STRATIFICATION AND SUBSEQUENT BEHAVIOR OF PLANT CELL ORGANELLES

G. BENJAMIN BOUCK, Ph.D.

From the Biological Laboratories, Harvard University, Cambridge. Dr. Bouck's present address is Biology Department, Yale University, New Haven

ABSTRACT

Living excised roots of pea were centrifuged at 20,000 g for 24 hours, and the behavior of organelles was followed by electron microscopy at various intervals after centrifugation. With these forces, organelles are not perceptibly or irreversibly damaged, nor is the viability of the whole root destroyed. Organelles stratify generally in the order of lipid (centripetal pole), vacuoles, smooth endoplasmic reticulum and dictyosomes, proplastids (without starch), mitochondria, rough endoplasmic reticulum, proplastids with starch. The nucleus distends from the vacuolar region to the extreme centrifugal pole of the cell, while the chromatin and nucleolus seek the centrifugal pole of the nucleus. During the redistribution of organelles the rough endoplasmic reticulum is among the first to reorient, and possible explanations for this are discussed. Mitochondria can be stretched elastically many times their original length, but proplastids seem fairly rigid. Small vacuoles, forced together during centrifugation, apparently may fuse to form a large unit. Lipid droplets, on the other hand, tend to remain separate. Dictyosomes and smooth endoplasmic reticulum layer in the same region of the centrifuged cell, indicating a density similarity between these two organelles.

Since its introduction in the early 1930's, ultracentrifugation has been recognized as a useful technique for the separation and collection of cell particulates in vitro. Thus Bensley and Hoerr (1934) by differential centrifugation isolated mitochondria from guinea pig liver and were able to investigate the fat content of this single cell component. This pioneering work was substantially refined by Claude (1941) and later by others so that today differential centrifugation of macerated cells has become a routine and reliable method for collection and characterization of cell components.

Ultracentrifugation has also been utilized for investigation of organelles within the living cell. Such studies have been undertaken to clarify a variety of cellular phenomena such as: 1) to examine the "relative specific gravities" of cell components. Luyet and Ernst (1934 a), using centrifuged onion roots, found that the nucleolus and chromatin settled at the centrifugal pole, that the cytoplasm could be stratified into two distinct layers, whereas the lipid droplets and vacuoles could be collected at the centripetal pole. Beams and King (1935) noted in bean roots a similar layering after centrifugation, but were more precise in their identification of cell particulates. 2) to establish the morphological reality of specific organelles, i.e. Golgi apparatus (Beams and King, 1934 a, 1935), spindle fibers (Schrader, 1934; Luyet, 1935; Beams and King, 1936 a), and nuclear inclusions (Beams and King, 1936 a). 3) to determine the viscosity of cytoplasm (Heilbrunn, 1926). 4) to follow gross changes induced in the cell or organism after stratification. For example, Harvey's (1932) centrifuged sea urchin
eggs would separate into half or quarter eggs, and each newly produced egg would then contain a particular cell fraction whose development could be subsequently followed. Whitaker (1940) studied the effects of centrifugation on the determination of polarity in *Fucus* eggs, while Guyer and Claus (1935) and Dornfeld (1937) demonstrated that ultracentrifugation need not impair the functional capacity of an organ or tissue. Beams (1949) examined the initiation of cyclosis after centrifugation in *Elocha* leaves, and Zalokar (1960) reported that *Neurospora* hyphae could recover following ultracentrifugation.

The advantages of coupling the results of ultracentrifugation with a detailed examination of the cytoplasm, *i.e.* by electron microscopy, have been realized by a number of investigators, primarily with animal cells (*e.g.* Afzelius, 1957; Pasteels *et al*., 1958; Gross *et al*., 1960; Beams *et al*., 1960; Zalokar, 1961). With such a coupling it is possible to clarify the physical properties of organelles, as suggested by earlier workers, and it is possible to determine as well the precise effect of strong centrifugal forces on fine structure. In addition, the order and speed of recovery of cell components can be followed as they redistribute throughout the cell. From such observations, a comprehension of the living organelle can be achieved without the problems introduced by destroying the intact viable cell.

Plant cells are especially useful for this kind of study since they undergo little distortion in shape during application of centrifugal forces. Furthermore, multicellular and differentiated tissue (such as root tips or stem apices) can be easily excised, centrifuged, and cultured, and are not seriously affected by their detachment from the rest of the plant body.

**MATERIALS AND METHODS**

"Rhondo" *Pisum sativum* (Asgrow) were grown in the dark in 6-cm Petri dishes containing filter paper soaked with Torrey’s (1955) medium. At the end of 3 days the roots, now about 1 cm long, were excised 4 mm from their tips. The excised tips either were fixed directly, or were transferred to sterile 5-ml cellulose centrifuge tubes containing 0.5 ml of hardened 3 per cent agar covered with 3.5 ml of liquid Torrey’s medium. Excised tips would then descend through the Torrey’s medium and come to rest lengthwise on the hardened agar, so that the cells would be centrifuged perpendicular to the long axis of the root, and any subsequent cross-section of the root would expose the layered organelles. The tubes were placed in a swinging bucket rotor and centrifuged at 20,000 g at 6°C in a Beckman model L ultracentrifuge. After 24 hours, the roots were removed from the centrifuge, and some were fixed directly for electron microscopy. Others were placed in the dark at room temperature, and growth (determined as an increase in root length) was periodically measured with an ocular micrometer fitted in the 10 X eyepiece of a Bausch & Lomb stereo binocular microscope.

For electron microscopy, roots were cut in a solution of 1 per cent osmium tetroxide buffered with potassium phosphates (Millonig, 1961 b) adjusted to a final molarity of 0.1 and a pH of 6.9. Fixation was carried out at 6°C for periods of 9 to 24 hours and was followed by three rapid rinses in distilled water, gradual dehydration in acetone, and finally three changes of propylene oxide (Luft, 1961) over a half-hour period. The final change of propylene oxide was diluted with an equal volume of a mixture containing 3 ml methyl nadic anhydride, 7 ml of

---

**Key to Labeling**

*Key to Labeling*

- ch, chromatin
- cr, collapsed vacuole
- d, dictyosome
- er, smooth endoplasmic reticulum
- rer, rough endoplasmic reticulum
- m, mitochondrion
- n, nucleus
- nl, nucleolus
- l, lipid
- p, proplastid
- s, starch
- v, vacuole
- w, cell wall

**Figure 1** Section through a “normal” cell from the root meristem of pea. Vacuoles at this stage appear as pairs of closely appressed membranes often in star-shaped configurations (inset). Note the relatively large nucleus (n) with its densely staining nucleolus and scattered chromatin. Proplastids (p) usually lack starch, and the endoplasmic reticulum is not extensively developed in these cells. v, cell wall. X 13,000; Inset. X 33,000.
Epon, and 0.15 ml DMP-30 (cf. Luft, 1961). The propylene oxide was allowed to evaporate overnight, leaving the Epon mixture. This was replaced with fresh Epon mixture in gelatin capsules, placed under vacuum for 1 hour, and finally polymerized at 45°C. Sections were cut with a diamond knife on either a Huxley or a Porter-Blum ultramicrotome. The sections were collected on copper grids, stained in Millonig's (1961 a) lead stain, and examined in a Siemens Elmiskop I electron microscope.

RESULTS

Since cells of the root tip vary considerably as to the size of the vacuole and as to the vacuole to cytoplasm volume, the degree of layering after ultracentrifugation was expected to be correspondingly variable in cells from different regions. Accordingly, two general types of cells were examined before and after centrifugation: (1) small rapidly dividing cells in which vacuolar fluids have not greatly accumulated; (2) larger cells in which the vacuoles have become markedly enlarged. All observations were made on cross-sections of the root.

I. The Normal Cell

Cells from the actively dividing region of the pea root tip contain an assemblage of proplastids, mitochondria, dictyosomes (Golgi apparatus), nuclei, and lipid droplets (Fig. 1). The endoplasmic reticulum seems not to be extensively developed, as there are few profiles discernible in any one section. Vacuoles appear as pairs of closely appressed membranes, often in star-shaped configurations (Fig. 1, inset). Within the proplastid, starch is usually absent but characteristic dense globuli and marginal invaginations can be identified. The nucleus contains scattered electron-opaque areas (chromatin) and a large granular nucleolus which frequently contains vacuolate regions. The irregular plasma membrane often encompasses areas containing particulate materials which are probably incorporated into the wall. The wall is not of uniform thickness, and regions which will later become pit fields can often be recognized at this early stage. The granular component of the cell ground substance is interpreted as ribonucleoprotein particles.

As the cell enlarges (Fig. 2), the vacuoles expand, presumably as the result of an accumulation of liquids, and ultimately the individual vacuoles fuse to form a single large sac. At this stage, starch can now be seen in many proplastids, and the internal tubules become more numerous. The walls of these cells have thickened somewhat, and the nucleus represents a smaller proportion of the total cell volume.

II. The Centrifuged Cell

Cells fixed immediately after centrifugation exhibit fairly consistent patterns of stratification (Figs. 3 to 5). At the centripetal (light) end of the centrifuged cell is collected a densely staining cap composed of individual lipid droplets. Below this an accumulation of vacuoles, then a mixed region of dictyosomes and smooth ER, a zone of mitochondria and proplastids without starch, and finally proplastids with starch and rough ER which are forced to the centrifugal (heavy) pole of the cell. The nucleus becomes quite distended as the denser chromatin and nucleolus settle towards the centrifugal pole while the less dense non-chromatin portions tend to collect at about the region of the vacuoles.

A. Lipid Zone

While there are indications that lipids collect fairly rapidly at the centripetal extremity, there, nevertheless, appears to be little if any fusion between adjacent droplets (Fig. 6). Centrifugal forces sufficiently high to alter the structure of organelles within the cell still fail to produce a fusion of individual droplets. This fact, first noted by Luyet and Ernst (1934 a), would seem to suggest that lipid exists in the cell in a rigid or insoluble state. The lipid droplets are seldom seen elsewhere in the centrifuged cell.

B. Vacuole Zone

In cells containing expanded vacuoles, the vacuole zone consists usually of a single, relatively large vacuole and a series of smaller vacuoles along the centrifugal margin of the large vacuole (Fig. 6). The collapsed vacuoles of meristem cells

Figure 2  Portion of two cells older than those in Fig. 1, illustrating the now swollen vacuoles (v), and the presence of starch (st) within the proplastid (p); rer, endoplasmic reticulum; d, dictyosome; l, lipid; m, mitochondrion; n, nucleus; w, cell wall. × 82,000.
also collect in the region below the lipid droplets (Fig. 5).

C. Smooth Endoplasmic Reticulum and Dictyosome Zone

The individual plates comprising the dictyosome usually do not seem disturbed as a result of centrifugation, and even the marginal vesicles associated with the dictyosome can still be identified (Fig. 7). Smooth ER and dictyosomes seem to mix freely, but there is some tendency for the dictyosome to respond as a denser organelle. Small segments of rough ER are sometimes found in this zone but, when present, are continuous with the cisternae of smooth ER.

D. Mitochondria and Proplastid Zone

Proplastids without starch are usually displaced centrifugally with mitochondria (Figs. 8 and 10), whereas proplastids with starch descend to the extreme centrifugal pole below the mitochondria. Mitochondria can apparently be stretched many times their original length (Figs. 5 and 8). Such stretching may occur either because of different densities within different parts of the mitochondrion or because the progress downward during centrifugation is impeded by some other organelle. Proplastids, when forced downward because of the greater mass of a contained starch grain, follow the starch to the centrifugal pole rather than just the portion containing the starch grain. The shape of the proplastid may be altered as a result of this migration downward, but the proplastid membrane itself seems inelastic. Other proplastid contents such as the globuli and tubules do not appear affected by these centrifugal forces.

E. Granular Endoplasmic Reticulum Zone

The rough ER usually occupies the centrifugal pole of the cell and often forms a striking mass of closely packed cisternae (Figs. 9 and 10). These cisternae layer as folded sheets or as concentric whorls, but in neither case are the attached ribosomes dislodged from their surfaces. The granular ER is resistant to displacement and apparently requires long periods of centrifugation for stratification at the centrifugal pole. However, when one considers the bulk of other organelles which the ER must bypass and displace, this circumstance is perhaps not too surprising.

F. The Nucleus

The intact nucleus apparently cannot be assigned to a single zone since it seems to behave as a closed system with its own internal layering (Figs. 4 and 5). The less dense part of the nucleus (nucleoplasm) tends to migrate toward the centripetal pole, while chromatin tends to collect towards the centrifugal pole. The nucleolus also descends centrifugally and ultimately collects against the extreme heavy pole of the nucleus. The distended appearance of the nucleus then is the result of portions of the nucleus responding in opposite directions to the applied forces.

G. Cell Wall, Plasma Membrane, and Ribonucleoprotein Particles

None of these elements is perceptibly affected by the treatment employed here.

III. The Recovery

Those roots transferred to nutrient media after centrifugation were allowed to grow for periods up to 3 weeks, and samples were fixed and examined at various intervals during that period. Recovery from centrifugation was apparently rapid, as the rate of growth of the centrifuged roots equaled that of the controls within 24 hours after removal from the centrifuge. Examination of the fine structure of roots fixed 2 hours after centrifugation showed some redistribution of organelles (Fig. 11). Lipid was found throughout the cell, while mitochondria, dictyosomes, and vacuoles showed partial redistribution. Proplastids remained predominantly at the centrifugal pole and the nucleolus exhibited no tendency towards a relocation within the nucleus. Most surprising, however, was the rapid recovery of the granular ER which within these 2 hours could be seen scattered throughout the entire cell. When one considers the difficulty in initially layering this component, presumably because of

Figure 3  Section through a group of cells near the root meristem which have been centrifuged at 20,000 g for 24 hours. X 1,500.
its large surface area, its rapid recovery would seem of some significance.

DISCUSSION

Ultracentrifugation of organelles within the living cell provides a means of in vivo manipulation of living components. By such manipulations it is possible to distribute components in a consistent and predetermined pattern, so that some basis is established for following their subsequent behavior. In addition, during the process of layering, the physical properties of organelles can be explored, i.e. their plasticity and elasticity, their relative densities, and their relative mobilities. The pattern in which these organelles layer and, more particularly, redistribute not only gives some understanding of the organelles themselves, but also may ultimately permit a revelation of the structural interrelationships among organelles which carry out coordinated activities such as wall formation and cell division.

Lipid droplets and vacuoles follow an expected distribution after centrifugation, assuming that vacuoles are highly hydrated. However, the difference in sizes among the vacuoles in the centrifuged cells (Fig. 4) is contrary to the usual condition found in uncentrifuged cells where in any given cell the vacuoles are all of about the same dimensions. It seems likely that during centrifugation many of the smaller vacuoles are forced together and fuse to form a single larger sac. Thus, the “squeezing out of cell sap” noted after centrifugation by Luyet and Ernst (1934 a) can probably be attributed to the coalescence of vacuoles individually too small to be seen with the light microscope. Beams and King (1935) also pointed out that vacuoles may fuse during centrifugation. The apparent ease with which vacuoles coalesce confirms the probability that this process occurs in normal cell development, and indicates that a fluid and dynamic membrane (tonoplast) surrounds the vacuole. Sections through cells centrifuged for only 2 hours at 20,000 g show lipid and vacuoles well layered, suggesting that there is little active resistance to the applied forces. Lipid and small vacuoles recover rapidly when removed from the centrifuge, and in the light of Beams’ (1949) observations it would appear likely that Brownian movement is the prime agent in their redisperal.

The dictyosomes and smooth ER stratify in the same region of the centrifuged cell (Fig. 7), indicating that they are probably of similar density. This density similarity does not seem to exist between the smooth ER and the rough ER where, despite obvious structural continuities, there can be, nevertheless, a clear separation by centrifugation. Such a condition might be attributed to the presence of attached RNP granules. These RNP granules, however, do not seem to be the limiting factor in the rather uniform packing of ER cisternae. The manner of packing suggests the existence of a zone of organization, not usually observed at the ER surface, which prevents adjacent centrifuged cisternae from being pushed directly against one another (Fig. 9).

It is difficult to reconcile the rapid recovery of rough ER with the observed relatively great mass and large surface area of this component. The rough ER requires long periods of centrifugation to force it to settle at the centrifugal pole, and one would expect proportionally long periods for redistribution. Since this is not the case, it must be assumed either that the ER is extremely mobile and under normal conditions can move easily throughout the cell, or that the ER becomes packed under tension by centrifugation and springs back when the force is relaxed. The latter would better explain the slowness of the initial layering, but would also imply a semi-rigid structure for the ER. The often observed folded or whorled appearance after centrifugation would also support the concept of a compressed system of cisternae.

The ability of the nucleus to be distended and stretched during centrifugation has been previously noted by Luyet and Ernst (1934 b) who conclude that “the coefficient of ductility is

Figure 4. A cell from a region of the root similar to that of Fig. 3, illustrating the general layering of organelles after centrifugation. From the centripetal pole (top of picture) the order of stratification is lipid (l), vacuoles (v), smooth ER and dictyosome zone (er & d), proplastids without starch (p), mitochondria (m), rough endoplasmic reticulum (rer), and proplastids with starch (arrow). Note the distended nucleus with the dense nucleolus (nl) at the centrifugal pole of the nucleus. × 6,600.
quite high in the nuclear membrane.” However, the nucleus cannot be stretched indefinitely since with sufficiently high force the nucleus separates into two portions. Beams and King (1936) reported that the nucleus responds quite differently in different cells of centrifuged chick embryos. They found that the nucleus was elastic in mesenchyme cells, but usually separated into two parts in red blood corpuscles. In pea roots, if modest forces (20,000 g) are used, the nucleus distends but never constricts to form two separate bodies. However, higher forces (100,000 g), even for relatively short periods (1 hour), often cause the nucleus to separate into two parts. Nuclei respond substantially alike in both highly vacuolate cells and those containing collapsed vacuoles. The materials within the nuclear membrane stratify well, especially the nucleolus which usually descends rapidly to the centrifugal pole. After centrifugation the nucleus tends to return to its spherical shape, but the nucleolus remains at the former centrifugal pole of the nucleus.

Beams and King (1934 b) observed that in rat liver cells the mitochondria seek the centrifugal pole during centrifugation, and that in bean roots (1935) a clean separation of mitochondria from plastids (without starch) may not always be achieved. The present investigation in pea roots confirms these observations, but the similarity in density is not matched by a similarity in elastic properties of the two organelles. Mitochondria that are stretched during centrifugation appear capable of regaining their original form, as stretched mitochondria are seldom seen shortly after removal of the roots from the centrifuge. Plastids, on the other hand, never appear appreciably deformed by centrifugation, but starch grains may rupture the plastid envelope if sufficient force is applied. The comparative rigidity of the plastid possibly reflects a three-dimensional internal molecular organization which may be necessarily rigid for efficient carbon fixation. The double membrane of the plastid envelope seems to be more electron-opaque than its counterpart in the mitochondrion, and this condition may also be indicative of a less flexible membrane in the plastid.

Roots can apparently recover and even develop secondary roots after centrifugation, but the initial lag period before growth could be detected suggests that organelles must reorient before coordinated activities such as cell division and wall deposition can occur. After the initial 24 hours recovery, the growth rates of centrifuged and control roots are about the same, and thus no observable irreversible damage seems to have taken place. Stratified Ascaris eggs apparently recover and may divide within 12 hours after centrifugation (Beams and King, 1936b), and, in fact, may even divide while being centrifuged at 150,000 g. Whitaker (1940) also reported that Fucus eggs usually produce rhizoids and divide while still stratified so that complete redistribution does not appear essential at least for cell division. Thus, different organisms or different cells from the same organism vary considerably as to the amount of centrifugal force they can withstand, as well as to the degree of redistribution necessary for normal cell functioning.

The temperature of the centrifuge tube seems greatly to affect the final recovery of the root. Preliminary experiments with pea roots performed at room temperature, using the same g forces (20,000) and the same period of time (24 hours), result in prolonging the initial lag period as much as 7 days. These roots are characterized at first by a helical growth, but ultimately grow in the normal manner. The same kind of growth pattern can also be achieved by using higher g forces for shorter periods (100,000 g for 1 hour) at 6°C, but often organelles are damaged at these forces.

The author wishes to express his sincere appreciation to Dr. Keith R. Porter, in whose laboratory the work was carried out, for originally suggesting this problem, and for following its subsequent progress. The assistance of Dr. Myron Ledbetter who performed

**Figure 5** Portion of a cell whose vacuoles (v) are not markedly distended, showing that the order of stratification is similar to that of cells with distended vacuoles (see Fig. 4 for comparison). A mitochondrion (arrow) has been greatly stretched during centrifugation. Within the nucleus, the chromatin (ch) and the nucleolus (nl) seek the centrifugal pole. × 15,000.

---

The Journal of Cell Biology • Volume 18, 1963
some of the initial experiments is also gratefully acknowledged.
This investigation was undertaken during tenure of a postdoctoral fellowship from training grant USPHS 2G707 to Dr. K. R. Porter.

Received for publication, January 23, 1963.

BIBLIOGRAPHY


LUYET, B. J., and ERNST, R. A., On the comparative specific gravity of some cell components, Biodynamica, 1934 a, 1, 1.


TORREY, J., On the determination of vascular pat-

Figure 6 The centripetal pole of a centrifuged cell which is occupied by lipid (l) and vacuoles. Some of the lipid droplets have become trapped in a projection of a large vacuole. There appears to be no fusion between individual lipid droplets, but vacuoles apparently can coalesce when forced together. X 12,000.

Figure 7 The dictyosome (d) and smooth endoplasmic reticulum (er) zone of the stratified cell. Small vesicles may still be seen associated with margin of the dictyosome. er, vacuole. X 35,000.


**Figure 8** The proplastid and mitochondria zone. Mitochondria may be greatly stretched (arrow) during centrifugation, but proplastids (p) seem to have a fairly rigid structure. × 18,000.

**Figure 9** A portion of centrifuged rough endoplasmic reticulum showing attached ribosomes. Note that the spacing of adjacent cisternae is fairly uniform, but not limited by the ribosomes. Such a condition may reflect the existence of a zone of organization either not preserved or not directly visible along the ER surface. × 66,000.

**Figure 10** The centrifugal pole of a stratified cell. Note the collection of rough ER cisternae (rer) folded over on itself. The nucleolus (nl) also seeks the centripetal pole. p, proplastid; m, mitochondrion. × 17,500.
Figure 11 A portion of three cells 2 hours after centrifugation. Lipid (l) is now well distributed. Mitochondria (m) and rough ER (rer) can be seen scattered throughout most of the cell. However, the nucleolus (nl) remains at the centrifugal pole of the nucleus, and the proplastids are slow to reorient. er represents a group of vacuoles which have collapsed during the preparation of the tissue. d, dictyosome; v, vacuole. X 15,000.