Physiological Effects of Gibberellic Acid. VII. Electron Microscopy of Barley Aleurone Cells[1, 2]

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Introduction

Earlier work indicated that barley endosperm, freed from the embryo, responds to gibberellic acid (GA_3) treatment with a loss in dry weight accompanied by release of reducing sugars and protein nitrogen (19, 20, 21). These results lead to the conclusion that the effects of the embryo and GA_3 on the endosperm are identical, and suggest that endogenous gibberellin may be the hormone produced in the embryo which initiates the breakdown of endospermal reserves.

In an attempt to trace the action of GA_3 to a locus within the barley endosperm it was shown that the aleurone layer was the tissue directly affected (23). Isolated segments of aleurone tissue, peeled away from the starchy endosperm, have demonstrated the ability to produce and release α-amylase as a result of GA_3 treatment. The results prompted the conclusion that it is this amylase, as it diffuses from its site of production in the intact germinating seed which causes the release of reducing sugar by acting on starch in the inner portion of the endosperm.

Identification of the aleurone layer as the cellular locus of GA_3 action, and the development of techniques for its isolation have made it possible to follow, visually, the GA_3-induced changes within this tissue. This report deals with ultrastructural manifestations of GA_3 treatment, as observed with the electron microscope. A preliminary report has been presented elsewhere (12).

Methods

Tissue. Seed of *Hordeum vulgare* L. var. Naked Blanco Mariout, generously supplied by Dr. Schaller, University of California at Davis, was used. The intact seed was sterilized in 5 % $Ca(OCl)_2$ for 2 hours. The embryo and basal end of the endosperm were cut off and the remainder incubated in water at 30° for 24 or 48 hours. During this time the water was changed several times. After a final rinsing, cuts were made lengthwise down the flanks of the grain, and the aleurone layer (with pericarp) was stripped from the upper surface of the endosperm. Groups of 5 to 10 aleurone segments were incubated at 30° for up to 42 hours in either 5 ml water or GA_3 (20 or 100 μg/ml).

Electron microscopy. Aleurone segments were taken after various incubation periods and fixed 2 to 12 hours in 3 % veronal-buffered $KMnO_4$ (pH 7.4) at room temperature. Tissue was also fixed in 2 % veronal-buffered OsO_4 (pH 7.4), 10 % neutral formaldehyde followed by the same OsO_4 fixation, and in 2 % OsO_4 vapors in a closed container. None of the osmium techniques produced as clear an image as that provided by $KMnO_4$. OsO_4 may have prevented satisfactory embedding since osmium-fixed tissue was always difficult to cut. Removal of the pericarp before or after fixation failed to improve embedding of OsO_4-fixed tissue.

The aleurone segments were dehydrated in graded alcohols, moved through 2 changes of propylene oxide and embedded in Epon 812 according to the method of Luft (15). Sections were cut on an LKB ultramicrotome and examined and photographed in a Philips 200 electron microscope.

Results

Ultrastructure of Mature Dry Aleurone Cells. Tissues were sectioned at right angles to the plane of the pericarp and aleurone layers. Since the cells of the different aleurone layers varied slightly in appearance, only those from the outermost layer immediately adjacent to the pericarp are illustrated here. Nevertheless, some variability, which can be attributed to irregular fixation, remained even in this layer, both in the dry and hydrated tissues. All aleurone cells have thick cell walls and dense cytoplasm at maturity.

A survey of the cytoplasm and wall of part of a cell in the outermost layer of a dry barley grain is shown in figure 1. The most obvious features of these cells are the lack of membrane clarity and the presence of 3 types of spherical organelles. The clearest are dense bodies about 1μ in diameter which are invariably compressed and damaged during sec-

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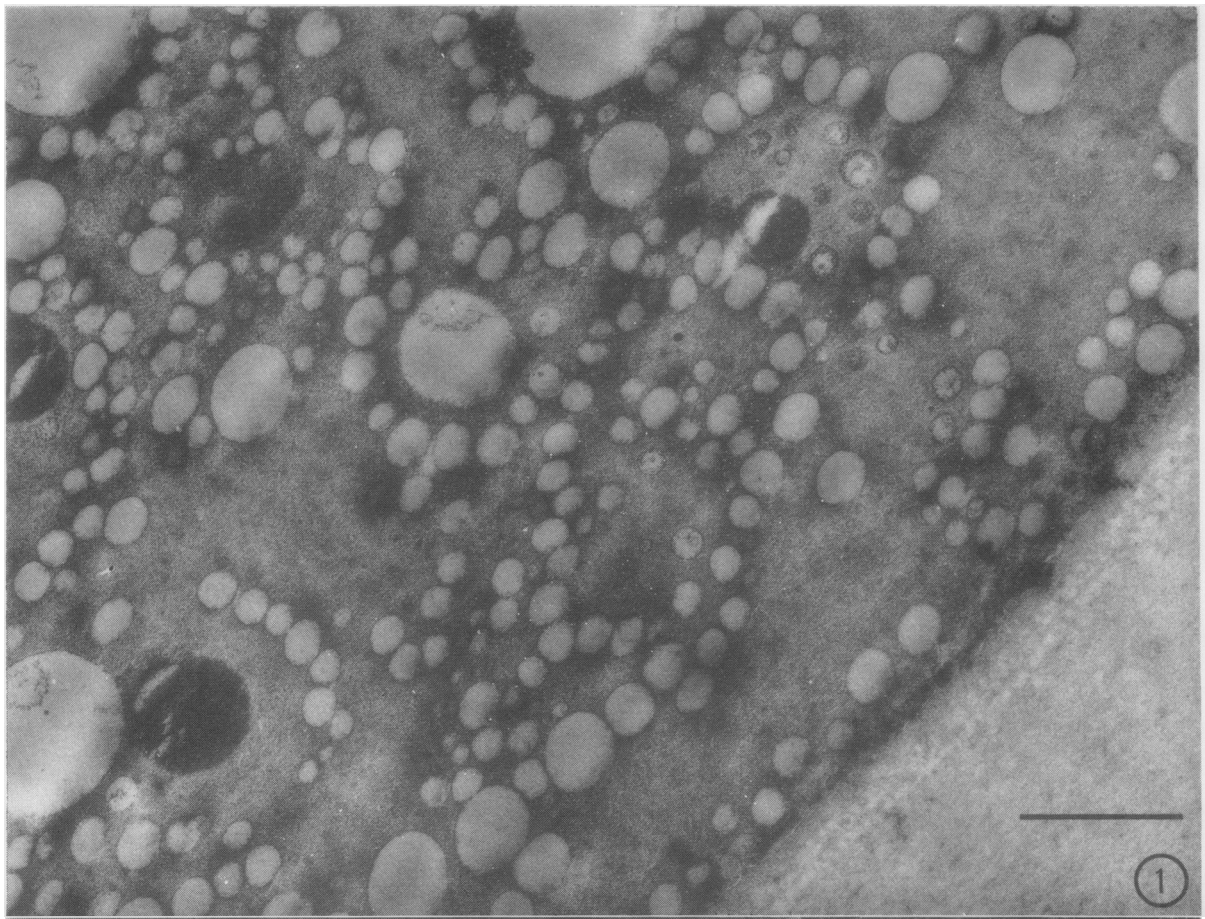
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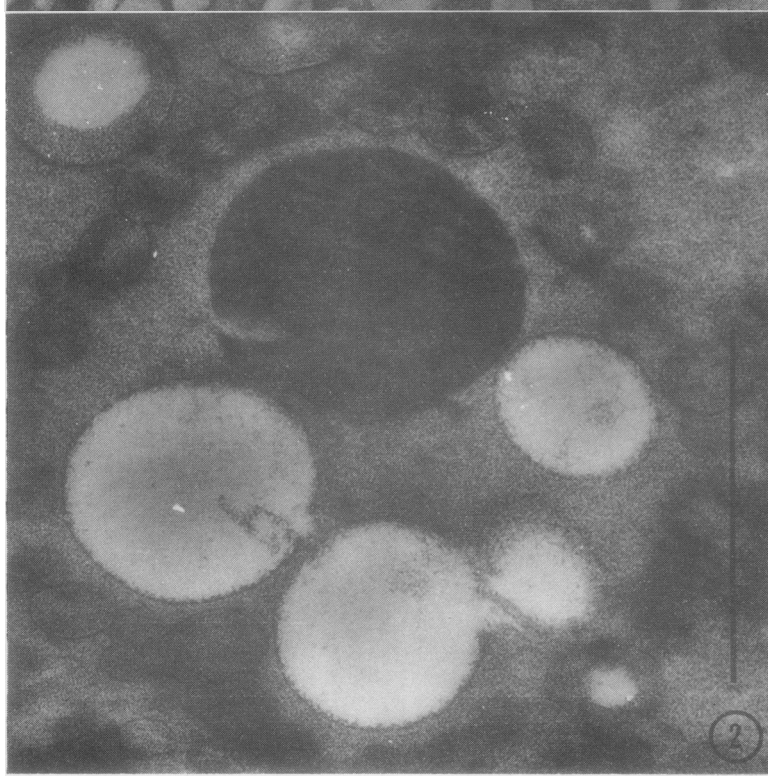
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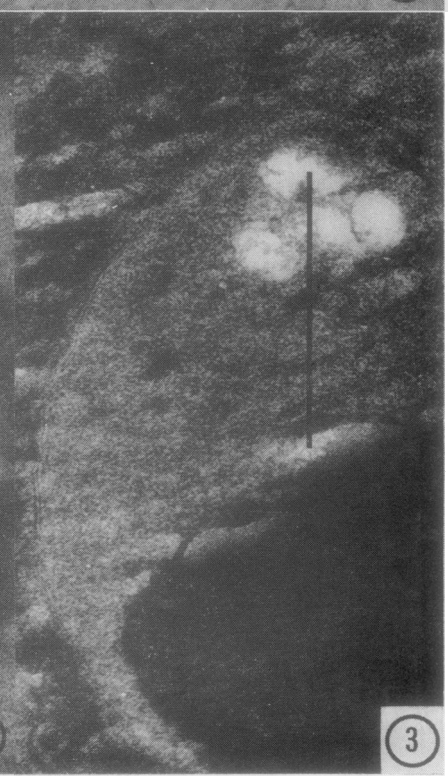
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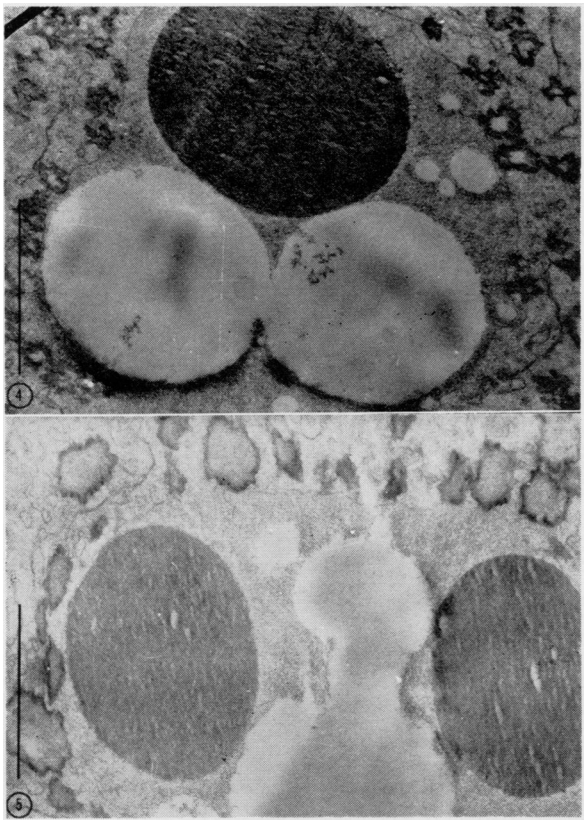

FIG. 4, 5. Aleurone grains 8 hours after beginning of experiment. FIG. 4. Control, showing results of hydration. Two types of inclusions, matrix and membrane. Spherosomes now have irregular profiles, stain darkly except for the center and often lie appressed to the membrane. FIG. 5. GA_3 treated, similar to control except for larger size of spherosomes. Scale lines represent 1μ.

tioning. The second are electron transparent objects in the same size range. The third are generally smaller ovoid bodies ranging about 0.2μ in diameter which occur in bead-like chains or layers and along the cell wall. These bodies are usually electron transparent but occasionally their contents are preserved.

The relationship between these 3 organelles, and their arrangement in groupings, is clearly illustrated in figure 2. The groupings are usually composed of 1 or 2 of the densely staining spheres and 2 to several of the 1μ electron transparent spheres. These 2 types of globular bodies are embedded in a matrix which is surrounded by a unit membrane (figure 3). We shall refer to this grouping (i.e., 2 types of inclusions, matrix and membrane) as an aleurone grain (2). Surrounding the aleurone grains are the smaller ovoid bodies which are also surrounded by a unit membrane. These bodies have recently been described by Peveling (24) and Frey-Wyssling et al. (6) and will be called spherosomes in conformity with their nomenclature.

Plastids, mitochondria, Golgi bodies, and other bodies, though present in dry aleurone, are rarely seen since their membranes are indistinct against the unusually dense cytoplasmic ground substance. Starch, although present during the early stages of development (2), is not present at maturity or at any subsequent stage.

Ultrastructural Effects of GA_3 Treatment. The technique (23) employed in securing isolated, biochemically reactive aleurone segments imposes certain conditions on the cells. The endosperm was first hydrated, and the effects of hydration are quite striking (compare fig 1 and 6). The aleurone was then peeled away from the starch-bearing cells, and although no differences which can be attributed directly to this procedure have been observed, some changes might be expected to arise due to changes in wall pressure. In the experiment to be described in detail endosperm were hydrated for 48 hours before aleurone removal. The aleurone segments were then incubated in water or GA_3 for varying periods of time.

An increase in the clarity of cytoplasmic bodies and of membrane structure in general were the most immediate effects of hydration. Another effect was the change in shape and $KMnO_4$-staining properties of the spherosomes which surround the aleurone grain and line the cell membrane. They lose their oval character and assume an irregular shape while also becoming considerably more electron dense with $KMnO_4$ staining. The entire aleurone cell swells and the aleurone grains, now less regular, also undergo swelling. (Although no pictures illustrating this stage are included, the effects of hydration may be appreciated by comparing figures 1 and 6.)

It is at about this stage that the aleurone segments were removed for treatment. During the early period of the experiments, there were few changes, if any, in the control aleurone segments and as a consequence figures 4 and 6 may be taken as representative of aleurone segments at 1 hour as well as 8 hours. Some of the subsequent changes noted occurred in both control and treated tissue but the tempo of these changes was always faster in treated material. In addition, GA_3-treated tissue fixed better and sec-

FIG. 1, 2, 3. Sections of cells from the outermost layer of dry barley aleurone fixed in $KMnO_4$. FIG. 1. General survey of the cytoplasm with a cell wall at the lower right. FIG. 2. Higher power image of an aleurone grain. Two types of globular inclusions are clearly distinguishable. At the top is a single, dense sphere which always shows compression from sectioning. Below are several electron transparent spheres. Surrounding these is a ring of spherosomes the contents of which have fixed better than those in figure 1. FIG. 3. A detail of the periphery of an aleurone grain to show the membrane which surrounds the matrix of an aleurone grain. Spherosomes at left outside the membrane have been distorted by sectioning. FIG. 1, 2. Scale line represents 1μ. FIG. 3. Scale line represents 0.5μ.

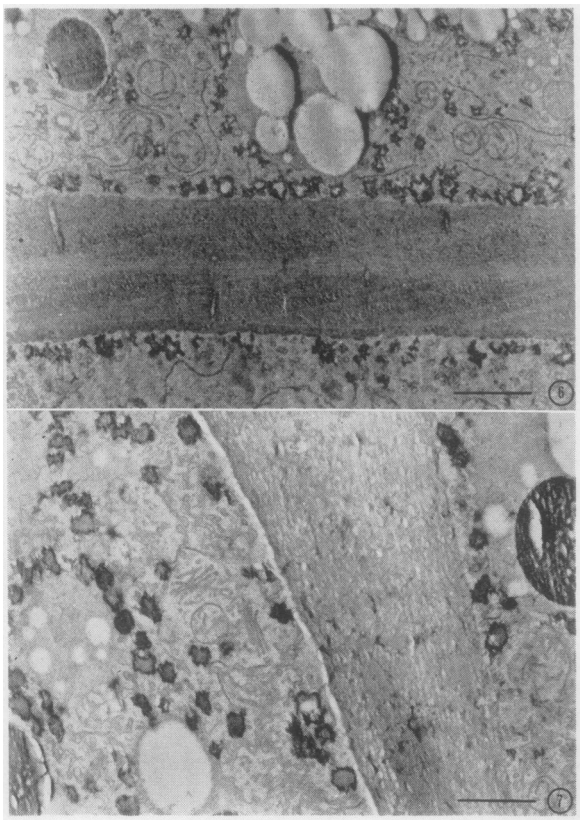

FIG. 6, 7. Survey of cytoplasm and cell walls 8 hours after beginning of experiment. FIG. 6. Control. Usual cytoplasmic components—mitochondria, Golgi apparatus, and endoplasmic reticulum are clear. Spherosomes arranged along the cell wall. FIG. 7. GA_3-treated. As above but spherosomes larger and not so regularly arranged along the cell wall. Extension of the aleurone grain membranes beginning. Scale lines represent 1μ.

tioned more easily than the controls.

By the end of 8 hours (figs 4–7) several effects of treatment were apparent. The aleurone grain had begun to increase in size. In contrast to the control, in which no marked change in this organelle occurred at any time, expansion of the aleurone grain in the treated tissue continued and became more pronounced with time. In addition, a consistent difference between the now dense spherosomes of the treated and control tissues could be seen. In the former, they became larger and appeared less dense than those of the controls.

Frequently, though not well illustrated in any figure, the membrane of the aleurone grain adjacent to the spherosomes could be seen projecting toward the spherosomes. No indication of continuities between the membranes of the 2 different structures, however, has been observed.

Examples of 18 hour tissue are shown in figure 8 and 9. Little change was apparent in the control samples. In the GA_3-treated tissue, the membranes and matrix of the aleurone grains had become greatly extended, and these organelles began to fuse with one another so that the contents of more than one grain were enclosed within a single membrane. The electron transparent inclusions of the aleurone grains of treated cells also began to extend, and because of thin sectioning appeared to be fragmented. These bodies eventually disappeared in the treated tissue, while largely maintaining their integrity in the controls.

One difference between control and treated tissue, the rate of change of which was increased by GA_3, was a decrease in number of spherosomes. This occurred in both series but was often seen in treated cells, particularly along the cell membrane, as early as 8 hours after treatment (fig 6, 7).

By 24 hours, (fig 10, 11), the unit membranes surrounding the original aleurone grains in GA_3-treated tissue had frequently extended into layered profiles which were indistinguishable (with $KMnO_4$ fixation) from those of endoplasmic reticulum. The fragmented, electron-transparent inclusions (fig 9) had almost completely disappeared (fig 11).

42 hour tissue is illustrated in figures 12 and 13.

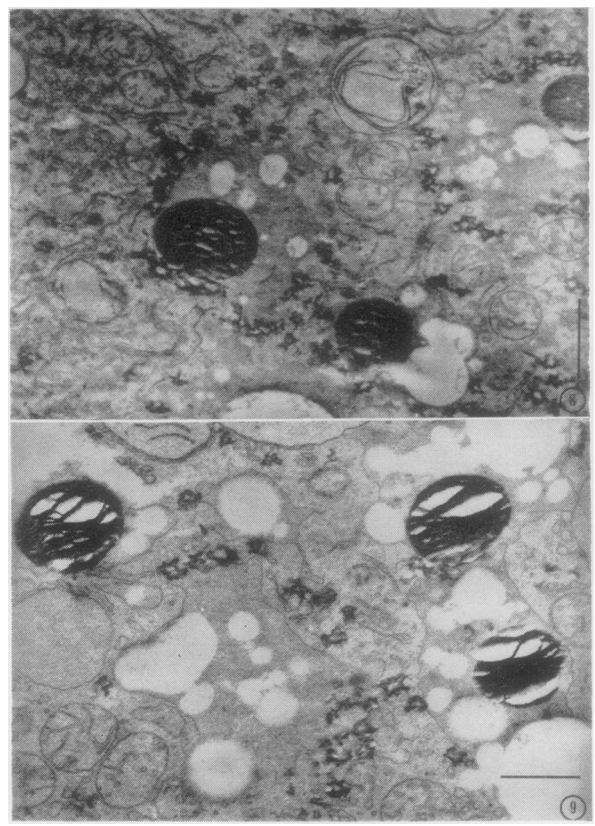

FIG. 8, 9. Survey of cytoplasm 18 hours after the beginning of the experiment. FIG. 8. Control. Like the 8 hour control. One proplastid visible. FIG. 9. GA_3 treated. Extension and fusion of aleurone grain membranes to form large vacuolar systems. Number of spherosomes decreasing. Electron transparent inclusions are beginning to ramify. Scale lines represent 1μ.

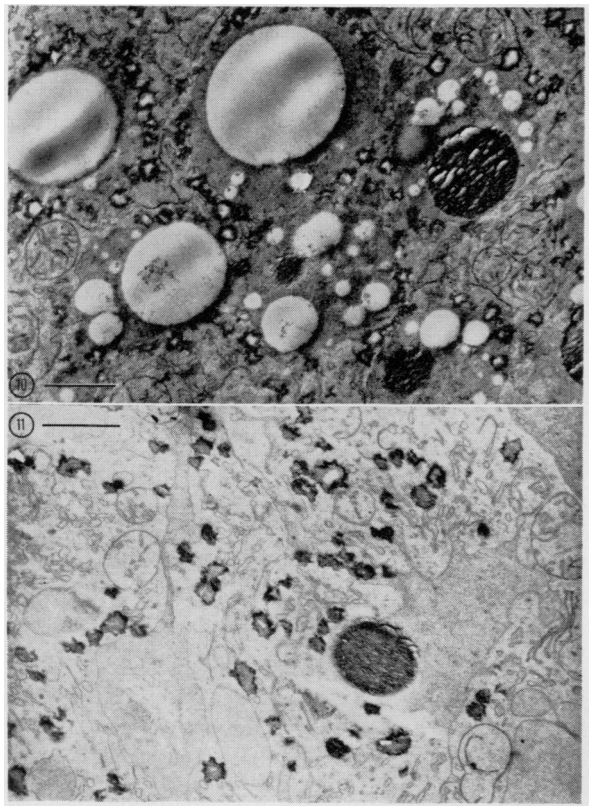

FIG. 10, 11. Survey of cytoplasm 24 hours after beginning of the experiment. FIG. 10. Control. General appearance of all organelles is similar to the 8 hour control. FIG. 11. GA_3 treated. Enormous extension of membranes of aleurone grains. At left center, membrane profiles indistinguishable from endoplasmic reticulum. Electron transparent inclusions of aleurone grains have disappeared. Scale lines represent 1μ.

The cells expanded considerably by this time. Aside from a decrease in number of organelles visible in the microscope field which might be expected to accompany cell swelling, the control cells appeared much as they did at 8 hours (fig 6). The spherosomes were no longer so frequent, or arranged around the aleurone grains, but the aleurone grains themselves were essentially unchanged.

The GA_3-treated tissue (fig 13) underwent a further modification which was not observed in control tissue. The walls of most cells exhibited distinct signs of erosion, and the fusion of aleurone grains led to the formation of increasingly larger, vacuolar areas. In other experiments with higher concentrations of GA_3, these differences were even more pronounced. Vacuoles formed from aleurone grains may fill almost the entire cell and destruction of cell walls and cytoplasm may be so drastic as to make interpretation nearly impossible.

Discussion and Conclusions

Aleurone tissue and aleurone grains in particular were the object of considerable investigation in the late 19th century. Sachs (*27*) stated that ". . . so-called aleurone-grains" were among the few contents of living plant cells known before 1840. The term seems to have been restricted in usage to distinct granules containing proteinaceous substances usually interpreted as reserve material. Frequently aleurone grains were described as having various inclusions within them such as crystals of protein (10), calcium oxalate (9), the double phosphate of calcium and magnesium (calcium and magnesium phytate or phytin) (25). They were also reported to contain other types of noncrystalline inclusions such as fat (28) or even anthocyanin pigments (3). It seems likely that a heterogeneous population of bodies have been called "aleurone grains" and that there may be, in fact, more than one basic stucture, with more than one mode of evolution and/or dissolution.

The derivation and fate of aleurone grains have

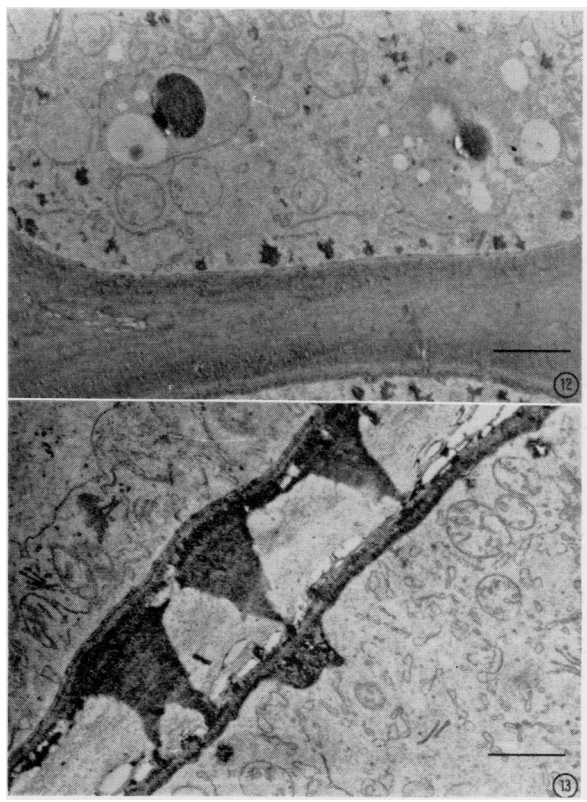

FIG. 12, 13. Survey of aleurone cell cytoplasm 42 hours after the beginning of the experiment. FIG. 12. Control. Membranes around aleurone grains still only slightly extended. Spherosomes many fewer than in 24 hour control but still arranged along the cell wall. FIG. 13. GA_3 treated. Extensive erosion of the cell wall. Spherosomes almost completely lost. Scale lines represent 1μ.

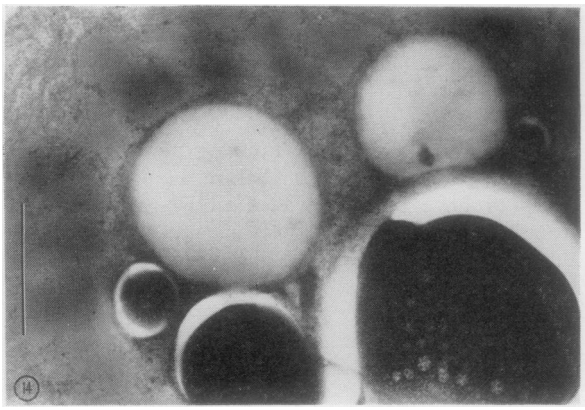

FIG. 14. Detail of an aleurone grain from control tissue fixed in OsO_4 1 hour after beginning of the experiment. The 2 lighter spheroidal inclusions at the top are the same as the very dense inclusions in $KMnO_4$-fixed material shown in the previous 13 figures. The 3 spheroids below which contain dense shrunken material are electron transparent in $KMnO_4$-fixed tissue. The matrix of the aleurone grain is clear but membranes are indistinguishable. Profiles of several spherosomes are outlined by the granular matrix and cytoplasm. Scale line represents 0.5μ.

also been the subjects of prolonged controversy. In early work with cereals Lüdtke (13) concluded that aleurone grains of the Gramineae were undifferentiated. Guillermond (7), however, reported that cereal aleurone grains were, indeed, heterogeneous. Mottier (17) reported that aleurone grains in the aleurone layer of maize arise from cytoplasmic primordia rather than within vacuoles, and shortly after, Dangeard (4) concluded that in maize and other cereals, as in legumes, etc., aleurone grains arise in vacuoles and, in turn, give rise to vacuoles. Wieler (31) and Muschik (18) on the other hand both suggest that the grains may have other than a vacuolar origin. In the only previous electron microscopic investigation, Buttrose (2) concluded that wheat aleurone grains arise as deposits within vacuoles, thus bringing a century's investigations full circle. The electron micrographs of aleurone cells of barley are strikingly similar to those of wheat.

The chemical constitution of the inclusions of the cereal aleurone grain has not, as yet, been identified with certainty. Guillermond (8) states that there are 1 or 2 protein bodies and one to several "globoids" in each aleurone grain of *Zea mays*. He reports that the globoids are composed of phytin, whereas Salmon (28) presents histochemical evidence that lipid is a component of the globoids of Cucurbita aleurone grains. There have been only a few analytical studies of the composition of the aleurone layer but 2 points emerge clearly. The hand-dissected wheat aleurone layer contains about 20 % protein (11) and between 6 and 20 % fat (16, 29), concentrations high enough to expect discrete organelles containing these constituents to be present. Figure 14 is a photograph of a portion of an aleurone grain of dry barley fixed with osmium. Numerous observations of osmium- and $KMnO_4$-fixed aleurone grains indicated that the electron density of the 2 types of inclusions can be reversed by these fixatives. Although there is no definite agreement about the types of compounds stained by these fixatives, it is possible that the dense bodies in $KMnO_4$-fixed material are proteinaceous (18, 30) while the dense bodies in the OsO_4-fixed tissue are lipoid (1). Through analogy with aleurone grains of legumes, (5) and because of the high protein content of these cells, it is likely that the matrix is also largely proteinaceous. The composition of the spherosome is also uncertain although Frey-Wyssling, et al. (6) suggest that similar bodies in other tissues consist of fat and protein.

Since the aleurone grain has always been considered a reserve body which supplies metabolites to the germinating embryo, it is not surprising to find that the changes induced by GA_3 clearly involve aleurone grains. These organelles expand, with their membranes extending into layered profiles resembling endoplasmic reticulum. One of the inclusions, possibly lipoidal, fragments and disappears while the other inclusion, the protein body, is more resistant to change. The grains themselves coalesce and give rise to vacuoles which eventually occupy most of the cell volume. At a later stage the cell walls also demonstrate evidence of erosion, and this, too, may be the initial step in a continuous process resulting in their complete breakdown.

The earliest effect of GA_3 as yet observed, seems to be on the spherosomes. They increase in size and lessen in density by 8 hours after the initiation of treatment, although subsequently, they, also, decrease in number and eventually disappear. α-Amylase measurements made on similarly-treated tissue indicated that enzyme production could be detected after 12 hours and continued at an increasing rate to at least 48 hours. It is difficult, at this time, to associate the observed cytological changes with the biochemical data. An important point in this regard is the fact that the barley aleurone layer is about 3 cells thick. Hormone-induced changes would undoubtedly occur first in the innermost layer since it is least restricted by the pressures of the surrounding cell walls, while the cells under observation in this work were, for the sake of uniformity, in the outermost confined layer.

Several points made by Buttrose (2) are important in considering the above observations. He demonstrated that during the early stages of development of the aleurone cells (at 10 days after anthesis) starch is present although no aleurone grains are visible. The starch, however, disappears by about 18 days after anthesis at which time aleurone grains are quite evident. If dissolution of this starch is due to α-amylase, then the mechanism for α-amylase production exists in the aleurone cells as early as 18 days and may be directly or indirectly related to the aleurone grain. Due possibly to the desiccation

accompanying maturation, or to a lack of endogenous gibberellin, the cells lose the ability to continue the production of this enzyme, and α-amylase production is not reestablished until either embryo action, or exogenous GA_3 restores the cells to their preexisting state. The observed effect of GA_3 on the aleurone grain and its membrane is of interest in this regard. By 18 hours after treatment (fig 9) it is possible to observe the attenuation of portions of the matrix and an enormous extension and convolution of the vacuolar membranes (fig 11) resulting in profiles resembling endoplasmic reticulum. The location of protein synthesizing sites on membranes has been established in both plants and animals (*26*), and an attractive hypothesis is that the aleurone grain membranes are, in fact, the location of microsomal particles capable of protein synthesis once the extension process is initiated by GA_3. Unfortunately no observations on the location of such particles in this tissue have been made since osmium-fixed material is so difficult to embed and section. It must also be pointed out that what is usually designated as endoplasmic reticulum in other plant cells is sometimes visible in dry barley aleurone cells and in all control as well as GA_3-treated preparations pictured here (fig 4, 6, 8, 10).

Unpublished information of Buttrose indicates that the changes which occur in wheat aleurone cells during germination are identical with those observed in the present work during GA_3 treatment of isolated barley aleurone segments. This strongly supports the hypothesis put forward earlier (*22*) that an endogenous gibberellin acts during germination as an endosperm mobilizing hormone, and initiates the processes controlling the hydrolysis of endospermal reserves.

Summary

The ultrastructure of the cytoplasm of the cells of isolated barley aleurone tissue has been investigated by electron microscopy with particular emphasis on changes induced by hormone (gibberellic acid) treatment. Characteristic cell structures include aleurone grains, limited by a unit membrane, which contain 2 types of spherical inclusions. Spherosomes surround the aleurone grains and are ranged along the plasma membrane. Plastids, mitochondria, endoplasmic reticulum, and Golgi structures are also present. The changes in aleurone tissue incubated in gibberellic acid are compared with those of water controls. Gibberellic acid-treated aleurone diverges from the relatively static controls in enlargement, fusion, and vacuolization of the aleurone grains, disappearance of globoids within the aleurone grains, almost complete disappearance of the spherosomes and extensive erosion of the cell walls.

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Carotenoids of Wild Type and Mutant Strains of the Green Alga, Chlamydomonas reinhardi [1, 2]

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Chlamydomonas reinhardi is an excellent organism for research in photosynthesis for there are several mutant strains with impaired photosynthesis (21), as well as a number of mutant strains in which the pigments are visibly altered (25). In view of the recent investigations on the photosynthetic competence of these mutant strains (17, 19, 20) we felt it was desirable to obtain a complete description of their carotenoid pigments.

In a preliminary study of the carotenoid pigments of *C. reinhardi*, Sager and Zalokar (26) separated 15 carotenoid fractions from their light-grown wild type strain. They were able to identify α-carotene, β-carotene and lutein. When grown in the dark, the wild type strain produced only 6 fractions, including the 3 identified in the light. In contrast, a pale green mutant strain (no. 95) which dies when grown either photosynthetically or heterotrophically in the light, produced only α-carotene and β-carotene in the dark.

In the present study, 9 different carotenoid pigments could be identified in light-grown cultures of the wild type strain of *C. reinhardi*. Dark-grown wild type cultures contain 7 of these pigments, have less total pigment, and have a markedly decreased ratio of β-carotene/α-carotene. The latter 2 properties are also characteristic of the mutant strains described here.

Materials and Methods

Organisms. Six strains of *C. reinhardi* were used in the experiments reported; wild type (strain *137c*), a mutant strain of spontaneous origin, *y-2*, and 4 UV-induced mutant strains, *ac-16*, *ac-21*, *ac-115* and *ac-141* (18).

Wild type *C. reinhardi* is green when cultured in the light or dark, whereas *y-2* is green when cultured in the light but yellow when cultured in the dark. The mutant strain is, therefore, phenotypically similar to the yellow in the dark or yellow strain described by Sager (24). This yellow phenotype results from the inability of the mutant strain to synthesize chlorophyll in the dark. During the course of this investigation, it became apparent that we were working with mixed cultures of wild type and *y-2*. Cell population studies were therefore carried out on cultures grown

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