STUDIES ON SEEDS

II. Origin and Degradation of Lipid Vesicles in
Pea and Bean Cotyledons

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ABSTRACT
At least two kinds of lipid vesicles are present in pea and bean cotyledons which can be recognized at seed maturity on the basis of whether they do or do not interassociate into lipid vesicle sheets. Those that do interassociate into sheets are also characterized by (a) their association with plastids or plasma membranes during dormancy, and (b) the unique transformation into flattened saccules that they undergo during the first few days of seed germination. These interassociated (or composite) lipid vesicles have been found in only a few seeds and may be restricted to certain classes of plants and/or certain states of cellular development.

Lipid vesicle-to-saccule transformation is predominantly confined to the germinating seed. However, some lipid vesicle-derived saccules are already present in some cells even before the seed reaches maturity. These partially transformed vesicles and saccules remain unchanged over dormancy, and then resume their transformation when the seed is germinated. This suggests that some stages of seed germination are already underway before the seed reaches maturity and are only resumed at seed germination.

The lipid vesicles that do not interassociate into sheets (i.e., the simple lipid vesicles) are present in all tissues at all states of cellular development. These vesicles do not undergo any conspicuous structural changes during development.

INTRODUCTION
This report is concerned with the reserve lipids (principally triglycerides) of seeds, which are easily recognized as distinct protoplasmic inclusions occurring as fluid droplets of various sizes either dispersed in the cytoplasm or aggregated into larger masses. Reserve lipids of this type are common in seeds (4, 6, 10), spores, and embryos, but are also found to a lesser extent in meristematic cells and in differentiated vegetative cells (3, 6, 13).

"Typical" droplets or vesicles of reserve lipids are spherical in form and about 0.1–2.0 µ in diameter. They are bounded by an interfacial structure (possibly a membrane) which is clearly visible in good electron microscope preparations (9, 16).

Occasionally, lipid vesicles are closely associated with mitochondria (5, 7, 24), endoplasmic reticulum (22, 23, 24), or other lipid vesicles (13), but most often they appear free in the cell cytoplasm. Some lipid vesicles may be equivalent to the spherosomes seen by light microscopy (2, 8, 11, 19–21, 25), thus implying that these vesicles have a lysosomal function as well as acting simply as a pool of reserve lipid. Previous reports have illustrated lipid vesicle form in various tissues (7, 9, 13, 15).

Reserve lipids are thought to be elaborated directly by the cytoplasm (6) or by portions of the endoplasmic reticulum (22–24). Time-sequence
Wyssling et al. (8) proposed that spherosomes are well documented in plant tissues as it is in animal rough and smooth elements of the endoplasmic reticulum (22–24), and possibly also mitochondria (24). In these tissues (liver, heart, and mammary glands) the esterified lipids are primarily triglycerides which appear in the cytoplasm as small droplets bounded by an interfacial structure or membrane (23). The membrane is presumably derived from the endoplasmic reticulum (23).

The pathway of triglyceride synthesis is not as well documented in plant tissues as it is in animal tissues, although it seems unlikely that the mechanisms would be significantly different from those in animal tissues. An example of a plant lipid-synthesizing system was described by Frey-Wyssling and his collaborators in relation to the formation of spherosomes (8). These highly refractile bodies (as viewed by phase-contrast microscopy) are reactive with lipid stains (2, 8, 11, 19–21, 25) and with chemicals that demonstrate various hydrolytic enzymes (2, 8, 11, 19–21, 25). Frey-Wyssling et al. (8) proposed that spherosomes are formed from the endoplasmic reticulum in an immature state, containing enzymes for lipid synthesis but little or no reserve lipid. As lipid synthesis takes place, these immature bodies evolve first into spherosomes and then into vesicles of reserve lipid. At maturity, these vesicles of reserve lipid probably contain few or no enzymes (8, 9).

Many seeds store substantial quantities of reserve lipids which can be isolated as intact vesicles by relatively simple procedures. By and large, these lipids are synthesized during the last few weeks of seed development and then utilized by the embryo during the first few days of germination. Because of this separation of metabolic events, cellular changes accompanying lipid synthesis and utilization can be followed accurately.

Pea and bean cotyledons were chosen for these studies because they are similar morphologically, biochemically, and developmentally and, therefore, can be used more or less interchangeably. In practice, however, most of the information on isolated lipids has come from bean cotyledon and most of the information on structure has come from pea cotyledon, because bean cotyledons have given the most uniform isolates, and pea cotyledons have been the most amenable for ultrastructural analysis. In all of this work, however, both cellular systems have been compared to ascertain that the information presented is characteristic of both seed types.

Two other points regarding the choice of a cellular system for study should be mentioned. They have to do with the amount and the kinds of lipids that the seed accumulates. Pea and bean are particularly useful for developmental studies because the changes accompanying lipid vesicle formation and degradation are well defined, and because the number of lipid vesicles formed is sufficiently small so that structural changes can be easily followed. Both pea and bean contain at least two structurally identifiable kinds of lipid vesicles. This diversity adds markedly to the value of these studies since it may lead eventually to an understanding of the mechanisms which control lipid synthesis and form.

For clarity, the work reported in this paper, in the accompanying paper, and in two papers yet to be published has been divided into four parts: techniques for fixing seeds, changes accompanying seed development and germination, isolation and form of lipid vesicles, and chemical composition of lipid vesicles at one stage during germination. Most of the information relates to the lipid vesicles which we have called “composite” because these vesicles have not been described before and because they are unique enough that changes in their form can be related to stages in development.

**MATERIALS AND METHODS**

For developmental studies, peas (var. Alaska) and beans (var. Topcrop) were grown in the garden in spring and early summer. The seeds were harvested at various developmental stages until they were mature and the seed pods were brown in color.

For the germination studies, seeds from commercial sources were used. Care was taken to obtain untreated seeds, since even slight residues of fungicides affect the structure of the cells during early stages of germination. For short germination times (1–4 hr), the seeds were soaked in shallow dishes of water at room temperature and in the light. The seeds were not covered with water since this inhibits germination. For longer germination times, the seeds were presoaked as above and then transferred to moist vermiculite in an incubator and germinated in the dark at 27°C. For test purposes, a few seeds were germinated in the light, but this did not seem to alter the sequence of events reported here.

In their preparation, fixation, and embedding, the seeds were handled as described in the accompanying paper (16). In most instances, this involved prefixa-
Figure 1  Micrograph of pea cotyledon late in seed development. Protein bodies (PB) are fully formed at this developmental stage but lipid vesicles have not yet accumulated at the cell surface or adjacent to the plastids. Newly formed lipid vesicles are spherical and single (i.e., not interassociated), and may be closely appressed to the outer surface of the endoplasmic reticulum (see arrows). \( \times 30,000 \).
tion in a mixture of acrolein, paraformaldehyde, and glutaraldehyde in 0.05 M collidine buffer, and post-fixation in collidine-buffered OsO₄. The tissues were dehydrated in a series of acetone solutions, embedded in an Epon-Araldite epoxy resin mixture (12), and viewed with a Philips EM-200 electron microscope.

RESULTS

Kinds of Lipid Vesicles

Lipid vesicles (see Figs. 1-10) are present in most cells of the cotyledon throughout development, dormancy, and germination, but they do not constitute a major cellular constituent until the seed approaches maturity. Near maturity the seed synthesizes great quantities of reserve lipids (Fig. 1) which are stored as small vesicles over dormancy (Fig. 2). These vesicles become a conspicuous component of the cell because they are present in such large numbers and because some of them occupy a unique position in the cell (Figs. 2, 3).

At seed maturity, the lipid vesicles can be divided into two general classes according to structural and developmental differences (see Fig. 3). One class of lipid vesicles is called “simple” because they are similar to those in most other plant and animal cells (see arrow, Fig. 3). Simple lipid vesicles do not form close associations with one another and do not appear to undergo any conspicuous transformations. The other class of lipid vesicles is called “composite” because they form into sheets as the seed approaches dormancy (Fig. 2). These sheets of lipid vesicles are characteristically located near the cell surface or adjacent to the plastids (Figs. 2, 3, 5). The composite lipid vesicles undergo a unique kind of transformation during the first several days of dormancy (see Figs. 6-9).

Lipid Vesicle Development

It has always been difficult to demonstrate and correlate structural and developmental changes in lipid vesicles, a problem which was also encountered in the present study. This difficulty is illustrated by the simple lipid vesicles which are always present in the cell and never seem to undergo any change except a gradual enlargement or diminution in size. Because these changes are gradual, it is very difficult to relate lipid vesicle form to cellular activity.

The composite lipid vesicles are more amenable to study because definite stages in development and/or degradation can be followed. These vesicles appear to be synthesized in cytoplasmic regions containing tubular endoplasmic reticulum and Golgi apparatus (Fig. 1). They first appear as small, individual, spherical vesicles (Fig. 1) which are free in the cytoplasm or close to the outer surface of an element of the endoplasmic reticulum. They do not appear to form in the lumen of the endoplasmic reticulum or in the Golgi apparatus.

It is important to point out that these lipid vesicles are not interassociated at this developmental stage; they are single vesicles that are free in the cytoplasm and look very much like the simple lipid vesicles described above. They can be related to the composite lipid vesicles only because they appear in such great numbers and because they are more uniform in size and are usually smaller than the simple lipid vesicles.

As they mature, the composite lipid vesicles increase in size, but otherwise do not change significantly in appearance. They seem to remain in the region in which they are formed and do not migrate to the cell surface or to the plastids until the seed is almost mature.

Ultimately, the composite lipid vesicles come to reside at the cell surface or adjacent to the plastids. The sequence of their interassociation is not clear, but the evidence suggests that they migrate as individual vesicles to the surface to which they will attach. When they reach this surface they elongate and interconnect and/or interassociate with each other and with the membranes of the cell surface or the plastids (Figs. 2, 3, 5). By the time the cell reaches dormancy, the composite lipid vesicles have formed into sheets at the cell surface or adjacent to the plastids. They remain in one of these positions until the seed is germinated.

Degradation of Lipid Vesicles

Simple lipid vesicles are present during germination but no special or unique transformations can yet be associated with them. Any structural changes that might be associated with their turnover are too small to be defined.

Changes in composite lipid vesicles are less difficult to follow; i.e., these vesicles are transformed into thin saccules and then disappear. This transformation takes only 2-4 days.

The most striking aspect of lipid vesicle transformation is the formation of saccules bounded by membranes which often appear more dense and more distinct than other membranes of the cell.
FIGURE 2  Micrograph of dormant pea cotyledon showing the alignment of lipid vesicles along the cell surface. The same alignment pattern is also found around the plastids but is not illustrated in this figure (however, see Fig. 5).  X 32,000.
FIGURE 3  Micrograph of pea cotyledon showing the form of composite lipid vesicles at an early stage of seed germination. Multilayers of lipid vesicles are common only in regions where several plastids (P) are localized as they are here. A simple lipid vesicle is shown at the arrow. Protein body (PB). X 28,000.

FIGURE 4  Sheets of lipid vesicles remain intact even after homogenization and isolation, and have the same form that they do in vivo. This preparation is from bean cotyledon after the seed had been soaked in water for 2½-3 hr. A simple lipid vesicle is shown at the arrow. X 24,000.
(Figs. 6–8). These saccules are probably flattened sacs, since no tubular profiles of them have been seen in thin section. They are always about 150 Å in over-all thickness, but may vary considerably in apparent length and/or width. It is not known whether these variations in size are the result of different planes of section through a saccule or of real differences in saccular dimensions.

Each composite lipid vesicle is eventually converted into a saccule, but the process is not synchronous. Many transformational states can be seen in each cell, and even in a single sheet of lipid vesicles (see Figs. 6 and 8). The rate of transformation seems to be proportional to the rate at which the seeds germinate. In pea and bean cotyledons, the transformations are usually complete in 2–4 days. After they are formed, the saccules remain interassociated for a while and then disappear from the cytoplasm (Fig. 9).

Discussion

Reserve lipids of most seeds are present in the form of spherical droplets which are clearly separated from the cytoplasmic ground substance by a thin interfacial structure. In suitable electron micrographs, this interfacial structure appears relatively dense and, therefore, is thought to be a bounding membrane. This interpretation is supported by the following observations: (a) In tissue sections, a membrane, or at least a dense residue, is visible at the lipid-cytoplasm interface even after lipids have been extracted from the tissues (9). (b) Lipid droplets which become packed together, as they do in high-fat seeds and some meristematic tissues (13), do not fuse together. (c) In these seeds, the thin interfacial structures bounding the lipid droplets are continuous with the more distinct membranes bounding the flattened saccules (Fig. 6). (d) In negatively stained preparations, the interfacial structures of the lipid droplets are continuous with membrane fragments (17). Therefore, until further information is available, it is assumed that these lipid droplets are bounded by a membrane and that they should be called lipid vesicles rather than lipid droplets.

The composite lipid vesicles appear to be synthesized in association with, but external to, the endoplasmic reticulum. We have not yet found any instance in which pools of reserve lipids appear inside the endoplasmic reticulum, or in which lipid droplets are continuous with, or appear to be budding from, the endoplasmic reticulum. Therefore, if reserve lipids are formed in the lumen of the endoplasmic reticulum, as suggested by the work of Stein and Stein (22–24), then they must be transported to lipid vesicles in packets which are below the resolution limit of the microscope. At this time, it seems more reasonable to assume that lipid synthesis occurs external to, or on the surface of, the endoplasmic reticulum, or even in spherosomes as suggested by Frey-Wyssling et al. (8).

Simple lipid vesicles seem to be present in all seeds at all developmental stages, but no conspicuous transformations are associated with them. In many seeds (particularly the high-fat seeds such as those of the peanut and the castor bean), simple lipid vesicles increase or decrease significantly in number (and occasionally also in size) during development and germination, respectively, but otherwise do not change in form, density, or general appearance. However, in pea and bean cotyledons most of the increase in lipid during seed development seems to come from composite, rather than simple, lipid vesicles. The simple lipid vesicles are never a conspicuous feature of the cytoplasm and, therefore, are not a very useful tool for ultrastructural analysis.

Simple and composite lipid vesicles are biochemically and structurally similar (1, 17, and Fig. 3). However, it is not yet possible to determine what differences may actually exist between them. The factors responsible for interassociation and for the vesicle-to-saccule transformation described in these reports will be considered elsewhere. It has been assumed that differences probably exist from the time of vesicle inception, but there is no real evidence to prove this. It will be necessary to find out first how the two classes of vesicles differ and then to analyze the vesicles for these differences at each developmental stage.

It was thought initially that the transformation of lipid vesicles into saccules might be a mechanism for rapidly synthesizing smooth endoplasmic reticulum (14). More recent work, however, has shown that the saccular membranes are significantly different structurally from those of the endoplasmic reticulum or from those of other membrane-bounded components of the cell. It may even be that, since they disappear shortly after they are formed, and since they do not appear to be common to all seeds (see below), the saccules represent a breakdown product, or lipid vesicle residue, with no metabolic function at all.
It is common for lipid vesicles in seeds to be aligned with the cell surface (for example, see Fig. 10) or around protein bodies (4, 10, 18, results unpublished), though the factors which cause this are unknown. In most instances, however, these aligned vesicles do not seem to be interconnected or interassociated and, therefore, do not undergo a transformation of form. In the course of our studies, we have looked at soybean, peanut, pumpkin, watermelon, squash, castor bean, maize, various beans, and pea, and have found composite lipid vesicles and lipid vesicle-derived saccules only in bean and pea. Although this sampling is not yet very large, it does indicate that the occurrence of composite lipid vesicles may be a relatively unique event that is restricted to certain tissues or stages of development. It also implies that only composite lipid vesicles are capable of transformation into saccules and that these conditions are probably related.

Membrane changes accompanying saccule formation (see Fig. 6) can be correlated with sedimentation properties and protein-lipid ratios of the composite lipid vesicles at various transformational stages (see references 1 and 17 for details). In brief, these data show that the buoyant density and the protein-lipid ratio of the saccules are greater than those of lipid vesicles, thus suggesting that there is either an increase in protein or a decrease in lipid (or both) during conversion of the composite lipid vesicles into saccules. We believe that at least some part of these transformations must be associated with membrane changes if the data are to fit the ultrastructural observations. Thus, it seems reasonable to suggest that the increased thickness and electron opacity of the saccular membranes compared with the lipid vesicle membranes are due to a net accumulation of protein on, or in, the membrane of the saccule.

The reasons why lipid vesicles accumulate close to the plastid and plasma membranes are not clear. The data only suggest either that plasma and plastid membranes become similar (in the sense that they bind or attract the composite lipid vesicles), or that two classes of composite lipid vesicles exist such that one class binds to the plasma membrane and the other class binds to the plastid membrane. We presently favor the first hypothesis since all of the composite lipid vesicles are similar in form and development and do not appear to be subdivided into classes.

We have implied in the previous discussions that lipid vesicle development and interassociation, and lipid vesicle transformation into saccules, are neatly restricted to seed development and germination, respectively. This is not entirely true since lipid fractions from dormant seeds contain some saccules as well as the more usual spherical form of lipid vesicles. This situation is significantly more marked in bean preparations than in pea preparations, and is interpreted to mean that lipid vesicle transformation begins shortly after the vesicles become interassociated, even if the seed has not yet reached dormancy. Insofar as we can determine, these partially transformed vesicles and saccules remain essentially unchanged over dormancy and then resume their transformation when the seed is germinated. This sequence of events suggests that some stages of seed germination are already underway before the seed reaches maturity and are only resumed at seed germination.

Various other data regarding composite lipid vesicle development and interassociation, and lipid vesicle transformation into saccules, are not covered in this discussion.

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**Figure 5** If homogenization is gentle, then the composite lipid vesicles remain attached to the plasma membrane (17) or to the plastids. × 10,000.

**Figure 6** Micrograph of a pea cotyledon from a germinating seed showing several stages in the formation of lipid vesicle-derived saccules. These vesicles and saccules are next to the plasma membrane, but the same transformational stages also take place in lipid vesicles adjacent to the plastids. Note that the membranes of the saccules are often thicker and more electron opaque than those of the lipid vesicles. × 100,000.

**Figure 7** Micrograph of a pea cotyledon during seed germination showing a region where all of the composite lipid vesicles have been converted into saccules. In pea cotyledon, the complete transformation of composite lipid vesicles takes between 2 and 4 days, depending upon how fast the seed germinates. × 80,000.

**Figure 8** A micrograph of an isolated lipid vesicle-saccule sheet from bean cotyledon showing that the two structural forms are still interconnected. × 80,000.

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FIGURE 9 Micrograph of pea cotyledon after the seed has germinated for about 8 days. Composite lipid vesicles and/or sacculi are no longer present. Only simple lipid vesicles remain in the cytoplasm. \( \times 35,000 \).

FIGURE 10 This micrograph from the embryo of germinating maize is included to show that lipid vesicles in these tissues also line the cell surface. These vesicles, however, do not appear to be interassociated and are not transformed into sacculi. These embryos were prefixed in glutaraldehyde-paraformaldehyde (16) and postfixed in \( \text{KMnO}_4 \). \( \times 8500 \).
vesicles are given in subsequent reports (1, 17) and
will not be discussed here. The over-all picture,
however, reveals that a unique class of lipid
vesicles exists in these tissues which can be recog-
nized at seed maturity. These vesicles are capable
of interassociating into sheets and of being trans-
formed into other structural entities.

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