three-day-old seedlings could be explained by the digestive action of protein bodies which are known to be the intracellular site where the digestion of cytoplasmic structures occurs.

Biochemical and electron microscopic results showed that most proteins were degraded after four days of seedling growth, which means a nutritive loss of protein. However, the increase in ascorbic acid and hydrolysis of raffinose and stachyose which cause flatulence problem have been reported (Suberbie et al., 1981; Smith and Circle, 1972). Furthermore, to overcome the flavor problems of soybeans, the germination process can help improve the odor and flavor scores of products, since lipoxygenase activity is decreased during germination.

It is evident that, from the results of the electron microscopic study, proteins were the more easily available substrate for seedling growth than lipids which were still present even during the latest stages of
seedling growth. Mobilization of food reserves from soybean cotyledons occurred first from epidermal and vascular bundle cells.

From the findings of subcellular distribution of lipoxygenase in the cotyledons, we come to realize how sensitive and effective the immuno-electron microscopic method is in determining the exact location of lipoxygenase. Furthermore, the direct immunogold labelling method, which was introduced by DeMey et al. (1981) by using anti-goat immunoglobulin, was shown to be a simple and sensitive method for immunocytochemistry when stable immunogold complexes are prepared. Furthermore, the application of low temperature embedding medium Lowicryl K4M® preserved the antigenic determinants and enhanced structural observations.
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APPENDIX: FIGURES

Key to Symbols

ER (Endoplasmic reticulum)
G (Golgi body)
gly (Glyoxysome)
LB (Lipid body)
M (Mitochondrion)
N (Nucleus)
P (Plastid)
PB (Protein body)
Ph (Phytochrome)
PLB (Prolamellar body)
SG (Starch grain)
V (Vacuole)
Figure 1. Germinating seed and seedling development showing growth of radicle, elongation of hypocotyl, and unfolding of cotyledons
Figures 2-3. 1.5 hr. Cotyledon.

2. Epidermis, hypodermis, and storage parenchyma; 457X

3. Precambium and storage parenchyma; 442X
Figure 4. 6 day. Cotyledon showing vacuolated epidermis and storage parenchyma; 434X

Figure 5. 9 day. Cotyledon showing vascular bundles and storage parenchyma; 552X
Figure 6. Basic scheme of the "direct" and "indirect" methods used for EM immunocytochemistry (Plattner and Zingsheim, 1983)
Figure 7. Gel filtration chromatography of soybean lipoxygenase
Figure 8. DEAE-Sephadex chromatography of soybean lipoxygenase
Figure 9. Rechromatography of fractions rich in lipoxygenase-2 and -3 obtained from first DEAE-Sephadex chromatography
Figure 10. Change of lipoxygenase activity and protein content in AmSoy during germination and seedling growth
Figure 11. Changes of lipoxygenase activity and protein content in A_5 during germination and seedling growth.
Figure 12. 1.5 hr. Mitochondria (M) in epidermal cell; 27,490X.

Figures 13-14. 1.5 hr. Storage parenchyma cells are packed with protein bodies (PB) and Lipid bodies (LB). Note appearance of shattered globoids and globoid area (arrows); 12,318X, 15,706X.
Figures 15-16. 9 hr. Globoids in radicle. Note a displaced globoid (arrow); 11,851X
Figure 17. 9 hr. Lipid bodies are packed around nucleus (N) and protein bodies in epidermis; 11,998X

Figure 18. 9 hr. Plastid (P) with membrane structure appears in epidermis; 20,369X

Figure 19. 9 hr. Protein bodies in storage parenchyma cells stain more intensely than ones in hypodermis; 5,868X
Figures 20-23. 9 hr. Endoplasmic reticulum (ER) wrapped around lipid bodies in storage parenchyma cells; 50,620X, 50,419X, 60,507X
Figure 24. 9 hr. Protein bodies showing different intensities of staining in one cell; 720X

Figures 25-26. 9 hr. Irregular shape of plastids containing electron transparent regions (arrowhead) and phytochrome (Ph). Note cytoplasmic invaginations with ribosomes and lipid bodies (arrows);
Figure 27. 30 hr. Protein bodies stain intensely and stacks of rough endoplasmic reticulum (ER) and golgi body (G) are present in adaxial epidermal cell; 32,400X

Figure 28. 30 hr. Flocculent protein bodies (PB) are seen in vascular bundle cell; 30,553X
Figure 29. 30 hr. Mitochondria (M) with rudimentary cristae in adaxial epidermal cell; 44,170X
Figure 30. 30 hr. Internal degradation of protein bodies in adjacent cells of vascular bundles; 9,659X

Figure 31. 30 hr. Irregular shape of protein bodies is observed. Note appearance of small electron transparent region (arrowhead) in protein bodies and a starch grain (SG) in plastid; 6,059X
Figures 32-33. 30 hr. Various stages of protein body degradation in vascular bundle cells; 25,791X, 14,528X
Figure 34. 30 hr. Internal degradation of protein body in vascular bundle cell; 20,989X
Figures 35-36. 2 day. Fusion of protein bodies in storage parenchyma cells; 36,005X, 27,698X
Figure 37. Swollen protein bodies (PB) and glyoxysomes (gly) are seen in storage parenchyma cell; 34,440X
Figure 38. 2 day. Glyoxysome with crystalline inclusions (arrowheads) appears in storage parenchyma cell. Lipid bodies are appressed to glyoxysome; 59,864X

Figures 39-40. 3 day. Irregular shape of protein bodies; few starch grains are seen in storage parenchyma cells; 4,150X, 4,200X
Figure 41. 3 day. Epidermis and storage parenchyma with numerous starch grains; 434X

Figure 42. 3 day. Protein bodies (PB) become vacuolated in epidermal cell; 23,919X
Figure 43. 3 day. Starch grains (SG), long endoplasmic reticulum, and glyoxysomes (gly) associated with endoplasmic reticulum are seen in epidermal cell; 32,277X
Figure 44. 5 day. Parallel array of endoplasmic reticulum and numerous mitochondria are seen in epidermis. Protein body is almost vacuolated; 38,456X

Figures 45-46. 5 day. Various sizes of protein bodies with flocculent materials are seen in vascular bundle cells; 14,222X, 14,696X
Figures 47-48. 6 day. Protein bodies (PB) in adjacent cell to vascular bundles are partially vacuolated; 697X, 13,630X
Figures 49-50. 6 day. Vesicles (arrows) in protein bodies appear as empty single membrane coated spheres which are interpreted as resulting from digestion of sequestered material leaving a digestion resistant limiting membrane (Herman et al., 1981); 16,001X, 19,126X
Figure 51. 7 day. Epidermal cells contain one single vacuole in each cell; 442X

Figure 52. 7 day. Significant reduction of lipid bodies in storage parenchyma cells are seen; 10,718X
Figures 53-54. 7 day. Protein bodies with different matrix in storage parenchyma; 8,739X, 6,024X

Figure 55. 7 day. Elongated mitochondria in storage parenchyma cell; 30,530X
Figures 56-58. 7 day. Starch grains (SG) in plastids are degrading and start to disappear in storage parenchyma cells; 31,104X, 23,018X, 27,495X
Figure 59. 7 day. Glyoxysomes closely associated with endoplasmic reticulum; 47,425X
Figures 60-61. 7 day. Pleomorphic mitochondria (M) in storage parenchyma cells; 36,757X, 36,911X
Figure 62. 7 day. Glyoxysome (gly) with crystalline inclusions in epidermal cell; 35,550X

Figure 63. 7 day. Mitochondria with swollen cristae in epidermal cell; 56,664X
Figure 64. 7 day. Elongated mitochondria in epidermal cell; 28,156X

Figures 65-67. 7 day. Glyoxysomes (gly) appear near to lipid bodies and mitochondria. Note plastids begin to disintegrate (arrows) in epidermal cells; 21,047X
Figure 68. 8 day. Vascular bundle cells with numerous mitochondria (M) and electron transparent vacuoles (V); 16,771X
Figures 69-70. 8 day. Vascular bundle cells showing degrading starch grains (SG) in plastids and various sizes of electron transparent vacuoles (V); 11,422X, 18,176X
Figures 71-72. 8 day. Secondary wall formation begins in vascular tissue. Note degenerating mitochondria (arrow); 17,295X, 9,896X
Figure 73. 9 day. Tonoplast breakdown in epidermal cell; 16,634X

Figure 74. 9 day. Degeneration of plastids (P) containing prolamellar body (PLB) and osmiophilic bodies (arrow) in epidermal cell; 19,210X
Figure 75. 9 day. Intact vacuolar membrane is observed in storage parenchyma cell. Note that lipid bodies (LB) show an affinity to plasma membrane; 19,293X

Figure 76. 9 day. No vacuole is seen in epidermal cell; 22,273X
Figure 77. 9 day. Concentric figure of endoplasmic reticulum (arrowhead) appears in epidermal cell; 20,245X

Figure 78. 9 day. Plastids (P) disintegrate and dark and swollen mitochondria appear in epidermal cell; 28,497X
Figures 79-82. 9 day. Glyoxysomes (gly) with flocculent matrix are associated with lipid bodies or endoplasmic reticulum; 59,969X, 68,141X, 31,227X, 47,152X
Figure 83. 10 day. Dark and swollen mitochondria (M) appear in vascular bundle cell; 31,568X

Figure 84. 10 day. Tomoplast breakdown is seen in some vascular bundle cells; 15,467X
Figure 85. Distribution of marker enzyme activities of homogenates from three-day-old seedlings on a linear sucrose gradient (13 - 60%)
Figure 86. Distribution of lipoxygenase and acid phosphatase activities of homogenates from one-day-old seedlings on a sucrose gradient (13 - 60%)
Figure 87. Distribution of lipoxygenase and acid phosphatase activities of homogenates from five-day-old seedlings on a sucrose gradient (13 - 60%)
Figure 88. 1 day. Storage parenchyma cell of AmSoy after incubation with anti-lipoxygenase-1-Ig G-gold complexes shows gold labels in cytoplasm; 50,384X
Figure 89. 1 day. Storage parenchyma cell of $A_5$ after incubation with anti-lipoxygenase-2-IgG-gold complexes shows gold labels in cytoplasm. No association with lipid bodies (LB) is found; 77,571X
Figures 90-91. 3 day. Storage parenchyma cells of AmSoy. No gold label is seen in protein body (PB) and mitochondria (M)

90. Anti-lipoxygenase-2-IgG-gold complexes; 67,228X
91. Anti-lipoxygenase-1-IgG-gold complexes; 67,228X
Figures 92-93. 3 day. Storage parenchyma cells of A. No gold label is seen in protein body (PB), lipid bodies (LB), mitochondria (M), and endoplasmic reticulum (ER)

92. Anti-lipoxygenase-2-IgG-gold complexes; 60,238X

93. Anti-lipoxygenase-1-IgG-gold complexes; 50,680X
Figures 94-95. Controls incubated with nonimmune-gold complexes

94. 5 day. Storage parenchyma cell of A$_5$. Few gold labels of nonspecific binding are seen in A$_5$; 38,782X

95. 3 day. No nonspecific binding is seen in storage parenchyma cell of AmSoy; 38,377X
Figures 96-97. 5 day. Storage parenchyma cells. No gold label is seen in glyoxysomes (gly) and plastid (P)

96. Anti-lipoxygenase-2-IgG-gold complex (A$_2$); 23,905X

97. Anti-lipoxygenase-1-IgG-gold complex (AmSoy); 44,910X
Figure 98. 7 day. Storage parenchyma cell of A5. Weak gold labelling is seen in cytoplasm after incubation of anti-lipoxygenase-2-IgG-gold complex; 30,778X
Figures 99-100. 1 day. Vascular bundle cells. Gold labels are only seen in cytoplasm

99. Anti-lipooxygenase-2-IgG-gold complexes ($A_2$); 34,292X

100. Anti-lipooxygenase-1-IgG-gold complexes (AmSoy); 67,594X
Figures 101-102. 3 day. Vascular bundle cells of A₅

101. Control incubated with nonimmune-gold complexes; 31,950X

102. Gold labels (anti-lipoxygenase-2-IgG-gold complexes) are seen in cytoplasm and protein body (PB); 67,599X
Figure 103. 3 day. Vascular bundle cell of AmSoy. Most gold labels (anti-lipoxygenase-1-IgG-gold complexes) are seen in cytoplasm and only a few bindings occur in protein bodies (PB). No gold label is seen in mitochondria (M) and lipid bodies (LB); 49,760X
Figures 104-105. 5 day. Vascular bundle cell. Gold labels are only seen in cytoplasm.

104. Anti-lipoxygenase-2-IgG-gold complex (A₂); 52,503X
105. Anti-lipoxygenase-1-IgG-gold complex (AmSoy); 33,272X
Figures 106-107. 7 day. Vascular bundle cells. No gold label is seen in mitochondria (M), vacuoles (V), and plastids (P).

106. Anti-lipoxygenase-2-IgG-gold complex (A2); 37,200X

107. Anti-lipoxygenase-1-IgG-gold complex (AmSoy); 49,762X
Figures 108-109. 1 day. Epidermal cells. Weak gold labelling is seen in cytoplasm


109. Anti-lipoxygenase-1-IgG-gold complexes (AmSoy); 58,893X
Figure 110. 3 day. Epidermal cell of A. Numerous gold labels (anti-lipoxygenase-2- IgG-gold complexes) are seen in cytoplasm. No gold label is seen in mitochondria (M), protein body (PB), and lipid bodies (LB); 34,496X
Figure 111. 3 day. Epidermal cell of AmSoy after incubation with anti-lipoxygenase-1-IgG-gold complexes; 50,384X
Figures 112-113. 5 day. Epidermal cell. No gold label is associated with endoplasmic reticulum (ER) and vacuole S (V)

112. Anti-lipoxygenase-2-IgG-gold complexes (A₂); 45,179X
113. Anti-lipoxygenase-1-IgG-gold complexes (AmSoy); 41,640X
Figures 114-115. 7 day. Epidermal cell. Weak gold labelling occurs in cytoplasm

114. Anti-lipoxygenase-2-IgG-gold complexes (A2); 59,700X
115. Anti-lipoxygenase-1-IgG-gold complexes (AmSoy); 62,361X