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Intracellular Localization of Lipoxygenases-1 and -2 in Germinating Soybean Seeds by Indirect Labeling with Protein A-Colloidal Gold Complexes

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ABSTRACT

Soybean lipoxygenases-1 and -2 were localized intracellularly in seeds at various stages of germination by indirect labeling of cryosections with protein A-colloidal gold complexes. Two sizes of gold particles (Au^5 and Au^{16}) were used in single- and double-labeling experiments. In primary leaves, lipoxygenases are demonstrated to occur in vacuolating parenchyma cells but not in massive, nondifferentiated cells. In cotyledons, both isoenzymes are localized in the cytoplasm of storage parenchyma cells and in an aberrant type of protein bodies, occurring in hypodermis and vascular bundle sheath cells. No association has been found with either protein bodies in storage parenchyma cells or lipid bodies, mitochondria, and other organelles in any type of cell. The possible significance of lipoxygenase in the metabolism of storage lipids and its possible function as a regulatory enzyme are discussed on the basis of the random distribution throughout the cytoplasm of storage parenchyma cells and the course of biochemical processes during seed germination.

Lipoxygenase (EC 1.13.11.12) oxidizes unsaturated fatty acids containing a 1,4-*cis*,*cis*-pentadiene system to conjugated hydroperoxy acids. Seeds of soybean (*Glycine max* [L.] Merr.) contain significant amounts of the isoenzymes lipoxygenase-1 and -2 (respectively, 1.4 and 2.8 mg/g dry weight) (16). Linoleic acid and structurally related fatty acids are substrates for both lipoxygenase-1 and lipoxygenase-2. Besides, lipoxygenase-2 is able to oxidize methyl linoleate, and mono- and trilinolein (1, 9, 13). With 1.8 mM linoleic acid as substrate in a standard polarographic assay, lipoxygenase-1 shows maximal activity at pH 9.0 and lipoxygenase-2 at pH 6.6. The activity of lipoxygenase-2 at pH 9.0 increases if the substrate concentration is lowered or Ca^{2+} is added (17, 25). Product-specificity of the reaction with lipoxygenase-2 also is higher at pH 9.0 (21) from which we suggest a pH 9.0 optimum for both isoenzymes *in vivo*. During the first 2 d of seed germination, lipoxygenase activity is maximal but the precise physiological function of the enzyme is not clear up to this time (24). It is unlikely that lipoxygenase plays a role as metabolic enzyme since mobilization of storage fat starts only after 3 d of germination. To gain a better insight into the physiological role of lipoxygenases we decided to localize the isoenzymes in tissues of soybean seeds during germination. It appears from fractionation studies with soybean seeds that lipoxygenases are soluble enzymes, but it is still an open question if lipoxygenases are localized in fragile cell organelles or in the cytosol. Therefore, we introduced the application of immunocytochemical techniques for the localization of lipoxygenases-1 and -2. Immunofluorescence labeling of paraffin-embedded sections of germinating seeds shows that both lipoxygenases-1 and -2 are predominantly localized in the cotyledonary storage tissues (24). Lipoxygenase-1 and, to a greater extent, lipoxygenase-2 can also be demonstrated to occur in protein body-like organelles in vascular bundle sheath parenchyma and abaxial hypodermis of cotyledons, after 2 or 3 d of germination (24). In leaves, lipoxygenases (especially type-2) can be shown after 1 d of germination (24). If lipoxygenases are localized in cell organelles with a distinct metabolic function, a physiological function of the enzymes can be postulated. Therefore, we examined the intracellular localization of lipoxygenases in tissues of germinating soybean seeds. We used an indirect labeling procedure for immunoelectron microscopy with protein A-collodial gold complexes. Preservation of antigenicity and attainability of lipoxygenases in sections was obtained by cryosectioning.

MATERIALS AND METHODS

Plant Material. Seeds of soybean (*Glycine max* [L.] Merr. var. Williams) and of pea (*Pisum sativum* L. var. 'Bliss Abundance' and 'High Germination') were externally sterilized in 70% ethanol for 10 s, rinsed with running tap water for 10 min, and soaked for 50 min in oxygen-flushed bi-distilled H_2O. Seeds were then layered on wet filter paper on cotton wool in a plastic box covered with glass. The box was placed in the dark at 26°C. Day 1, 2, and 3 correspond to an incubation time, respectively, of 25, 50, and 74 h.

Tissue Processing. Tissues of germinated seeds of soybean and pea were cut with a razor blade into approximately 1 mm^3 blocks in fresh fixative at 4°C. As fixative 1% (w/v) glutaraldehyde and 1% (w/v) paraformaldehyde in PBS (0.05 M Na-phosphate buffer [pH 7.4] containing 0.09 M NaCl) was used. The tissue blocks were fixed for 20 h at 4°C. Fixed blocks were rinsed twice in PBS and twice in 0.05 M Tris/HCl buffer (pH 7.2). The tissue blocks were then soaked in 1.2 M sucrose in 0.05 M Tris/HCl buffer (pH 7.2) for at least 24 h at 4°C. Subsequently, they were permeated with 10% (w/v) gelatin in 0.05 M Tris/HCl buffer (pH 7.2) containing 1.2 M sucrose for 30 min at 37°C. Chilled tissue blocks were placed on specimen holders, frozen in melting nitrogen and stored in liquid nitrogen.

Cryosectioning. Frozen tissue blocks were cryosectioned according to the method of Tokuyasu (19) but with the following modifications. Sections of 0.07 to 0.10 μm were cut with a dry

glass knife in a Reichert OMU-4/FC-4 cryoultramicrotome at a temperature between −60 and −80°C. Sections were picked up with a droplet of 2.3 M sucrose in PBS in a small loop of platinum wire and transferred to nickel grids, which had been covered with a carbon-coated parlodion film. The grids were placed upside down on 1% (w/v) agarose in distilled H_2O to remove the sucrose.

For light microscopy, semithin sections (0.5 μm) were prepared under the same conditions and transferred to degreased microscope cover glasses coated with 1.5% (w/v) gelatin in 0.05% (w/v) $KCr(SO_4)_2$.

Enzyme Purification and Antibody Production. Soybean lipoxygenases-1 and -2 were isolated according to Finazzi-Agrò *et al.* (5) and Galpin *et al.* (7). The two isoenzymes were homogeneous on 9% (w/v) PAGE (22). Lipoxygenase-2 was separated from traces of lipoxygenase-1 by affinity-purification over a column with lipoxygenase-1 antibodies coupled to Sepharose 4B (Pharmacia Fine Chemicals) (22). Rabbits were immunized by five monthly intradermal injections of approximately 500 μg lipoxygenase in an emulsion of 0.15 M NaCl with an equal volume of Freund's complete adjuvant (Miles Laboratories Inc.). Ten d after the last booster injection, the rabbits were bled by heart puncture. Sera were stored at −40°C.

Preparation of Specific Antibodies. Immunoglobulin G (IgG) fractions of antisera were isolated by precipitation of the sera with 0.15 M caprylic acid according to Steinbuch *et al.* (18). The 10,000 *g* supernatant was dialyzed against PBS at 4°C.

Anti-lipoxygenase-1 IgG was chromatographed over a column with lipoxygenase-1 coupled to Sepharose 4B as described previously (23). Affinity-purified anti-lipoxygenase-1 IgG was then mixed with lipoxygenase-2 coupled to Sepharose 4B and gently shaken for 60 h at 4°C to remove the IgG which possibly cross-reacts with lipoxygenase-2 in immunocytochemical procedures.

Anti-lipoxygenase-2 IgG was first affinity-chromatographed over a lipoxygenase-2/Sepharose 4B column and then gently mixed with lipoxygenase-1/Sepharose 4B for 60 h at 4°C to remoce the IgG cross-reacting with lipoxygenase-1. Unadsorbed specific antibodies of the second affinity purification procedure were collected and used for immunocytochemistry. Affinity-purified antibodies were analyzed by Ouchterlony double-diffusion tests and by the enzyme-linked immunosorbent assay (ELISA) as described previously (23).

Immunolabeling Procedure. All immunolabeling procedures were performed at room temperature. In control experiments, anti-lipoxygenase IgG was replaced by either nonimmune serum or by unadsorbed IgG fractions from the lipoxygenase/Sepharose 4B immunoadsorbent columns.

Light Microscopy. Semithin cryosections (0.5 μm) on coated cover glasses were washed successively in PBS, PBS containing 0.05% (w/v) gelatin, and PBS containing both 0.3% (w/v) gelatin and 0.5% (w/v) BSA (PBS/gelatin/BSA solution). The sections were incubated in 25 μl of anti-lipoxygenase-1 IgG in PBS (40 μg/ml) or anti-lipoxygenase-2 IgG in PBS (100 μg/ml) for 30 min. After several washings with PBS/gelatin/BSA solution, the sections were incubated in sheep anti-rabbit IgG labeled with fluorescein isothyocyanate (FITC; Institut Pasteur, Paris), diluted 1:100 in PBS/gelatin/BSA solution. The sections were washed with PBS/gelatin/BSA solution and with PBS and mounted on degreased microscope slides in 10% (v/v) glycerol in PBS (pH 7.8) containing *o*-phenylenediamine (OPD, 1 mg/ml) to reduce fading of FITC (12). They were examined with a Zeiss microscope with fluorescence equipment and with a Zeiss photomicroscope III equipped with Nomarski optics and phase-contrast optics.

Electron Microscopy. Colloidal gold particles with an average diameter of 5 nm (Au^5) were obtained by reduction of tetrachloroauric acid with white phosphorus (4, 11). Larger particles with an average diameter of 16 nm (Au^{16}) were prepared by reducing tetrachloroauric acid with sodium citrate (6). Gold particles were adsorbed with staphylococcal protein A (Sigma) according to Romano and Romano (15). Before use, the protein A-colloidal gold complexes were diluted in PBS/gelatin/BSA solution to minimize nonspecific adhesion of the gold particles to the section surface. The use of two sizes of gold particles for double-labeling experiments has been described by Geuze *et al.* (8).

Ultrathin cryosections (0.07–0.10 μm) were washed for 10 min in PBS containing 0.05 M glycine followed by incubations for 10 min in PBS containing 2% (w/v) gelatin, and then in PBS/gelatin/BSA solution. Incubation with anti-lipoxygenase-1 IgG (40 μg/ml) or anti-lipoxygenase-2 IgG (100 μg/ml) lasted 60 min. The sections were then washed three times with PBS/gelatin/BSA solution for 1 min. Protein A-gold complexes were diluted just before use until the color was faintly pink. After an incubation time of 60 min, the sections were washed three times for 10 min with PBS/gelatin/BSA solution. Before staining, the sections were washed three times for 1 min in PBS, for 5 min in PBS containing 0.5% (w/v) glutaraldehyde, and three times for 5 min in bi-distilled H_2O. The sections were positively stained in 2% (w/v) neutralized uranylacetate (pH 7.4) and in 2% (w/v) acid uranylacetate (pH 4.0) for 2 min each (19). They were embedded in 1.4% methylcellulose (Fluka, type Methocel MC 25) containing 0.02% (w/v) acid uranylacetate for 1 min at 4°C and dried at room temperature. The sections were examined in a Philips EM 301 electron microscope. Stereo electron micrographs were obtained with a Jeol EM 200C microscope.

RESULTS

Cryosectioning. Preservation of the antigenicity and the ultrastructure in ultrathin cryosections of tissues of cotyledons and leaves was good. At the EM level, it was possible to identify nucleus, nucleolus, protein bodies, vacuoles, lipid bodies, proplastids with starch and phytoferritin, etioplasts, mitochondria, and ER. After 7 d of germination, cryosectioning and preservation of the ultrastructure of cotyledonary tissues was difficult because of the vacuolated cells. From day 1 to day 7 the results of immunoelectron microscopy and immunofluorescence microscopy on cryosections agreed with those obtained by immunofluorescence microscopy on paraffin sections (24).

Specificity of Anti-lipoxygenase-1 IgG. To check the specificity of affinity-purified anti-lipoxygenase-1 IgG, cryosections of cotyledonary storage parenchyma of two varieties of green pea, known to contain exclusively type-2 lipoxygenases (3, 23), were used. Gold-labeling, obtained after incubation of cryosections of pea parenchyma with anti-lipoxygenase-2 IgG, were identical with the results presented for soybean (Fig. 1A). The use of anti-lipoxygenase-1 IgG caused no labeling of parenchyma of pea seeds, showing the specificity of anti-lipoxygenase-1 IgG in immunocytochemical techniques.

Labeling with Protein A-Gold Complexes. Both Au^5 and Au^{16} particles were used in single-labeling experiments since the Au^{16} label could readily be detected at lower magnifications, while Au^5 labeling was more sensitive (8). Stereo electron micrographs, not shown here, proved that Au^{16} particles only caused a very superficial labeling. In double-labeling experiments (antibody-1/protein A-Au^5/antibody-2/protein A-Au^{16}) anti-lipoxygenase-1 IgG was used as the first immunolabel. Figure 1B shows a control experiment in which twice the same anti-lipoxygenase IgG was used and protein A-Au^{16} as second label. Au^{16} is almost absent, demonstrating that there is no interference between the various steps of the procedure.

Storage Parenchyma Cells of Cotyledons. Cotyledons of soybean comprise an epidermis, a distinct abaxial hypodermis, abaxial nearly isodiametric storage parenchyma, and adaxial elongated storage parenchyma. Vascular bundles, surrounded by

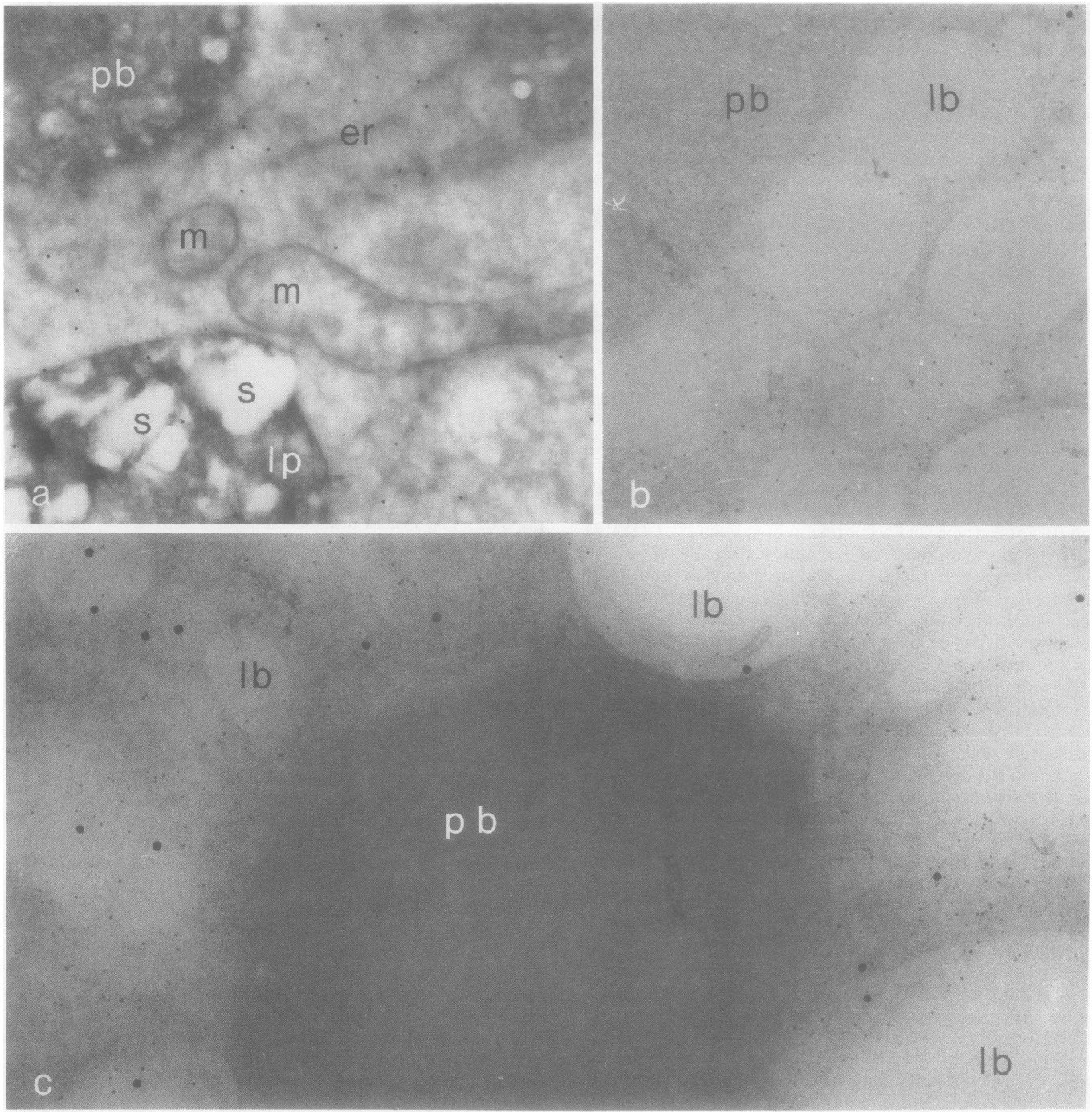

FIG. 1. Electron micrographs of storage parenchyma cells of pea and soybean cotyledons. anti-1, section incubated with anti-lipoxygenase-1 IgG; anti-2, section incubated with anti-lipoxygenase-2 IgG; Au^5, section labeled with protein A-Au^5; Au^{16}, section labeled with protein A-Au^{16}. A, Pea cotyledon after 26 h of germination with label in the cytosol (cyt), but no label in mitochondria (m), endoplasmic reticulum (er), or leucoplasts (lp), (anti-2/Au^{16}); B, 26-h-old soybean cotyledon after a control experiment with twice anti-1 as immunolabel (anti-1/Au^5, anti-1/Au^{16}); C, protein body (pb) surrounded by lipid bodies (lp) in a 75-h-old soybean cotyledon (anti-1/Au^5, anti-2/Au^{16}).

a parenchymatous bundle sheath, are situated along the boundary between adaxial and abaxial parenchyma.

Storage parenchyma cells of soybean contain numerous protein and lipid bodies. In early stages of germination, protein bodies are spherical with a diameter of 9 to 14 μm (Fig. 2D), but during germination they become more variable in shape and size and often fuse into a single entity (Fig. 2, E and F). Lipid bodies with diameters between 0.3 and 1.4 μm fill the cytoplasm between the protein bodies in early stages of germination (Figs. 1B and 3A). In 3-d-old cotyledons, a few proplastids containing starch grains can be discerned, as shown in Figure 3C. After 3 d of germination, the lipid bodies start to disappear and their concentration patterns point to affinity for protein bodies and cell membrane. There is no apparent affinity of lipid bodies for nuclei, mitochondria, or proplastids. At day 7 the storage parenchyma cell consists of a large central vacuole surrounded by a small zone of cytoplasm.

Immunofluorescence microscopy of semithin cryosections shows the presence of both isoenzymes in the cytoplasm from day 1 up to day 7 (Fig. 2, A and C). Immunoelectron microscopy

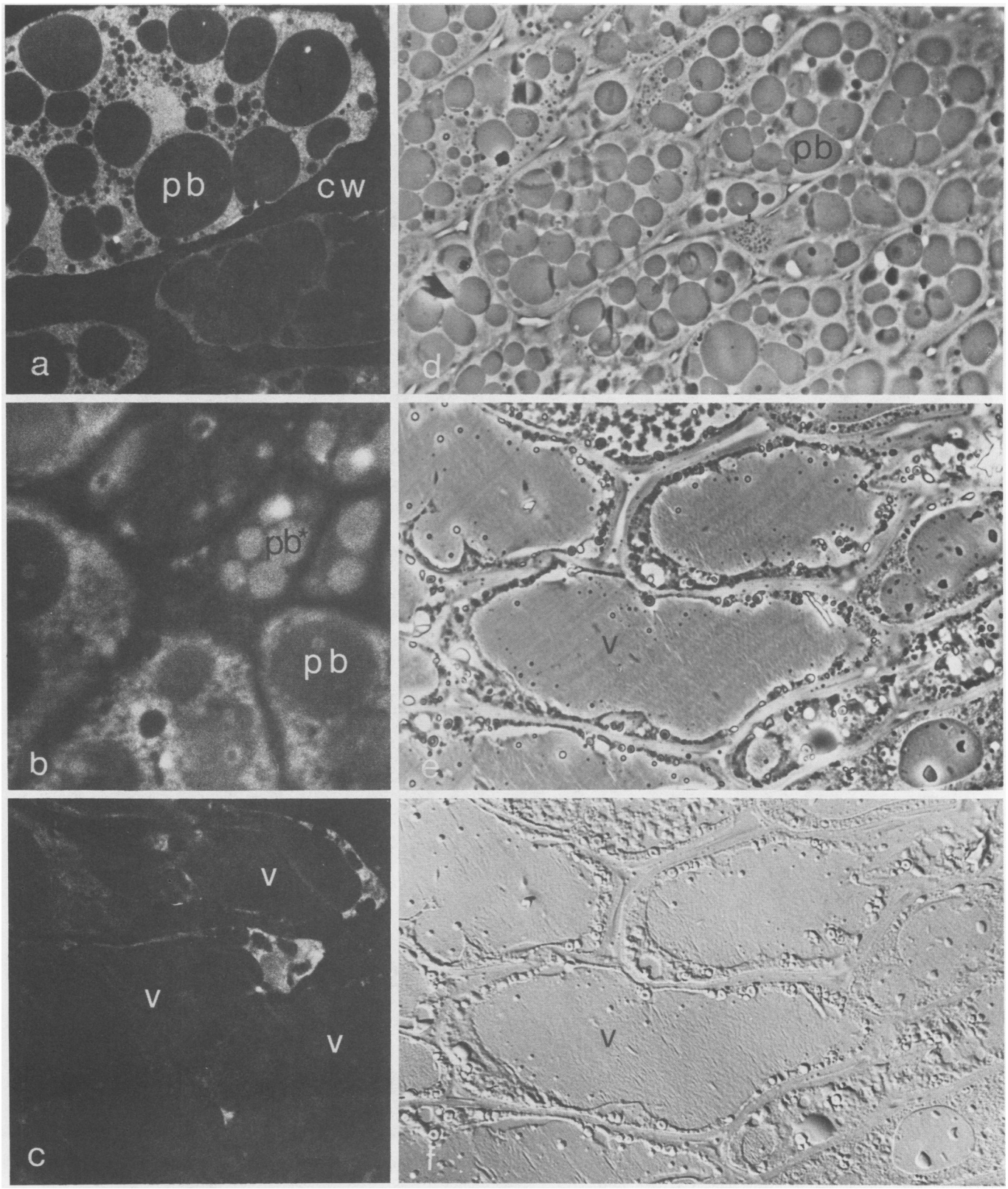

FIG. 2. Semithin cryosections of cotyledons of soybean seedlings. A, immunofluorescence in 26-h-old storage parenchyma cells (anti-1); B, 50-h-old vascular bundle sheath cells containing fluorescing protein bodies (pb*), (anti-2); C, storage parenchyma cells of 7-d-old cotyledons with fluorescing peripheral cytoplasm (anti-1); D, phase-contrast micrograph of 1-d-old storage parenchyma cells, showing large protein bodies; E, phase-contrast micrograph of 7-d-old storage parenchyma cells with coalescing protein bodies or a central vacuole; F, same section as E, photographed with Nomarski optics.

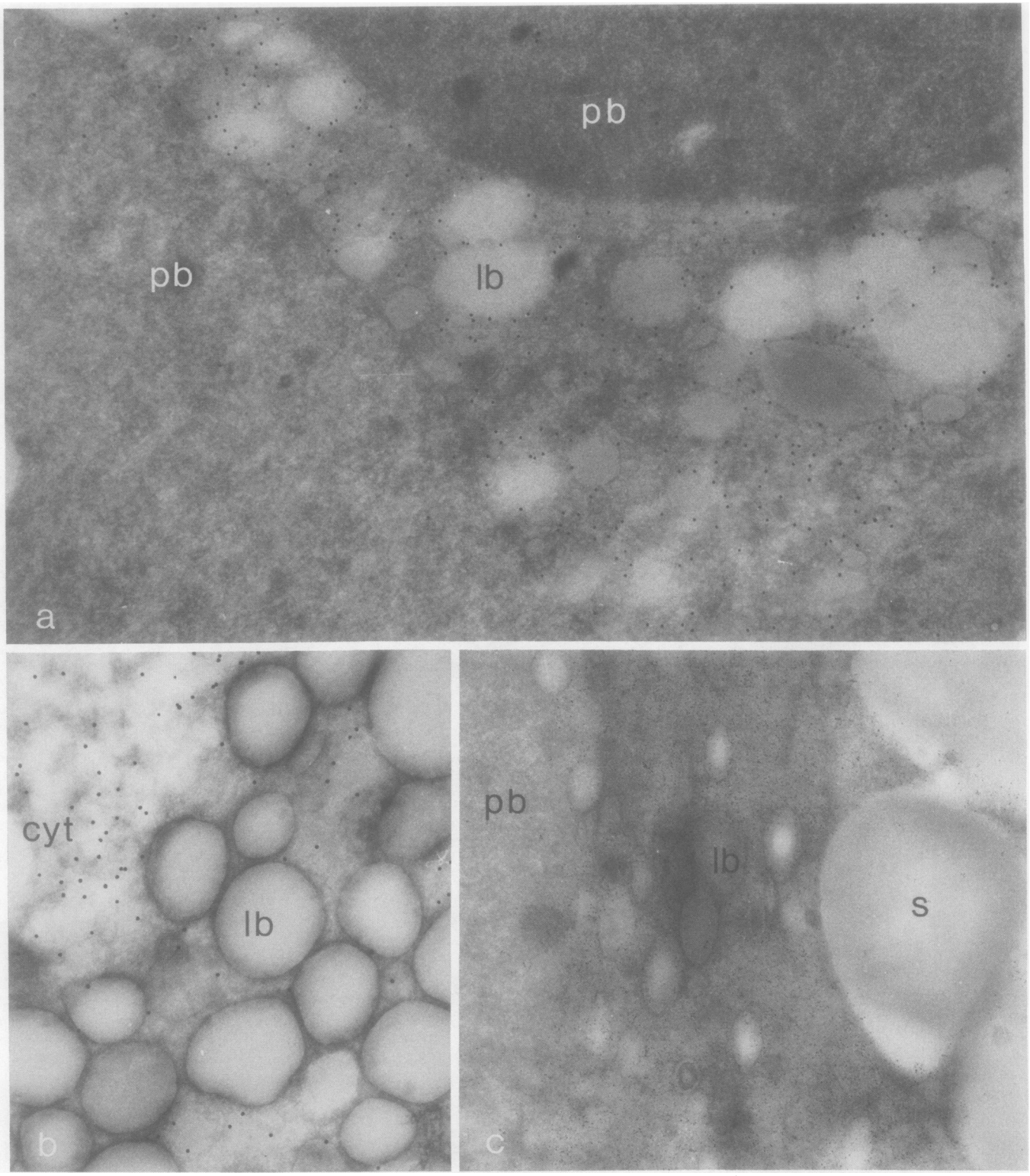

FIG. 3. Electron micrographs of storage parenchyma cells of soybean seedlings. A and B, 26-h-old cotyledon showing nonlabeled protein bodies and lipid bodies surrounded by highly labeled cytosol (anti-2/Au[16]); C, 50-h-old cotyledon with nonlabeled starch grains (s), (anti-2/Au[5]).

of ultrathin cryosections shows that both lipoxygenases-1 and -2 are randomly distributed throughout the cytoplasm (Figs. 1, B and C; 3, A, B, and C). No association has been found with either protein bodies, lipid bodies, mitochondria, or other organelles. In parenchyma cells of 3-d-old cotyledons, after incubation with anti-lipoxygenase-1 or -2 IgG, a high density of label is found in regions of cytoplasm without lipid bodies (Fig. 3B). After 7 d of germination, the cytoplasm of vacuolated cells forms a thin peripheral layer next to the cell wall and still proves to contain both types of lipoxygenase. The concentration of gold label in the cytoplasm is about the same as found in the cytoplasm of 1-d-old cotyledonary parenchyma cells.

Bundle Sheaths of Cotyledons. In parenchyma cells of vascular bundle sheaths some lipid bodies, many plasmodesmata and, at

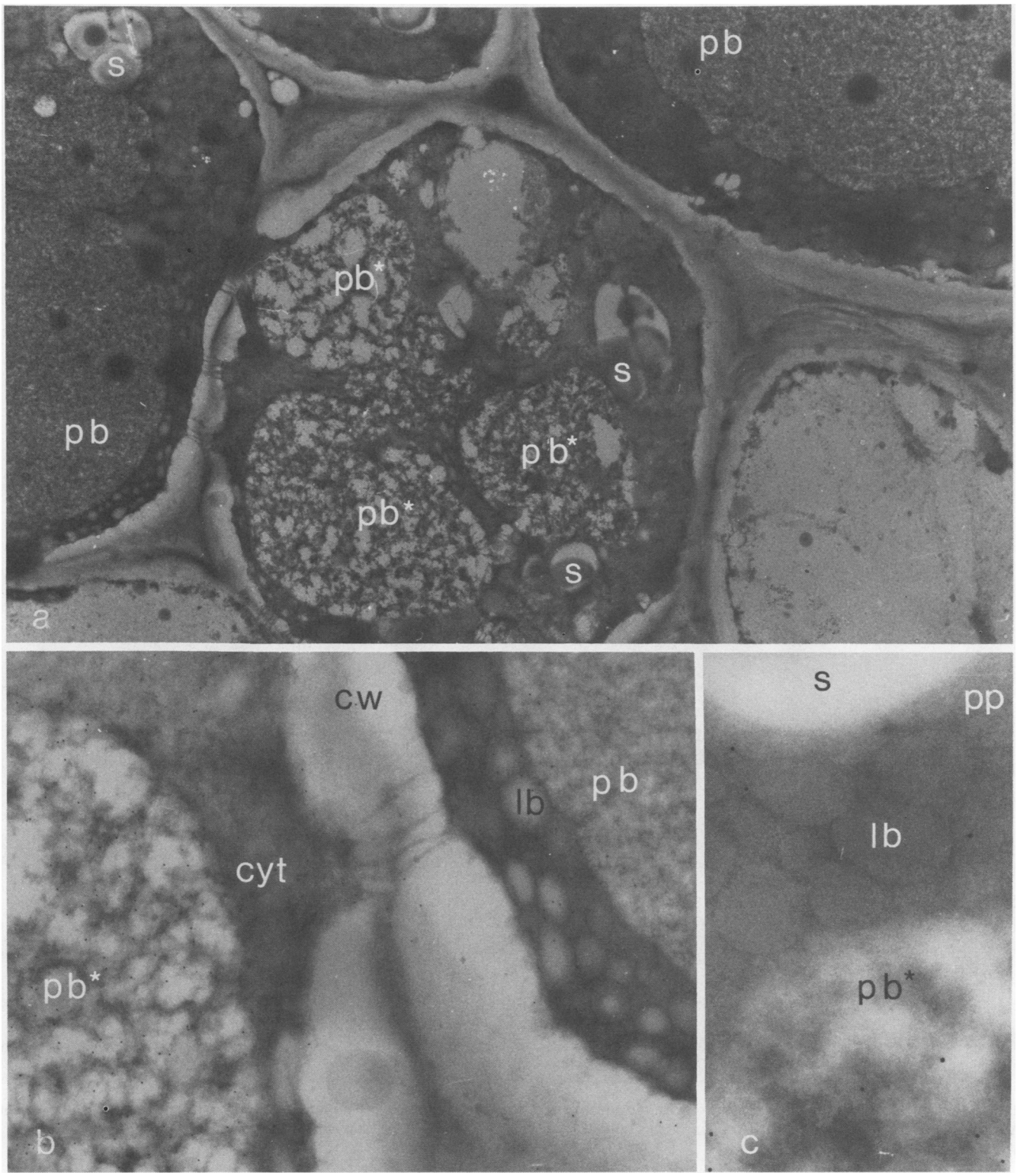

FIG. 4. Vascular bundle sheath cells of a 75-h-old soybean cotyledon with large nonfusing protein bodies (pb*) with flocky contents (anti-2/Au[16]).

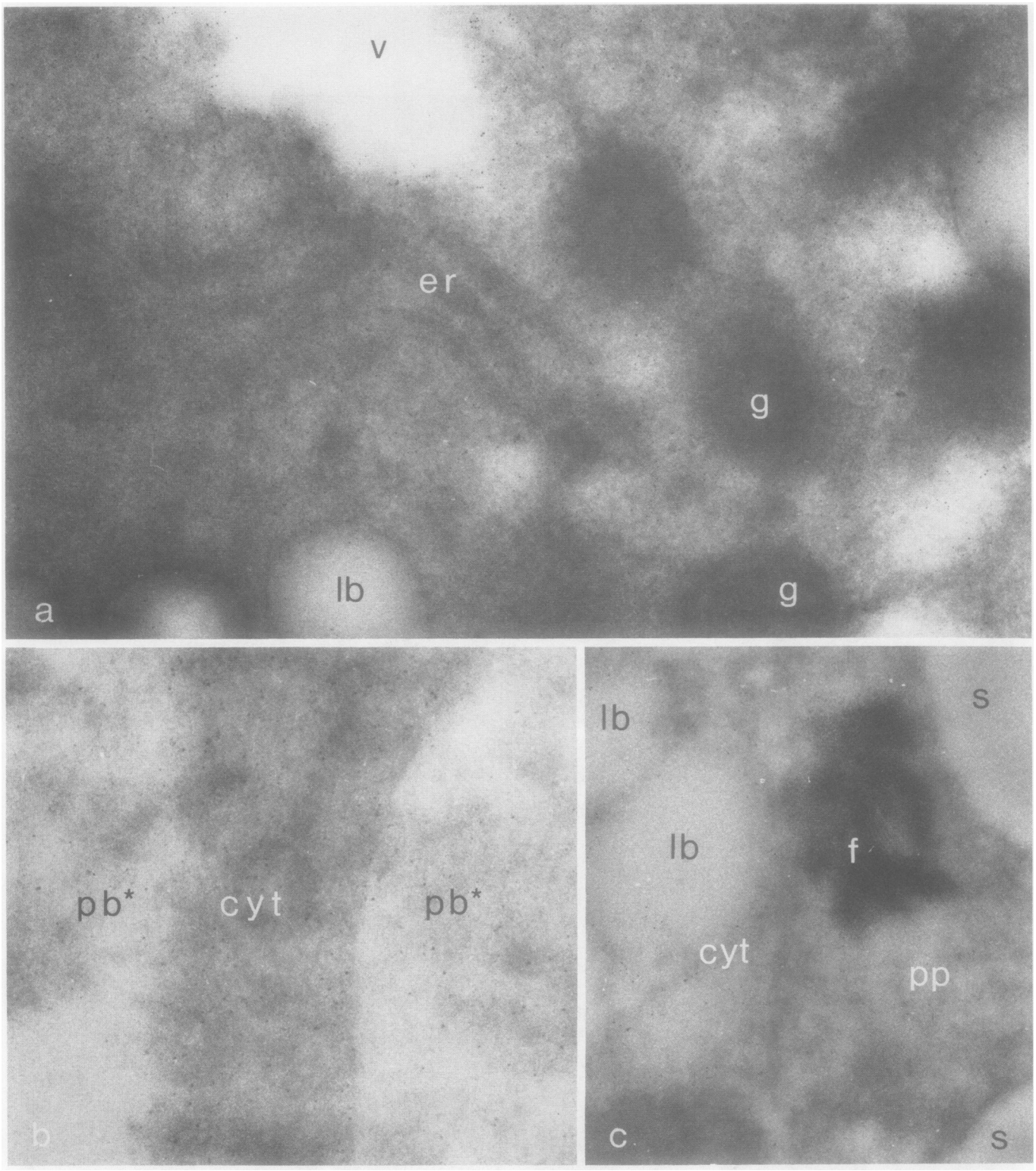

FIG. 5. Abaxial epidermis and hypodermis of 75-h-old soybean cotyledons. A, epidermis cell with an electron clear nonlabeled vacuole (v) (anti-2/Au^5); B, hypodermis cell with labeled protein bodies (anti-1/Au^5); C, a nonlabeled proplastid (pp) with starch and a paracrystalline deposit of phytoferritin (f), (anti-2/Au^5).

day 3, proplastids containing starch grains are found. During germination, protein bodies do not fuse to one entity like the protein bodies in storage parenchyma cells. After 3 d of germination, several protein bodies of variable diameter are found with electron-dense flocky contents. As can be seen in Figure 4A, their contents differ from the contents of aging protein bodies in storage parenchyma. The fluorescing large cell organelles in paraffin sections of cotyledons as described previously (24) are most probably identical to the aberrant type of protein bodies. Also, in semithin cryosections the latter fluoresce after labeling with anti-lipoxygenase-1 or -2 IgG (Fig. 2B), and in ultrathin cryosections gold-label is present predominantly in the aberrant

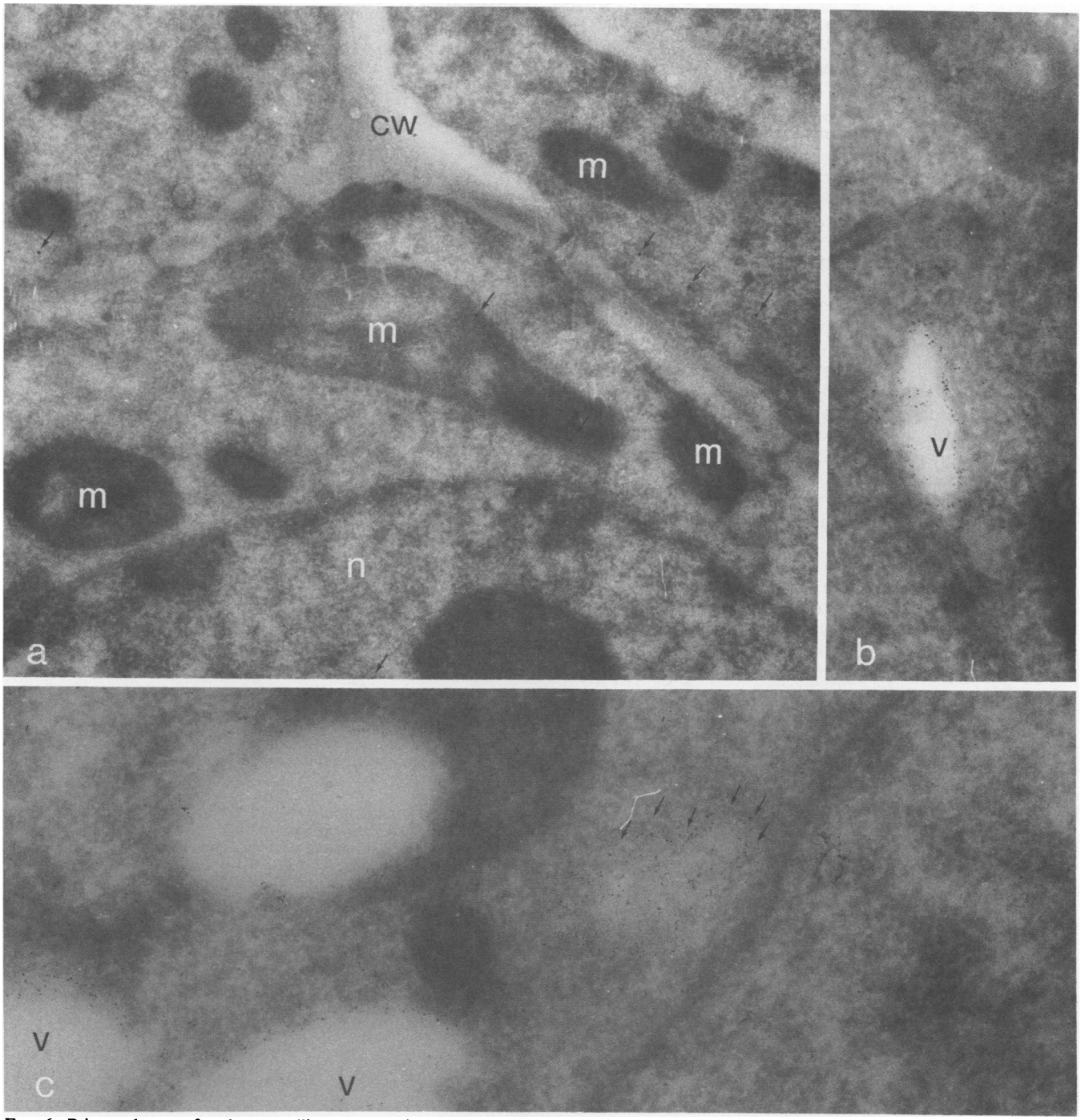

FIG. 6. Primary leaves of soybean seedlings. A, massive parenchyma cells showing hardly any labeling (arrows) after 75 h of germination (anti-1/Au[16]); B, the same section of A showing regions of parenchyma cells which become vacuolated with a high density of gold label (anti-2/Au[5]).

protein bodies (Fig 4, B and C). In all cases labeling is less intense after incubation with anti-lipoxygenase-1 IgG than after incubation with anti-lipoxygenase-2 IgG.

Epidermis and Hypodermis of Cotyledon. After 3 d of germination, cells of epidermis contain a few lipid bodies, many mitochondria, large quantities of ER, and vacuoles with electron-clear content. Small amounts of lipoxygenases-1 and -2 are present in the cytoplasm, and the enzymes are not associated to any cell organelle. Most of the gold label is found in regions of cytoplasm where vacuoles are about to be formed (Fig. 5A).

Hypodermal cells contain ER, mitochondria, proplastids rich in starch grains and phytoferritin, and the same kind of flocky-filled protein bodies as are found in bundle sheath cells. Lipoxygenase-1 and especially lipoxygenase-2 are found in the cytoplasm and in the protein bodies as shown in Figures 5, B and C.

Leaves. Young leaves comprise several tissues such as differentiating vascular strands, massive parenchyma cells with large nuclei, vacuolated parenchyma cells, and epidermis. Small quantities of lipoxygenase-1 and lipoxygenase-2 are found in parenchyma cells, especially in regions of cytoplasm which become vacuolated (Fig. 6). There is no association of lipoxygenases with mitochondria, ER, and etioplasts.

DISCUSSION

In cotyledons, immunoelectron microscopy shows that lipoxygenases-1 and -2 are both localized in the cytoplasm of storage

parenchyma. From day 1 up to day 7 of germination, lipoxygenases are present in the cytoplasm in a concentration remainig fairly constant because the density of gold label was about the same in all examined stages of germination. However, total enzyme activities at pH 6.6 and 9.0 decreases after 3 d of germination. From this, we conclude that decrease of the amount of enzymes coincides with the disintegration of the cytoplasm during germination, through which the total activity of lipoxygenase decreases but not its concentration in the cytosol. Random distribution of lipoxygenase in cytoplasm of storage parenchyma cells may point either to a metabolic function or to a regulatory function. A function might lie in the dioxygenation of fatty acids to make their transport from lipid bodies to glyoxysomes possible or to facilitate β-oxidation of *all cis*-polyunsaturated fatty acids. However, neither of these possibilities is very likely. Hypodermis and vascular bundle sheaths of epigeal germinators become photosynthetically active and their mitochondrial activity increases during germination (14). In those tissues, lipoxygenases are localized in protein bodies which do not fuse to form a central vacuole during germination. The amorphous contents of those protein bodies differ from the contents of protein bodies of storage parenchyma cells. They are probably identical with the aberrant type of protein bodies described by Tombs (20) to occur in cells around vascular bundles and in the epidermis layer of soybean cotyledons. Localization in the protein bodies possibly reflects a stage in the digestion of lipoxygenases originating from the cytoplasm. Similarly, Herman *et al.* (10) found that in cells of mung bean cotyledons protein bodies digested cytoplasmic structures. The suggested ingestion of lipoxygenases by protein bodies might serve to protect Cyt systems from possible unspecific and adverse effects of hydroperoxy linoleic acid (2).

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