Protoplasma (1988) 143: 51–62

PROTOPLASMA

© by Springer-Verlag 1988

Structure of Maize Protein Bodies and Immunocytochemical Localization of Zeins

C. R. LENDING, A. L. KRIZ[1], B. A. LARKINS*, and C. E. BRACKER

Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana

Received September 28, 1987
Accepted November 16, 1987

Summary

Zeins, the seed storage proteins of maize (*Zea mays* L.), are synthesized by membrane-bound polyribosomes and transported into the lumen of the endoplasmic reticulum in developing endosperm, where they assemble into protein bodies. To better understand the organization of protein bodies and the mechanism by which zeins are assembled, we have used immunolocalization to study their distribution within isolated protein bodies. In sections stained with uranyl acetate and lead citrate, the protein body matrix consists of light- and dark-staining regions with the darker stain predominating at the periphery and the lighter stain in the central region. Immunogold staining of the storage proteins in isolated protein bodies reveals a distinct segregation with α-zein localized in the light-staining region and β- and γ-zein localized in the dark-staining regions. However, the relative amounts and distribution of these proteins varies substantially among different protein bodies. These results indicate a more complex internal organization than has been previously observed, and suggest that spatial and/or temporal differences in zein synthesis account for this complexity.

Keywords: Immunolocalization; Zein; Protein bodies; *Zea mays;* Rough endoplasmic reticulum.

Abbreviations: BSA = bovine serum albumin; IgG = immunoglobulin G; PB = phosphate buffer; SDS = sodium dodecyl sulfate; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TTBS = Tween-20/tris-buffered saline; TBS-T = Tris-buffered saline/Tween-20; TBS-T-B = Tris-buffered saline/Tween-20/bovine serum albumin.

1. Introduction

The storage proteins in maize seeds comprise a group of alcohol-soluble proteins called zeins. These proteins are synthesized in the developing endosperm between 10 and 40 days after pollination and constitute approximately 50% of the total protein in the seed (LEE *et al.* 1976). Previous studies have shown that zeins are synthesized by membrane-bound polyribosomes and transported into the lumen of the endoplasmic reticulum where they assemble into protein bodies (KHOO and WOLF 1970, LARKINS and HURKMAN 1978). Although the mechanism of this assembly is unclear, it appears to be directed by interactions between zein proteins, since structures with physical characteristics similar to protein bodies form in *Xenopus laevis* oocytes injected with zein mRNAs (HURKMAN *et al.* 1981).

When separated by SDS-PAGE, maize storage proteins are resolved into polypeptides of Mr 27, 22, 19, 16, 14, and 10 kD (LARKINS *et al.* 1984). These proteins are traditionally fractionated according to their solubility in alcoholic solutions in the presence or absence of reducing agents such as β-mercaptoethanol (LANDRY and MOUREAUX 1970). Because of variation in extraction procedures, the nomenclature of these proteins is somewhat complex (WALL and PAULIS 1975, WILSON 1985). ESEN (1986) divided the proteins into groups designated α-, β-, and γ-zeins, that correspond to the Mr 22,000 and 19,000, the Mr 16,000 and 14,000, and the Mr 27,000 components, respectively. In general, these three groups of proteins differ in the primary amino acid sequences, as deduced from cDNA clones (ARGOS *et al.* 1982, PEDERSEN *et al.* 1986, PRAT *et al.* 1987). The Mr 10,000 protein is structurally different from the others and may constitute a distinct fourth group (J. MESSING, personal communication).

Although each of the zein types has been isolated from protein bodies (HURKMAN *et al.* 1981, TORRENT *et al.* 1986), little attention has been given to their organi-

[1] Current address: Department of Agronomy, University of Illinois, Urbana, IL 61801, USA.

* Correspondence and Reprints: Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907, USA.

zation within protein bodies. It is possible that once inside the lumen of the rough endoplasmic reticulum these proteins are distributed uniformly in the protein body. Alternatively, structural differences among the zeins may cause them to segregate into discrete regions within the protein body. γ-Zein was shown to be localized at the periphery of isolated protein bodies, although no ultrastructural differences were observed (LUDEVID *et al.* 1984). The quantitative variation of the three zein types within the population of protein bodies has not been examined, nor has the effect of location within the endosperm on protein body composition.

To better understand the organization and distribution of the zeins within protein bodies, we have used immunogold staining with antibodies directed against the α-, β-, and γ-zeins. This has enabled us to determine the location and distribution of these proteins within protein bodies isolated from developed maize endosperm. In general, the α-zeins occur in the central region of the protein body with the β- and γ-zeins located primarily at the periphery. This staining pattern correlates with the light- and dark-staining regions observed in material post-stained with uranyl acetate and lead citrate. However, the amount and distribution of these proteins varied substantially among different protein bodies.

2. Materials and Methods

2.1. Sources

Bovine serum albumin (BSA), Tris (Trizma base), Tween-20, and chloroauric acid were from Sigma Chemical Co., St. Louis, MO. Goat antirabbit IgG was from Miles Scientific, Naperville, IL. LR White resin was from Structure Probe, Inc., West Chester, PA. All other chemicals were reagent grade.

2.2. Purification of Zein Proteins

Embryos were removed from mature kernels of the maize inbred line W64A and the endosperms were pulverized to a fine meal in a ball mill. The meal was stirred in cold acetone (40 ml/g) for 2 hours to remove lipids, and dried by washing with anhydrous ether. The α-, β-, and γ-zein fractions were isolated from the meal according to ESEN (1986). Residual α-zein was removed from the β-zein fraction by gel filtration through a column of Sephadex G-75 as described by PHILLIPS and MCCLURE (1985).

2.3. Preparation of Antisera and IgG Purification

Individual zein fractions were dissolved at a concentration of 500 μg/μl in 6 M urea containing 2% (v/v) β-mercaptoethanol, emulsified with an equal volume of Freund's complete adjuvant, and injected subcutaneously into multiple sites of individual New Zealand White rabbits. Each rabbit received a second series of injections with Freund's incomplete adjuvant as emulsifying agent two to three weeks after the primary inoculation. In each instance, 500 μg of protein was used for the primary inoculation and 200 μg for the secondary inoculation. The rabbits were bled from the marginal ear vein two to three weeks after the secondary inoculation, and serum was prepared from the blood by standard methods (COOPER 1977). IgG was purified from serum essentially as described by PALMITER *et al.* (1971) except that chromatography through a Sephadex G-25 column was substituted for the final dialysis step.

2.4. Gel Electrophoresis and Western Blot Analysis

SDS-PAGE was performed according to LAEMMLI (1970) in separating gels which contained 13.5% (w/v) polyacrylamide. For western blot analysis, proteins were transferred from SDS gels to nitrocellulose (Schleicher and Schuell, Keene, NH) with the glycine electrode buffer of TOWBIN *et al.* (1979) in a BioRad TransBlot apparatus (BioRad, Richmond, CA). Electroblotting was performed at 250 mA for 2 hours at 4 °C. After transfer to nitrocellulose, the filters were agitated for 30 minutes in TTBS (10 mM Tris, pH 7.4, 140 mM NaCl, 0.15% (v/v) Tween 20) that contained 3% (w/v) nonfat dry milk, transferred to a solution of zein-specific antiserum that had been diluted 1 : 1000 in TTBS/3% milk, and incubated overnight with agitation. After the filters were washed in three changes of TTBS over a period of 45 minutes, protein bands reacting with zein-specific rabbit antibodies were identified by reaction with a goat anti-rabbit IgG horseradish peroxidase conjugate (BioRad) according to the manufacturer's specifications.

2.5. Isolation and Fixation of Protein Bodies

Protein bodies were isolated from 18-day post-pollination kernels of W 64 A by sucrose density gradient centrifugation as previously described (LARKINS and HURKMAN 1978). After the upper portion of the gradient was removed with a Pasteur pipet, the region containing protein bodies was removed, and approximately 500 μl were added to an ultracentrifuge tube containing ice-cold fixative. All steps prior to infiltration with plastic were performed on ice or at 4 °C. The primary fixative consisted of 1% (v/v) glutaraldehyde and 4% (w/v) freshly prepared formaldehyde in PB (50 mM KH_2PO_4/Na_2HPO_4, pH 7.0). Samples were gently mixed and pelleted by centrifugation at 20,000 rpm for 30 minutes in a SW 41 rotor (Beckman Instruments Inc., Palo Alto, CA). After centrifugation, the fixative was decanted, and the pellet was washed for five minutes with PB. The pellets were then washed three times with distilled water and post-fixed for two hours in 2% (w/v) osmium tetroxide in PB. Samples were dehydrated in a graded ethanol series, 20 minutes each, in 10, 30, 50, 70, 90, and then 100%. After an additional 20 minutes in fresh 100% ethanol, samples were progressively infiltrated with LR White resin in ethanol, with 20 minutes steps in 25, 50, 75, and then 100% LR White resin. Fresh resin was added two additional times, and samples were polymerized in open molds at 60 °C in a sealed oven purged with nitrogen. After 18 hours, samples were removed from the molds and thin sections were cut on a Reichert ultramicrotome. Sections were collected on copper grids coated with formvar and carbon for immunocytochemical staining.

2.6. Preparation of Colloidal Gold Conjugates

Goat anti-rabbit immunoglobulin (IgG) was complexed to colloidal gold as described by ROTH (1982). Briefly, chloroauric acid was reduced with a saturated solution of white phosphorus in diethyl ether. Goat anti-rabbit IgG was dialyzed against 2 mM sodium borate, pH 9.0, as described by DE MAY (1984). The amount of antibody required to stabilize the colloidal gold was determined by titration,

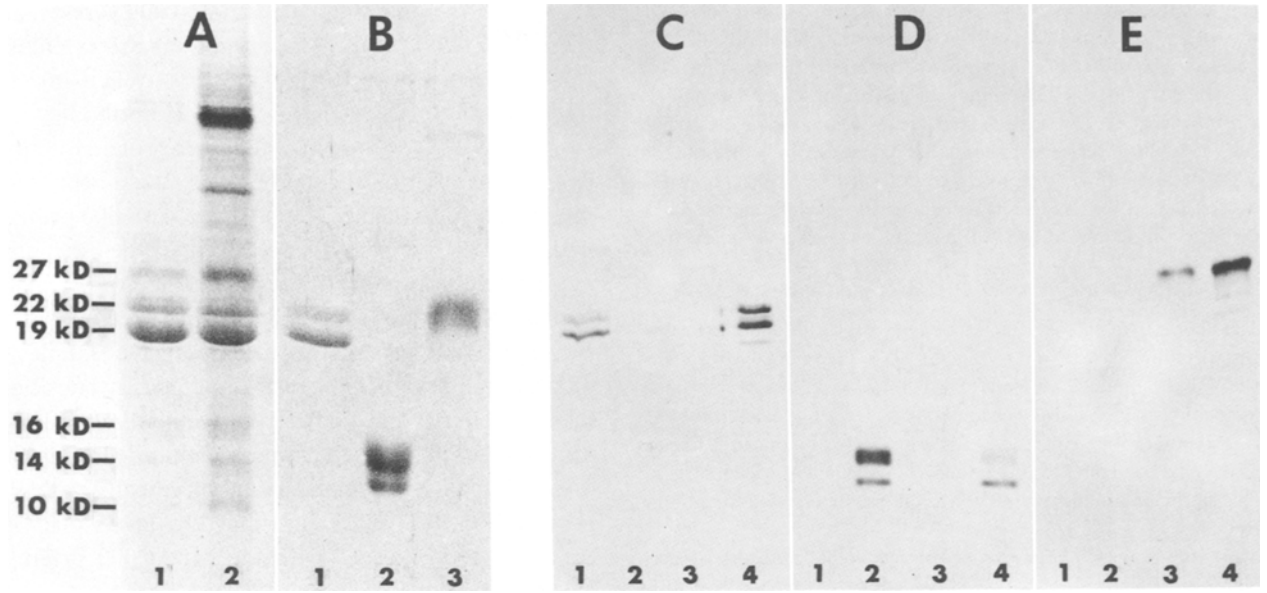


Fig. 1. Immunospecificity of α-, β-, and γ-zein antisera. (*A*) Five μg of zeins extracted with 70% ethanol plus 1% β-mercaptoethanol from mature endosperm of W64A (lane *1*), or 10 μg of endosperm protein extracted with 1% SDS containing 1% β-mercaptoethanol were separated by SDS-PAGE and stained with Coomassie blue (*B*). Two μg each of purified α-, β-, and γ-zein were separated by SDS-PAGE and stained with Coomassie blue. *C–E* Twenty ng of purified α-zein (lane *1*), β-zein (lane *2*), γ-zein (lane *3*), or 40 ng of total endosperm protein were separated by SDS-PAGE and transferred to nitrocellulose for immunodetection as described in Materials and Methods. The western blots were reacted with antisera specific for the α-zein (*C*), β-zein (*D*), and γ-zein (*E*)

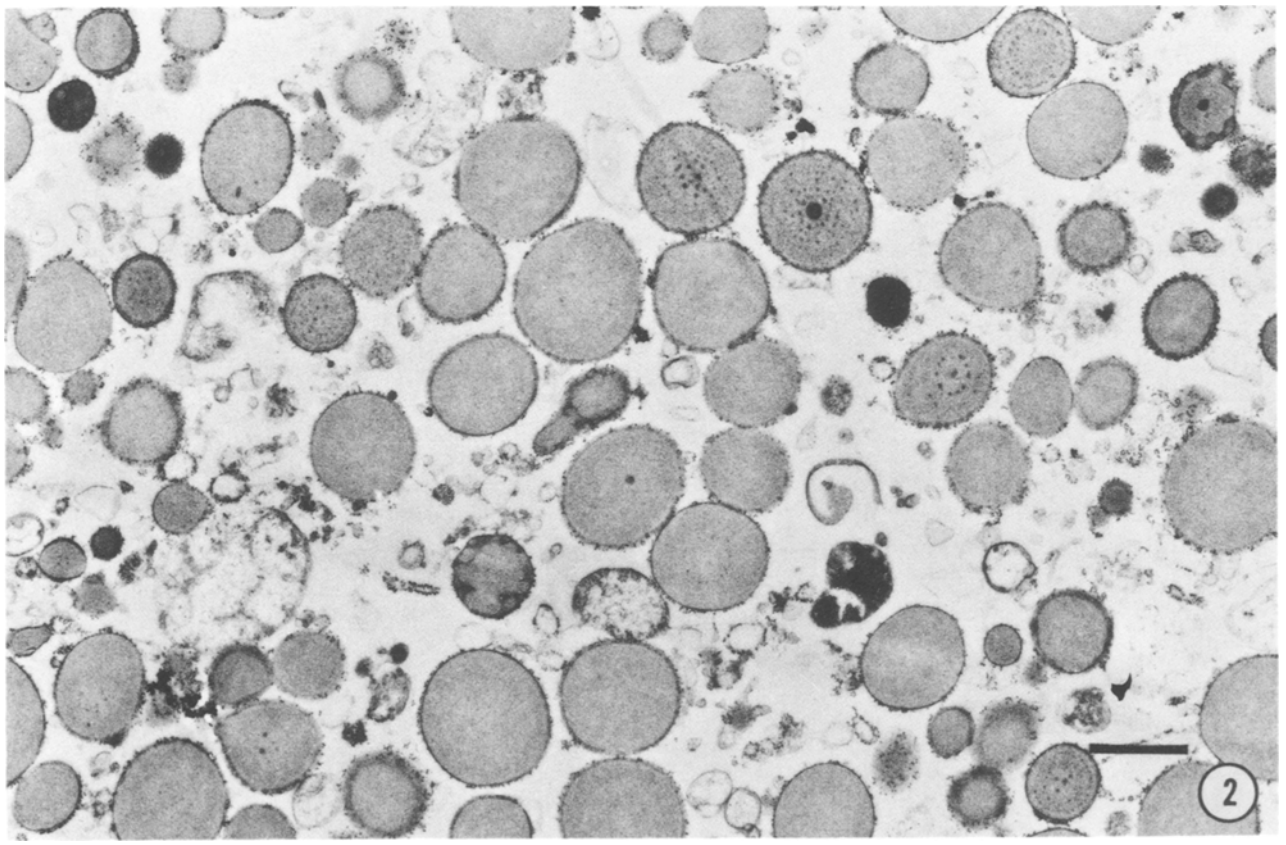


Fig. 2. Electron micrograph showing a low magnification field of protein bodies isolated from developing maize endosperm. Section stained with uranyl acetate and lead citrate. Membrane fragments, a few organelles, and other cell debris are found among the protein bodies. × 13,000. Bar = 1 μm

and a 20% excess was added. After stabilization, the colloidal gold was incubated with 10% (w/v) bovine serum albumin (BSA) and pelleted. The pelleted conjugates were separated by centrifugation in a 10–30% linear glycerol gradient containing 20 mM Tris-HCl, 150 mM NaCl, 1% (w/v) BSA, and 20 mM sodium azide (SLOT and GEUZE 1981). After centrifugation for 45 minutes at 41,000 rpm in a SW-41 rotor, one-ml fractions were collected. The gold conjugates were stored at 4 °C and remained stable for several months. Gold conjugates were diluted to a pale pink color immediately before use.

2.7. Quantitation and Sizing of Colloidal Gold Conjugates

The diameter of the various fractions of colloidal gold conjugates was determined with a LeMont OASYS image analysis system (LeMont Scientific, State College, PA). Micrographs of protein bodies immunostained with α-zein antibody followed by goat anti-rabbit/colloidal gold were enlarged 215,000 times, and the mean diameter of the gold conjugates was determined. Additionally, the number of gold particles bound per square micron was determined for several fractions.

Most sections were immunostained with colloidal gold with a mean diameter of 5 nm, since the labelling density was higher than with larger diameter gold particles.

2.8. Immunocytochemical Staining

Thin sections were stained by a technique modified from TITUS and BECKER (1985). Grids were floated section-side down, on 30 µl drops of reagents on a sheet of dental wax. All incubations were done at room temperature. Incubations and washes were done in 20 mM Tris-HCl, pH 8.2, containing 500 mM NaCl and 0.3% (v/v) Tween-20 (TBS-T). Grids were incubated for 10 minutes in TBS-T containing 1% (w/v) BSA (TBS-T-B). Grids were blotted and incubated for 2 hours in primary antibody (typically diluted 1 : 500) in TBS-T-B. The grids were blotted and incubated for 10 minutes in TBS-T-B, and washed by immersion for 1 minute in 60 ml of stirred TBS-T. After washing, grids were blotted and incubated for 1.5 hours in the colloidal gold/goat anti-rabbit solution diluted in TBS-T-B. After incubation in the colloidal gold conjugate, grids were blotted and incubated in two successive drops of TBS-T for 10 minutes each. Grids were rinsed in distilled water adjusted to pH 8.2 with NaOH and blotted dry. Sections were post-stained for 5 minutes in 2.5% (w/v) aqueous uranyl acetate, rinsed three times with distilled water, blotted dry, and examined with a Philips EM-400.

3. Results

3.1. Preparation and Characterization of Antibodies

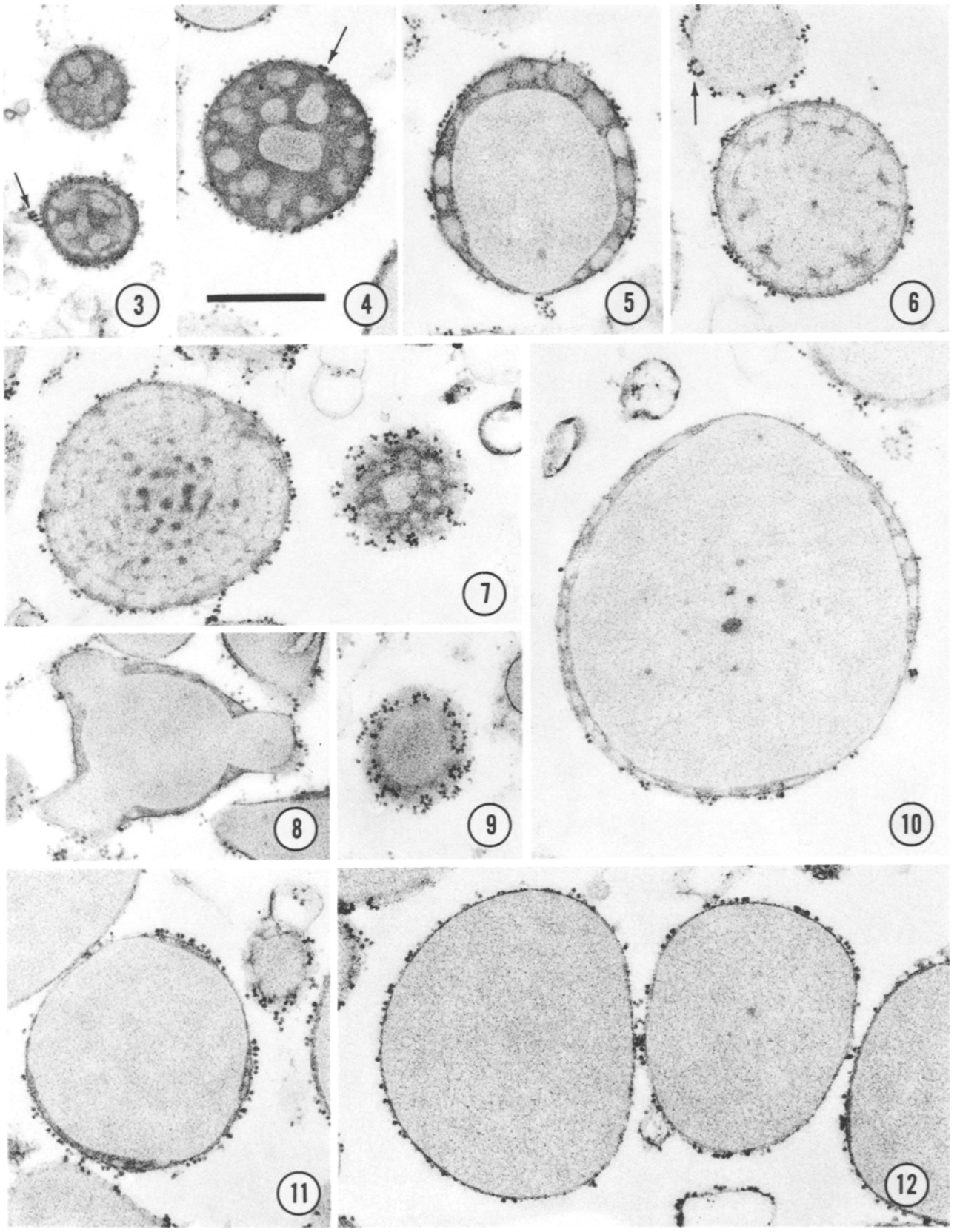
Using the procedure of ESEN (1986), we have prepared protein fractions corresponding to the Mr 22,000 and 19,000 α-zeins, the Mr 16,000 and 14,000 β-zeins, and the Mr 27,000 γ-zeins. These fractions are essentially free of cross-contaminating proteins. When injected into rabbits, these preparations elicit antibodies that react specifically with each of the three protein fractions (Fig. 1). Antibodies directed against the α-zeins react specifically with the Mr 22,000 and Mr 19,000 proteins with essentially no cross-reactivity with the other storage protein components (Fig. 1 *C*, lanes *1–4*). Antibodies against the β-zein fraction are specific for the Mr 16,000 and Mr 14,000 proteins (Fig. 1 *D*, lanes *1–4*), although in gels that contained high concentrations of total zein protein, some cross-reactivity with the γ-zein is detected (data not shown). Similarly, antibodies directed against the γ-zein react specifically with this protein (Fig. 1 *E*, lanes *1–4*), but low levels of cross-reactivity are observed with the Mr 16,000 β-zein in gels containing large amounts of total zein.

3.2 Structure and Internal Organization of Protein Bodies

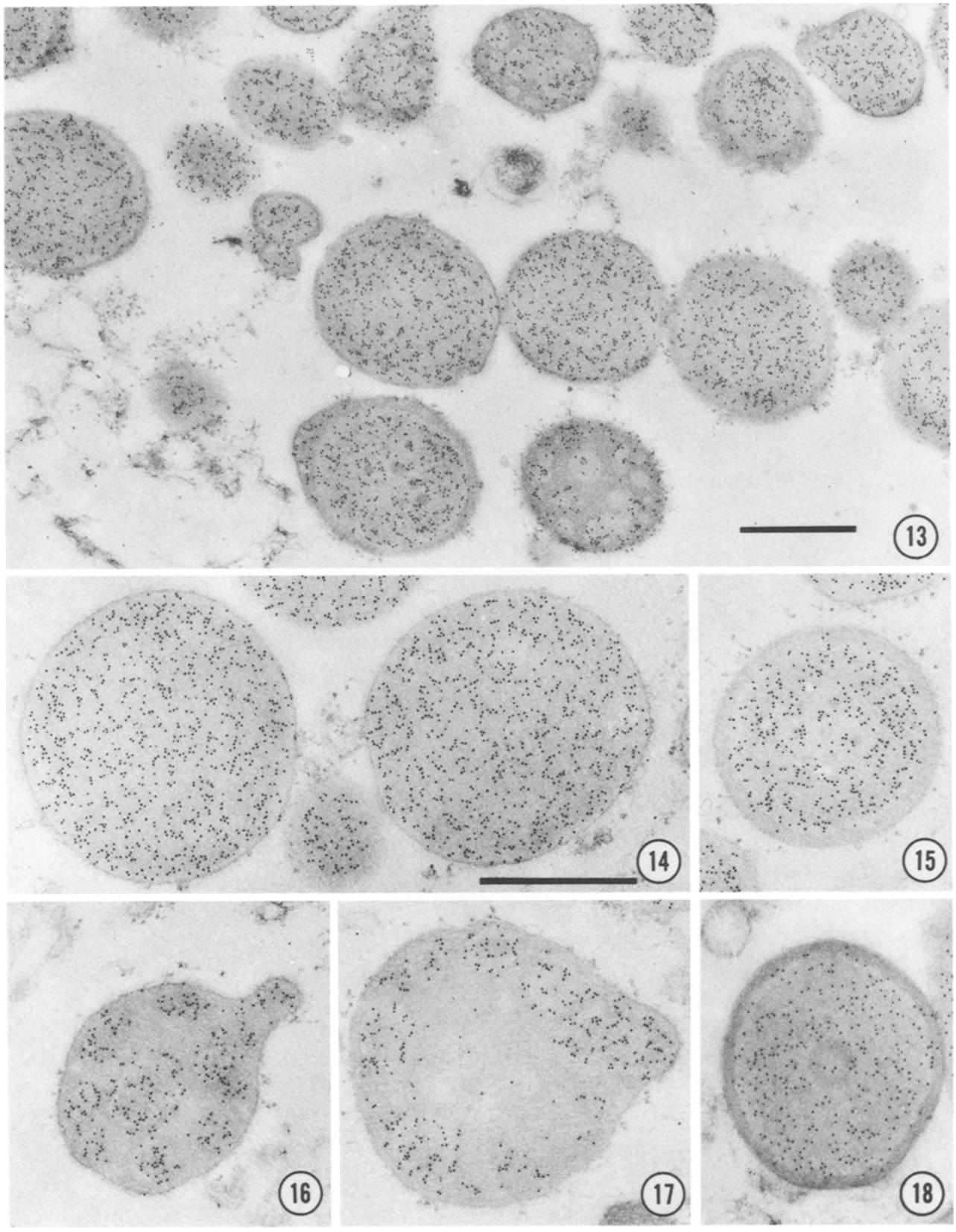
The majority of the protein bodies isolated from the inbred line W64A are nearly spherical structures ranging from 0.3 to 1.3 µm in diameter, with most falling between 0.8 and 1.2 µm (Fig. 2). Some protein bodies have irregular or more complex profiles (Figs. 8, 16, 17, 27, and 33), but these are distinctly in the minority. These organelles, which are formed within cisternae of rough-surfaced endoplasmic reticulum (RER), retain a coating of ribosomes on their surface (all figures). The distribution of ribosomes is irregular, but typical polysome configurations are observed on most protein bodies (*e.g.*, Figs. 5–7) and are especially clear in tangential sections (Figs. 7 and 9).

When the protein bodies are embedded in LR White resin and stained with uranyl acetate and lead citrate, as they are in this study, they reveal a more distinctive internal organization than those previously observed following embedment in epoxy resins (KHOO and WOLF 1970, LARKINS and HURKMAN 1978). The most obvious difference is the appearance of light- and dark-staining

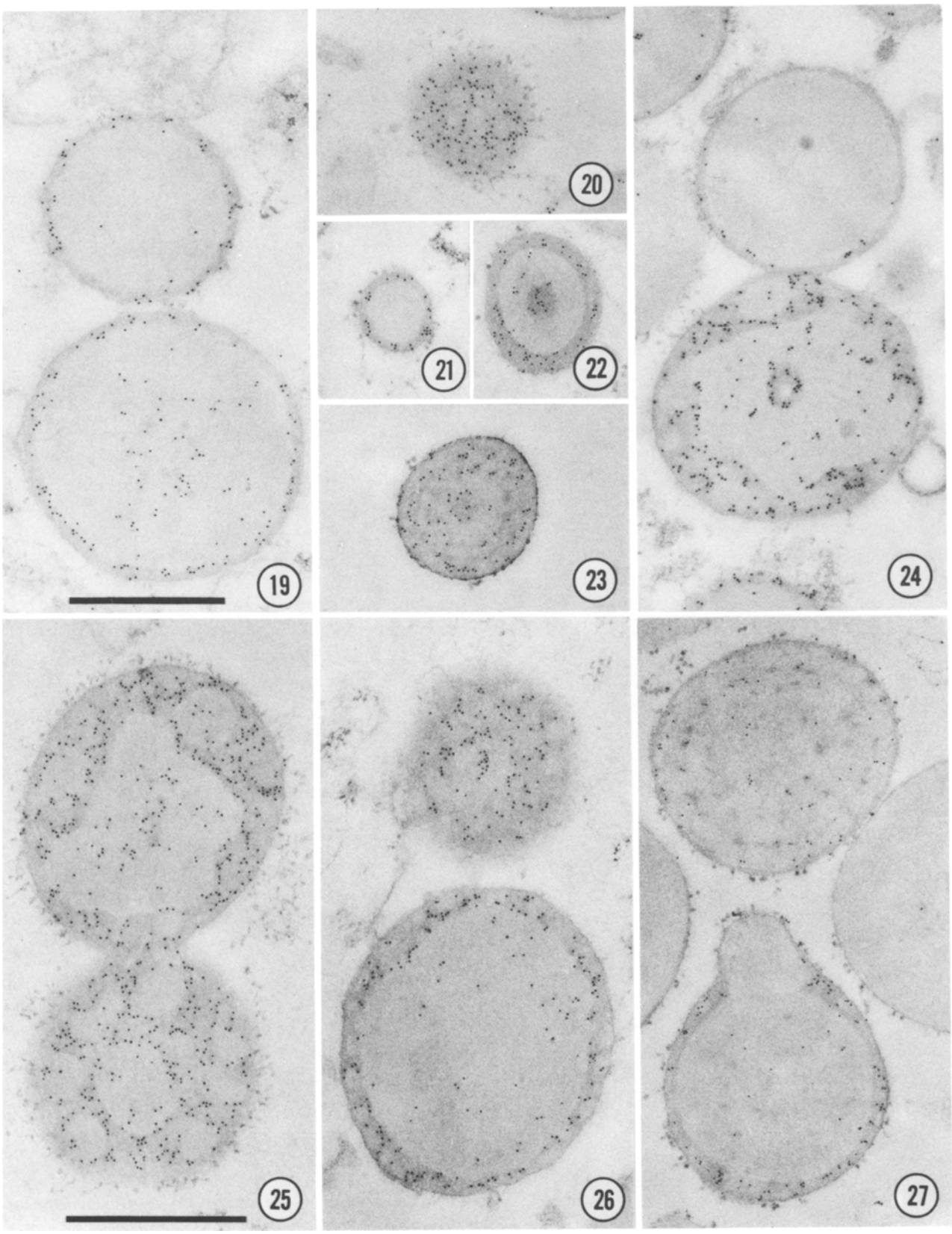
Figs. 3–12. Diverse staining patterns observed in isolated protein bodies post-stained with uranyl acetate and lead citrate. All × 42,000. Figs. 3 and 4. Protein bodies consisting of dark-staining matrix with lighter-staining locules. Ribosomes (arrows). Bar = 0.5 µm. Fig. 5. Peripheral layer of dark-staining material with embedded locules of lighter material. Fig. 6. Discontinuous pattern of dark material. Polyribosomes (arrow). Fig. 7. Concentric pattern of dark material (left). Tangential view (right) showing pattern of peripheral dark material and polysomes on surface of the protein body. Fig. 8. Irregular lobed profile of protein body with large central segment and smaller satellite globules at edge. Fig. 9. Near tangential section of protein body showing polyribosome pattern. Fig. 10. Large protein body with thin peripheral layer of dark-staining material containing lighter-staining locules. Note dark-staining islands near center of the protein body. Fig. 11. Protein body with discontinuous peripheral layer of dark-staining material. Note preferential association of polyribosomes with the peripheral dark material. Fig. 12. Common form of protein body from maize endosperm, consisting mostly of light-staining matrix. Only sparse amounts of the dark-staining material are evident



Figs. 3–12



Figs. 13–18. Protein bodies stained with anti-α-zein followed by goat anti-rabbit colloidal gold, demonstrating localization of α-zein. Fig. 13. Several protein bodies in a pellet, demonstrating the specific labelling pattern for α-zein and the low background labelling that was characteristic of these specimens. × 40,000. Bar = 0.5 μm. Fig. 14. Labelling pattern in which the gold marker is distributed uniformly throughout the protein bodies. × 55,000. Bar = 0.5 μm. Fig. 15. Gold marker distributed through the light-staining matrix but nearly absent from the peripheral darker-staining portion of the protein body. × 55,000. Figs. 16 and 17. Colloidal gold is clustered over the light-staining locules in the protein bodies but not the darker-staining matrix. × 55,000. Fig. 18. Distribution of colloidal gold over the light staining matrix but not over the peripheral dark portion or the darker inclusions near the center of the organelle. × 55,000



Figs. 19–27. Protein bodies stained with anti-β-zein followed by goat anti-rabbit colloidal gold, demonstrating localization of β-zein. Fig. 19. Gold localization over the dark-staining peripheral band and some of the central dark inclusions. × 55,000. Bar = 0.5 μm. Fig. 20. Tangential section intercepting periphery of protein body. Figs. 21 and 22. Small protein bodies with peripheral dark-staining material. Note central inclusion labelled by colloidal gold in Fig. 22. × 55,000. Fig. 23. Small protein body with concentric pattern of dark material and gold label. × 55,000. Fig. 24. Neighboring protein bodies with differing amounts of β-zein. The gold label lies mostly over the dark-staining material. × 55,000. Fig. 25. Protein bodies containing light-staining locules embedded in dark-staining matrix that bears the gold label. × 64,000. Bar = 0.5 μm. Fig. 26. Tangential section of protein body (top). Distribution of gold label over dark-staining peripheral material (bottom) and sparingly over light-staining inclusions in central portion. × 64,000. Fig. 27. Concentric pattern of staining and gold labelling (top). Peripheral localization of gold label over dark-staining material (bottom). Also note virtual absence of gold label in the protein bodies at left and right. × 55,000

regions within the protein bodies. The matrix appears granular and amorphous throughout but is distinctly organized into the two differently staining phases. Often, but not always, the polyribosomes appear to be preferentially associated with the darker-staining peripheral regions of a protein body (Fig. 11).

A wide range of staining patterns is observed within the protein body matrix. The smaller protein bodies consist largely of the darker-staining material and contain discrete pockets of light-staining matrix (Figs. 3 and 4). Among the larger protein bodies, the dark-staining material is usually observed along the periphery. Sometimes the dark material forms a substantial continuous peripheral layer with pockets of light-staining matrix embedded in it (Fig. 5). More commonly, the dark matrix occupies a thin layer at the periphery (Figs. 10 and 11). Discontinuous patterns of dark material range from the fragmented form in Fig. 6 to the concentric pattern in Figs. 2 and 7. We often see evidence of one or more nodules of dark-staining material near the center of the protein bodies (Figs. 2, 6, 7, 10, 12, 18, 22, 24, 29, and 31). Some protein bodies do not show any evidence of dark-staining matrix (Fig. 12). Because all these patterns, and others, are observed, it is apparent that no single form represents the typical protein body. However, the most common morphology within a population is one which has only a thin, peripheral region of dark-staining matrix material, as shown in Figs. 2, 11, and 12.

3.3. Localization of α-Zein

Localization of α-zein is generally limited to the light-staining areas of the protein bodies. Because the sections treated with antibodies and colloidal gold are post-stained only with uranyl acetate and not lead citrate, the differentiation of light and dark areas in these micrographs is not as pronounced as in Figs. 2–12 where both stains are used. Many protein bodies are uniformly labelled (Fig. 14), exhibiting morphology similar to those in Fig. 12. In others, a peripheral, dark-staining ring does not bind colloidal gold (Figs. 15 and 18), nor do the majority of dark, internal inclusions (Fig. 18), representing forms similar to those in Figs. 5 and 10. Still others contain light-staining pockets which bind colloidal gold amid a dark-stanining matrix which has little or no gold label (Figs. 16 and 17), representing the staining pattern seen in Figs. 2 and 3.

3.4. Localization of β-Zein

The localization of β-zein is complementary to α-zein. The dark-staining areas of the protein bodies are labelled, while the light regions contain little or no colloidal gold. β-Zein is commonly found in a peripheral band (Figs. 19, 21, 22, 26, and 27) and in the central inclusions (Figs. 19, 22, and 24). Oblique sections through the peripheral ring show the distribution of β-zein in tangential view (Figs. 20 and 26). In protein bodies with more complicated arrangements of dark- and light-staining regions, the dark areas are predominantly stained (Figs. 23–25 and 27). Some protein bodies show essentially no label (Fig. 27, left and right). The variety of antibody staining patterns is greatest for β-zein, and adjacent protein bodies sometimes contain strikingly different amounts of this protein (Fig. 24).

3.5. Localization of γ-Zein

γ-Zein shows a localization pattern similar to β-zein (Figs. 28–32), with nearly all the label located within the dark-staining areas. The labelling density within dark-staining regions is generally lower for γ than for β-zein, although it may not be evident from the particular selection of micrographs used in this article. Also, a larger proportion of the population shows little or no labelling for γ-zein (as in Fig. 28, right).

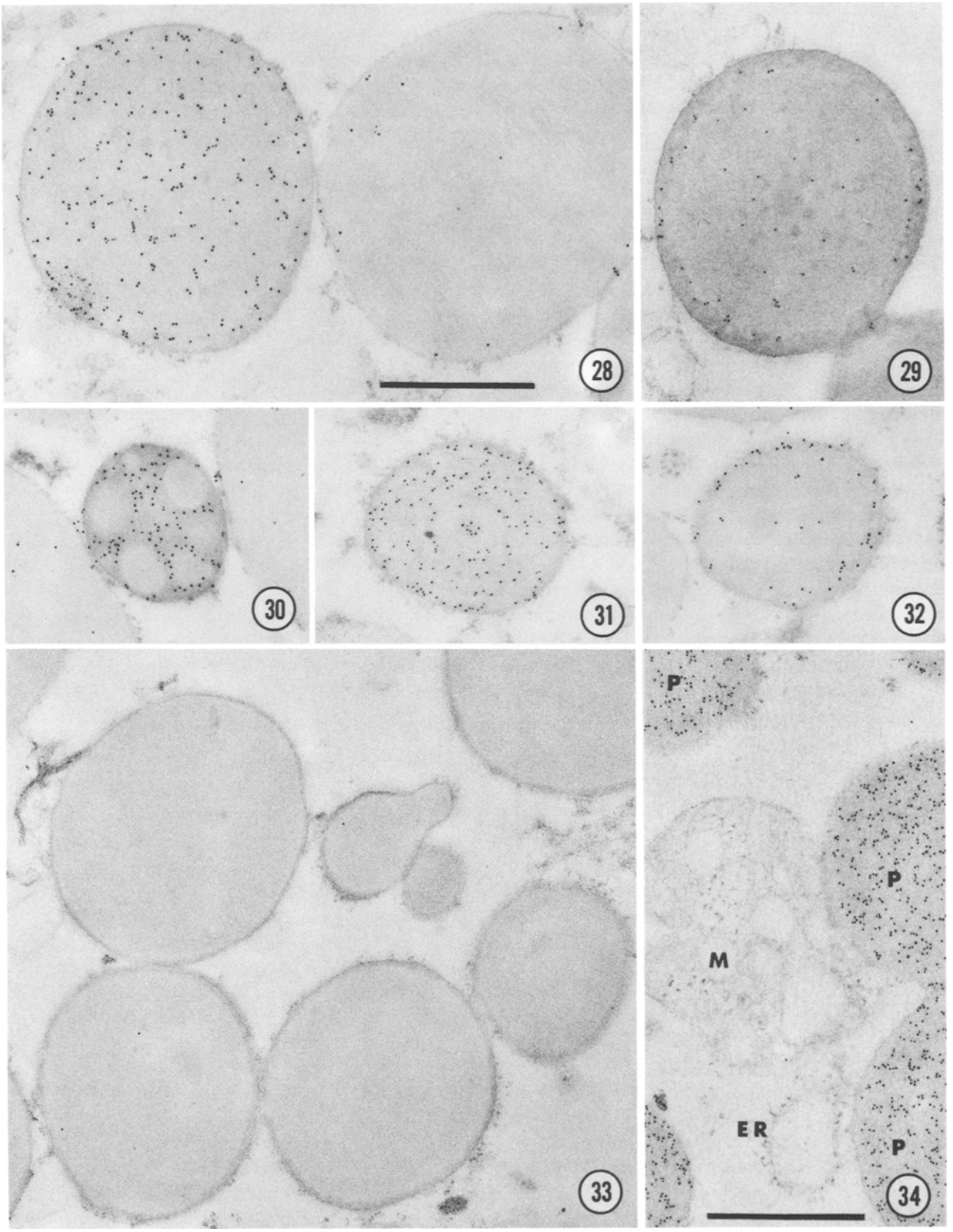
3.6. Controls

In all the preparations prepared by immunocytochemical staining, colloidal gold markes are limited almost exclusively to the protein bodies (Fig. 13). Where membranes, organelles, or other cellular materials are pres-

Figs. 28–32. Protein bodies stained with anti-γ-zein followed by goat anti-rabbit colloidal gold, demonstrating localization of γ-zein. All × 55,000. Fig. 28. Two protein bodies, showing extremes in labelling density for γ-zein. The label on the protein body at left is mostly over subtly dark-staining material. Bar = 0.5 μm. Fig. 29. Gold label over peripheral dark-staining material and few of the dark inclusions in the central region. Fig. 30. Distribution of gold label over the dark-staining matrix but not the lighter-staining locules. Fig. 31. Concentric pattern of staining and gold labelling pattern coinciding with pattern of dark-staining material

Fig. 33. Control treatment in which the section was treated with preimmune serum instead of anti-zein antibody before goat anti-rabbit colloidal gold. × 55,000

Fig. 34. Illustration of the level of background labelling over non-protein body organelles in a protein body fraction. The unlabelled organelles are endoplasmic reticulum (*ER*) and mitochondrion (*M*) among protein bodies (*P*). The section was stained with anti-α-zein followed by goat anti-rabbit colloidal gold. × 55,000. Bar = 0.5 μm



Figs. 28–34

ent among the protein bodies, background labelling is extremely low (Fig. 34). When nonimmune or preimmune serum is used instead of the primary antibody, the background or nonspecific labelling is very low, even over the protein bodies (Fig. 33).

4. Discussion

The ultrastructural examination of isolated protein bodies described here reveals a population of diverse sizes with variable internal organization. The majority of protein bodies consist of two distinct staining regions. The variations in staining patterns and the diversity of sizes are representative of protein bodies *in situ,* since we observe similar protein bodies and staining patterns in intact tissue prepared for electron microscopy by similar methods (unpublished data). It is likely that the differentiation of the light and dark areas is enhanced by embedment in LR White instead of epoxy resin. However, our main consideration for using LR white resin is the level of specific labelling with antibodies, which is several times greater than that observed with epoxy resin (unpublished data).

Polysomes appear to be preferentially associated with the darker-staining areas of protein bodies, although we can only speculate about why this occurs. Possibly, the β- and γ-zeins accumulate in the protein body after the α-zein. Therefore, most of the ribosomes are localized next to regions where high levels of β- and γ-zein are being synthesized in the 18-day post-pollination samples we studied. This correlates with the observation that α-zein mRNAs can be detected much earlier (12 days after pollination) than β- and γ-zeins (MARKS *et al.* 1985). However, the significance of the increase in polysomes associated with the dark-staining regions can be confirmed only by analysis of protein bodies and the associated polysomes at various stages of development.

In previous studies, maize endosperm protein bodies were generally observed to have a finely granular, uniformly stained matrix (KHOO and WOLF 1971, BURR and BURR 1976, LARKINS and HURKMAN 1978). However, at later stages of development (50-day post-pollination), KHOO and WOLF (1971) noted many protein bodies with dark-staining projections extending into the interior. The nature of this dark-staining material was not examined further.

Dark-staining inclusions similar to those described here have been reported by ADAMS and NOVELLIE (1975) in protein bodies from sorghum endosperm. TAYLOR *et al.* (1984) found that extraction of sorghum protein bodies, as well as maize protein bodies, with alcoholic solutions removed most of the contents, leaving behind a proteinaceous network that could be extracted when reducing agents were added to the solvent. From these experiments they concluded that the proteinaceous network corresponded to cross-linked kafirin in sorghum protein bodies and zein II (typically a mixture of α- and β-zein) in maize protein bodies. Our results suggest that the proteinaceous network observed by TAYLOR *et al.* (1984) corresponds to aggregates of the β- and γ-zeins. Furthermore, it seems likely that the network of proteins found in sorghum protein bodies are also cystine-rich, cross-linked proteins.

LEUDEVID *et al.* (1984) used immunogold affinity labelling to determine the location of the Mr 27,000 zein protein (glutelin II, γ-zein) in protein bodies. Their results were similar to those presented here, as γ-zein was localized primarily in the peripheral areas of isolated protein bodies embedded in Lowicryl. However, they did not observe the heterogeneity reported here in the distribution of this protein among the protein body population.

The specificities observed for antibodies directed against the α-, β-, and γ-zeins are consistent with what is known about the structures of these proteins. Based on primary sequences deduced from cDNA and genomic clones, the α- and γ-zeins constitute two structurally distinct groups of proteins (ARGOS *et al.* 1982, PRAT *et al.* 1987), and antibodies directed against them would not be expected to cross-react. However, β- and γ-zeins share structural similarities. There are matches of 10 residues each between amino acids 34 to 49 of the Mr 14,000 β-zein and amino acids 112 to 125 of the γ-zein; amino acids 61 to 73 of the Mr 14,000 β-zein and 130 to 141 of the γ-zein also have 10 matching residues (PEDERSEN *et al.* 1986). Even greater homology exists between the amino acid sequence of the Mr 16,000 β-zein and the γ-zein (PRAT *et al.* 1987). Therefore, a degree of cross-reactivity might be expected with the β- and γ-zeins and polyclonal antisera directed against them. But in spite of the similarities in primary amino acid sequences of these proteins, we observed a significant degree of specificity with antisera directed against each of these protein fractions (Fig. 1).

Because the protein bodies are isolated from a homogenate of total maize endosperm, we do not know the extent to which the variation in their protein composition reflects differences in proteins synthesized in different regions of the endosperm. Certain regions of maize endosperm contain more protein than others, and differences in cellular structure of normal and mu-

tant maize genotypes have been reported (WOLF *et al.* 1969). It is possible that the synthesis of certain types of zeins is more prevalent in some regions of the endosperm than others. Experiments using procedures similar to those described here are in progress to resolve this question.

Protein bodies in the endosperm of maize and sorghum differ from those in other cereals, such as wheat (PARKER and HAWES 1982), barley (CAMERON-MILLS and VON WETTSTEIN 1980), and oats (SAIGO *et al.* 1983), as they occur within the lumen of the rough endoplasmic reticulum (LARKINS and HURKMAN 1978, TAYLOR *et al.* 1985) rather than in the vacuole. In rice, protein bodies which contain prolamines are found in the lumen of the rough endoplasmic reticulum, while the globulin (glutelin) forms protein bodies within the vacuole (KRISHNAN *et al.* 1986). The mechanisms responsible for the transport and assembly of cereal prolamine proteins into protein bodies in different parts of the cell are unknown, but they presumably relate to transit sequences within the proteins themselves (KELLY 1985). Interactions between the α-, β-, and γ-zeins may be responsible for their assembly into protein bodies and retention within the rough endoplasmic reticulum rather than transport to other subcellular structures. When only the smaller molecular weight β-zein was synthesized in transgenic tobacco plants, the protein was transported into the vacuolar protein bodies rather than being retained in the rough endoplasmic reticulum (HOFFMAN *et al.* 1987).

This study reveals substantial variation in the organization of protein bodies in developing endosperm of maize. An understanding of the basis of this variation should provide valuable information regarding the process by which protein bodies are assembled during seed development. β- and γ-zein may play a regulatory role in determining the size of a protein body. Experiments in which different combinations of zein mRNAs are translated in *Xenopus laevis* oocytes indicate that β- and γ-zeins, in addition to α-zein, are required to produce protein bodies with a density similar to those isolated from endosperm (unpublished observations). We are now using immunocytochemical techniques to investigate both the spatial and temporal events leading to the formation of protein bodies *in vivo*.

Acknowledgements

We would like to acknowledge the excellent technical assistance of Mr. DICK BENTLAGE, Ms. JUDITH S. LINDELL, and Ms. KATY SHAW. We thank Dr. JOHN WALLACE for his insightful comments regarding this manuscript.

This research was supported by a grant from the National Institutes of Health (GM 36790) to BAL. This is journal paper number 11367 from the Purdue Agricultural Experiment Station. We acknowledge the Electron Microscopy Center at Purdue University.

References

ADAMS CA, NOVELLIE L (1975) Composition and structure of protein bodies and spherosomes isolated from ungerminated seeds of *Sorghum bicolor* (Linn.) Moench. Plant Physiol 55: 1–6

ARGOS P, PEDERSEN K, MARKS MD, LARKINS BA (1982) A structural model for maize zein proteins. J Biol Chem 17: 9984–9990

BURR B, BURR FA (1976) Zein synthesis in maize endosperm by polyribosomes attached to protein bodies. Proc Natl Acad Sci USA 73: 515–519

CAMERON-MILLS V, VON WETTSTEIN D (1980) Protein body formation in the developing barley endosperm. Carlsberg Res Commun 45: 577–594

COOPER TG (1977) The tools of biochemistry. John Wiley & Sons, NY, pp 270–274

DE MAY J (1984) Colloidal gold as marker and tracer in light and electron microscopy. EMSA Bull 14: 54–66

ESEN A (1986) Separation of alcohol-soluble proteins (zeins) from maize into three fractions by differential solubility. Plant Physiol 80: 623–627

HOFFMAN LM, DONALDSON DD, BROOKLAND R, RASHKA K, HERMAN E (1987) Synthesis and protein body deposition of 15 kD zein in transgenic tobacco seeds. EMBO J 6: 3213–3221

HURKMAN WJ, SMITH LD, RICHTER J, LARKINS BA (1981) Subcellular compartmentalization of maize storage proteins in *Xenopus* oocytes injected with zein messenger RNAs. J Cell Biol 89: 292–299

KHOO U, WOLF MJ (1970) Origin and development of protein granules in maize endosperm. Am J Bot 57: 1042–1050

LAEMMLI UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

LANDRY J, MOUREAUX MT (1970) Heterogeneite des glutelines du grain de mais: extraction selective et composition en acides amines des trois fractions isolees. Bull Soc Chem Biol 52: 1021–1037

LARKINS BA, PEDERSEN K, MARKS MD, WILSON DR (1984) The zein proteins of maize endosperm. TIBS 9: 306–308

— HURKMAN WJ (1978) Synthesis and deposition of zein in protein bodies of maize endosperm. Plant Physiol 62: 256–263

LEE KH, JONES RA, DALBY A, TSAI CY (1976) Genetic regulation of storage protein content in maize endosperm. Biochem Genet 14: 641–650

LUDEVID MD, TORRENT M, MARTINEZ-IZQUIERDO JA, PUIGDOMENECH P, PALAU J (1984) Subcellular localization of glutelin-2 in maize (*Zea mays* L.) endosperm. Plant Mol Biol 3: 227–234

MARKS MD, LINDELL JS, LARKINS BA (1985) Quantitative analysis of the accumulation of zein mRNA during maize endosperm development. J Biol Chem 260: 16445–16450

PALMITER RD, OKA T, SCHIMKE RT (1971) Modulation of ovalbumin synthesis by estradiol-17β and Actinomycin D as studied in explants of chick oviduct in culture. J Biol Chem 246: 724–737

PARKER ML, HAWES CR (1982) The Golgi apparatus in developing endosperm of wheat (*Triticum aestivum* M.). Planta 154: 277–283

PEDERSEN K, ARGOS P, NARAVANA SVL, LARKINS BA (1986) Sequence analysis and characterization of a maize gene encoding a high-sulfur zein protein of Mr 15,000. J Biol Chem 261: 6279–6284

PHILLIPS RL, MCCLURE BA (1985) Elevated protein-bound methionine in seeds of a maize line resistant to lysine plus threonine. Cereal Chem 62: 213–218

PRAT S, PEREZ-GRAU L, PUIGDOMENECH P (1987) Multiple variability in the sequence of a family of maize endosperm proteins. Gene 52: 41–49

ROTH J (1982) The preparation of protein A-gold complexes with 3 nm and 15 nm gold particles and their use in labelling multiple antigens on ultrathin sections. Histochem J 14: 791–801

SLOT JW, GEUZE HJ (1981) Sizing of protein A-colloidal gold probes for immunoelectron microscopy. J Cell Biol 90: 533–536

TAYLOR JRN, SCHUSSLER L, LIEBENBERG N V D W (1985) Protein body formation in starchy endosperm of developing *Sorghum bicolor* (L.). Moench seeds. S Afr J Bot 51: 35–40

— — — (1984) Location of zein-2 and crosslinked kafirin in maize and sorghum protein bodies. J Cereal Sci 2: 249–255

TITUS DE, BECKER WM (1985) Investigation of the glyoxysome-peroxisome transition in germinating cucumber cotyledons using double-label immunoelectron microscopy. J Cell Biol 101: 1288–1299

TORRENT M, POCA E, CAMPOS N, LUDEVID MD, PALAU J (1986) In maize, glutelin-2 and low molecular weight zeins are synthesized by membrane-bound polyribosomes and translocated into microsomal membranes. Plant Mol Biol 7: 393–403

TOWBIN H, STRAEHLIN T, GORDON T (1979) Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets. Proc Natl Acad Sci USA 76: 4350–4354

WALL JS, PAULIS JW (1978) Corn and sorghum grain proteins. In: POMERANG Y (ed) Advances in cereal science and technology, Vol II. American Association of Cereal Chemists, St Paul, MN, pp 135–219

WILSON CM (1985) A nomenclature for zein polypeptides based on isoelectric focusing and sodium dodecyl sulfate gel electrophoresis. Cereal Chem 62: 361–365

WOLF MJ, KHOO U, SECKINGER HL (1969) Distribution and subcellular structure of endosperm protein in varieties of ordinary and high-lysine maize. Cereal Chem 46: 253–263