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Synthesis and Deposition of Zein in Protein Bodies of Maize Endosperm[1]

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ABSTRACT

The origin of protein bodies in maize (*Zea mays* L.) endosperm was investigated to determine whether they are formed as highly differentiated organelles or as protein deposits within the rough endoplasmic reticulum. Electron microscopy of developing maize endosperm cells showed that membranes surrounding protein bodies were continuous with rough endoplasmic reticulum membranes. Membranes of protein bodies and rough endoplasmic reticulum both contained cytochrome *c* reductase activity indicating a similarity between these membranes. Furthermore, the proportion of alcohol-soluble protein synthesized by polyribosomes isolated from protein body or rough endoplasmic reticulum membranes was similar, and the alcohol-soluble or -insoluble proteins showed identical [^{14}C]leucine labeling. These results demonstrated that protein bodies form simply as deposits within the rough endoplasmic reticulum.

Messenger RNA that directed synthesis of only the smaller molecular weight zein subunit was separated from mRNA that synthesized both subunits by sucrose gradient centrifugation. This result demonstrated that separate but similar sized mRNAs synthesize the major zein components. *In vitro* translation products of purified mRNAs or polyribosomes were approximately 2,000 daltons larger than native zein proteins, suggesting that the proteins are synthesized as zein precursors. When intact rough endoplasmic reticulum was placed in the *in vitro* protein synthesis system, proteins corresponding in molecular weight to the native zein proteins were obtained.

During development of cereal seeds, storage proteins are synthesized in the endosperm and deposited in protein bodies (5, 9, 19). Based on electron micrographs of developing wheat endosperm, Morton and co-workers (20) concluded that these protein bodies originated in specialized protein-forming organelles (plastids) which contained polyribosomes distinct from those of the general ER. An ultrastructural study of developing maize seeds led Khoo and Wolf (11) to conclude that the endosperm protein bodies originated in vesicles produced by RER or formed at the enlarged ends of RER cisterna.

Recent experiments have demonstrated that protein bodies in maize (3, 13) and barley endosperm (2, 6) contain the major prolamine proteins and that these proteins are synthesized by membrane-bound polyribosomes. In some of these studies membrane-bound polyribosomes were isolated from the particulate fraction that pelleted between 500 and 37,000*g*[2] (2, 6, 13). In another study polyribosomes were isolated from a protein body fraction obtained by continuous sucrose gradient centrifugation (3). Because of the attachment of ribosomes to the membrane surrounding protein bodies, several investigators concluded that protein bodies are highly differentiated sites of storage protein synthesis (3, 20, 27), and furthermore that mRNAs isolated from "protein body" polyribosomes represent a homogeneous source of mRNAs (4). Since the particulate fraction that sediments between 500 and 37,000*g* contains a mixture of RER, protein bodies, and other membranous inclusions, polyribosomes obtained from this fraction may represent a heterogeneous sample of mRNAs.

To determine the origin of maize endosperm protein bodies and the heterogeneity of mRNAs associated with them, we have done ultrastructural and cell fractionation studies. Results from these studies as well as experiments analyzing *in vitro* products of purified zein mRNAs are reported here.

MATERIALS AND METHODS

Electron Microscopy. Sections of 19-day postpollination endosperm tissue from the maize (*Zea mays* L.) inbred line W64A were fixed 1.5 to 2 hr at room temperature in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer (pH 7.2). Samples were rinsed with buffer and postfixed 3 hr in 2% osmium tetroxide in the dark. Fixed specimens were rinsed in H_2O, soaked 8 to 12 hr in 0.5% aqueous uranyl acetate, dehydrated in acetone, and embedded in Epon. Thin sections were stained with lead citrate and viewed with a Philips EM 200 electron microscope. Isolated cell fractions were pelleted and treated similarly.

Cell Fractionation and Polyribosome Isolation. Developing maize seeds were frozen in liquid N_2 22 days after pollination and stored at −80 C (13). After homogenization of frozen kernels in 3 volumes of buffer A (0.2 M Tris-HCl [pH 8.5], 0.2 M sucrose, 60 mM KCl, 50 mM $MgCl_2$, 1 mM DTT), the extract was strained through cheesecloth and centrifuged in a Sorvall SS-34 rotor at 500*g* for 5 min. Total membrane-bound polyribosomes in the 500*g* supernatant (which contained a mixture of protein bodies and RER) were obtained by centrifugation at 37,000*g* for 10 min. The membranous pellet was suspended in buffer A containing 1% Triton X-100, and detergent-insoluble material was removed by centrifugation at 37,000*g* for 5 min. The supernatant fraction was layered over 4 ml of 2 M sucrose in buffer B (40 mM Tris-HCl [pH 8.5], 20 mM KCl, 10 mM $MgCl_2$) and polyribosomes were pelleted by centrifugation at 230,000*g* for 3 hr in the 65 rotor of a Beckman L5-65 ultracentrifuge.

A modification of the procedure of Burr and Burr (3) was used to isolate polyribosomes associated with protein bodies. Samples of 3 ml of the 500*g* supernatant fraction were layered on 200 to 800 mg/ml linear sucrose gradients in buffer A and centrifuged at 80,000*g* for 30 min in a Beckman SW 27 rotor. After removing the upper portion of the gradient by aspiration, the layer of protein bodies was recovered, diluted with buffer A minus sucrose, and pelleted at 37,000*g*. Polyribosomes were then isolated by the methods used for total membrane-bound polyribosomes.

Alternatively, protein body polyribosomes and polyribosomes of the RER were separated using discontinuous sucrose gradients. Samples of 20 ml of the 500*g* supernatant were layered on gra-

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[2] Abbreviation: *g* gravitational force determined at R_{av}.

dients consisting of 3 ml of 2.0 M sucrose, 7.5 ml of 1.5 M sucrose, and 8 ml of 0.5 M sucrose in buffer B. After centrifugation at 80,000*g* for 2 hr in a Beckman SW 27 rotor, the upper portion of the gradient was removed by aspiration, and membranes between the 0.5 and 1.5 M sucrose layers and protein bodies between the 1.5 M and 2 M sucrose layers were removed. Samples were diluted 5-fold with buffer A minus sucrose and pelleted by centrifugation at 37,000*g* for 1 hr. Polyribosomes were recovered from these fractions by the procedure used for total membrane-bound polyribosomes.

Enzyme Determinations. Maize kernels frozen 22 days after pollination were homogenized in 4 volumes of buffer C (0.25 M Tris-MES [pH 7.2], 0.25 molar sucrose, 3 mM EDTA, 1 mM DTT) and centrifuged 5 min at 500*g*. Samples of 2 ml were layered on 200 to 800 mg/ml sucrose gradients in buffer C and centrifuged for 2 hr at 80,000*g* in a Beckman SW 27 rotor. Gradients were scanned continuously at 254 nm with an ISCO model UA-5 absorbance monitor and fractionated into 0.4-ml aliquots with an ISCO model 640 gradient fractionator. NADH-Cyt *c* reductase was assayed at 25 C by following the reduction of Cyt *c* at 550 nm. The 3-ml reaction mixture contained 0.05 to 0.25 ml of the gradient fraction, 1.66 mM sodium cyanide, 120 μM Cyt *c*, 50 mM phosphate buffer (pH 7.5), and 230 μM NADH. The rate of Cyt *c* reduction was estimated with an extinction coefficient for Cyt *c* of 18.5 mM^{-1} cm^{-1} (8).

Cyt *c* oxidase was assayed by following the oxidation of Cyt *c* at 550 nm. Each 3-ml reaction volume contained 0.05 to 0.25 ml of the gradient fraction, 60 μM Cyt *c* reduced with Na-dithionite, 0.01% digitonin, and 50 mM phosphate buffer (pH 7.5). The rate of Cyt *c* oxidation was calculated according to Smith (26).

Isolation and Fractionation of Poly(A) RNA from Membrane-bound Polyribosomes. Methods for poly(A) RNA isolation were modified from those previously described (14). After elution of poly(A)-containing RNA in 10 mM HEPES (pH 7.5), the sample was heated to 60 C for 3 min and rapidly chilled to 4 C. Solid KCl was added to make the sample 0.3 M and the RNA was rehybridized to oligo(dT)-cellulose. This procedure was repeated three times, and the RNA was precipitated with 2.5 volumes of absolute ethanol after adding 0.1 volume of 3 M K-acetate.

Poly(A) RNA was analyzed on linear log sucrose gradients similar to those previously described (14). The RNA was heated to 60 C for 3 min, rapidly chilled to 4 C, and centrifuged at 120,000*g* for 17 hr in a Beckman SW 41 rotor. Gradients were scanned photometrically at 254 nm and 0.4-ml aliquots collected. After adding 0.5 ml of H_2O, 0.1 ml of 3 M K-acetate, and 1 A_{260} of tRNA, the RNA was precipitated with 2.5 volumes of absolute ethanol.

***In Vitro* Protein Synthesis.** A standard cell-free protein synthesis system (18) was prepared from wheat embryos. The complete system in a volume of 50 μl contained: 15 μl of wheat germ supernatant (1 A_{260} unit), 20 mM HEPES (pH 7.4), 2 mM DTT, 1 mM ATP, 20 μM GTP, 40 μg/ml creatine phosphokinase, 8 mM creatine phosphate, 2.5 mM Mg-acetate, 100 mM KCl, 0.25 μCi of [^{14}C]leucine (Amersham/Searle) and 25 μM 19 unlabeled amino acids. Samples were incubated at 25 C after one of the following was added: 0.75 to 1 A_{260} of polyribosomes suspended in H_2O, 2 to 4 μg of poly(A) RNA in H_2O, or 0.8 to 0.9 A_{280} of RER membranes in 10 mM HEPES (pH 7.5), 20 mM KCl, and 1 mM Mg-acetate. Reactions with mRNA were incubated for 60 min at 25 C and those with purified polyribosomes or RER were incubated for 30 min. Protein synthesis was terminated by the addition of 2 ml of cold 5% trichloroacetic acid or sufficient absolute ethanol to bring the final concentration to 70%. The percentage of ethanol-soluble protein synthesized *in vitro* was determined as previously described (13, 14). Hot 5% trichloroacetic acid-insoluble, hot 70% ethanol-soluble, or hot 70% ethanol-insoluble protein from the *in vitro* assays was dialyzed in an SDS buffer (0.05 M Tris-HCl [pH 6.9], 0.5% [w/v] SDS, and 1% [v/v] 2-mercaptoethanol) (16). Dried ethanol extracts of protein synthesis reactions containing RER membranes were washed with cold acetone before dialysis in SDS buffer.

SDS-Polyacrylamide Gel Electrophoresis and Fluorography. Radioactive samples were analyzed on slab gels with a SDS-polyacrylamide gel electrophoresis system similar to that described by Laemmli (12). Gels were 1.5 mm thick and consisted of a 9-cm running gel of 12.5% acrylamide (acrylamide/bisacrylamide = 75/1) in 3.75 mM Tris-HCl (pH 8.9), 0.058 mM TEMED, and 0.075% SDS. The running gel was overlaid with a 2.5-cm stacking gel of 5% acrylamide. Freshly prepared ammonium persulfate solution was added to a final concentration of 0.035% immediately before each gel layer was poured. Samples were applied in sample buffer (0.024 M Tris-HCl [pH 8.3], 1% SDS, 1% 2-mercaptoethanol, 0.002% bromophenol blue, and 10% glycerol). Electrophoresis was carried out at room temperature at 15-mamp constant current through the stacking gel and 25 mamp constant current through the running gel until the tracking dye reached the bottom of the gel. Gels were stained overnight in a solution of 0.1% Coomassie blue, 43% methanol and 8.6% acetic acid and destained in 22.5% methanol-7% acetic acid.

Zein isolated by ethanol extraction of protein bodies was used as a marker for the native protein. Radioactive proteins from *Escherichia coli* infected with φX-174 were kindly provided by T. Pollock of the Department of Biology, Purdue University, for use as radioactive mol wt standards.

Fluorography of dried gels was essentially as described by Laskey and Mills (15).

RESULTS

Endosperm cells of 19-day-old maize kernels were characterized by an extensive network of RER, starch grains, and by the presence of numerous protein bodies. The interconnecting cisternae of RER appeared discontinuous in cross-section (Fig. 1A), but surface sections (Figs. 1B and 2A) revealed that this discontinuity was due to the presence of pores. A striking feature of surface sections of RER was the presence of numerous ribosomes in polyribosome configuration (Figs. 1B and 2A). Protein bodies were prominent in endosperm cells (Fig. 1A); they were circular to ovoid in profile, had a finely granular, uniformly stained matrix, and were bounded by a membrane. Ribosomes and polyribosomes were associated with protein body membranes and were especially distinct in protein bodies sectioned obliquely (Figs. 1B and 2A). The protein bodies were closely associated with RER (Fig. 1A), and in some instances protein body membranes were directly connected with RER (Fig. 1B).

To determine if membranes associated with protein bodies had properties of ER, the 500*g* supernatant was analyzed on a continuous sucrose gradient, and the distribution of Cyt *c* reductase was monitored. This enzyme is commonly used as a marker for ER (7, 17, 23). As indicated in Figure 3, the gradient revealed three peaks of Cyt *c* reductase activity. The region of highest activity had the greatest UV absorbance and remained near the top of the gradient. This region corresponded to stripped RER, since it was displaced to a greater density when Mg was included in the gradient (data not shown). The second zone of Cyt *c* reductase activity had little UV absorbance and corresponded to mitochondria, since it also had Cyt *c* oxidase activity. The third peak of Cyt *c* reductase activity corresponded to a zone of high UV absorbance (light scattering). When a sample from this region of the gradient was examined with the electron microscope it was found to contain protein bodies (Fig. 2B), indicating that the enzyme activity was associated with protein body membranes.

When the 500*g* supernatant was centrifuged on discontinuous sucrose gradients consisting of layers of 0.5, 1.5, and 2.0 M sucrose in buffer B, two prominent light scattering zones were formed. One band, between 0.5 and 1.5 M sucrose was yellow, and exam-

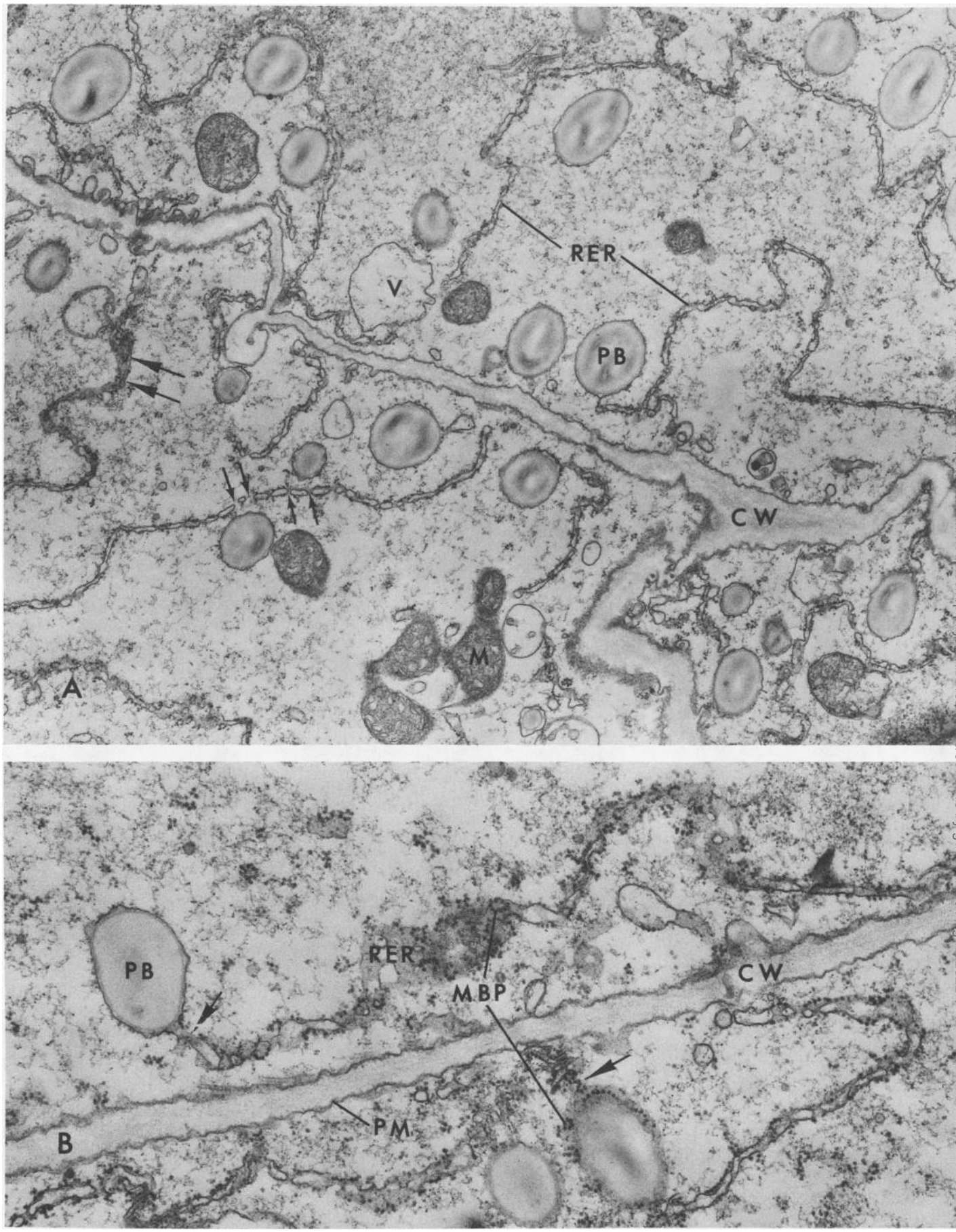

FIG. 1. Electron micrographs of 19-day maize endosperm cells. A: pronounced features of the endosperm cell were rough endoplasmic reticulum (RER), protein bodies (PB), and mitochondria (M). RER is discontinuous in cross-section (small arrows) but is continuous in surface sections (large arrows) (× 17,800). B: cross- and oblique sections of protein bodies show continuity between protein body membranes and extended RER cisternae (arrows). Membrane-bound polyribosomes (MBP) are evident in surface sections of RER and membranes of protein bodies (see also Fig. 2A). CW: cell wall; PM: plasma membrane; V: vacuole (× 33,500).

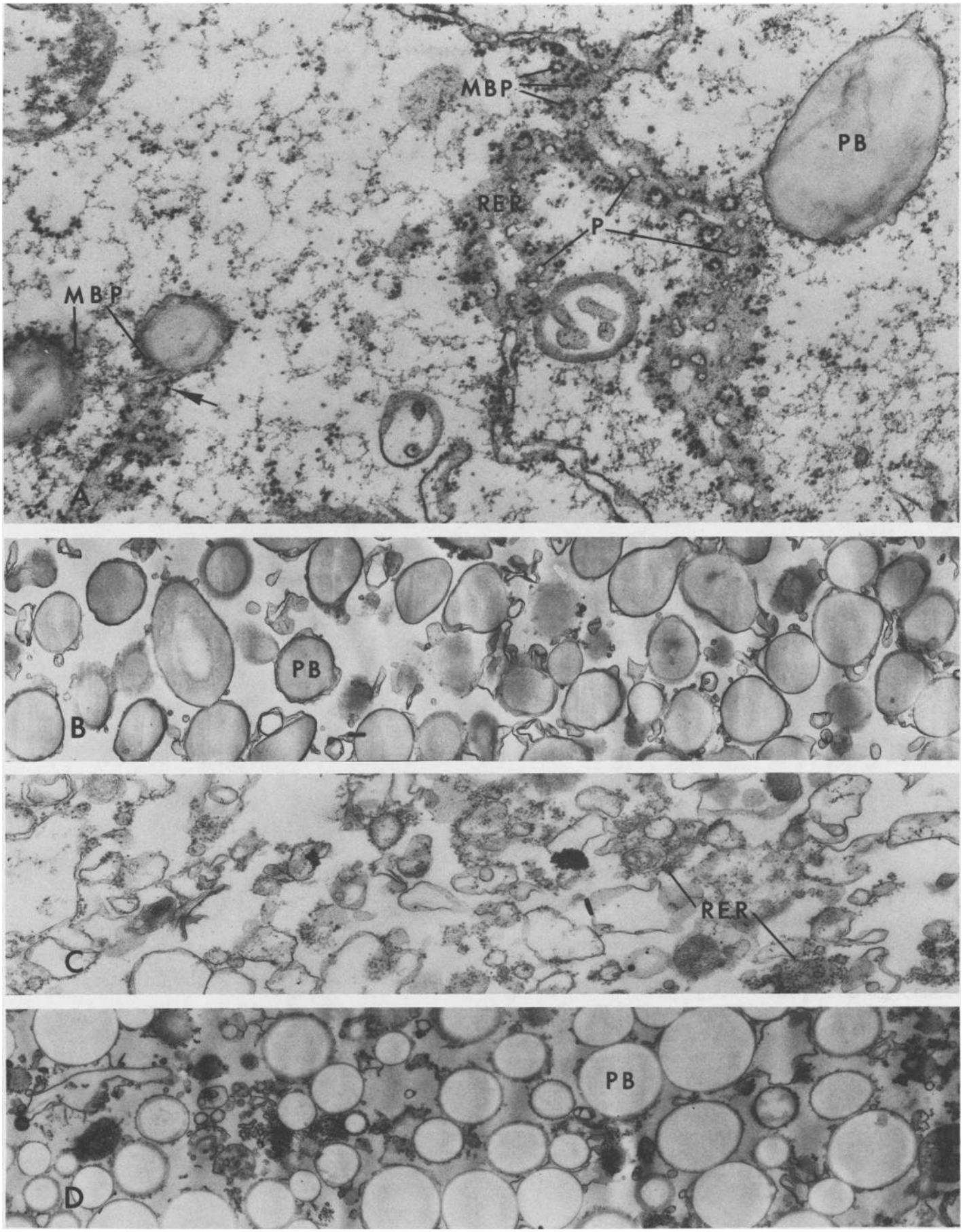

FIG. 2. Electron micrographs of maize endosperm. A: rough endoplasmic reticulum (RER) in surface section was characterized by membrane-bound polyribosomes (MBP) and numerous pores (P) (× 39,000); B representative sample of protein bodies (PB) from a linear sucrose gradient assayed for Cyt *c* reductase (ribosomes are not associated with membranes because of EDTA in the grinding buffer) (× 20,000) C: fraction enriched in RER sedimenting between 0.5 and 1.5 M sucrose in discontinuous sucrose gradient (× 25,000). D: protein body fraction sedimenting between 1.5 and 2.0 M sucrose from a discontinuous sucrose gradient (× 20,000).

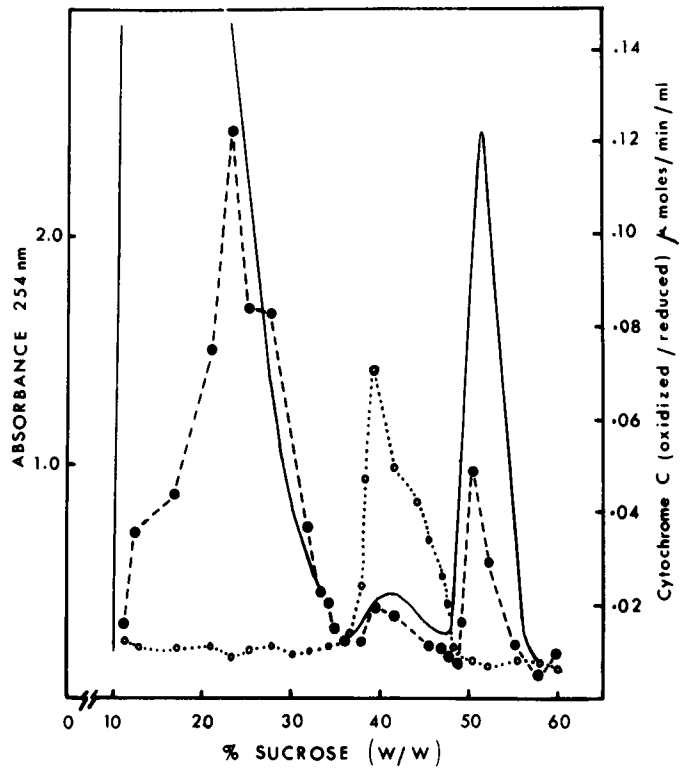

FIG. 3. Distribution of Cyt *c* reductase and Cyt *c* oxidase activity after sucrose gradient centrifugation of maize endosperm extract. Methods for gradient fractionation and assay of enzyme activity are described under "Materials and Methods." (——): absorbance at 260 nm; (– – –): Cyt *c* reductase activity; (.....): Cyt *c* oxidase activity.

Table I. Comparison of ^{14}C-leucine incorporation into total and ethanol-soluble protein synthesized by membrane-bound polyribosomes

Procedures for polyribosome isolation and in vitro protein synthesis are described in "Materials and Methods." Fractions A and B correspond to polyribosomes derived from rough endoplasmic reticulum and protein bodies, respectively, from discontinuous sucrose gradients. A mixture of A and B was obtained by isolating polyribosomes from membranes pelleted between 500 and 37,000*g*. Polyribosomes in fraction PB were obtained from protein bodies recovered from a continuous sucrose gradient. Each 50 μl assay contained approximately 0.85 A_{260} of polyribosomes in Experiment 1, and 0.62 A_{260} in Experiment 2. The radioactive counts were the average of triplicate assays and are expressed as cpm/50μl reaction.

	Fraction A		Fraction B		Fractions A & B		Fraction PB	
	Total cpm	% ethanol soluble	Total cpm	% ethanol soluble	Total cpm	% ethanol soluble	Total cpm	% ethanol soluble
Exp. 1	116,000	58	106,000	61	92,000	70	---	---
Exp. 2	74,000	45	58,000	50	------	---	53,000	52

ination with the electron microscope showed it to be enriched in RER with no visible evidence of protein bodies (Fig. 2C). The absence of protein bodies was also confirmed by inability to detect zein proteins in ethanol extracts of this fraction. The second band, which was between 1.5 and 2.0 M sucrose layers, consisted primarily of protein bodies. Both ribosomes and polyribosomes were bound to the membranes surrounding protein bodies (Fig. 2D).

Several experiments were carried out to determine if there were differences between these two populations of membrane-bound polyribosomes. Groups of 50 kernels were homogenized in buffer A, and membrane-bound polyribosomes recovered from discontinuous sucrose gradients. The average recovery of polyribosomes from both fractions was 32 A_{260} units when 12 samples (10.5 g tissue) were extracted. Of this total, 20 A_{260} units were isolated from the RER and 12 A_{260} units were from protein bodies. Polyribosome profiles of the two fractions were identical, with polyribosomes containing 8 to 9 ribosomes as the most abundant size-class.

A comparison of the amount of ethanol-soluble protein synthesized *in vitro* by these polyribosomes is presented in Table I. In one experiment the protein synthesized by polyribosomes from RER, protein body membranes, or a mixture of both, was 58, 61, and 70% ethanol-soluble, respectively. In another experiment comparing polyribosomes from RER with those from protein bodies, 45 and 50%, respectively, of the products were ethanol-soluble.

No differences in the ethanol-soluble proteins synthesized by these isolates could be detected by fluorography after SDS-polyacrylamide gel analysis. We therefore analyzed the ethanol-insoluble radioactive proteins. The ethanol-insoluble proteins synthesized by polyribosomes from RER (Fig. 4A) or protein bodies (Fig. 4B, 4PB) appeared to be identical. The size distribution of proteins in these samples was heterogeneous, although there were distinct bands whose mol wt corresponded to the major ethanol-soluble proteins. Neither the source nor the method by which the membrane-bound polyribosomes were isolated qualitatively altered the pattern of labeling.

A sucrose gradient analysis of the mRNA isolated from a mixed membrane fraction is presented in Figure 5. The mRNA, which sedimented at approximately 13S under these conditions (14), was separated into small fractions and translated *in vitro*. An analysis of hot acid-insoluble products synthesized by the fractionated mRNA is shown in Figure 6. The synthesis of neither of the major zein components was directed by mRNA in fraction 10. The

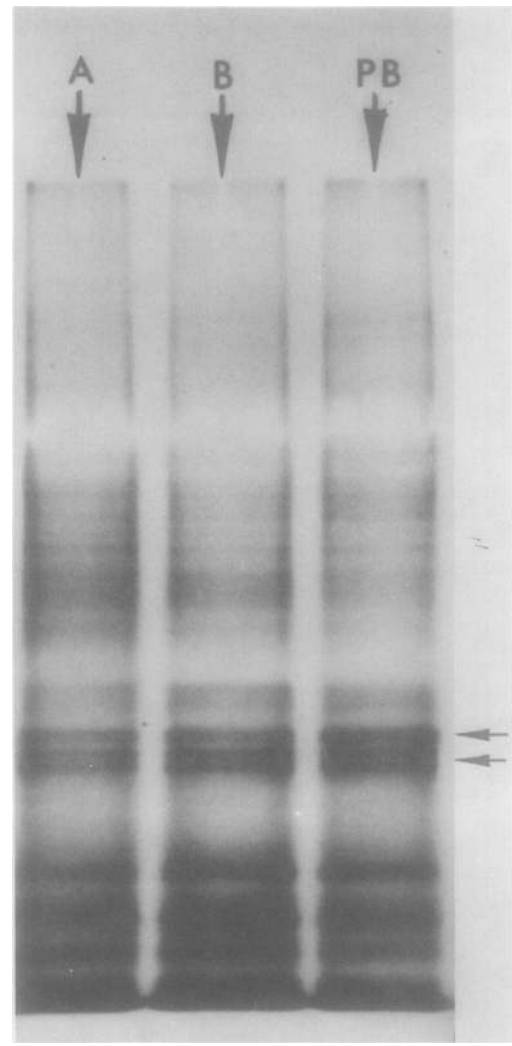

FIG. 4. SDS-polyacrylamide gel analysis of alcohol-insoluble proteins synthesized by membrane-bound polyribosomes from (A) RER; (B) protein bodies from discontinuous sucrose gradients; and (PB) protein bodies from continuous sucrose gradients. Methods for polyribosome isolation, *in vitro* protein synthesis, and fluorography of SDS-polyacrylamide gels are described under "Materials and Methods." Samples contained approximately equal amounts of radioactivity. Arrows indicate position of major ethanol-soluble radioactive proteins.

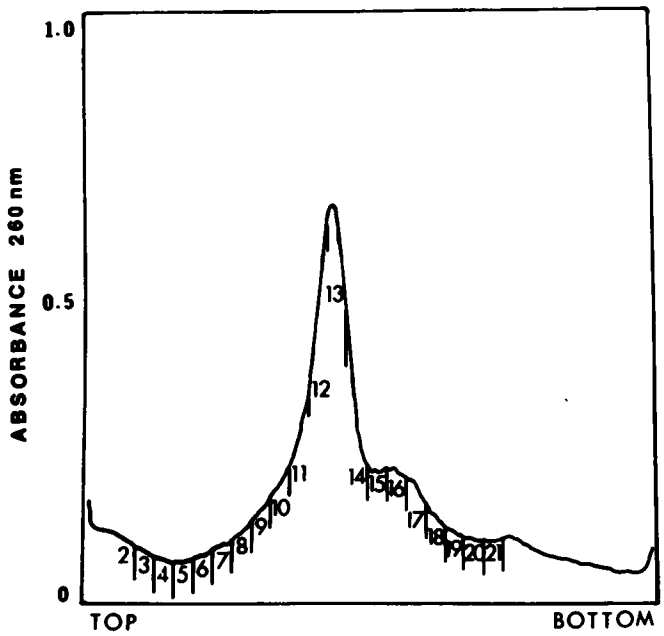

FIG. 5. Sucrose gradient analysis of poly(A)-containing RNA isolated from total membrane-bound polyribosomes. RNA was heated to 60 C for 3 min and rapidly chilled before layering on linear log sucrose gradients. After centrifugation at 120,000*g* for 17 hr gradients were fractionated into 0.4-ml aliquots and the RNA precipitated.

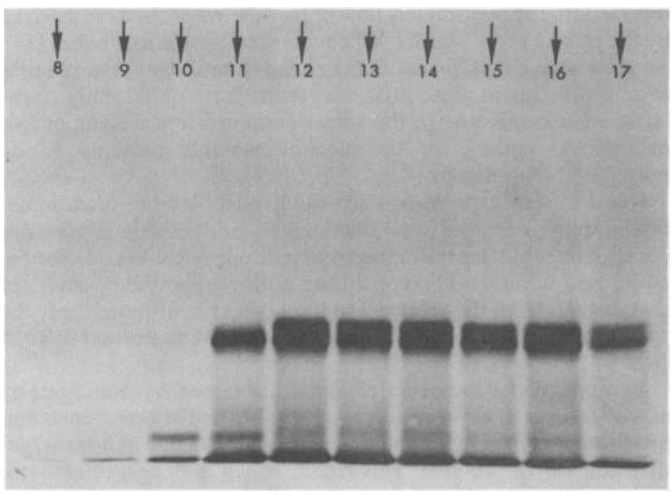

FIG. 6. SDS-polyacrylamide gel analysis of the hot 5% trichloroacetic acid-insoluble proteins synthesized by fractionated mRNA. Sample numbers correspond to the gradient fractions designated in Figure 5. Samples contained approximately equal amounts of radioactivity except for reactions that had little [^{14}C]leucine incorporation into protein. The x-ray negative was exposed for 20 hr at −80 C.

mRNA in fraction 11 directed synthesis of only the smaller zein component, while that in fraction 12 (and several subsequent fractions) directed synthesis of both zein components. These proteins corresponded to the major zein components as previously reported (13, 14); however, they migrated more slowly in the gel than the native proteins. Compared to labeled ϕX-174 proteins (Fig. 7), the mol wt of the native proteins (indicated by the arrows) were 19,000 and 22,000 daltons and the proteins synthesized *in vitro* were 21,000 and 24,000 daltons.

Slightly larger transcripts of the two major zein components were synthesized when either mRNA or polyribosomes stripped from the ER were added to the *in vitro* protein-synthesizing system. To determine if ER was involved in the reduction in protein size, we compared the alcohol-soluble proteins synthesized by polyribosomes attached or stripped from RER membranes. The alcohol-soluble proteins synthesized by purified polyribosomes (Fig. 8A) were slightly larger than those synthesized by polyribosomes attached to ER membranes (Fig. 8B).

DISCUSSION

In an early study of storage protein synthesis in wheat endosperm, Morton and co-workers (20) concluded that protein bodies originated in specialized protein-forming organelles (plastids). A similar situation has been suggested for maize endosperm (3), and that isolation of polyribosomes associated with protein bodies permits isolation of homogeneous preparations of zein mRNA (4).

Our results favor the interpretation of Khoo and Wolf (11), who postulated that maize protein bodies are formed as prolamine deposits within the ER. This conclusion is based on observation of continuity between protein body membranes and RER, localization of Cyt *c* reductase activity in protein body and RER membranes, similarity in the proportion of alcohol-soluble protein synthesized, and identical labeling of proteins *in vitro* by protein body and RER polyribosomes.

Although it must be pointed out that in cross-section direct membrane continuity between protein body and RER membranes is not common (Fig. 1), such observation is hindered by the presence of pores in the RER (Figs. 1B and 2A). If protein bodies originate as localized dilations along the RER (11), the number of observed continuities would be statistically small.

We observed two peaks of Cyt *c* reductase activity in sucrose gradients corresponding to RER and protein body membranes. Although this finding indicated that membranes surrounding protein bodies are closely related to ER, it does not prove that polyribosomes on protein body membranes are identical to those on RER. Asymmetric distribution of RER exists in the pancreatic exocrine cell (21), as well as plant cells during cell plate formation (22). Such segregation has been cited as a possible indication of heterogeneity in polyribosome binding sites on the RER (25).

We tested for variation among membrane-bound polyribosome populations by comparing the amount of alcohol-soluble protein synthesized. While this proportion varied among experiments, the proportions were similar within any single experiment. In an

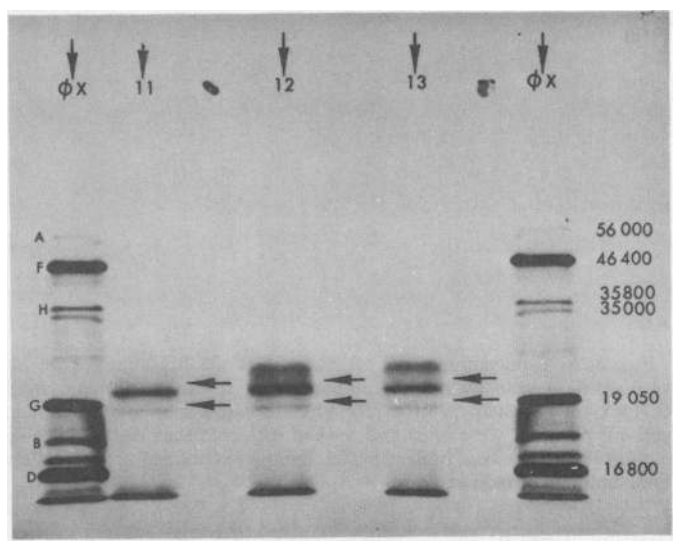

FIG. 7. Comparative migration of proteins synthesized by fractionated mRNA and ϕX 174 proteins. Samples 11 and 13 contained approximately 30,000 cpm [^{14}C]leucine, and sample 12 contained approximately 40,000 cpm. Horizontal arrows indicate the location of stained native zein proteins. Mol wt of the ϕX 174 proteins, A, F, H, G, B, and D were based upon nucleotide sequence calculations of Sanger *et al.* (24).

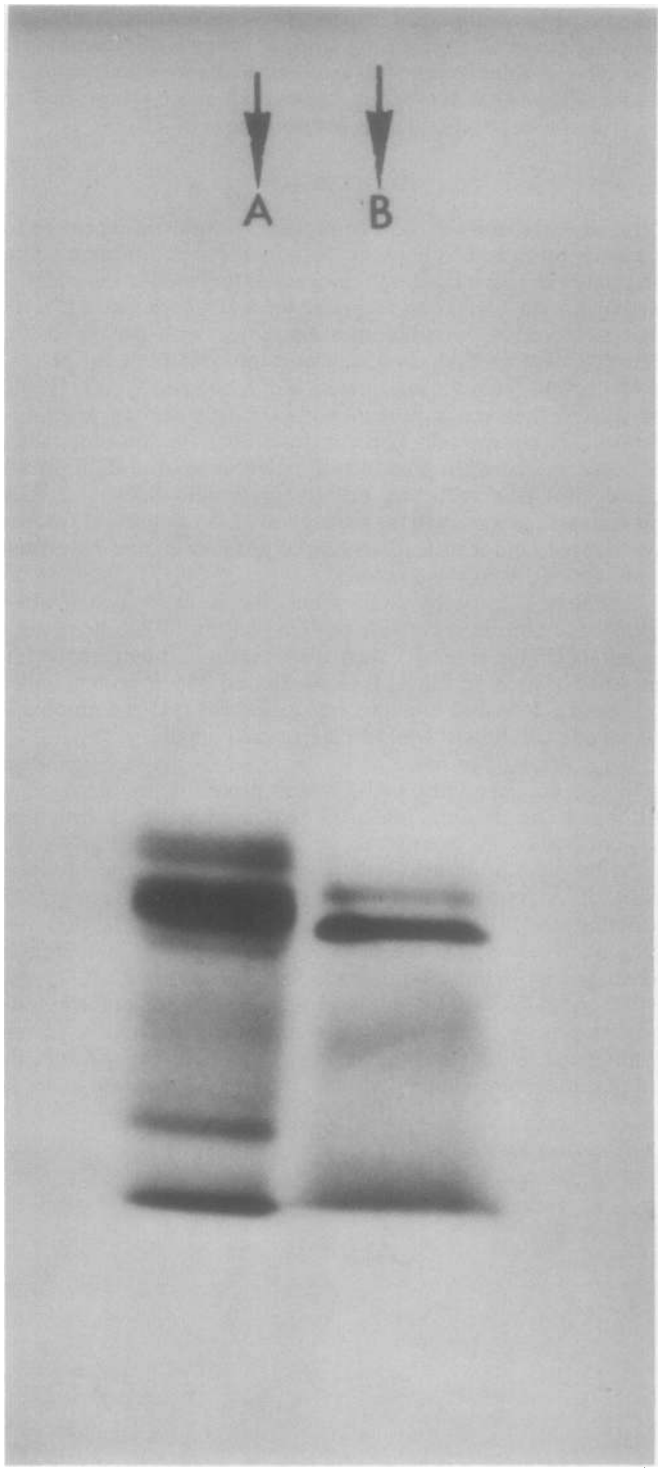

FIG. 8. SDS-polyacrylamide gel analysis of alcohol-soluble proteins synthesized *in vitro* by membrane-bound polyribosomes isolated from RER (A) or attached to RER (B). Ethanol extracts of *in vitro* protein synthesis reactions were dried and washed with cold acetone before being solubilized and dialyzed in SDS buffer. Samples contained approximately equal amounts of radioactivity.

experiment comparing polyribosomes from RER, protein bodies, or a mixture of the two, the percentage of alcohol-soluble counts was slightly greater for the mixed sample. To insure that this similarity was not a result of incomplete separation of RER and protein bodies with discontinuous sucrose gradients, we also compared these fractions with polyribosomes from protein bodies isolated from continuous gradients. Although the proportion of ethanol-soluble protein was less in this experiment (Table I), there was no significant difference resulting from the method by which protein bodies were isolated.

Since the similarity between alcohol-soluble proteins synthesized by different polyribosome preparations may result from selection for zein proteins by the extraction procedure, we also analyzed the alcohol-insoluble proteins on SDS-polyacrylamide gels. Samples from either the RER (Fig. 4A) or protein body polyribosomes (Fig. 4, B and C) contained a heterogeneous distribution of [^{14}C]leucine-labeled proteins. The pattern of labeling between these fractions was identical, and was independent of whether polyribsomes were from protein bodies isolated by continuous or discontinuous gradient centrifugation. The presence of labeled proteins in these samples of similar mol wt to the major zein proteins may reflect incomplete extraction of zein by ethanol.

Our results suggest that homogenization of developing endosperm results in fragmentation of RER, and because of the greater density of protein bodies, two fractions of membrane-bound polyribosomes are recovered. The 60 to 40% distribution of polyribosomes between RER and protein bodies is therefore probably an artifact of tissue homogenization. A similar population of polyribosomes, hence mRNAs, exists in both fractions. Although zein mRNAs are predominant in these polyribosomes, other mRNAs are also present. Quantitative recovery of these mRNAs requires isolation of the total membrane-bound polyribosomes.

The mRNA from total membrane-bound polyribosomes showed a homogeneous size distribution in sucrose gradients (Fig. 5). When this mRNA was fractionated and translated *in vitro*, only the major zein subunits were evident (Fig. 6). We previously reported that approximately 90% of the labeled protein synthesized by the poly(A) RNA with [^{14}C]leucine was soluble in ethanol (14). As yet we have insufficient data to determine if the predominance of zein proteins in these products results from preferential translation of zein mRNAs in the wheat germ cell-free system, or loss of mRNAs coding for the alcohol-insoluble proteins during poly(A) RNA isolation.

Results from experiments in which mRNA from membrane-bound polyribosomes was fractionated and translated *in vitro* demonstrate that separate, but similar sized, mRNAs code for the major zein subunits. This conclusion is also supported by evidence that mRNA from the *opaque*-2 mutant, which synthesizes only the smaller zein component, has the same mol wt as normal mRNA (10).

Because of the increased resolution obtained by fluorography of SDS-slab gels, we were able to determine that *in vitro* translation products of zein mRNAs were approximately 2,000 daltons larger than the native proteins. This result, which is in agreement with the preliminary report of Burr *et al.* (4), suggests that the storage proteins are synthesized as precursors, similar to proteins of animal mRNAs derived from RER (25). It has been shown in several instances (25) that such precursors contain an extra N-terminal amino acid sequence. This sequence is postulated to initiate polyribosome attachment to RER, and is subsequently removed by proteolytic cleavage (1, 25). When such mRNAs are translated *in vitro* in the presence of RER membranes, proteolytic cleavage occurs and proteins of correct mol wt are obtained (1).

In order to determine if RER membranes were involved in processing zein proteins, we compared alcohol-soluble products from reactions containing RER and detergent-solubilized polyribosomes (Fig. 8). Although reactions containing RER gave less total [^{14}C]leucine incorporation than those containing purified polyribosomes, sufficient product was obtained for analysis by concentration of alcohol-soluble protein. A comparison of proteins from these reactions on SDS-polyacrylamide gels indicated that products of RER were of smaller mol wt. Although the reduction in size of the larger zein component in Figure 8B was more apparent than that of the smaller component, both radioactive

bands coincided with the native proteins. The reduction in size of the smaller zein component was less apparent as a result of more intense labeling of this protein by the purified polyribosome fraction (Fig. 8A). The alteration in protein size did not appear to result from nonspecific proteolytic activity or incomplete mRNA translation since proteins of distinct mol wt were synthesized, and these results were very reproducible. Although we have insufficient data to conclude that a portion of the N-terminal amino acid sequence was removed, this seems a reasonable explanation.

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