STUDIES ON SEEDS

III. Isolation and Structure of Lipid-Containing Vesicles

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ABSTRACT

Two structurally distinct lipid vesicles are present in pea and bean cotyledons during the first few days of germination. Both were isolated by sucrose density gradient centrifugation without significant morphological changes.

Lipid vesicles of one type were elongated into a sausage-like or flattened-saccular shape, and were interassociated into sheets which were usually one vesicle thick. These sheets remained intact during homogenization and centrifugation, because some of the lipid vesicles in the sheet were interconnected through their bounding membranes, and because there seemed to be a bonding substance between adjacent vesicles. These vesicles were called "composite" lipid vesicles to distinguish them from the more usual, or "simple," lipid vesicles of other plant and animal tissues.

Lipid vesicles of the other type were usually larger than the composite lipid vesicles and were always spherical in form. These vesicles remained single and did not interassociate into sheets. They were probably equivalent to the simple lipid vesicles of other tissues.

INTRODUCTION

Ultrastructural studies show that most reserve lipids accumulate within the cell cytoplasm in distinctive membrane-bounded vesicles which may vary in size, depending on the type of tissue, the stage of development, and the kind of lipid (3–6, 8, 11, 12). These vesicles are bounded by a thin interfacial structure which we believe is a limiting membrane (10; but see also reference 4). Lipid vesicles usually remain discrete and seldom fuse, even when their external surfaces come into contact (see Figs. in references 3–6, 8, and 12). Lipid vesicles appear to be components of all cells, although their number, size, distribution, and chemical composition vary (3–6, 8, 11, 12).

In many instances, it is not necessary, or even desirable, to use high-fat seeds to study lipid synthesis or lipid vesicle formation and degradation. Because of their massive accumulations of lipid vesicles, high-fat seeds are often too dense for radioactive or color labels, or fixatives, to penetrate, and too crowded internally for the investigator to differentiate minor developmental changes with the electron microscope. In contrast, low-fat seeds can be handled easily, yet have lipid vesicles and/or developmental patterns like those of high-fat seeds. We have found that pea and bean cotyledons are particularly interesting for studies of lipid vesicles in that these seeds contain two distinct types of lipid vesicles (10), each of which can be isolated in highly purified form. We illustrate the form of these vesicles for only one developmental stage, which corresponds more or less to an "average" point in lipid vesicle catabolism during seed germination. The procedures outlined, however,

are equally applicable to all stages of seed development.

MATERIALS AND METHODS

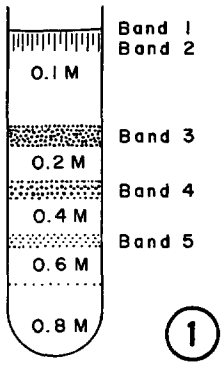
Bush bean (*Phaseolus vulgaris* var. Topcrop), pea (*Pisum sativum* var. Alaska), and soybean (*Glycine max* var. Harosoy) were used after they had been soaked in water for 24 hr. The cotyledons were placed in cold homogenization medium (50 mM Tris maleate, pH 6.5 with 0.8 M sucrose, 1% dextran [average mol wt 280,000], and 5 mM $MgCl_2$) and homogenized for 20–30 sec in a Polytron Model PT10, type "OD" (Brinkmann Instruments Inc., Westbury, N. Y.) operating at minimum speeds. The homogenate was filtered through Miracloth (Johnson & Johnson Filter Products Div., Chicago, Ill.) and centrifuged for 15 min at 17,300 *g*. The material floating on top was resuspended and placed under a discontinuous gradient consisting of zones containing 0.1 M, 0.2 M, 0.4 M, and 0.6 M sucrose prepared in 50 mM Tris maleate, pH 6.5, with 1% dextran (average mol wt 280,000), and 5 mM $MgCl_2$. The gradient was centrifuged for 2 hr at 35,000 rpm in an IEC B-60 ultracentrifuge with an SB-283 rotor. The number and positions of the bands are illustrated in Fig. 1.

For electron microscopy, the bands were either prefixed in glutaraldehyde, pelleted by centrifugation, postfixed in OsO_4, and embedded in an Epon-Araldite epoxy resin mixture as described previously (7, 9); or they were washed (without prefixation) in distilled water, mixed with neutralized phosphotungstic acid (PTA), and sprayed onto carbon-coated specimen grids.

RESULTS

The initial work in lipid isolation utilized a linear gradient with the same density spread as the step gradient shown in Fig. 1. The results were not satisfactory, however, because the lipid vesicles would not form distinct bands, even with extended periods of centrifugation. Because of these results, the linear gradient was abandoned in favor of the step gradient shown in Fig. 1.

The gradient of Fig. 1 divides the lipid vesicles into two general classes which correspond to the simple and composite lipid vesicles described in other papers (9, 10). The simple lipid vesicles are spherical, are 0.5–3.0 μ in diameter, are *not* interassociated, and are confined almost entirely to band 1 (i.e., the pellicle on the gradient surface). Band 1 is in intimate contact with band 2 and is slightly contaminated with portions of band 2 as shown in Fig. 2. Further purification was achieved, however, by subsequent washes in dilute buffer or water.



All figures except Figs. 5 and 12 are from bean cotyledon.

FIGURE 1 A diagram illustrating the approximate distribution of lipid vesicles in a fraction from bean cotyledon after the seed has soaked for 24 hr. The simple lipid vesicles (see Fig. 2) are almost totally confined to band 1 of the gradient. The small composite lipid vesicles (see Figs. 3–5) occupy bands 2–5.

Bands 2–5 (see Figs. 3 and 4 for representative fields) consist primarily of sheets of lipid vesicles which correspond to the composite lipid vesicles mentioned above and described in other reports (10). These vesicles are in various stages of transformation into saccules (see reference 10), and, therefore, range widely in size and form. The more spherical forms are usually around 0.1 μ in diameter, and the saccular forms may be a micron or more in maximum length. In all cases, both the spherical and the saccular forms are intermixed and interconnected into sheets.

Bands 2–5 are usually contaminated by a few spherical lipid vesicles of the simple type. Further purification by additional washing and centrifugation is not effective in eliminating these vesicles.

A few membrane fragments may also be present in all of these bands containing composite lipid vesicles, and particularly in band 5. These membranes are all smooth surfaced, and ribosomes are not present. No mitochondria or other organelles are present in bands 2–4, although they may be present occasionally in band 5.

Fig. 5 is included to illustrate the effects of short homogenization times and low Polytron speeds on the appearance of the composite lipid vesicles. Under these conditions, each sheet of composite vesicles is lined on one side with a single or a double "unit" type of membrane. These membranes are smooth surfaced and resemble plasma and plastid membranes, respectively.

The complexity of the composite lipid vesicles is

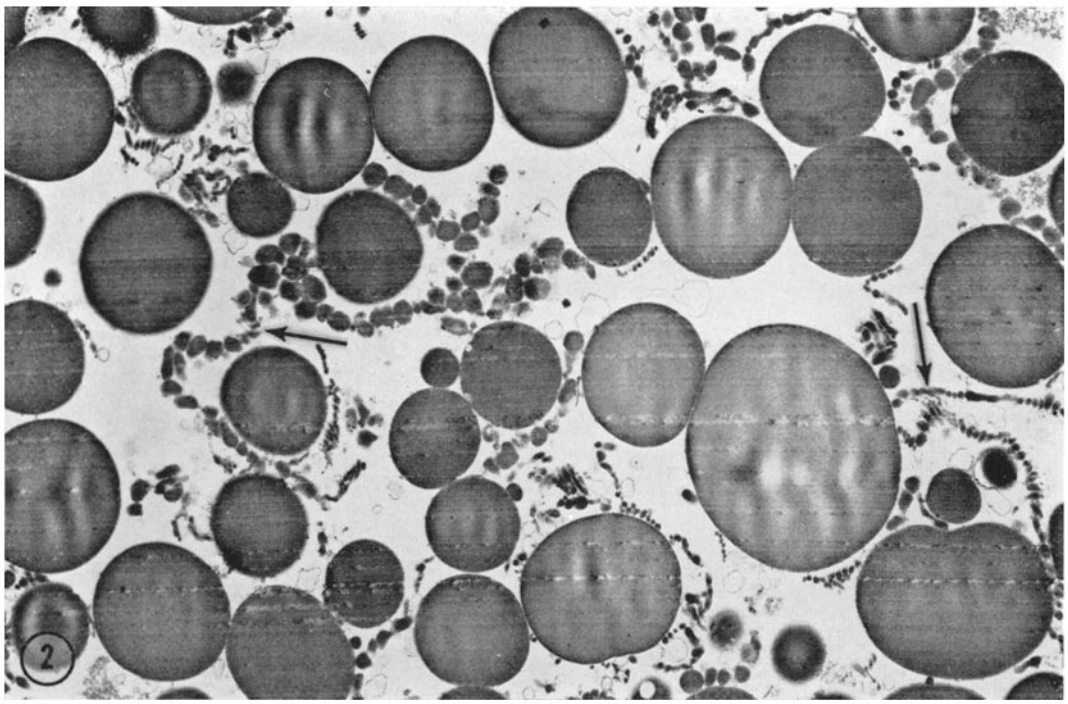


FIGURE 2 Simple lipid vesicles from band 1 (Fig. 1) of the gradient. These vesicles are usually between 0.5 and 3.0 μ in diameter and always appear as individual vesicles (i.e., they never seem to be connected). Band 1 is a pellicle which lies on top of band 2, and it cannot be removed from the gradient without also carrying along some composite lipid vesicles from band 2 (see *arrows*). × 11,000.

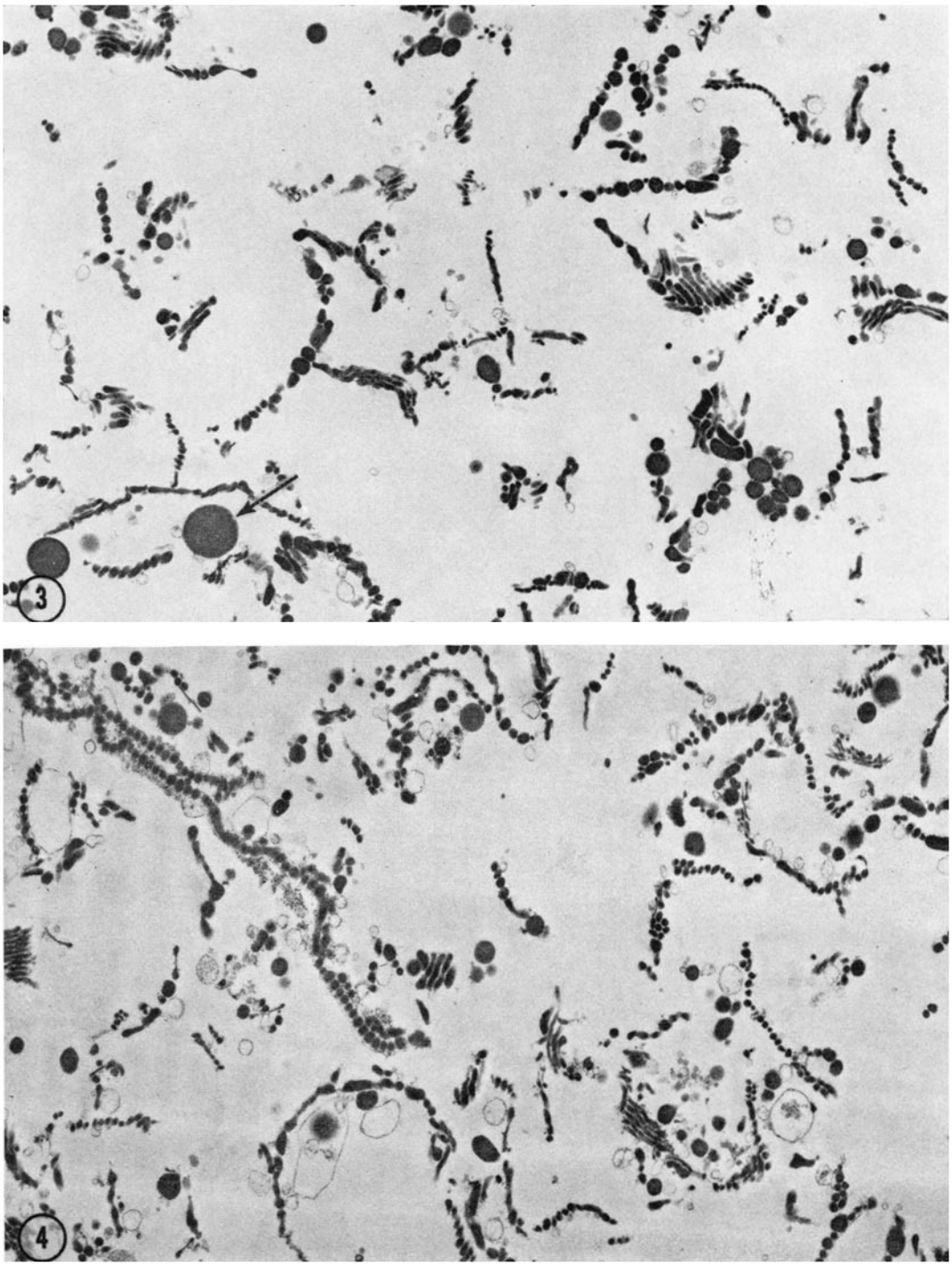
especially evident when they are viewed by the method of negative staining. The lipid vesicles easily withstand PTA, and their elongate and/or sheetlike form allows them to lie quite flat over the supporting carbon film (see Figs. 6–9). All of the vesicles are closely appressed to one another, and some of them seem to be interconnected at their end points into a common junction (Figs. 8, 9) which looks like a form of membrane (reference 2 and Fig. 9). These groups usually consist of less than eight vesicles distributed outward from the common junction (Figs. 7–9). So far as we know, lipid vesicles are only connected at one of their end points and are not connected to two or more groups.

DISCUSSION

The bands containing composite lipid vesicles are remarkably uniform in general appearance and differ only in the relative amounts of lipid vesicles and saccules that are present. The reason for this kind of distribution is that the transformation of lipid vesicles is not synchronous, and many transformational stages are always present in the total fraction and even in individual lipid vesicle sheets. The number of saccules in a vesicle sheet determines where the sheet will sediment in the gradient. This relationship causes the lower bands to be predominantly saccular and to represent a later developmental stage than the upper bands.

Lipid vesicles appear to be relatively sturdy, and easily survive homogenization and centrifugation. Isolation media are not critical, and they (i.e., the lipid vesicles) can be handled for several hours without significant structural change. The isolated lipid vesicles have the same form as they do in vivo.

Fig. 5 illustrates the fact that single or double "unit" type membranes are bound to one side of the composite lipid vesicle sheets. This is the same relationship that these vesicles show in vivo to the plasma and plastid membranes (10), and indicates that there is a real and possibly functional binding between plasma and plastid membranes and the membranes of the lipid vesicles.



FIGURES 3 and 4 Bands 2–5 consist predominantly of composite lipid vesicles and a minor contaminant of small, simple lipid vesicles (see *arrow*). The primary difference between bands 2 and 5 is related to the degree of lipid vesicle–to–saccule transformation that has taken place. Saccules are heavier than lipid vesicles and equilibrate lower in the gradient. Therefore, the band in which a lipid vesicle sheet will appear depends upon how many saccules, or partial saccules, are present in the sheet. Fig. 3, band 2, × 15,000. Fig. 4, band 3, × 15,000.

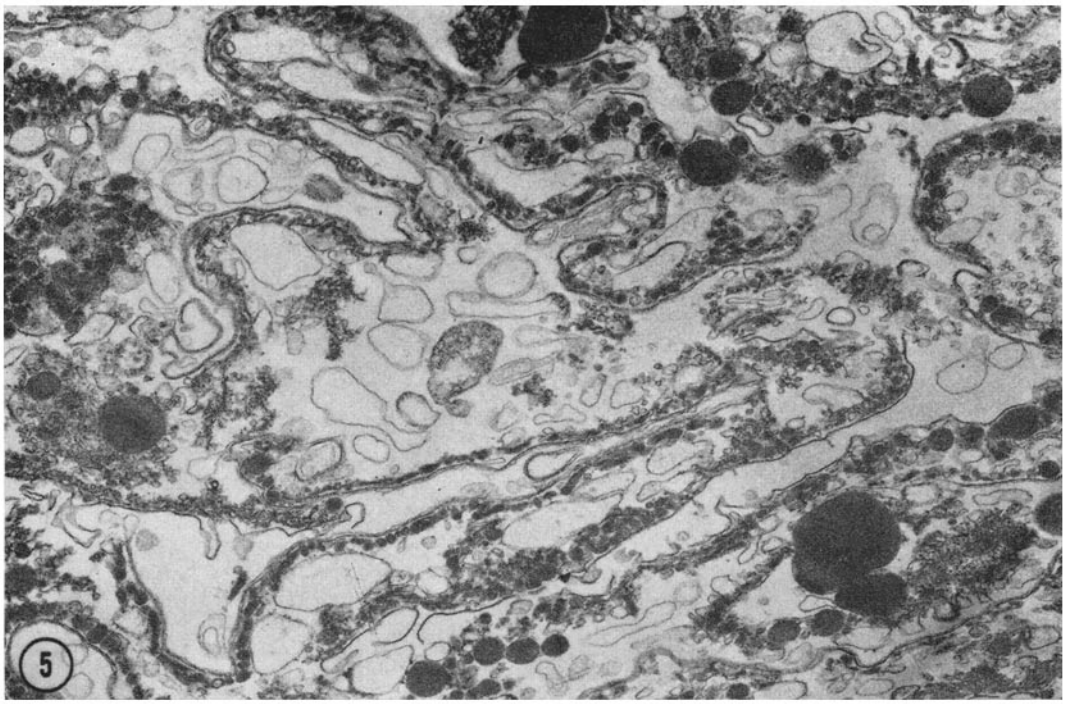


FIGURE 5 Plasma or plastid membranes associated with a lipid vesicle sheet can be preserved intact if the homogenization stresses are low. This fraction from pea cotyledon shows many of these vesicle sheet–to–membrane associations. Fraction from pea cotyledon. × 25,000.

It is difficult to completely disperse the composite lipid vesicles. This implies that they are bound together either by direct interconnections or by substances which fill the space between them. We feel that both conditions exist, but that the latter is probably responsible for the greater binding force. This is based upon the following arguments: (*a*) Some lipid vesicles are bound together by direct membrane-to-membrane continuity through a membrane junction like that illustrated in Figs. 7–9; (*b*) These membrane junctions might be segments of plasma or plastid membranes to which the lipid vesicles bind at seed maturity (10); (*c*) These membrane bonds are relatively weak because they occur only at occasional spots along the lipid vesicle sheet; (*d*) Membrane-to-membrane interconnection cannot account for the vesicle-sheet stability, since only groups of vesicles are interconnected. This implies that there are other mechanisms such as binding substances that hold the groups of lipid vesicles together. These substances appear to occur between each adjacent vesicle (see Fig. 10), and could account for the difficulty in separating the individual vesicles from the sheets.

The simple lipid vesicles in these tissues are structurally equivalent to lipid vesicles in most other kinds of tissues. They do not seem to undergo any unique kinds of transformations nor associate with other cellular constituents. They are characterized biochemically in an accompanying report (1), but very little can be said about them structurally.

Composite lipid vesicles do not seem to be present in most of the other seeds that we have examined (10), at least as judged by thin sections of the tissues. In several instances, we have tried to isolate composite lipid vesicles from seeds other than bean or pea, but the results have been consistently negative (for example, see Fig. 11). The observations further support the hypothesis that composite lipid vesicles are restricted to certain tissues or to certain stages of development.

The information in this report represents prelim-

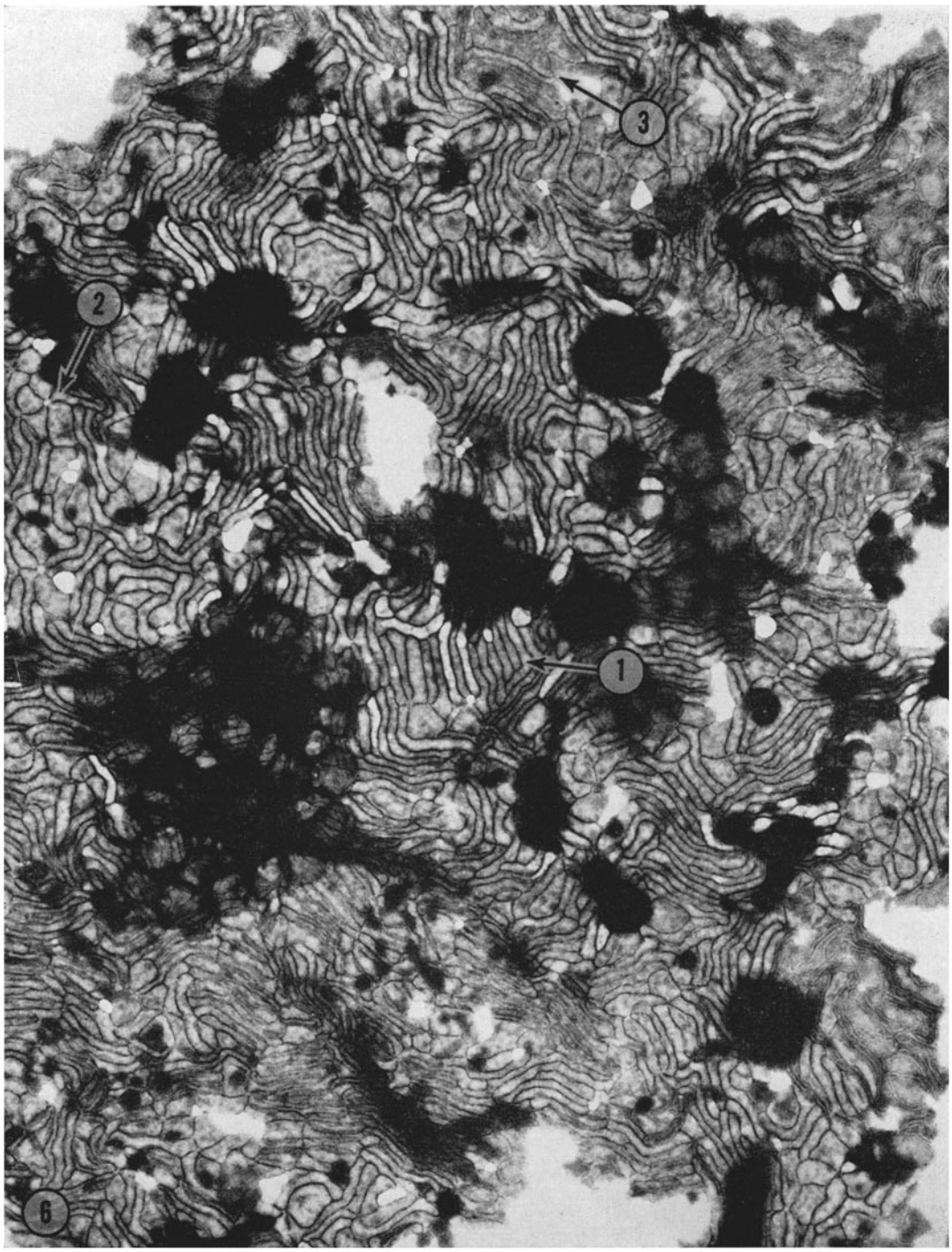
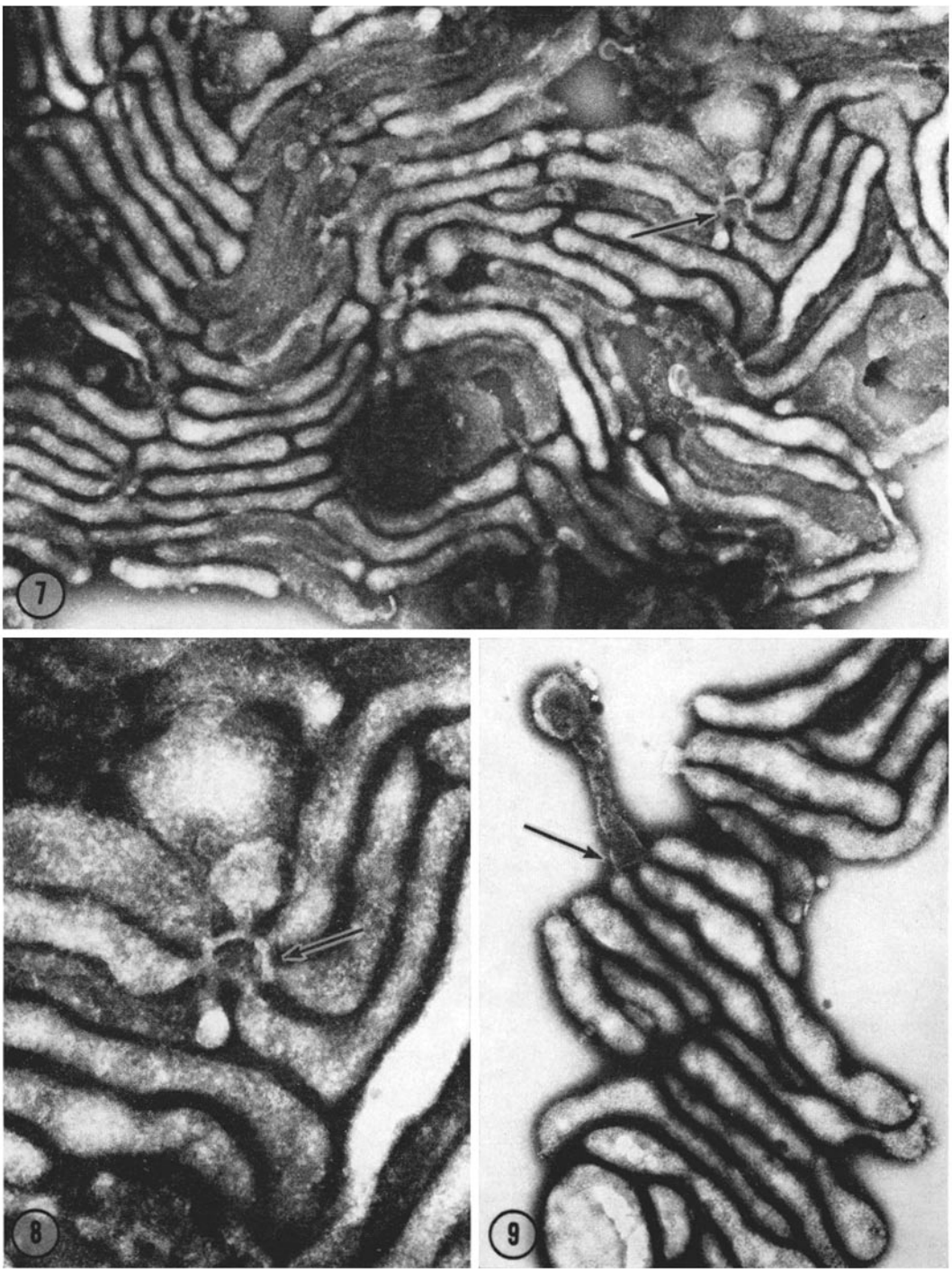


FIGURE 6 The elongate form and dense packing of composite lipid vesicles is easy to see in negatively stained preparations. Various transformational stages can be seen in almost all lipid vesicle sheets. An "average" form of vesicle for this developmental stage (24 hr of germination) is shown at arrow no. *1*. An earlier stage in which the vesicles are still nearly spherical is shown at arrow no. *2*, and a later stage is shown at arrow no. *3*. × 25,000.



FIGURES 7–9 These figures show the end point interconnections common to the composite lipid vesicles. These connections often seem to have an intermediate membrane structure as a common juncture point (see *arrows*). It is possible that these intermediate membranes are fragments broken off from the plasma or plastid membranes to which the lipid vesicle sheets may have been attached. Fig. 8 is an enlargement of the upper right portion of Fig. 7. Fig. 7, × 45,000; Fig. 8, × 110,000; Fig. 9, × 48,000.

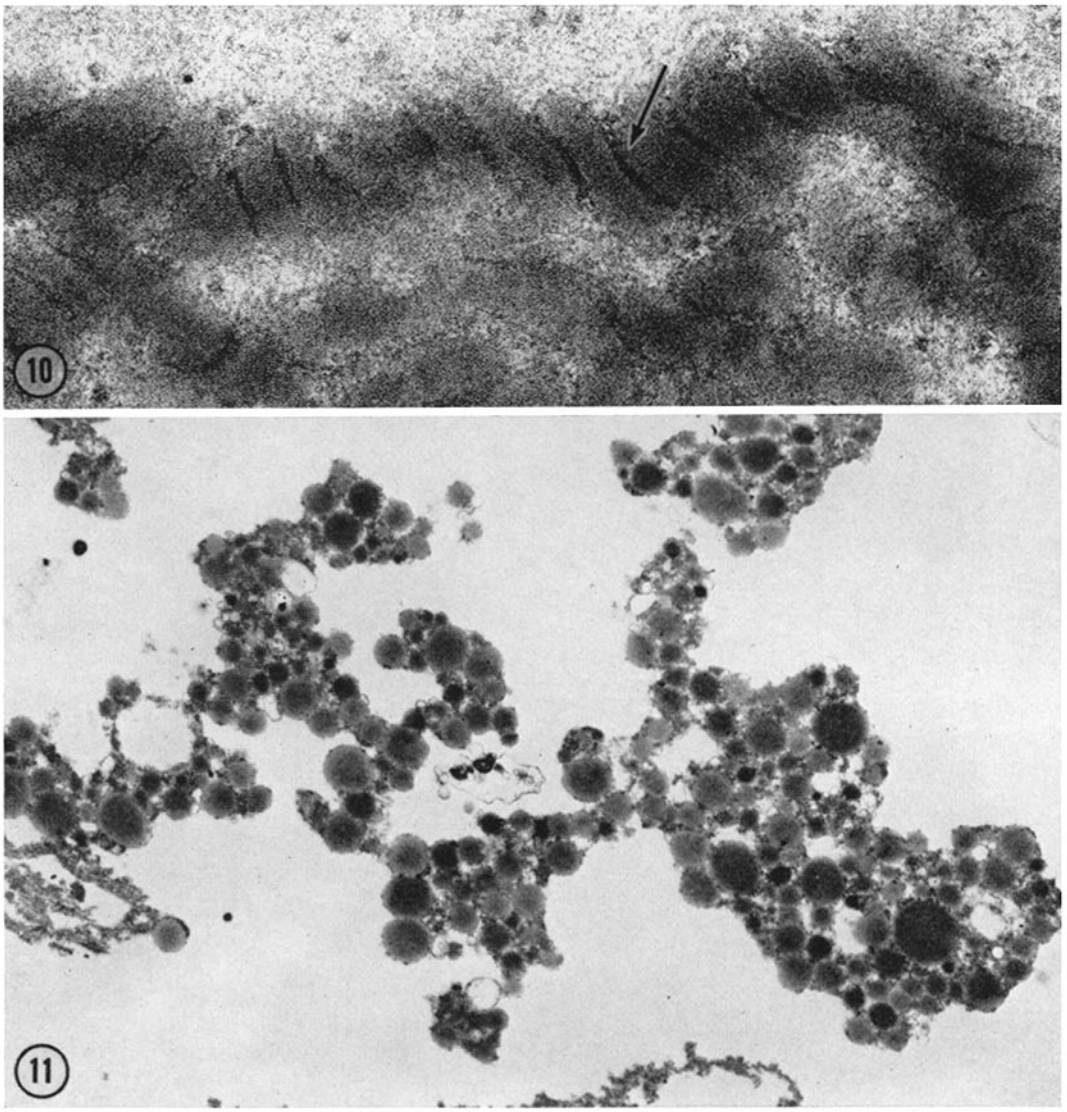


FIGURE 10 In OsO_4-fixed and sectioned material, a dense material is often visible between lipid vesicles (see *arrow*). We feel that this material may be a binding substance which helps to hold the lipid vesicles together. × 100,000.

FIGURE 11 Lipid vesicles have been isolated from germinating seeds other than pea and bean cotyledon, but composite lipid vesicles were not present. This is a typical fraction from soybean cotyledon. × 13,000.

inary work on the isolation and form of lipid vesicles, and covers only one stage in seed germination. Other developmental stages, as well as lipid vesicles of other seeds and/or tissues are presently being studied. The isolation procedures and fixation techniques described here and in reference 9 are also applicable to these studies and are being used without significant modification.

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