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IMMUNOHISTOCHEMICAL LOCALIZATION OF α-AMYLASE IN BARLEY ALEURONE CELLS

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SUMMARY

α-Amylase was localized in aleurone cells of barley using immunohistochemical methods. Anti-α-amylase antibody was produced by rabbits immunized with enzyme purified from malt diastase and Himalaya variety barley seeds. Immunoelectrophoresis showed that the antibodies to both antigens were immunologically similar, therefore, they were used interchangeably in the localization of α-amylase. Fluorescence of 8–10-μm sections of freeze-substituted and paraffin embedded, gibberellic acid (GA)-treated aleurone tissue incubated with rabbit anti-α-amylase IgG and rhodamine-conjugated goat-anti-rabbit IgG is localized in the cytoplasm, the nuclear region and the innermost portion of the cell wall. Cytoplasmic immunofluorescence is not associated with a specific organelle but rather is diffusely distributed. The fluorescence of the nuclear region, however, is intense and in thinner (4–5-μm) sections is associated not with the nucleoplasm but with the nuclear envelope and perinuclear region of the cytoplasm. Fluorescence of the cell wall is confined to the inner boundary of the wall corresponding to the resistant wall layer. The immunofluorescent properties of non-GA-treated cells are quantitatively different; fluorescence of these sections is low and diffuse and is particularly reduced in the nuclear region. Electron microscopy shows that GA-treatment results in the proliferation of endoplasmic reticulum (ER) in the perinuclear region of the cell. We suggest that the α-amylase localized by immunofluorescence in the perinuclear region of the cell is localized in this ER produced in response to GA treatment. Immunohistochemical localization of α-amylase in cells zonated by centrifugation also suggests that the enzyme is intimately associated with the perinuclear area.

INTRODUCTION

Aleurone cells of barley (*Hordeum vulgare L.*) synthesize and release a wide spectrum of hydrolytic enzymes in response to treatment with gibberellic acid (GA) (Yomo & Varner, 1971). The synthesis of α-amylase, ribonuclease and β-1,3-glucanase has been shown to be *de novo* (Bennett & Chrispeels, 1972; Filner & Varner, 1967). Although many of the events associated with GA-induced, *de novo* enzyme synthesis have been documented (Evins, 1971; Evins & Varner, 1971; Jones, 1969*a*, *b*), little is known of the subcellular site of enzyme synthesis or of the mechanism of its secretion.

Electron microscopy has shown that enzyme production is correlated with the proliferation of endoplasmic reticulum (ER) in barley aleurone, and autoradiography indicates that protein synthesis is associated with this ER (Jones, 1969*b*; Chen & Jones, 1974*a*, *b*). Cytological techniques have not shed light on the secretion mechanism. There have been several reports of vesicles in these cells which could participate in the secretion process, but proof that these organelles contain secreted hydrolases is

lacking (Jones, 1969*b*; Vigil & Ruddat, 1973). Cell fractionation experiments also provide equivocal evidence for the participation of vesicles in enzyme secretion from cereal aleurone tissue. In barley, less than 10% of the extractable enzyme can be pelleted from cell homogenates (Jones, 1972) although in wheat aleurone 80% of the enzyme in the tissue can be sedimented by centrifugation at forces up to 100000 g (Gibson & Paleg, 1972, 1975).

An attempt to localize α-amylase in barley aleurone by specific antibody labelling was reported by Jacobsen & Knox (1973). Using immunofluorescence these workers suggested that α-amylase synthesis is associated with the envelope of the aleurone grains, but they were unable to determine the mechanism of its secretion. Although the localization technique used by Jacobsen & Knox (1973) offered the specificity lacking in other approaches to this problem, the limited resolution of immunofluorescence with fresh sections did not allow accurate localization of the antigen.

In this paper we report on the localization of α-amylase in aleurone cells of barley using rabbit anti-amylase gamma globulin and rhodamine-conjugated sheep anti-rabbit gamma globulin in freeze-substituted, paraffin-embedded tissue.

MATERIALS AND METHODS

Tissue preparation

Seeds of barley (*Hordeum vulgare* L. cv. Himalaya, 1973 harvest) were prepared according to the procedures of Chrispeels & Varner (1967). After imbibition on sand for 3 days, half-seeds were incubated in 10 mM $CaCl_2$ and 2 mM sodium acetate buffer, pH 4·8 with or without 5 μM GA (Jones & Varner, 1967). Aleurone layers were removed from the half-seeds at the end of the desired incubation time as described by Chrispeels & Varner (1967).

Tissue ultracentrifugation

Aleurone cells were zonated by ultracentrifugation of tissue pieces (Jones, 1969*c*). Our method was modified to facilitate the centrifugation of a greater number of tissue pieces by providing a larger flat surface in the centrifuge tubes. Approximately 5 ml of epoxy resin were added dropwise into the bottom of No. 302237 cellulose nitrate tubes (Beckman-Spinco, Palo Alto, Cal., USA) and allowed to polymerize for 48 h at 40 °C. After cooling, tubes were filled with distilled water and 2–4-mm^3 aleurone pieces allowed to sink to the flat epoxy surface. Tissue was centrifuged at 100000 g for 1 h at 4 °C prior to freeze substitution.

Freeze substitution

Centrifuged and non-centrifuged aleurone layers were cut into 1–2-mm^3 pieces, quenched in partially solidified Freon-22 in a liquid N_2 bath, then dropped into capped tubes of anhydrous methanol in a dry ice-acetone bath. Substitution was at -96 °C for 10 days with 4 changes of methanol, then one change (1 day) of methanol at -30 °C, and one at 4 °C. The methanol was replaced by 50% toluene/50% methanol (v/v) for 24 h, then 100% toluene (2 changes) for 24 h. The tissue was then brought to room temperature, embedded in paraffin and stored at 4 °C until sectioned.

Antibody production

Two antigens were purified for antibody production using the methods described previously (Chen & Jones, 1974*a*): (1) α-Amylase from Himalaya barley seeds was obtained by incubating 2000 half-seeds in 5 μM GA for 36 h. Following incubation, the medium was removed and the secreted α-amylase purified. (2) Malt diastase (Nutritional Biochemicals Co., Cleveland, Ohio,

USA) was extracted with 100 mM sodium acetate buffer (pH 4·8) at 4 °C for 24 h and the extract purified as described by Chen & Jones (1974*a*) except that molecular sieve chromatography on Biogel P-60 (Bio-Rad Laboratories, Richmond, Ca, USA) was substituted for anion exchange chromatography on Cellex D (Bio-Rad Laboratories).

Rabbits were initially inoculated with purified antigen mixed with an equal volume of Freund's complete adjuvant to give a final antigen concentration in the inoculum of 3 mg ml^{-1} for Himalaya antigen, and 6 mg ml^{-1} for malt (Chen & Jones, 1974*a*). After 4–5 weeks rabbits were boosted and bled from the ear vein at approximately weekly intervals for 2 months. Immune and non-immune sera were purified (Chen & Jones, 1974*a*).

Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the methods of Fairbanks, Steck & Wallach (1971). Gels were stained for protein with 0·05 % Coomassie brilliant blue in 35 % trichloroacetic acid and diffusion destained in 7 % acetic acid. For carbohydrate analysis gels were stained with periodic acid–Schiff's reagent (Fairbanks *et al.* 1971). Stained gels were scanned using a Gilford (Gilford Inst. Labs., Oberlin, Ohio) system adapted to a Beckman DU spectrophotometer.

Immunoelectrophoresis was performed according to the procedures of Scheidegger (1955). Antigens were electrophoresed in 50 mM barbitol-acetate buffer (pH 8·6) at 4 °C for 90 min at 250 V with a current of 10 A (6 slides/run). Incubation time with antiserum varied to provide optimal precipitin formation. After washing and drying, electrophoretograms were stained with amido black (9 g amido black/1500 ml acidic methanol (methanol:glacial acetic acid:water; 45:10:45, v/v)) for 30 s and washed with 10 % acetic acid.

Immunofluorescence

Paraffin sections (8–10 or 4–5 μm) were floated on to base- and ethanol-washed glass microscope slides which had been evenly coated (by dipping) with a thin film of 2 % gelatin. The sections were allowed to expand and the gelatin dried at 40 °C for 5 min. The sections were then deparaffinized with xylene (2 × 15 min) and gradually hydrated through an ethanol series. After 2 (10-min) changes of staining buffer (phosphate-buffered saline: 0·15 M phosphate, 0·1 M NaCl, pH 7·8), sections were incubated in 1–2 mg/ml rabbit anti-amylase IgG for 30 min at room temperature in a moist chamber, washed twice (10 min each) with staining buffer then incubated in 1 mg/ml rhodamine-conjugated IgG fraction goat anti-rabbit IgG (Cappel Laboratories, Downingtown, PA) for 30 min at room temperature in a dark moist chamber. After washing twice (10 min each) with staining buffer, sections were washed with glass-distilled water and coverglasses mounted with water or 50 % glycerol. The following staining controls were routinely prepared: sections incubated with staining buffer only, rhodamine conjugated goat anti-rabbit IgG only, rabbit anti-α-amylase IgG immune serum only, and non-immune rabbit IgG plus rhodamine-conjugated goat anti-rabbit IgG.

Light microscopy

Sections were examined and photographed (Kodak Tri-X film) with a Leitz Orthoplan fluorescence microscope with 100 × oil immersion objective and epi-illumination from an Osram 200-W, ultra-high-pressure mercury vapour lamp with 2-mm BG38, BG36 and S546 excitation filters and K610 and K580 barrier filters. Each section was also photographed with bright-field and phase-contrast oil immersion optics. After fluorescence microscopy some sections were stained for 1 min with 0·5 % toluidine blue-O in 0·1 M phosphate buffer, pH 6·8, washed with water and photographed with bright-field optics.

Electron microscopy

Aleurone tissue was fixed and embedded according to the procedures of Jones & Price (1970). Sections were stained with saturated aqueous uranyl acetate followed by Reynolds' lead citrate and examined with a Zeiss EM 9A.

RESULTS

Antibody production

Two antigens were used to induce production of anti-α-amylase antibody. Because of the availability of commerical malt diastase our effort was concentrated on purification of antigen from this source. The polyacrylamide gel electrophoresis pattern of the purified malt α-amylase is shown in Fig. 1. Only 1 band of absorbance shows after staining the gel with either Coomassie brilliant blue or periodic acid–Schiff's reagent. Immunoelectrophoresis of the anti-malt α-amylase IgG with malt α-amylase

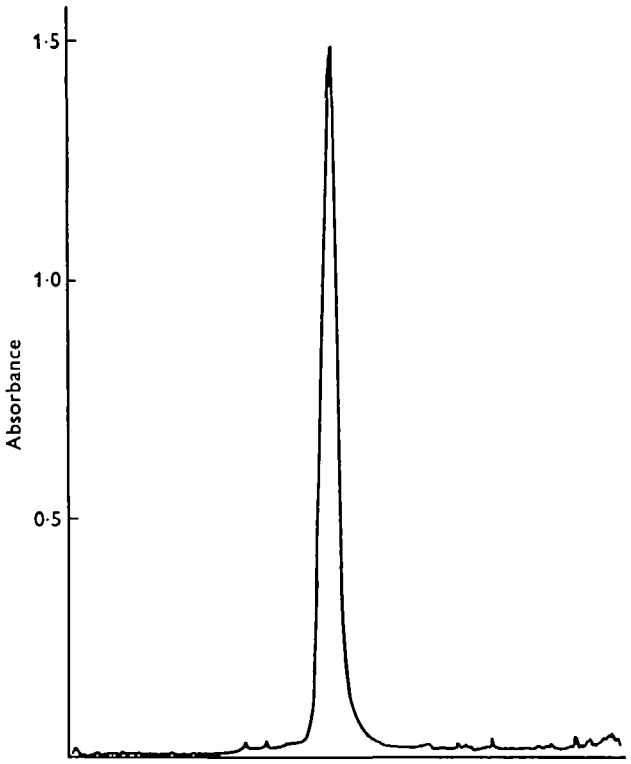

Fig. 1. Densitometric scan of SDS-polyacrylamide gel electrophoretogram of α-amylase antigen purified from malt diastase. Gel stained with Coomassie brilliant blue.

and purified α-amylase secreted from Himalaya barley aleurone is shown in Fig. 2A. One major precipitin band is observed for each antigen and the positions of the bands indicate that the antigens are immunologically similar. A minor precipitation band was always observed on immunoelectrophoresis plates of malt antigen, suggesting that it consisted of more than one immunologically distinct protein (Fig. 2A). Non-immune serum did not form precipitin arcs with these antigens. Immunodiffusion confirmed that the malt and secreted α-amylases reacted identically with anti-malt α-amylase antibody.

Immunohistochemical localization of α-amylase

Under the microscopy conditions described for rhodamine fluorescence, autofluorescence of unstained sections of freeze-substituted aleurone tissue is negligible (Fig. 3B). In cells zonated by centrifugation autofluorescence is visible only in the aleurone grain (protein body) region of the cell. Unstained and non-immune IgG controls were included in all experiments. The fluorescence shown in Fig. 3B is representative of all the staining controls.

Sections were stained with rhodamine following incubation in antibody to either Himalaya (Figs. 2C, E, 3I) or malt α-amylase (Figs. 2G, 3B, D, G, 4B, D, F, H). Fluorescence from GA-treated tissue sectioned at 8–10 μm and including only aleurone grains is confined to the cytoplasm surrounding the grains (Fig. 2C). When transverse sections include the nucleus (Fig. 2D), a zone of intense fluorescence corresponding to the nuclear region is observed (Fig. 2E). In sections of plasmolysed tissue the innermost boundary of the cell wall is brightly fluorescent (Figs. 2E, 4H).

Fluorescence of sections from non-GA-treated aleurone after incubation with anti-α-amylase IgG and the rhodamine conjugate is shown in Fig. 2G. The bright fluorescence seen in GA-treated tissue is absent, although both nuclei and aleurone grains are clearly visible in the bright-field micrographs of these cells (Fig. 2F).

To increase the resolution of fluorescence at the light-microscope level, aleurone cells were zonated by ultracentrifugation prior to freeze substitution (Fig. 3). In centrifuged cells, aleurone grains are localized centrifugally and spherosomes (lipid bodies) centripetally (Fig. 3A). Nuclei, ER, mitochondria and other small organelles sediment in a zone between aleurone grains and spherosomes (Jones, 1969*c*). Immunochemical localization of α-amylase in GA-treated zonated cells is shown in Figs. 3D, G, 4B. In sections cut parallel to the plane of centrifugation, non-fluorescent aleurone grains are seen at the centrifugal pole while spherosomes are observed at the centripetal pole. Nuclei, which are intensely fluorescent, are located toward the centripetal end of the cell as seen in bright-field micrographs (Fig. 3C, E), and confirmed by staining with toluidine blue (Fig. 3F). It is apparent (Fig. 3D, G) that intense fluorescence is indeed confined to the nuclear region of the cell. Thus, although small organelles sediment to the zone of cytoplasm surrounding the nucleus, immunofluorescence of these structures is considerably less intense than that of the nucleus (Figs. 3D, G, 4B). When GA-treated tissue is cut at right angles to the plane of centrifugal force, similar fluorescence of the nuclear region is observed (Fig. 3I).

To determine whether fluorescence associated with the nuclear region of the cell represented the nucleoplasm or the nuclear membrane and perinuclear cytoplasm, thinner (4–5 μm) serial sections of the tissue were prepared. Sections of uncentrifuged, GA-treated tissue which include median sections of the nucleus clearly show that fluorescence is associated with the periphery of the nucleus, not the nucleoplasm (Fig. 4D, F, H). The broad ring of fluorescence surrounding the nucleus suggests that antigenicity is not confined to the nuclear membrane alone but is also associated with the cytoplasm adjacent to the nuclear membrane.

Electron microscopy

The ultrastructure of nuclei and perinuclear regions of GA- and non-GA-treated cells is shown in Fig. 5. Non-GA-treated cells have few ER lamellae adjacent to the nuclear membrane while GA-treated cells are characterized by the presence of stacked ER lamellae in the perinuclear region. The formation of ER in the perinuclear region of aleurone cells is a GA-dependent response. Thus, with increasing duration of GA treatment up to 24 h there is an increase in the development of this membrane system (Fig. 5).

DISCUSSION

The specificity of immunohistochemical enzyme localization is primarily dependent upon the purity of the immunizing antigen. In this study both Himalaya and malt α-amylase were purified until only 1 band of protein could be detected by SDS-polyacrylamide-gel electrophoresis. Since antibodies to the two antigens are immunologically similar (Fig. 2A), they were used interchangeably in this work. The presence of a minor precipitin arc in immunoelectrophoretograms of malt α-amylase (Fig. 2A) suggests that at least 2 groups of immunologically distinct α-amylases are present in this preparation. Jacobsen & Knox (1973) also report the occurrence of 2 different proteins in their α-amylase preparation from GA-treated barley aleurone layers. The absence of a distinct second precipitin arc in immunoelectrophoretograms of our Himalaya preparation probably results from a lower concentration of this antigen in the purified IgG fraction applied to the agar gel.

Our initial immunohistochemical experiments were conducted with fluorescein isothiocyanate-conjugated anti-rabbit IgG; however, green autofluorescence of the tissue made this probe less than ideal. Native red fluorescence after excitation of aleurone tissue with green light is negligible, thus rhodamine was the fluorescent conjugate of choice.

In GA-treated barley aleurone, α-amylase is localized by immunofluorescence in the cytoplasm, the region of the cell around the nucleus, and the inner region of the cell wall (Figs. 2–4). In contrast to the observations of Jacobsen & Knox (1973) we could find no fluorescence associated with the aleurone grain membrane nor with the protein body matrix (Figs. 2, 3). That the enzyme which is diffusely distributed throughout the cytoplasm is not associated with a specific organelle is also shown by zonation of cells by ultracentrifugation. Thus, after centrifugation, the cytoplasm surrounding the protein bodies, nuclei and spherosomes still retains diffuse fluorescence (Figs. 3D, G, I, 4B). If α-amylase were located on the aleurone grain membrane as suggested by Jacobsen & Knox (1973), then intense fluorescence in the centrifugal pole of the cell would be expected.

Sections of uncentrifuged, GA-treated tissue which included nuclei show intense fluorescence in the nuclear region. Median sections of the nucleus indicate that fluorescence is associated with the perinuclear region rather than the nucleoplasm itself (Fig. 4D, F, H). Further evidence that α-amylase is concentrated in the perinuclear

region is shown by immunohistochemical localization in centrifuged cells (Figs. 3D, G, I).

Although light-microscopy does not provide sufficient resolution to localize α-amylase at the organelle level, electron microscopy suggests that the enzyme is associated with the perinuclear ER and nuclear membrane. Autoradiography has shown that protein synthesis occurs on the rough ER of GA-treated aleurone cells (Chen & Jones, 1974*b*). Furthermore, the formation of ER in aleurone tissue is a characteristic response to GA (Evins & Varner, 1971; Jones, 1969*a*, *c*) and much of this new ER is associated with the nuclear envelope (Fig. 5). Therefore it seems reasonable to conclude that α-amylase is located in this endomembrane system.

Since numerous ER lamellae are found in the cytoplasm distant from the nucleus, such specific localization of enzyme in the perinuclear region suggests that the synthesis of α-amylase is obligately dependent on the formation of a new endomembrane system from the nuclear envelope. Based on their studies of the activities of phosphorylcholine cytidyl transferase and phosphorylcholine-glyceride transferase in GA-treated aleurone tissue, Johnson & Kende (1971) have suggested that new ER formation is also a prerequisite for hydrolase synthesis.

The mechanism of α-amylase release from aleurone cells remains to be resolved. The membrane flow model proposed by Morré, Keenan & Huang (1974) envisages the conversion of ER to secretory organelles via a series of transition elements. Our immunohistochemical observations do not support the involvement of such an organelle system in the aleurone cell. Since ultracentrifugation provides distinct zonation of small transitionary elements and dictyosomes in the central region of the aleurone cell, the existence of a high local concentration of α-amylase in this region of the cell would be apparent. It is clear from Figs. 3D, G, I, and 4 that α-amylase-containing organelles do not occur in GA-treated aleurone cells of barley. Gibson & Paleg (1973, 1975) have reported the isolation of lysosomal particles from wheat aleurone cells which may be involved in α-amylase secretion. We suggest that the lysosomal particles isolated by Gibson & Paleg may represent fragments of the α-amylase-containing perinuclear ER system.

The immunofluorescence properties of the aleurone cell wall can be clearly visualized in GA-treated plasmolysed tissue (Figs. 2E, 4H). That part of the cell wall adjacent to the protoplast which is fluorescent corresponds to the layer described as the resistant wall (Taiz & Jones, 1973). Varner & Mense (1972) have suggested that the aleurone cell wall acts as a barrier to α-amylase movement. Localization of α-amylase in the resistant wall lends support to this hypothesis.

The immunohistochemical staining of non-GA-treated aleurone tissue is quantitatively different from that of the GA-treated tissue (Fig. 2G). A low level of α-amylase is clearly present in the cytoplasm of these cells confirming the observation of Jacobsen & Knox (1973) that water control cells contain a low level of α-amylase which is antigenically identical to that found in GA-treated tissue. The absence of bright perinuclear fluorescence in non-GA-treated cells also supports the hypothesis that synthesis of α-amylase is dependent upon the formation of new ER from the nuclear envelope.

The evidence presented above supports the hypothesis that release of α-amylase from the aleurone cell occurs without the participation of secretory organelles. We suggest that α-amylase is synthesized on new ER proliferated from the nuclear envelope, released from the endomembrane system into the cytoplasm, and passes to the exterior of the cell by a mechanism analogous to the facilitated diffusion of small molecules. The transport of α-amylase by such a mechanism into zymogen granules of the rat pancreas has been described (Liebow & Rothman, 1972). After release from the protoplast, diffusion of α-amylase through the cell wall may be modified by the resistant wall.

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Fig. 2. A, immunoelectrophoretogram of Himalaya (upper) and malt (lower) antigens against malt anti-α-amylase IgG fraction. The minor precipitin arc of malt antigen can be seen above the main arc. B-E, bright-field (B, D) and corresponding immunofluorescence (C, E) photomicrographs of 8-10-μm sections of GA-treated tissue incubated with Himalaya antibody. F, G, bright-field (F) and immunofluorescence (G) photomicrographs of non-GA-treated tissue stained with malt antibody. *ag*, aleurone grain; *c*, cell wall; *n*, nucleus; *rw*, resistant wall.

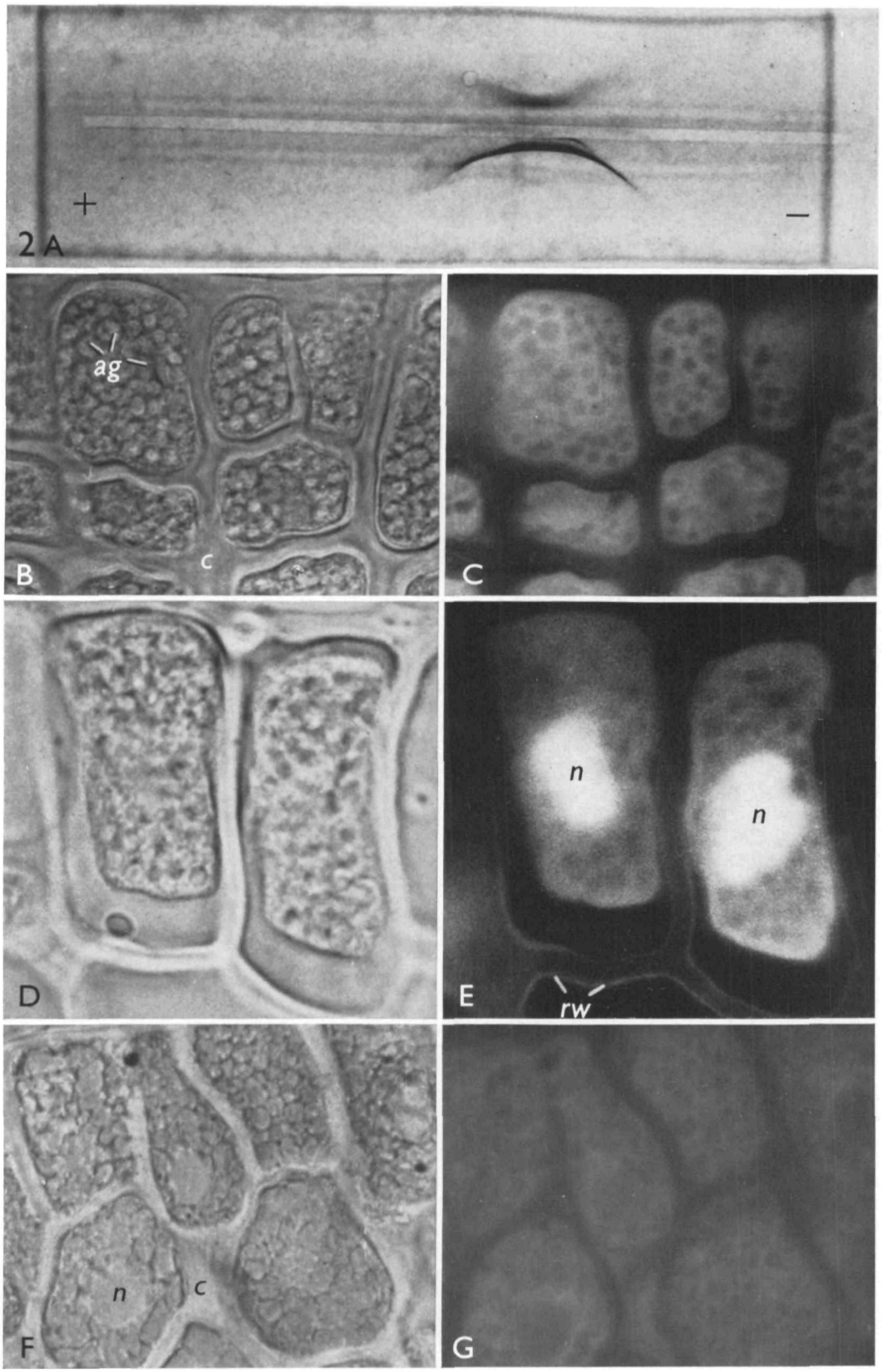
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Fig. 3. Photomicrographs of 8–10-μm sections of centrifuged aleurone tissue. A, B, non-GA-treated, unstained tissue viewed under bright-field (A) and fluorescence (B) optics showing low level of autofluorescence. C–I, GA-treated tissue: C, D, section incubated with malt antibody; C, bright field; D, immunofluorescence. E–G, section incubated with malt antibody: E, bright field; F, toluidine blue-stained, bright field; G, immunofluorescence; H, I, section incubated with Himalaya antibody: H, bright field; I, immunofluorescence. *ag*, aleurone grain; *c*, cell wall; *n*, nucleus; *s*, spherosomes.

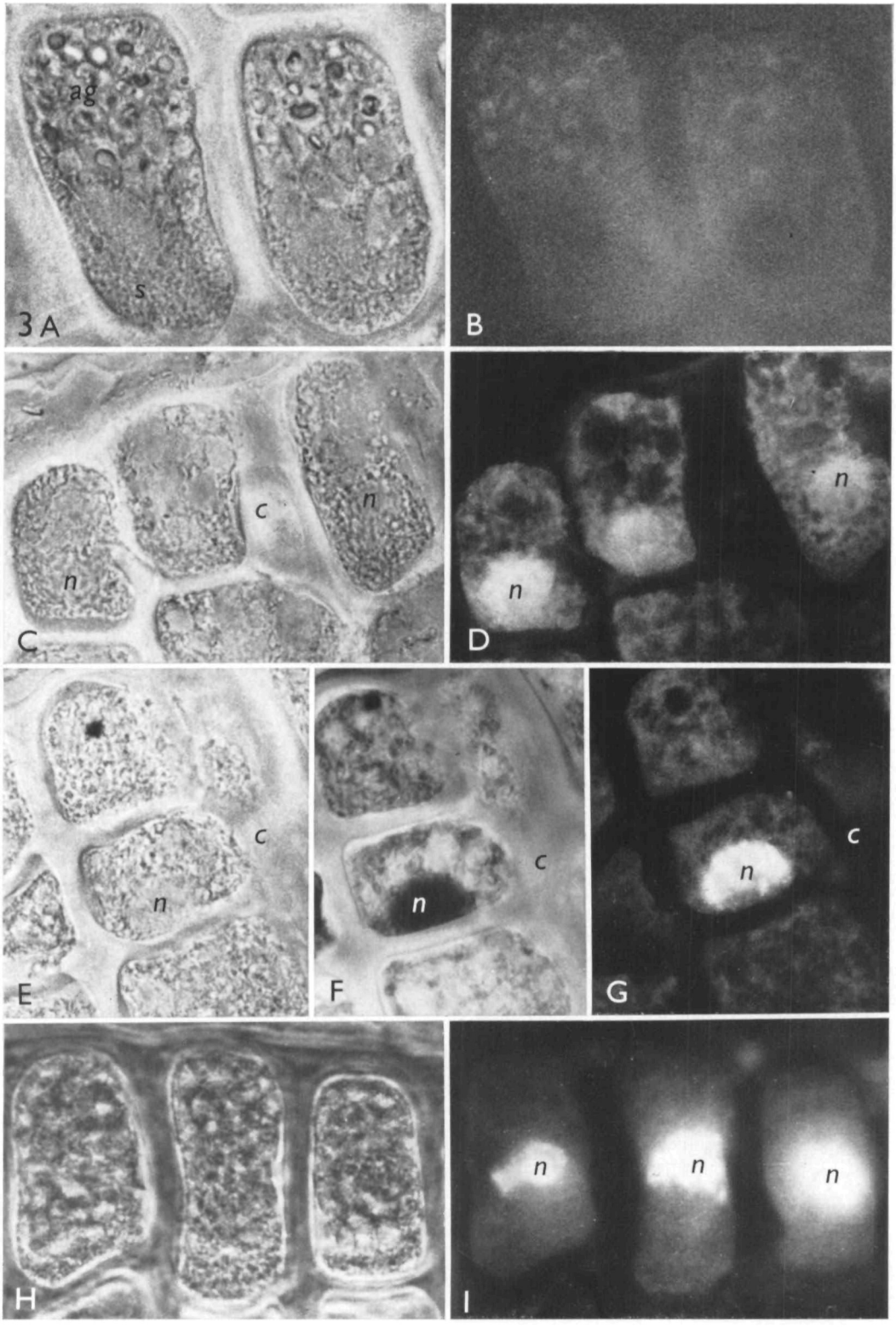

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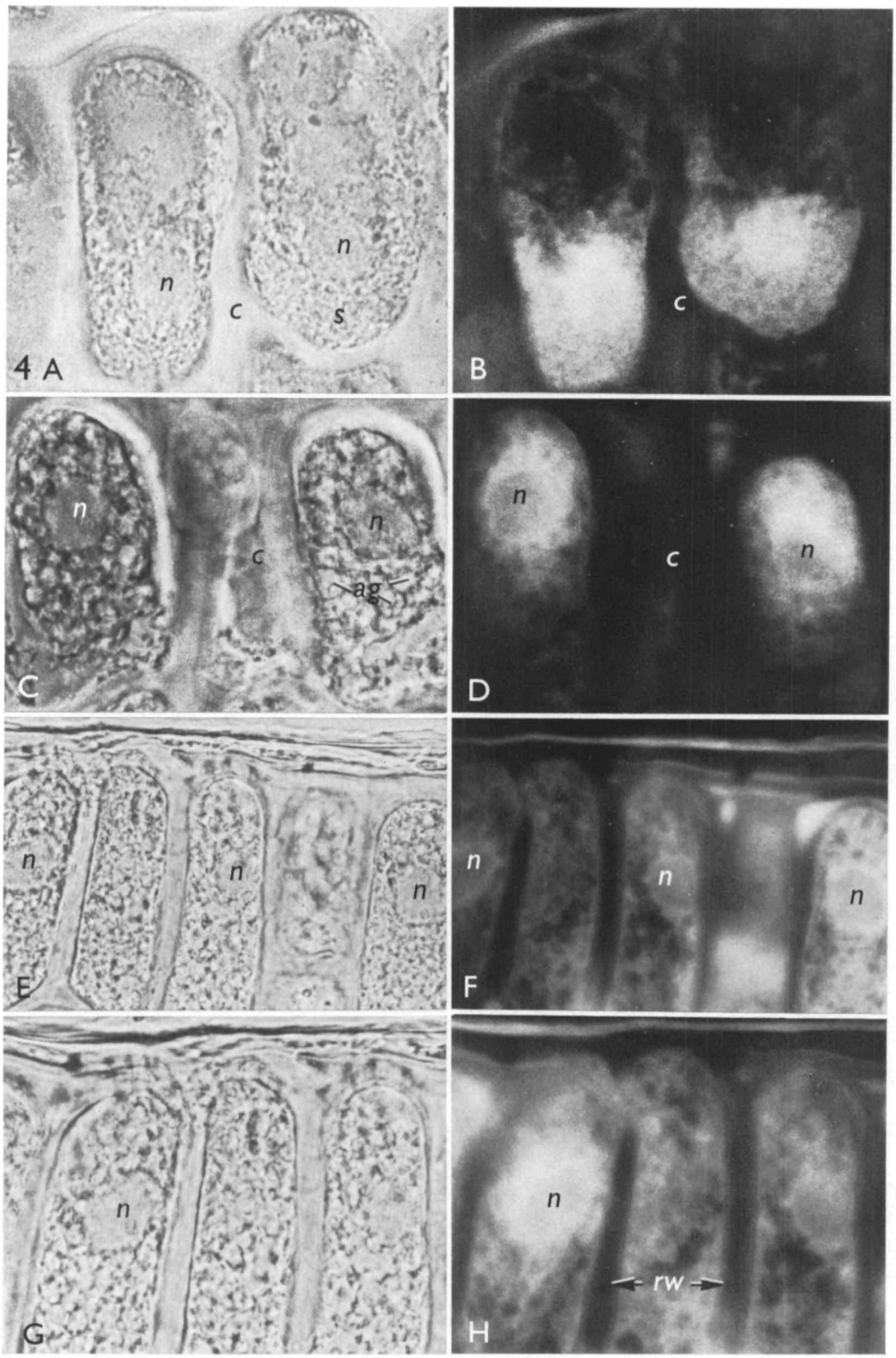
Fig. 4. Photomicrographs of sections of GA-treated tissue incubated in malt antibody. A, B, bright field (A) and immunofluorescence (B) of centrifuged tissue sectioned at 8–10 μm. C–H, bright-field (C, E, G) and corresponding immunofluorescence (D, F, H) micrographs of non-centrifuged tissue sectioned at 4–5 μm. *ag*, aleurone grain; *c*, cell wall; *n*, nucleus; *rw*, resistant wall; *s*, spherosomes.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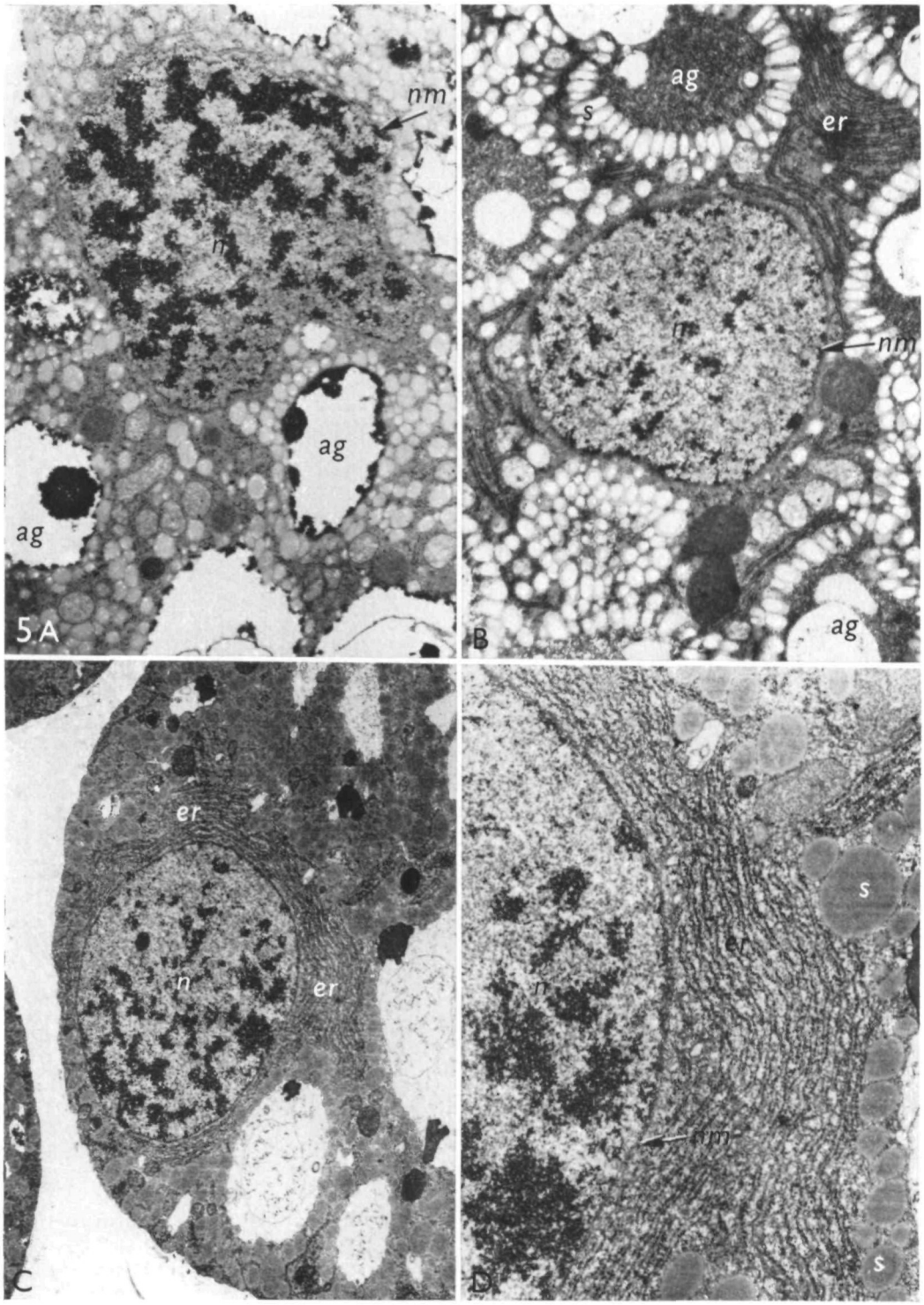

Fig. 5. Electron micrographs of non-GA- (A), 10-h GA- (B), and 24-h GA-treated (C, D) aleurone tissue. A, ×7500; B, ×8000; C, ×6750; D, ×13000. *ag*, aleurone grain; *er*, endoplasmic reticulum; *n*, nucleus; *nm*, nuclear membrane; *s*, spherosomes.