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Enzymic Mechanism of Starch Breakdown in Germinating Rice Seeds[1]

11. ULTRASTRUCTURAL CHANGES IN SCUTELLAR EPITHELIUM

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ABSTRACT

The ultrastructural changes occurring in the scutellar epithelium cells of rice seeds have been studied during germination and early seedling growth. During this time, several prominent structural changes occur, including (a) formation, development, and proliferation of organelles such as mitochondria, rough endoplasmic reticulum, free ribosomes, and Golgi apparatus; (b) folded structural modification of plasmamembranes in later stages; and (c) conspicuous decrease in lipid-storing spherosomes. Glyoxysome-like electron dense particles are detectable but their formation is much less prominent. It is conceivable that all these structural changes are related to the enhancement of the metabolic activities of the epithelial cells including the synthesis of hydrolytic enzymes such as α-amylase and their secretion into the endosperm tissues. Some enzyme activities characteristic of mitochondria and glyoxysomes have been determined using the crude scutellar extracts, and the results dealing with the low activities of the glyoxylate cycle enzymes and palmitoyl-coenzyme A oxidase appear to indicate that fatty acid breakdown is possibly via mitochondrial β-oxidation, although we reserve a definitive conclusion on the glyoxysomes being nonfunctional in fatty acid oxidation in rice seedlings.

It has recently been demonstrated that the initial site of formation of α-amylase in germinating rice seeds is the single layer epithelium cells of the embryonic scutellum (28). This finding is based on histochemical experiments using a thin starch-film technique, and by the same method it has been shown that essentially an analogous mechanism operates in other starch-bearing cereal seeds such as barley, wheat, rye, oat, and maize (29). Using immunological and microscopy techniques, Gibbons (13) has independently reached the same conclusion for α-amylase production in barley seeds. It must be stressed that this observation provides a source of α-amylase in addition to the formation of α-amylase that occurs in the aleurone layers of cereal seeds when triggered by GA_3 from the embryonic tissues (15, 37). Historically there have been some indications that in barley and maize the scutellum is a site of amylase formation (4, 11). It now appears that the epithelial cells play a crucial role in the overall metabolic events in cereal seeds at the onset of germination, *i.e.* (a) formation of hydrolases, including α-amylase; (b) secretion of these macromolecules into the endosperm; and (c) absorption of low molecular substances, such as saccharides and amino acids, produced by these enzymes from reserves in the endosperm. To gain insight into the functional aspects of the epithelial cells of rice seeds during the germination period, it is of interest first to investigate their ultrastructural changes. It will be recalled that the fine structure of the barley aleurone cells has been extensively studied in relation to the synthesis of α-amylase (18, 19), and the formation of RER was implicated as being one of the early events relating the effect of GA_3 to enzyme formation. We now report on EM[2] observations of the epithelium and concurrent analysis of some enzyme activities in relation to the formation and development of organelles.

MATERIALS AND METHODS

Growth of Plants and Preparation of Scutellum. Rice seeds (*Oryza sativa* L. cv. Kimmazé) soaked overnight in distilled H_2O at 4°C were germinated in a dark chamber for stated periods at 30°C. Germinating seeds were harvested at each stage, and scutellum tissues were dissected from the seeds for EM studies, lipid analysis, and enzyme assays (see below).

EM Studies. For EM studies, dry seeds were scraped flat on each side on a sand paper, soaked overnight in distilled H_2O at 4°C, or germinated at 30°C. Seed samples were then cut transversely through the embryo-containing region. These specimens were fixed for 24 h at 4°C in a modified Karnovsky's fixative (2% paraformaldehyde, 4% glutaraldehyde, 80 mM phosphate buffer [pH 7.0], and 20 mM collidin buffer [pH 7.0]) (20). The dissected tissues were washed overnight in 0.2 M phosphate buffer (pH 7.3) and postfixed in 50 mM phosphate buffered 2% OsO_4 solution at 25°C for 8 h with one change of the solution. Samples were further stained with 0.5% aqueous uranyl acetate overnight at 4°C. Samples dehydrated in a graded acetone series were infiltrated and embedded in Spurr's resin (34). Sections were cut with a glass knife on a Porter-Blum ultramicrotome. The thin sections were doubly stained with saturated aqueous uranyl acetate solution (38) and 0.2% lead citrate solution (31), and examined in a Hitachi HV 12A electron microscope at 75 kv.

For the scanning EM studies, scutellar tissues dissected from the germinated seed were fixed using a modified Karnovsky's fixative (12 h at 4°C) (see above). The postfixed samples were dehydrated in serially graded ethanol and finally in an isoamyl alcohol solution. After drying, standard technique was employed for scanning EM using a JEOL-SEM.

Determination of Triacylglycerol. The lipid analysis was carried

[1] Dedicated to the memory of Dr. Sterling Hendricks. I met Dr. Hendricks in the Philippines for the first time in 1962, when I initiated work on starch metabolism. It is my fond memory to recall his warm encouragement of our research (T. A). Paper No. 10 of the series is Reference 23 by Miyata *et al.*

[2] Abbreviations: EM, electron microscope; Pal-CoA, palmitoyl-CoA.

out by the method reported by Folch *et al.* (12) with slight modification. Two hundred dissected scutellar tissues at appropriate stages were homogenized in 5 ml of 20 mM glycylglycine buffer (pH 7.4) containing 1 mM EDTA. The whole homogenates were extracted overnight with 10 vol of chloroform:methanol (2:1, v/v). The extracts were washed three times with 0.2 volumes of 0.1% $MgCl_2$, followed by centrifugal separation with the upper aqueous phase being discarded. The lipid fraction was dried and dissolved in 5 ml of chloroform, transferred on to a 1 g silicic acid column, which had been prepared by using chloroform. The column was eluted with 20 ml of chloroform, and the effluents containing the original chloroform solution and eluant were combined. The simple lipid fractions thus collected were dried and redissolved in 30 ml of diethylether, extracted three times with 10 ml of 4% Na_2CO_3 solution. The final simple lipid fractions were dried and redissolved in 5 ml of hexane. The samples were then applied to a 1 g silicic acid column, which had been washed successively with 5 ml each of diethylether, 15% benzene in hexane, and hexane. The column was eluted stepwise with 5 ml of hexane, 10 ml of 15% benzene in hexane, and 20 ml of 5% diethylether in hexane. The eluate from the final eluting solvent, which contains triacylglycerol, was evaporated to dryness *in vacuo* at about 50°C and weighed. The triacylglycerol content is expressed as μg per scutellum.

Preparation of Crude Extracts and Enzyme Assays. Two hundred dissected scutella were homogenized in 5 ml of 20 mM glycylglycine buffer (pH 7.4) containing 1 mM EDTA. The homogenates were centrifuged at 20,000*g* for 20 min, and the supernatant fractions obtained by further centrifugation (15,000*g*, 120 min) served as the crude enzyme preparations, which were subjected to the following enzyme assays. (a) Fumarase activity was measured by the method of Hatch (14); (b) catalase activity was determined according to Lück (22); (c) activities of isocitrate lyase and malate synthetase were assayed by the method of Dixon and Kornberg (10); and (d) Pal-CoA oxidase activity was assayed by the spectrophotometric method of Hryb and Hogg (17), except that 15 mM phenol was used instead of 25 mM β-hydroxybenzoic acid, and Triton X-100 was eliminated. A molar extinction coefficient of 6.4×10^3 (500 nm) was used for antipyrylquinoneimine. To determine the enzyme activity (O_2 uptake) with an oxygen electrode (Hansatech, England) (*cf.* 9), the same reaction mixture devoid of phenol and antipyrylquinoneimine was added to a reaction chamber and incubated at 30°C. (e) Pal-CoA dehydrogenase activity was assayed by the method of Hryb and Hogg (17), except that Triton X-100 was eliminated. Pal-CoA used in these assays was kindly donated by H. Beevers. All enzyme activities determined were expressed as nmol substrate utilized per scutellum per h.

RESULTS

Ultrastructural Change. Our primary interest has been focused on the ultrastructure of the epithelial cells during the stages of germination and early seedling growth.

Figure 1 represents the morphological structure of scutellar epithelium cells in dry (A), imbibed in H_2O (B; 12 h at 4°C), and germinated (C and D; 12 h at 30°C) seeds. A unique feature of the epithelial cells at this early stage is an abundance of lipid-storing bodies (spherosomes or oleosomes) localized near the cell wall. A number of vacuoles, mitochondria, and plastids are also visible. However, the internal cristae structure of mitochondria is not clearly recognizable. There is a thick fibrous layer between the epithelium and the endosperm tissue, which is clearly distinguishable from the epithelial cell wall (A and C).

Conspicuous intracellular structural changes at the organellar level start during incubation for 24 to 48 h and onward (Fig. 2). Among several notable features are (a) proliferation of mitochondria and development of the internal cristae structure (A and B), (b) proliferation of RER and ribosomal particles (A and B), (c) structural change in nuclei as visualized from the swollen shape and the appearance of the nuclear membrane (A and B), (d) development of plastids enclosing starch granules, (e) formation and development of Golgi stacks and vesicles (B), and (f) decline in number of spherosomes and their inward translocation (A and B). As can be seen in Figure 2A, a few glyoxysome-like electron-dense particles are visible at certain stages, but the development of this organelle appears to be less conspicuous in the cell. In contrast, the formation and development of mitochondria and RER continue during prolonged incubation (8 d) (D), while many of the spherosomes still remain. Structural change in the cell wall is also prominent, and the fibrous boundary layer becomes definitely thinner, presumably because of the hydrolytic breakdown of the cell wall polysaccharides by β-glucanase (*cf.* 13).

It is conceivable that the proliferation of RER, mitochondria, and Golgi apparatus in the epithelium closely reflects the enhancement of the protein-synthesizing activities, production of amylase, and other hydrolase molecules and their subsequent secretion into the endosperm tissues. It can be emphasized, however, that in addition to the secretion of macromolecules, the epithelium also functions in the inward active transport of low mol wt substances, such as sugars and amino acids, from the endosperm. The folded structure of the plasmamembranes (Fig. 3A) is thought to be advantageous for this activity. The scanning EM picture given in Figure 2E also shows this feature of the cell surface facing the endosperm.

In general, the EM pictures indicate that as germination and seedling growth progress there occur conspicuous formation and development of membrane structures in the rice seed epithelium. This structural change is distinguishable from that in the adjacent scutellar parenchymatous cells. A typical structure of the latter after 4 days incubation is shown in Figure 3B. Although there are a number of spherosomes, vacuoles, and mitochondria in the scutellum, the development of RER is much less prominent in comparison with that in the epithelium.

Depletion of Reserve Fat Content. From EM pictures described above, it is evident that the reserve fat in the epithelium, stored in the form of spherosomes, starts to break down at the onset of germination. Results of a parallel time-sequence analysis of triacylglycerol support this interpretation (Fig. 4A). Moreover, consistent with the ultrastructural observation, it is notable that approximately one half of the fat still remains in the tissue in later stages (about 80 μg/scutellum, after 2.5 d; and about 30 μg/scutellum, after 6.5 d incubation).

Mitochondrial and Glyoxysomal Enzyme Activities. As a step in correlating ultrastructural changes in the epithelium to possible metabolic reactions *in situ*, we have determined some enzyme activities characteristic of mitochondria and glyoxysomes during the germination stage. There are two possible metabolic pathways for the breakdown and utilization of fat reserves in epithelial cells. One is mitochondrial β-oxidation of fatty acids. ATP formed in this process will eventually be utilized for energy-requiring processes such as protein synthesis, but a second pathway which cannot be excluded, is the transformation of fat to carbohydrate by glyoxysomes as in the endosperm tissues of oil seeds (3). These two alternatives can be evaluated from EM pictures showing proliferation of mitochondria (*cf.* Fig. 2, A and B, and Fig. 3A) and the presence of glyoxysome-like particles (*cf.* Fig. 2A).

Results of time-sequence analysis of the organellar enzyme activities are presented in Figure 4, B and C. For the purpose of comparing enzyme activities with metabolic capacity of the tissue, specific activities of individual enzymes are expressed as per scutellum per h. However, as it was difficult to excise scutella from seedlings at a very early stage, we have determined enzyme activities only from 2.5 d onward. As shown in Figure 4B, activity of fumarase, a marker enzyme of mitochondria, was found to be

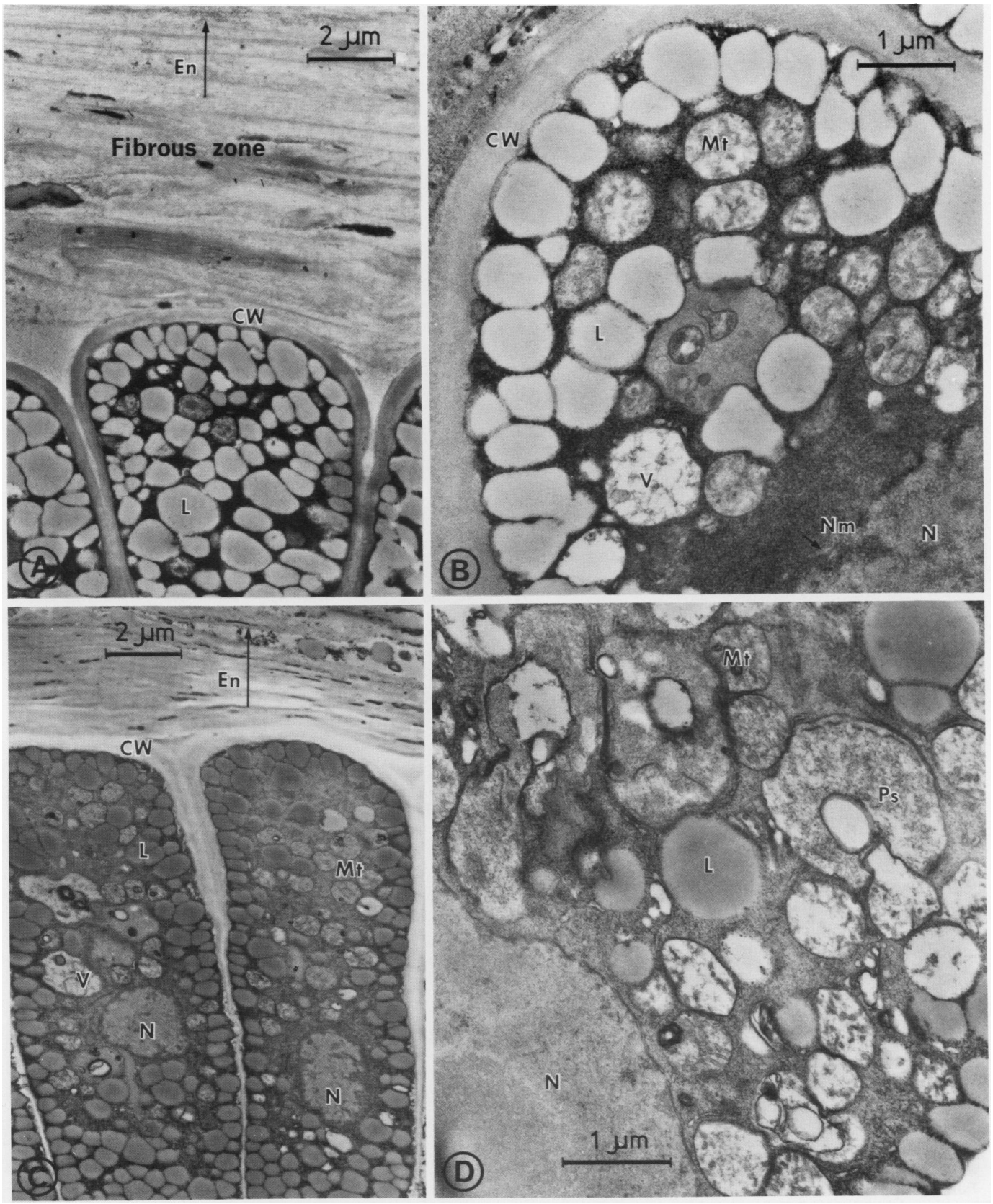

FIG. 1. Electron micrographs of scutellar epithelium cells in rice seeds. A, Dry; B, imbibed in H_2O overnight at 4°C; C and D, incubated for 12 h at 30°C. Note the presence of abundant lipid bodies (spherosomes or oleosomes) (L); thick, fibrous layer between epithelium cell wall (CW) and endosperm (En); and the undeveloped mitochondria (Mt) lacking the clear internal cristae structure. Other symbols are: nucleus (N); nuclear membrane (Nm); plastid (Ps); vacuole (V).

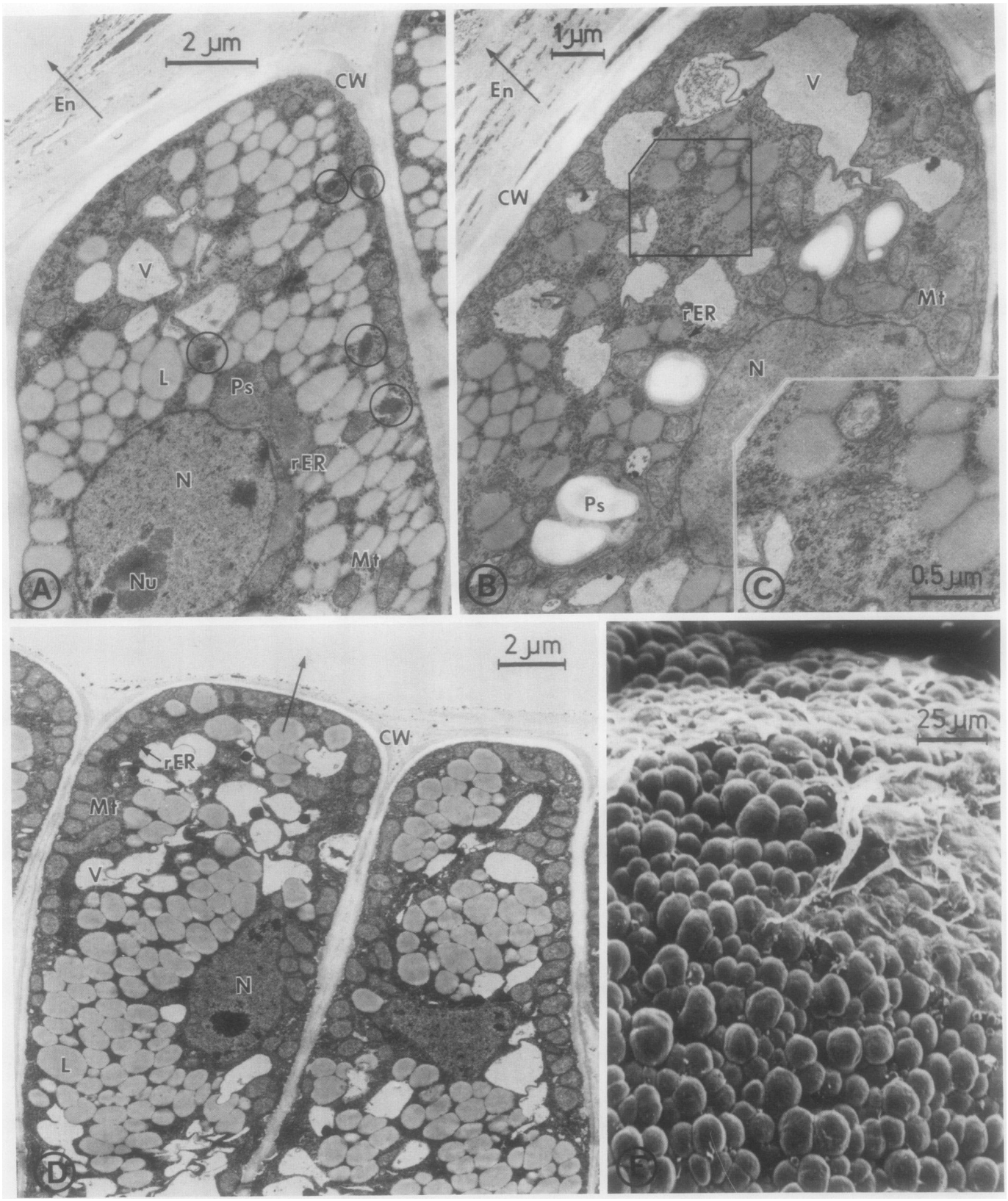

FIG. 2. Electron micrographs of ultrastructural changes in scutellar epithelium cells during germination. A, B, and C, Germinated for 2 d at 30°C; D, germinated for 8 d at 30°C; E, scanning electron micrograph of the epithelial cells at 2-d germination stage (30°C). Note the prominent development of membrane structures as well as cell organelles such as the formation of the internal cristae structures in mitochondria (Mt) and RER (rER). Glyoxysome-like particles are circled in A. Golgi apparatus as can be seen in the boxed in portion of B is enlarged in C to clearly show the vesicular structures. Upon progress of seedling growth (D), both decrease in spherosomes (L) and their inward translocation as well as the digestive breakdown of the fibrous layer are clearly seen. Scanning micrograph (E) shows the intestinal villi-like structure of the epithelial cells. Other symbols are: cell wall (CW); vacuole (V); plastid (Ps); nucleus (N); nucleolus (Nu); endosperm (En).

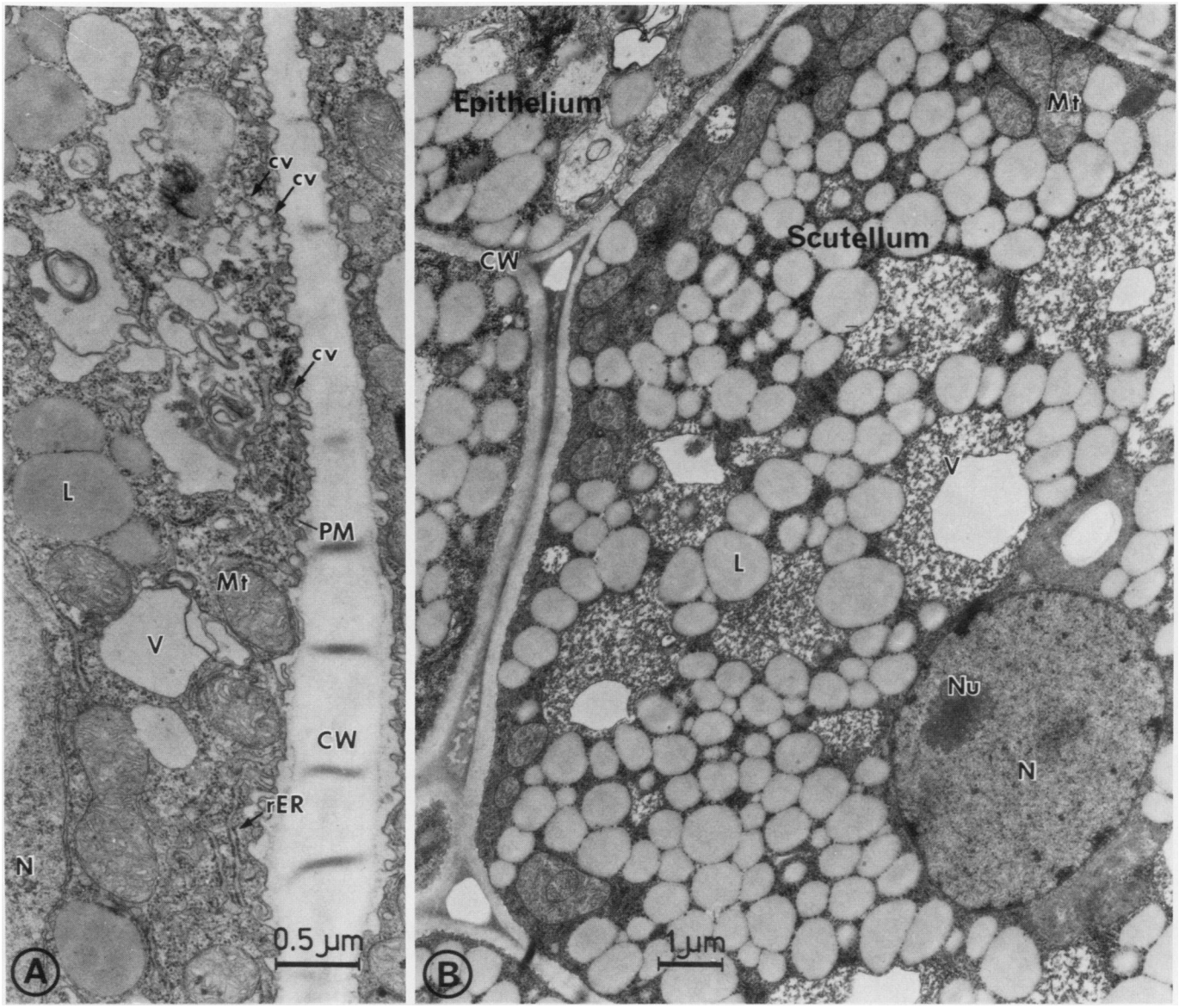

FIG. 3. Electron micrographs showing comparative membrane structures of scutellar epithelium (A) and scutellum proper (B). A and B, incubated for 4 d at 30°C. Note in A, well-developed RER (rER) and mitochondria (Mt). Note also the presence of vesicular structures (CV), presumably representing the condensing vacuoles. Folded structure of the plasmamembranes (PM) is another distinctive feature. In contrast to epithelium, development of membranous structure such as RER is much less conspicuous in scutellum, as shown in B. Other symbols are: spherosome (L); vacuole (V); nucleus (N), nucleolus (Nu).

high at an early stage (2.5 d) and declined gradually thereafter. The trend of catalase is comparable to that of fumarase, its activity being markedly high (Fig. 4C). It is currently accepted that catalase is specifically localized in the glyoxysomes (or peroxisomes) in living cells. However, activities of two other typical glyoxysomal enzymes engaged in the fat-carbohydrate conversion, *i.e.* isocitrate lyase and malate synthetase, were found to be low throughout the 1-week test period.

To examine the two different pathways of fatty acid oxidation, either by mitochondrial or glyoxysomal β-oxidation, we measured the activities of Pal-CoA dehydrogenase and Pal-CoA oxidase, respectively (17). As shown in Figure 4B, the activity of Pal-CoA dehydrogenase was clearly detectable, though its specific activity was considerably lower than that of fumarase. In contrast, Pal-CoA oxidase activity was not detectable either by spectrophotometric or by O_2-electrode assay methods. By employing the various assay conditions, so far we have been unable to detect glyoxysomal enzyme activities, except catalase activity.

DISCUSSION

The ultrastructure of cereal seeds has been studied by many investigators. However, in contrast to the several reports dealing with the fine structure of ungerminated rice and other starchy seeds (1, 2, 5, 6, 24, 27, 30, 35), relatively few studies deal with structural changes during and following germination, except those by O'Brien and associates (32, 33, 36) who have given a detailed description of the scutellum in barley and other cereals. Because the scutellar epithelium is functionally important in the production and secretion of α-amylase and other hydrolytic enzymes, crucial to the digestive breakdown of the reserve macromolecules in the endosperm tissues, specific attention has been focused on the ultrastructure of this unique cell, anatomically distinguishable from the scutellum proper even by light microscope inspection. Some of the structural features of epithelium such as the presence of spherosomes are consistent with reports by Bechtel and Pomeranz (1, 2) on ungerminated rice seeds, but results of our present

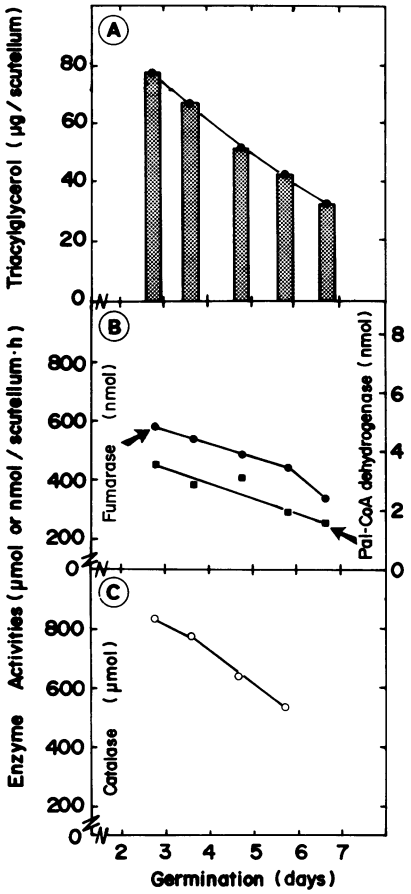

FIG. 4. Triacylglycerol content in scutellar tissues of rice seedlings (A) and changes in mitochondrial (B) and glyoxysomal (C) enzyme activities. Scutellar tissues dissected from the germinating rice seeds at the indicated incubation time were subjected to the triacylglycerol analysis by the method described in "Materials and Methods." Scutella were also ground to prepare the crude extracts, subjected to the respective enzyme assays as explained in "Materials and Methods." B, Mitochondrial enzyme—fumarase and Pal-CoA dehydrogenase. C, glyoxysomal enzyme—catalase.

investigation have demonstrated that striking structural changes occur in the epithelial cells.

We have recently reported the cell-free synthesis of the α-amylase molecule directed by the specific mRNA isolated from the scutellum of germinating rice seeds (23). Therefore, the full development of the membrane structures, *e.g.* RER and ribosomes, in the epithelium as evidenced may well indicate enhancement of the protein-synthesizing capacity in the cell. α-Amylase is a typical gylycoprotein, and mechanism(s) of the enzymic glycosylation involved in the formation of the mature enzyme molecule from the precursor form and the subsequent secretory processes (segregation and discharge) from the epithelium cells into the endosperm are of great importance for future investigations (8). In this context, therefore, it is interesting to note that there occurs a prominent development of the Golgi apparatus (stacks, vesicles, and condensing vacuoles) in the epithelium (*cf.* Fig. 2, B and C, and Fig. 3A), which is presumably required for the secretion of α-amylase.

In the present investigation, we have also attempted to examine the metabolic pathway in the epithelium relating to the fat-carbohydrate conversion. There is a serious drawback in this experimental approach; that is, epithelium, scutellum, and embryonic axis comprising the whole scutellar tissue are inevitably homogenized, and the specific enzyme activities determined are expressed as per scutellum. Therefore, the values given will be far below the actual metabolic capacity of the epithelium *per se*. It is generally believed that the glyoxylate cycle is specifically operating in seedlings, in which the transformation of fatty reserves to carbohydrate (sucrose) occurs, such as in the castor bean endosperm or peanut cotyledon (3, 7). However, there exists in fact a large amount of fat reserve in the epithelium as well as in the scutellum of the starch-bearing cereal seeds (6, 16, 24, 25), and Longo and Longo (21) reported the presence of the glyoxylate cycle in the maize seed scutellum. Earlier, we found that active synthesis of sucrose occurs in the rice scutellum (26), and it was simply assumed that the monosaccharides produced by the amylolytic breakdown of the reserve starch in the endosperm tissues are transported to the scutellum, where they are resynthesized to sucrose.

From the results of Figure 4A, it can be roughly calculated that the rate of fat consumption in the scutellar tissue is about 10 μg/d (0.5 nmol/h). Although we have found that activities ofthree glyoxysomal enzymes, *e.g.* Pal-CoA oxidase, isocitrate lyase, and malate synthetase, are low or barely detectable by various assay methods, relatively low enzyme activity is sufficient for catabolizing the measured triacylglycerol. On the other hand, two mitochondrial enzymes, *i.e.* Pal-CoA dehydrogenase and fumarase, are evidently present in the scutellar extracts. Taken altogether, we speculate that fatty acid is primarily oxidized by the mitochondrial β-oxidation coupled with the TCA cycle. Indeed, the proliferation of mitochondria is the most prominent ultrastructural feature of the epithelial cells observed (Figs. 2D and 3A). However, there remains a question whether or not the fat reserve is degraded solely by the mitochondrial pathway and subsequently converted to sucrose.

Formation and development of glyoxysomes seem to occur (*cf.* Fig. 2A), and we have found that activities of catalase, a glyoxysomal marker enzyme, are relatively high. We cannot totally exclude the presence of other glyoxysomal enzymes essential for fatty acid utilization in the epithelium, which might be detected by further refinement of assay methods. Therefore, until this important question is settled by future experiments, we reserve final judgment. The following possibilities are still open: (a) only mitochondrial, (b) only glyoxysomal β-oxidation, or (c) both are responsible for fatty acid oxidation in the rice seedling.

Note added in proof: As to the glycosylation reaction of the precursor molecule to make up the mature form of α-amylase, we have demonstrated the formation of non-glycosylated polypeptide molecule in the rice seed scutellar tissues treated with the antibiotic Tunicamycin (Miyata S, T Akazawa 1982 Plant Physiol 70: 147–153).

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