Immunocytochemical Localization of the Wheat Storage Protein Triticin in Developing Endosperm Tissue

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ABSTRACT

A variety of antisera raised against cereal storage proteins were used in an immunocytochemical study of wheat endosperm protein bodies. Antisera raised against α-gliadin, γ-gliadin, a high molecular weight glutenin subunit, barley C-hordein, and oat 12S globulin labeled the matrix of the wheat endosperm protein bodies but failed to label the electron-dense inclusions within protein bodies. The type of plastic resin used to embed the samples had little effect on the amount and location of the gold label. Immunocytochemistry using antibodies made against triticin, a storage protein with leguminlike characteristics, labeled both the matrix and the dense inclusions. These inclusions contained several times more label than matrix protein. The embedding media greatly affected the amount of labeling obtained when using antitriticin; a low-viscosity epoxy resin gave less labeling than did an acrylic-based plastic. We conclude from this study that triticin is located primarily in the dense inclusions of protein bodies.

Two major groups of storage proteins, the gliadins and the glutenins, are found in wheat endosperm. These proteins are synthesized by membrane-bounded polysomes and secreted into the rough endoplasmic reticulum. Early in endosperm development, the proteins are secreted into vacuoles, while later in development the rough endoplasmic reticulum itself becomes the reservoir for the storage proteins. Prolamins in wheat and in *Haynaldia villosa* are localized in endosperm protein bodies (Kim et al 1988, Krishnan et al 1988). Recently another class of storage proteins with leguminlike properties was identified in wheat endosperm (Singh and Shepherd 1985, 1987). The proteins, originally called triplet proteins and now referred to as triticin, are synthesized between 8 and 21 days postanthesis (Singh and Shepherd 1987). While it is known that triticin is concentrated in the protein body fraction of isolated wheat protein bodies (Payne et al 1986, Singh and Shepherd 1987), it is not known whether triticin is secreted as a separate granule or mixed with the other storage proteins as a heterogeneous component. Similarly, it is not known whether the pathways of secretion for triticin are similar to those for the gliadins and glutenins.

The present study uses immunocytochemistry to identify the location of triticin in developing wheat endosperm. We present data indicating that triticin is concentrated in discrete electron-dense inclusions within starchy endosperm storage protein bodies.

MATERIALS AND METHODS

The wheat (*Triticum aestivum* cv Highbury) used for this study was grown either in field plots or in glasshouses at Rothamsted Experimental Station, Harpenden, England, during the spring and summer of 1987. Individual heads were tagged at anthesis and harvested at various times during development, typically 2, 4, 7, 9, 11, 14, 18, 21, 24, 28, 35, 42, and 50 days after flowering (DAF). Samples were fixed and embedded in Spurr resin (Spurr 1969), as previously described (Bechtel et al 1982a), using 2% glutaraldehyde (v/v) and 2% paraformaldehyde (w/v), followed by postfixation in 1% buffered osmium tetroxide. Two other sets of samples were fixed in either 2% paraformaldehyde or 2% glutaraldehyde and embedded in LR White resin (The London Resin Co. Ltd., Basingstoke, Hampshire, England). LR White resin was polymerized by either thermal polymerization or ultraviolet light photopolymerization methods and, in either case, osmium tetroxide postfixation was omitted. Sections were cut with glass and diamond knives and picked up on copper grids for routine transmission electron microscopy or on gold or nickel grids for immunocytochemistry.

Immunocytochemistry was conducted on grids that were floated on successive drops of reagents on dental wax sheets in petri dishes lined with moistened filter paper at room temperature. All water used for microscopy was deionized and glass distilled. Grids were first rinsed on a drop of distilled water for 10 min, followed by 30 min on saturated sodium metaperiodate, 5 min on distilled water, 15 min on 3% bovine serum albumin [BSA], and 1.5 hr on primary antibodies (crude antiserum dilution varied from 1:20 to 1:1,000, depending upon which antiserum was used). They were given two 5-min rinses on buffer (0.5% BSA, 0.05% Tween 20, 0.5*M* NaCl, and 0.01*M* sodium dihydrogen phosphate buffered to pH 7.2 with 0.01*M* sodium monohydrogen phosphate). Grids were then placed for 1.5 hr on gold-conjugated secondary antibody (typically goat-anti-rabbit [GAR] immunoglobulin G [Janssen AuroProbe EM, Amersham Corp., Arlington Heights IL], 5, 10, or 15 nm diluted 1:50, for polyclonal antibodies, or goat-anti-mouse immunoglobulin G, also diluted 1:50, for monoclonal antibodies). They were then given two 5-min rinses of phosphate-buffered saline (0.01*M* phosphate buffer in 0.15*M* NaCl at pH 7.2) and finally two 5-min rinses on water. Grids were dried with filter paper and the sections were usually viewed in the electron microscope unstained to avoid staining artifacts that might be interpreted as colloidal gold. Two controls were also conducted: preimmune sera or a buffer replacement of the primary serum.

A variety of antibodies was used to localize storage proteins in the developing wheat caryopsis. The polyclonal antibodies against wheat and barley storage proteins, in the form of crude sera, were those raised against α-gliadin, γ-gliadin, a high molecular weight (HMW) glutenin subunit, and barley C-hordein. Specificities of the antisera have been reported previously using laser nephelometry and purified test proteins (Festenstein et al 1984, 1985, 1987). The properties of the antiserum to 12S oat globulin have been reported previously (Matlashewski et al 1982); it was shown by Singh et al (1988) to react with triticin, but also with gluten proteins, including HMW glutenin subunits. All antisera were retested for the present study using immunoblotting following sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis and lactate PAGE separations of gliadins and glutenins (data not shown). This showed that the antibodies to γ-gliadin and HMW glutenin subunits had the widest reactions and that both appeared to react with each group of gliadins and the low molecular weight glutenin subunits. The HMW glutenin subunit antiserum reacted with only the HMW glutenin subunits, however. The C-hordein antiserum reacted with each group of gliadins, and the polyclonal α-gliadin antibodies with a number of bands on SDS-PAGE, which included gliadins and some low

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molecular weight glutenin subunits. Similar analyses of a monoclonal antibody raised against A-gliadin showed much greater specificity, with single major reacting bands and several minor ones on SDS-PAGE and in the α-gliadin region of lactate-PAGE gels. This is consistent with the reacting proteins being α-type gliadins. Immunoblotting of the triticin polyclonal antiserum showed a broad reaction with gliadins and glutenins, including the HMW subunits of glutenin. The latter is consistent with the presence of some sequence homology (Singh et al 1988).

Previous results using Protein A-gold to label wheat endosperm showed that it gave less intense labeling than did gold-conjugated secondary antibodies, exhibited nonselective but specific binding of wheat storage proteins, and gave increased background staining, even at dilutions down to 1:100 (Bechtel, 1990). It was therefore not used in this study. Counts of colloidal gold particles were conducted on micrographs by randomly placing a mask over the inclusions and matrix and counting the gold particles exposed by the mask. Counts ranged from one to five particles per square micrometer for the matrix and three to 10 particles per square micrometer for the inclusions.

RESULTS

A variety of antisera were used to localize storage proteins in the developing wheat caryopsis. These included polyclonal antisera raised against α-gliadin, γ-gliadin, a HMW glutenin subunit, barley C-hordein, 12S oat globulin, and a monoclonal antibody made against A-gliadin (Figs. 1–6). Specific labeling of the protein body matrix was obtained using all of these antisera. Both the mono- and polyclonal anti-α-gliadins, the barley anti-C-hordein, and the anti-12S oat globulin gave light-specific labeling of the protein body matrix (Figs. 1, 4–6). The γ-gliadin and HMW glutenin antibodies yielded dense-specific labeling of the matrix (Figs. 2 and 3). None of the antisera consistently labeled the electron-dense inclusions as intensely as the protein body matrix typically found in developing wheat protein bodies. Preimmune serum and buffer controls did not show nonspecific labeling of the protein bodies (Figs. 7 and 8). Similar results were obtained whether samples were embedded in Spurr or LR White resins (data not show).

Antitriticin antiserum labeled the endosperm embedded in Spurr resin only lightly (Fig. 9). The label was present over the protein body matrix and inclusions, with the latter more intensely labeled. The Spurr resin-embedded samples exhibited very low background labeling, however (Fig. 10). Greater density of label was achieved when samples were embedded in LR White resin and the osmium tetroxide postfixation step was omitted (Fig. 11). The inclusions labeled more intensely than the protein body matrix. Even with the extremely heavy labeling obtained using LR White resin, background staining was minimal (Fig. 12). Quantitative counts of the number of colloidal gold particles over the matrix and inclusions were conducted on LR White-embedded samples. Counts showed that the inclusions had at least twice as much label as the corresponding protein body matrix (Table I). Labeling was heaviest at the 14 DAF stage (the earliest stage in which large numbers of inclusions were present and the labeling could be counted) and decreased during development. Concomitantly, the labeling of the matrix also decreased during the same period. The ratio of number of gold particles on inclusions to those on the protein body matrix remained a constant 2.1–2.3 for 14–21 DAF but increased to 3.6 at 24 DAF.

Double labeling with antitriticin and anti-HMW glutenin subunit antibodies was conducted on LR White-embedded UV-cured 21-DAF wheat using 5- and 10-nm gold-GAR, respectively. When HMW glutenin subunit antibodies were used first and antitriticin second, there were 2.8 times more 5-nm gold particles than 10-nm particles on the protein body matrix (Fig. 13). If triticin antibodies were used first, followed by anti-HMW glutenin subunit antiserum, the ratio of 5- to 10-nm gold particles was 10.7 for the matrix protein (Fig. 14). In both cases, the number of 5-nm gold particles on the inclusions was similar, and no 10-nm gold particles were observed on the inclusions (Figs. 13 and 14).

DISCUSSION

Protein bodies of wheat starchy endosperm are composed of a matrix of storage proteins in which a variety of inclusions are embedded (Parker 1980, Bechtel et al 1982b). Electron-dense spherical inclusions in protein bodies of wheat have been long recognized (see Parker, 1980 for a review), but little information has been obtained on their composition. Protein bodies stained with Sudan black B gave an intense positive reaction for lipid at the protein body periphery (Parker 1980). The inclusions, however, are resistant to digestion by a variety of proteases (Parker 1980, Bechtel et al 1982b). This information led to speculation that they represented a lipoprotein complex (Parker, 1980) or that they are the last-added protein and for some unexplained reason are resistant to enzymatic extraction (Bechtel et al 1982b). A preliminary search was conducted to try to locate the cytoplasmic source of the inclusions, but none could be conclusively identified. We are currently conducting experiments to try to identify the cytoplasmic origin of these electron-dense inclusions.

The use of a variety of antisera raised against several wheat and barley storage proteins and against the 12S "legumin" globulins of oats failed to identify any immunoreactive proteins in the dense inclusions, although each antiserum reacted with the homogeneous protein body matrix. These results were not altered even when LR White resin was substituted for Spurr resin, despite the fact that Spurr resin has been shown to adversely affect immunocytochemical labeling (Craig and Goodchild, 1982).

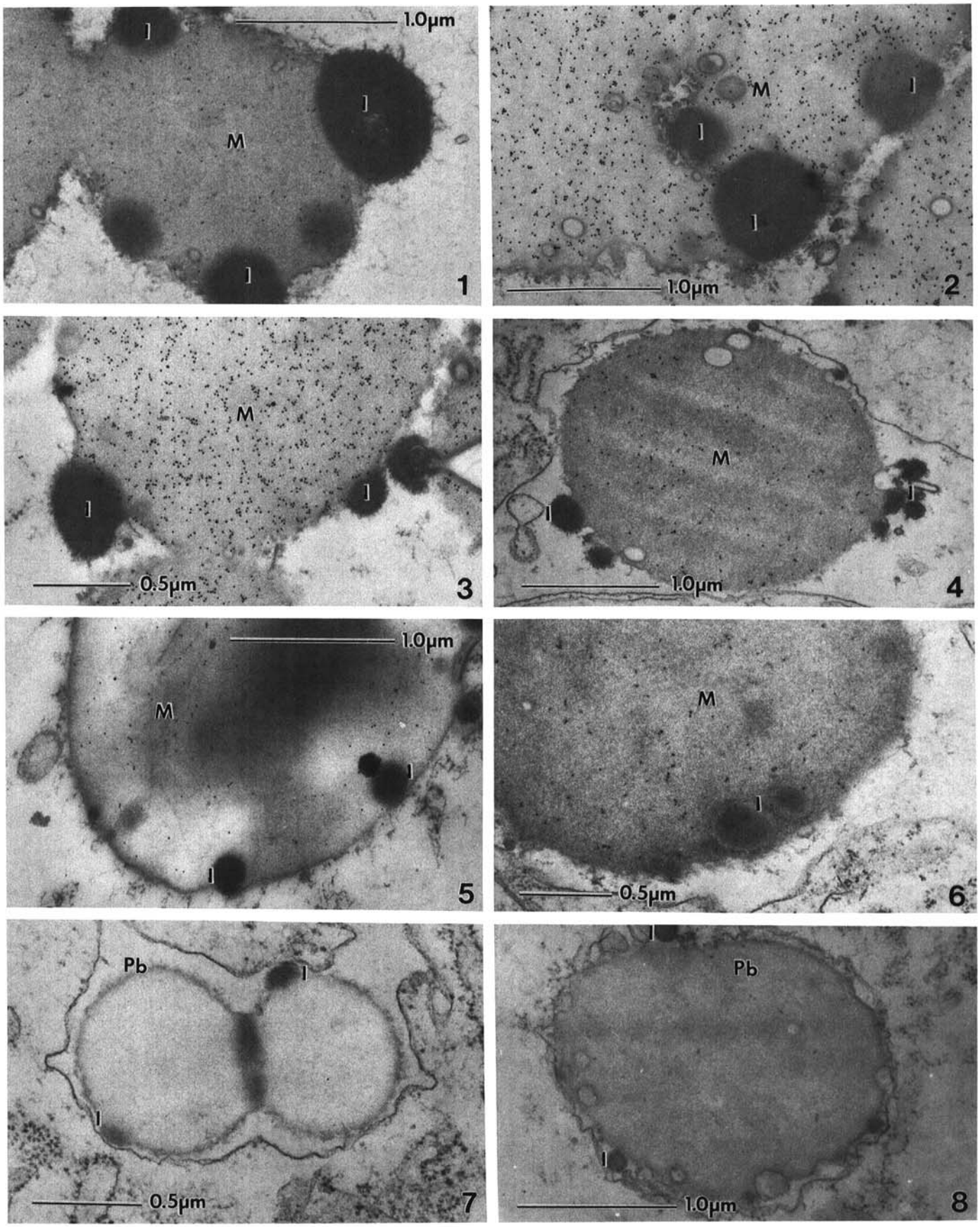
The only antiserum that consistently reacted with the dense inclusions was that to triticin, although this also labeled the protein body matrix to a lesser extent. The amount of labeling obtained using the triticin antiserum was affected by the embedding medium used. LR White resin consistently gave better labeling for both the dense inclusions and matrix than did Spurr resin. We have no explanation why some antibodies give less intense labeling with Spurr resin while others seem unaffected, but a possible explanation is that masking or blocking of antigenic determinants on certain proteins occurs when they are embedded in Spurr resin.

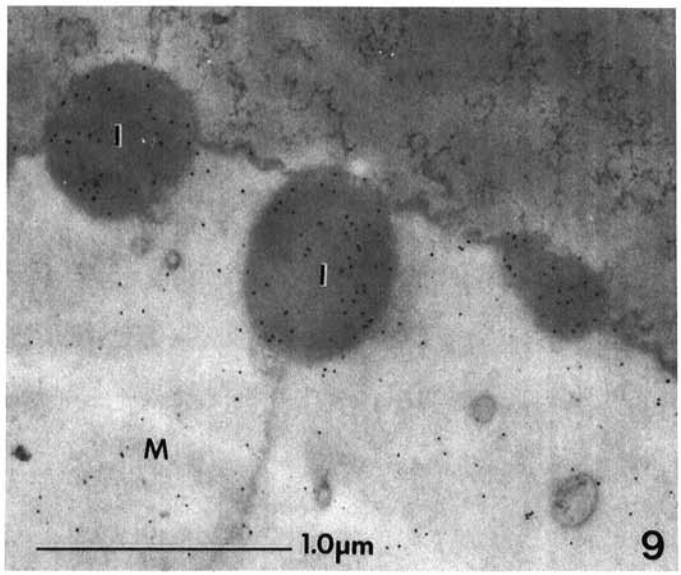
Segregation of different types of proteins within protein bodies has been previously observed in pea embryos (Craig 1986). In this case, the core of the protein granules consisted of globulins, while the periphery contained a sulfur-rich albumin (PA 1). Wheat protein bodies exhibit a different type of segregation; prolamins and glutenins occupy the protein body matrix, while discrete inclusions contain the leguminlike storage protein triticin.

There is sufficient homology between triticin and HMW subunits of glutenin to account for the labeling of the matrix proteins with the antitriticin antibodies, and this possibility is supported by the double labeling experiments. These show that the labeling of the matrix proteins with antitriticin can be reduced by a previous reaction with anti-HMW subunit. The broad reac-

Figs. 1–8. Immunocytochemistry of wheat storage proteins. **1,** wheat endosperm protein body at 11 days after flowering (DAF) embedded in LR White resin and labeled with anti-α-gliadin/gold-goat-anti-rabbit (gold-GAR), showing light labeling of protein matrix (M) but the lack of label on inclusions (I) (×30,780). **2,** LR White-embedded 14-DAF wheat protein body labeled with anti-γ-gliadin and gold-GAR, yielding heavy matrix labeling and no inclusion labeling (×29,700). **3,** nine-DAF LR White-embedded protein body labeled with anti-high molecular weight (HMW) glutenin subunit/gold-GAR, showing lack of inclusion label (×36,000). **4,** protein body from 18-DAF wheat embedded in LR White resin and stained with anti-C-hordein/gold-GAR. Light labeling of matrix but no inclusion labeling was achieved (×26,300). **5,** wheat endosperm (18 DAF) fixed in paraformaldehyde only, embedded in LR White resin, and labeled with anti-12S oat globulin/gold-GAR. Labeling is lightly distributed over matrix but not inclusions (×30,700). **6,** wheat protein body processed as in Fig. 2 but labeled with a monoclonal anti-A-gliadin and gold-goat-anti-mouse. Note lack of label on inclusions (×35,100). **7,** wheat protein body (Pb) with inclusions processed as in Fig. 2 but labeled with preimmune serum instead of primary antibody. Note lack of label (×40,400). **8,** protein body and inclusions processed as in Fig. 4 and treated with buffer instead of primary antibody, showing lack of labeling (×30,700).

tion of triticin antiserum with gliadins and glutenins, including the HMW subunit, suggests that the labeling of the protein body matrix may be due to cross reactivity. The general reduction in the amount of labeling as the endosperm tissue ages can possibly be explained by changes in penetration of the larger protein bodies by the plastic embedding media. The important observation, however, is that the triticin antiserum is the only antiserum that reacts with the dense inclusions. This is all the more profound when one considers the broad range of specificities of the antisera used in this study. Thus, the only epitopes being recognized in

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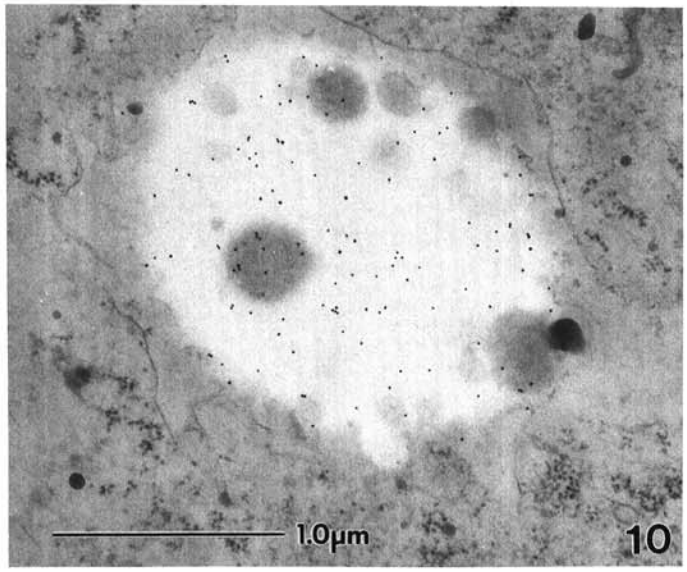
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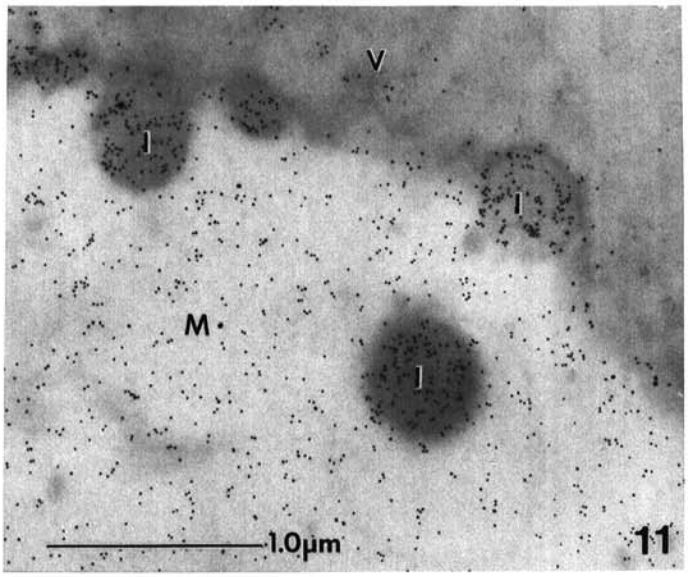
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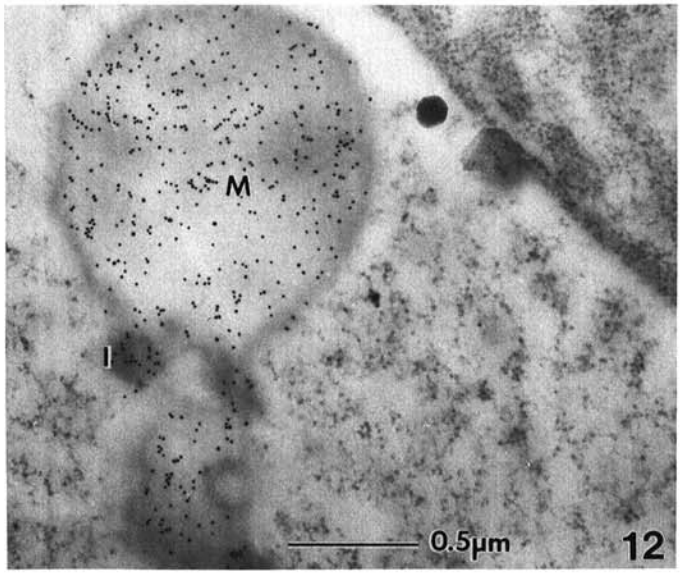
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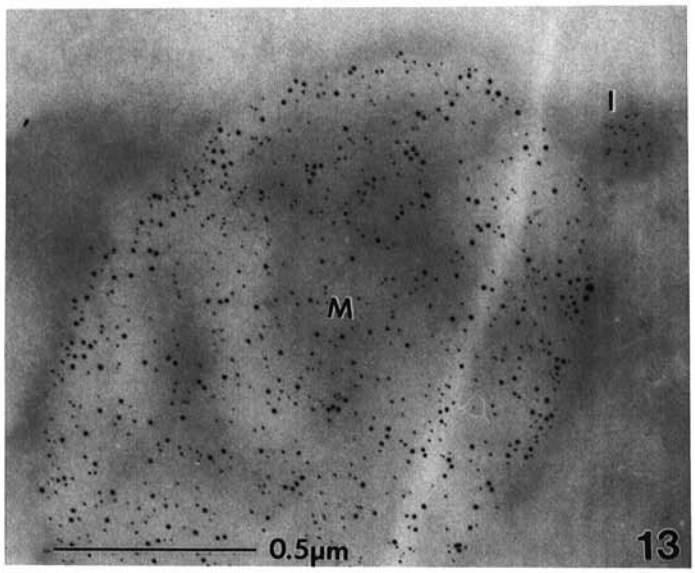
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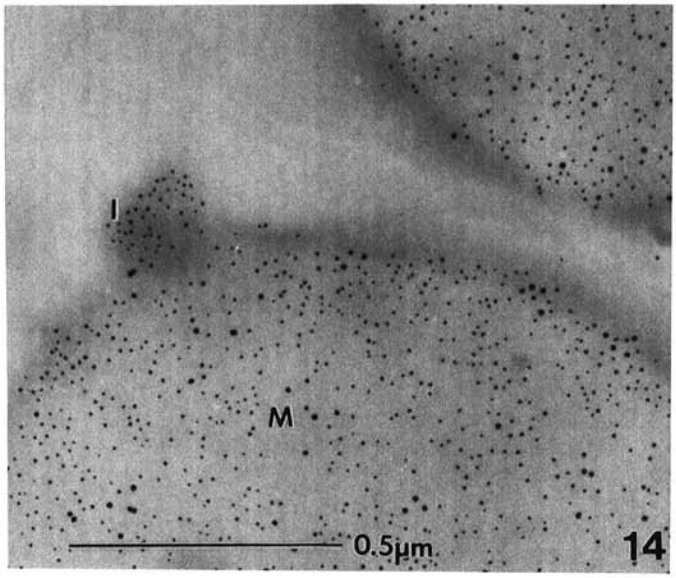
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the inclusions are those of triticin.

The reaction pattern of the anti-12S oat globulin is more difficult to explain. The 12S oat globulin, rice glutelins, and triticin are related to the 11S legumin-type globulins in many plant groups, and the 12S globulin antibodies react with triticin on immunoblots (Singh et al 1988). However, this antiserum also reacted strongly with a number of gluten proteins, most notably the HMW glutenin subunits (Singh et al 1988). It would therefore be expected to label the protein body matrix and inclusions, whereas it labeled only the former. The explanation may lie in differences in epitope presentation or masking in the two phases.

In conclusion, our results are consistent with all or most of the triticin being located in the electron-dense inclusions in wheat protein bodies, but we cannot rule out the possibility that some is also present in the homogeneous protein body matrix. Since the origin of the electron-dense inclusions has not been determined, we cannot tell whether the deposition mechanism parallels that of other storage proteins in the protein body matrix.

TABLE I
Quantitative Counts of Antitriticin, Gold-Labeled Wheat Sections[a]

Days After Flowering	Number of Particles per Square Micrometer		Ratio of Inclusions to Matrix
	Matrix	Inclusions	
14	3.3	7.2	2.2
18	2.6	5.9	2.3
21	2.7	5.6	2.1
24	1.4	5.1	3.6

[a] Minimum of 55 counting areas per sample. Approximately 13 μm^2 of inclusions and 90 μm^2 of matrix protein were measured for each sample day.

Figs. 9–14. Immunocytochemistry of triticin localization in wheat endosperm. **9,** wheat endosperm at 11 days after flowering (DAF) embedded in Spurr resin and labeled with antitriticin and gold-goat-anti-rabbit (gold-GAR), showing light localization on both matrix (M) and inclusions (I) (×33,100). **10,** conditions as in Fig. 9, showing extremely low background labeling of cytoplasm (×30,200). **11,** wheat endosperm embedded in LR White resin at 18 DAF labeled with antitriticin and gold-GAR, showing increased labeling in both matrix and inclusions. Note labeling in the vacuole (V) (×28,400). **12,** protein body from 21-DAF wheat embedded in LR White resin and UV-polymerized. The heavy gold label from antitriticin/gold-GAR is restricted to matrix and inclusion (×34,400). **13,** wheat endosperm processed as in Fig. 12 and double labeled, first with anti-HMW glutenin subunit/10-nm gold-GAR and then with antitriticin/5-nm gold-GAR. Note that the inclusion has only 5-nm gold label and the matrix has both 5- and 10-nm label. The section was unstained (×54,000). **14,** same sample as in Fig. 13, but labeling reversed (i.e., antitriticin/5-nm gold-GAR followed by anti-HMW glutenin subunit/10-nm gold-GAR). Note that the inclusion has only 5-nm gold label and the matrix has much more 5-nm than 10-nm gold label. The section was unstained (×72,000).

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