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Cell-free Synthesis of Globulin by Developing Oat (*Avena sativa* L.) Seeds[1]

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ABSTRACT

The primary storage protein synthesized during oat (*Avena sativa* L.) groat development is a globulin. Polysomes were isolated from oat groats 12 days after anthesis. These polysomes directed the incorporation of radioactive amino acids into protein in a cell-free protein synthesis system containing wheat germ supernatant. The Mg^{2+} optimum was 4 mM, the pH optimum was 6.8, and the amount of amino acid incorporation depended on polysome concentration. Incorporation of amino acids was linear for about 10 min and approached a maximum after 20 min. Using the initiation inhibitor, T-2 toxin, it was determined that about 36% of the amino acid incorporation was due to the initiation of new polypeptide chains. The *in vitro* product co-electrophoresed with authentic oat groat globulin on polyacrylamide-sodium dodecyl sulfate (SDS) gels. The cyanogen bromide peptides of the *in vitro* product partially corresponded with those from authentic globulin when electrophoresed on polyacrylamide-SDS gels. These data suggest that the *in vitro* product is primarily oat globulin. The polysome population was separated into membrane-bound and free polysomes. Membrane-bound polysomes synthesized about twice the amount of protein as did free polysomes. Products synthesized *in vitro* on both types of polysomes were essentially the same.

The mature oat (*Avena sativa* L.) groat (dehulled seed) contains approximately 15 to 20% protein, depending on cultivar and on environmental conditions during seed maturation. The storage protein of oats is composed primarily of a salt-soluble globulin fraction, whereas most cereals contain primarily alcohol-soluble prolamins. Globulin increases in a linear fashion with time from about 4 to 16 days after anthesis, and constitutes 50% of the storage protein by the 12th day (19). Under reducing and denaturing conditions (25), oat globulin separates into two major bands with mol wt of 31,000 and 21,000 daltons (17). In addition, from one to three heavier bands (mol wt about 56,000) are always present (17).

Until recently, little was known about storage protein biosynthesis in developing seeds. Boulter (2) and Danielson (4) suggested that developing seeds would be a good system in which to study protein biosynthesis because of the relative homogeneity of the storage protein products. Rabson and co-workers (20) found that the rate of protein synthesis in a cell-free system from maize (*Zea mays* L.) kernels varied with stage of development. Extracts from kernels 0 to 10 days after pollination showed higher rates of amino acid incorporation than those taken before pollination or 10 days after pollination. Morton and Raison (14) reported *in vitro* amino acid incorporation by extracts of developing wheat (*Triticum aestivum* L.) endosperm. Beevers and Poulson (1) not only elucidated the chronology of protein synthesis and deposition in developing pea (*Pisum sativum* L.), but they determined that albumins were synthesized early in development whereas globulins were formed later. Polysomes from French bean (*Phaseolus vulgaris* L.) cotyledons (24) and from maize kernels (8, 10, 11) directed the synthesis of G1 protein (analogous to legumin) and zein, respectively, when translated in the wheat germ cell-free protein synthesis system (13). Burr and Burr (3) showed that polysomes isolated from protein bodies of immature maize kernels synthesized zein *in vitro*.

The objectives of this study were to develop a cell-free protein synthesis system from developing oat groats and to characterize the products of synthesis.

MATERIALS AND METHODS

Plant Culture. Oat seeds (*Avena sativa* L., cv. Goodland) were planted six per pot (plastic, 11 × 14.5 cm) in a 1:1 peat-vermiculite mixture. Plants were grown in an environmental chamber programmed for a 16-hr photoperiod, 28,000 lux illuminance at the top of the plants, and a day/night temperature regime of 23/18 C. One week after sowing, plants were thinned to four/pot. Plants were irrigated daily with nutrient solution (18). Panicles were tagged at anthesis and were harvested 10 to 12 days hence. Caryopses (groats) were removed from the florets and stored in liquid N_2 for later use, or in some cases they were homogenized immediately without freezing. Freezing and storing oat groats in liquid N_2 before polysomes were extracted had no deleterious effects on their amino acid-incorporating ability.

Extraction of Polyribosomes. The procedure followed was modified from that of Davies *et al.* (5). Groats were homogenized with a chilled mortar and pestle. The homogenization buffer contained 250 mM sucrose, 200 mM tris-HCl (pH 8.5), 60 mM KCl, 30 mM Mg acetate, and 5 mM 2-mercaptoethanol. One g fresh weight of groats was used/3 ml of buffer. The homogenate was filtered through two layers of cheesecloth, solid sodium deoxycholate was added to a concentration of 0.3% (w/v), and the homogenate was centrifuged at 27,000*g* for 15 min at 4 C. The supernatant was then layered over a 3-ml sucrose cushion (1.5 M sucrose, 40 mM KCl, 10 mM Mg acetate in 40 mM tris-HCl [pH 8.5]) and centrifuged for 2 hr at 270,000*g* (avg) at 4 C in a type 65 rotor of a Spinco[4] model L2-65B ultracentrifuge.

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After centrifugation, the pellets were rinsed twice with a small amount of sterile distilled H_2O and suspended in 40 mM tris-HCl (pH 8.5) containing 40 mM KCl, 10 mM Mg acetate, and 20% (w/v) glycerol (suspension buffer). Washed polysomes were prepared by resedimenting the suspended polysomes for 2 hr through an identical sucrose cushion and by resuspending them in suspension buffer described above. Polysomes were stored in liquid N_2 until use. A portion of the preparation was diluted for absorbance readings. Average A_{260}/A_{280} absorbance ratio was 1.22. One A_{260} unit contains 50 μg rRNA/ml, based on $A_{260}^{0.1\%\ RNA} = 20$ (7).

Isolation of Membrane-bound and Free Polysomes. Membrane-bound and free polysomes were isolated as described by Larkins and Dalby (11) from groats homogenized in the same buffer used above. Membrane-bound and free polysomes were pelleted and suspended as described above; they were not washed.

Sucrose Gradient Analysis of Polysomes. Polysomes were layered on 15 to 26% (w/v) isokinetic sucrose gradients (15). The sucrose was dissolved in the suspension buffer without glycerol. The gradients were centrifuged at 200,000*g* (avg) in a Spinco SW 40 rotor for 90 min. Gradients were fractionated with an ISCO model 184 density gradient fractionator and the absorbance at 254 nm was recorded with an ISCO UA4 analyzer.

Cell-free Protein-synthesizing System and Assay. The standard reaction procedure for the incorporation of amino acids into protein was modified from that of Sun *et al.* (24). The 50-μl reaction mixture contained 25 mM ATP, 0.25 mM GTP, 4 mM Mg acetate, 5 mM P-enolpyruvate, 40 mM HEPES (pH 8 with NH_4OH), 45 mM K-acetate, 2 μCi of [^{3}H]leucine, 2 μCi of [^{3}H]valine, 2 μCi [^{3}H]phenylalanine, 0.025 mM of each of the other 17 protein amino acids, 10 μl of wheat germ S23[5] extract, and 10 μl of polysomes. Wheat germ S23 was prepared according to Marcus *et al.* (13) except that 1 g PVP (Polyclar AT)/11.25 ml extraction buffer was added prior to homogenization. The extract contained 28 mg/ml protein as determined by the method of Lowry *et al.* (12) using BSA as a standard. Aliquots of wheat germ S23 were stored in liquid N_2 until use. The reaction mixture was incubated for 40 min at 30 C; 10-μl aliquots were removed at 0, 10, 20, and 40 min. Samples were prepared for scintillation counting according to Sun *et al.* (24). Radioactivity of the protein was counted in scintillation fluid (4 g PPO/l toluene) in a Beckman liquid scintillation system. Counting efficiency for ^{3}H was 26.8%.

Preparation of Authentic Oat Globulin. Sixteen g mature oat groats were ground in a Udy cyclone mill (Tecator/Udy, Boulder, Col.). The flour was defatted with 100 ml cold acetone and oven-dried, *in vacuo*, at 60 C. Protein was extracted from the defatted sample by homogenization in 10 volumes of extraction buffer (1 M NaCl, 0.05 M Na-phosphate [pH 7], 1% [v/v] 2-mercaptoethanol). The sample was initially homogenized in a Sorvall Omni-Mixer and then stirred for 2 hr (room temperature). After centrifugation at 27,000*g* for 15 min (room temperature), $(NH_4)_2SO_4$ was added to 60% of saturation (4 C) and the mixture was centrifuged at 27,000*g* for 15 min (4 C). The $(NH_4)_2SO_4$ precipitate was redissolved in extraction buffer by stirring overnight. Any undissolved protein was pelleted and the supernatant was dialyzed (4 C) against 0.1% (v/v) 2-mercaptoethanol (1 hr) and against two changes of distilled H_2O (30 hr). After dialysis, the precipitated globulin was collected by centrifugation, the pellet was suspended in distilled H_2O and lyophilized.

Preparation of Product and Characterization by Polyacrylamide Gel Electrophoresis. Samples of the cell-free protein synthesis reaction were prepared for electrophoresis according to Sun *et al.* (24). In some cases (for membrane-bound and free polysomes), the reaction mixture was centrifuged at 65,000*g* (avg) for 30 min in a Beckman airfuge in order to pellet polysomes and nascent polypeptides prior to preparation for electrophoresis. However, unpublished data showed no difference in the profiles of radioactivity from centrifuged and noncentrifuged samples. A sample (100 μl) of the total radioactive polypeptides obtained above was applied to a 10% (w/v) polyacrylamide-SDS gel (0.65 × 6.8 cm, in quartz tubes) (26) along with 100 μg authentic oat globulin. Authentic oat globulin was dissolved in dissociation buffer (0.1 M phosphate buffer [pH 7.1], 1.5% [w/v] SDS, and 1.5% [v/v] 2-mercaptoethanol) and heated at 60 C for 15 min before electrophoresis. Electrophoresis was conducted at 5 mamp/tube for the first 30 min, then 8 mamp/tube until the tracking dye reached the bottom of the gels. Products from membrane-bound and free polysomes were electrophoresed on 10% (w/v) polyacrylamide-SDS gels (0.65 × 6.8 cm) using the buffer system of Laemmli (9) at 5 mamp/tube until the tracking dye reached the bottom.

After electrophoresis, the gels were scanned at 280 nm (if they contained authentic globulin), and then cut into 1-mm slices and dispensed with 0.3 ml H_2O into scintillation vials by use of a Gilson aliquogel fractionator. Gel slices were kept at room temperature overnight to elute radioactive protein. The next day, 5 ml of scintillator (2:1 toluene-Triton X-100, v/v, containing 3.2 g PPO and 67 mg of POPOP/l) was added and the samples were counted.

Formation and Characterization of Cyanogen Bromide Peptides. A sample (200 μl) of the cell-free protein synthesis reaction mixture was lyophilized after 40 min of incubation. Cyanogen bromide peptides were formed by making a 10% (w/v) solution of either authentic globulin or radioactive product in 70% (v/v) formic acid containing 4 M urea and 67 mg/ml CNBr (6). The solution was incubated at room temperature for 24 hr in the dark. The reaction was stopped by adding 10 volumes of H_2O, and the solution was lyophilized. Radioactive CNBr peptides were dissolved in 200 μl dissociation buffer (see above). Authentic globulin CNBr peptides were made to a concentration of 1 mg/0.1 ml in the same buffer. Both samples were heated at 60 C for 15 min. Samples of the radioactive CNBr peptides (100 μl) and of authentic globulin CNBr peptides (500 μg) were electrophoresed on 12.5% (w/v) polyacrylamide-SDS gels (0.65 × 6.8 cm) (9) at 5 mamp/tube until the tracking dye reached the bottom. Gels containing radioactive peptides were sliced into 1-mm slices and counted as described above. Gels containing authentic globulin peptides were stained with Coomassie blue and scanned at 580 nm.

Glassware, Buffers, and Chemicals. All glassware used for the isolation of polysomes, the preparation of wheat germ S23, and the protein synthesis assay was heated in 1% (w/v) SDS at 60 C for 15 min and rinsed with sterile distilled H_2O. Buffers were autoclaved or passed through Millipore filters to prevent bacterial contamination. All biochemicals were purchased from Sigma Chemical Co. Isotopes (L-[4,5-^{3}H]leucine, 60 Ci/mmol; L-[alanine-3^{3}H]phenylalanine, 16 Ci/mmol; L-[2,3-^{3}H]valine, 12.5 Ci/mmol) were obtained from New England Nuclear. RNase-free sucrose was purchased from Schwarz/Mann.

RESULTS AND DISCUSSION

Polysome Profiles from Developing Oat Seeds. A typical profile of polysomes extracted from developing oat groats and analyzed on isokinetic sucrose gradients (23) is shown in Figure 1A. When a pH 8.5, high ionic strength (200 mM tris-HCl) extraction buffer was used (5), and precautions were taken against RNase contamination, this type of profile was consistently achieved. The susceptibility of mRNA connecting the ribosomes to pancreatic RNase (0.1 μg/ml) (Fig. 1B) indicated that the polysomes were not a product of nonspecific aggregation.

The largest polysome resolved (Fig. 1A) was a 9-mer; how-

[5] Abbreviation: S23: post 23,000*g* supernatant.

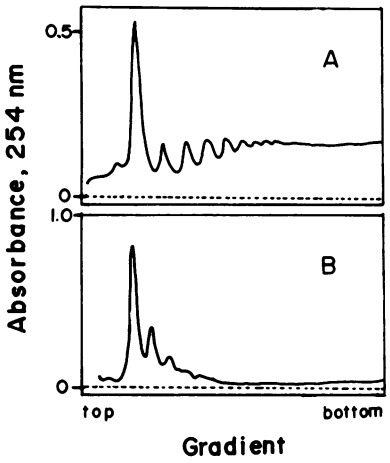

FIG. 1. Sucrose density gradient profiles of polysomes from developing oat groats. Polysomes (2.6 A_{260} units) were extracted and layered on isokinetic 15 to 26% sucrose gradients, centrifuged and analyzed. A: Typical polysome preparation; B: polysome preparation treated with 0.1 μg/ml RNase at 30 C for 5 min prior to centrifugation.

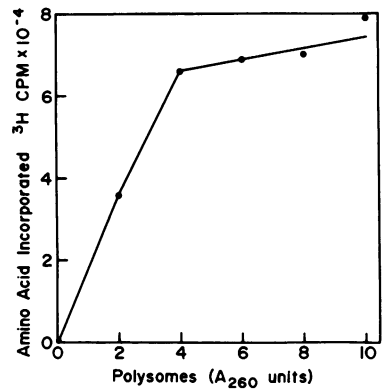

FIG. 2. Effect of oat polysome concentration on protein synthesis in a wheat germ cell-free system. Varying amounts of washed polysomes were used in a 50-μl reaction volume, and the reactions were stopped after 40 min.

ever larger polysomal material (that is, mRNA with more polysomes attached to it) was present "lower" in the gradient but was not resolved. Using the relationship of Noll (15) for bacterial ribosomes, it can be calculated that a 9-mer could accommodate 740 nucleotides. If approximately 200 of these nucleotides can be attributed to a 3'-polyadenylic acid sequence, that leaves 540 nucleotides which would code for a polypeptide of mol wt 18,000 daltons (assuming an average mol wt of 100 for each amino acid). A 9-mer of larger (80S) eukaryotic ribosomes could, presumably, code for a somewhat larger polypeptide. Thus, it is possible that polysomes of this size could synthesize the 21,000 dalton subunit of oat globulin, and the larger polysomes, which are present on the gradient, but unresolved, could code for the 31,000 dalton subunit.

Optimization of Amino Acid-incorporating Ability. The cell-free translation system had a pH optimum of 6.8 and a Mg^{2+} optimum of 4 mM (data not shown). The reaction was dependent on polysome concentration up to 4 A_{260} units/50 μl reaction mix (Fig. 2). At higher concentrations of polysomes, other factors seemed to be limiting. Incorporation of ^{3}H-amino-acids was linear for approximately 5 to 10 min (Fig. 3) and approached a maximum after about 20 min.

Rates of endogenous amino acid incorporation by oat ribosomes were low, and were decreased by washing (Table I). Wheat germ S23-dependent amino acid incorporation was much higher than the endogenous level, and was 30% higher with washed polysomes as compared to unwashed. This phenomenon was also reported by Sun *et al.* (24).

Evidence for Polypeptide Chain Initiation. The amount of polypeptide chain initiation taking place in this system was estimated by adding T-2 toxin, an initiation inhibitor (21) to the reaction mixture. T-2 toxin inhibited amino acid incorporation by 36% (Table II). This value (greater than the 29% inhibition by T-2 toxin obtained for bean polysomes by Sun *et al.* [24]) indicates that over one-third of the amino acid incorporation could be attributed to initiation of new polypeptide chains, while the remaining activity apparently resulted from the completion of nascent polypeptide chains.

Product Analysis. Electrophoresis of authentic oat globulin on 10% (w/v) polyacrylamide-SDS gels resulted in three bands identified by *A* at 280 nm (Fig. 4, arrows) or by staining with Coomassie blue (see Fig. 7). The heaviest polypeptide (mol wt 56,000 daltons) is the narrow band near the top of the gel; two lighter polypeptides (mol wt 31,000 and 21,000 daltons) appear as bands approximately equal in size lower in the gel (17). When the product from *in vitro* amino acid incorporation was co-electrophoresed with authentic globulin, radioactive peaks of the product corresponded with all three globulin peaks of the A_{280} scan of authentic globulin (Fig. 4). Although other storage protein fractions are being synthesized 10 to 12 days after anthesis, their rate of synthesis is considerably less than that of globulin (19). Of the total amount of radioactivity placed on the gel, 87% of the counts co-electrophoresed with the globulin

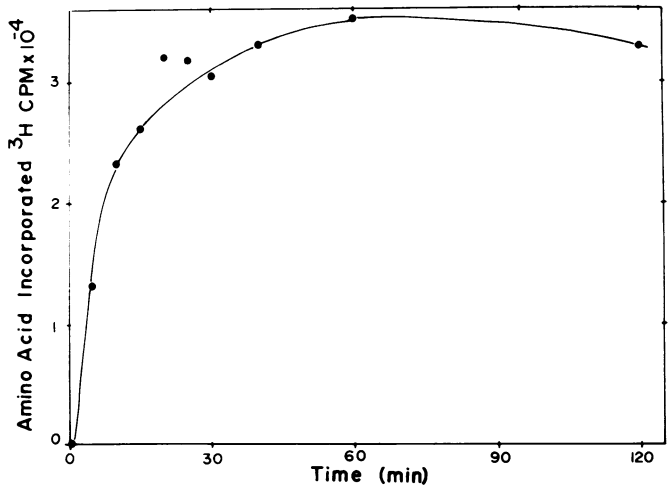

FIG. 3. Time course of oat polysome-dependent amino acid incorporation in the wheat germ cell-free system. The reaction mixture (110 μl) contained 12.0 A_{260} units of unwashed polysomes. Aliquots (10 μl) of the reaction mixture were removed at specified time points.

Table I. *Effect of Wheat Germ S23 on Oat Polysome-Dependent Amino Acid Incorporation*

The standard reaction mixture is described under "Materials and Methods." Each reaction volume (50 µl) contained 1.6 A_{260} units (10 µl) of washed or unwashed polysomes. The incorporation values for 10 µl samples are corrected for zero time and endogenous control values.

Cell-Free Extract Used	Incorporation
	cpm
Unwashed polysomes	4,690
Washed polysomes	2,509
Wheat Germ S23	4,661
Unwashed polysomes + wheat germ S23	27,747
Washed polysomes + wheat germ S23	36,107

Table II. *Effect of T-2 Toxin on Amino Acid Incorporation Dependent on Oat Polysomes*

The standard reaction mixture is described in "Materials and Methods." Each reaction mixture (50 µl) contained 4.7 A_{260} units of washed polysomes. The incorporation values for 10 µl samples are corrected for zero time and endogenous control values.

Cell-Free Extract	Incorporation
	cpm
Washed polysomes + wheat germ S23	84,370
Washed polysomes + wheat germ S23 + T-2 Toxin (0.1 µg)	54,391

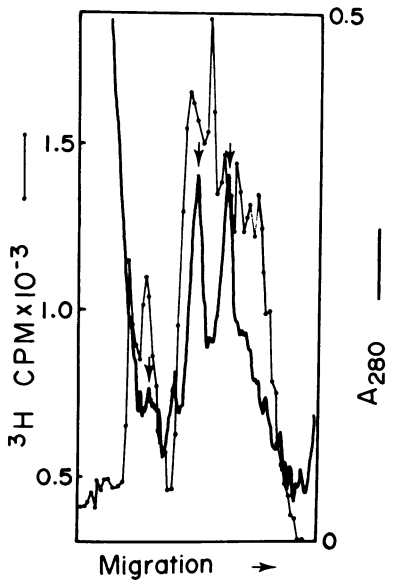

FIG. 4. Polyacrylamide-SDS gel (10% w/v) analysis of the products from *in vitro* protein synthesis with oat polysomes. The radioactive product and authentic globulin were prepared for electrophoresis. Samples of 100 µl and 100 µg, respectively were applied to the gel.

subunits. These data suggest that the major *in vitro* protein synthesis product of oat polysomes is oat globulin.

Two possible explanations for the large (56,000 dalton) peptide are: it could be a distinct polypeptide, not structurally related to the other two polypeptides, or it could be a precursor of the two smaller peptides that is posttranslationally cleaved to produce them. The large polypeptide may be present in small amounts in globulin extracts from the mature seed because of incomplete posttranslational processing. This last possibility is suggested by the data in Figure 4, which show a proportionally greater amount of the heavy polypeptide in the *in vitro* product than in the authentic globulin extracted from mature oat seeds. One way of determining if the 56,000 dalton polypeptide is structurally related to the smaller polypeptides would be to separate each polypeptide and to compare tryptic or cyanogen bromide peptide maps or amino acid composition of each one. Alternatively, one could isolate the large polypeptide and determine if it is cleaved by extracts from oat seeds to form the two smaller polypeptides.

Analysis of Cyanogen Bromide Cleavage Products. For more certain determination that the *in vitro* product was oat globulin, product polypeptides and authentic globulin were cleaved at methionine residues with cyanogen bromide (CNBr) and the resultant peptides were mapped by electrophoresis on 12.5% (w/v) polyacrylamide-SDS gel columns (9). Considering only the two major polypeptides (combined mol wt, 52,000 daltons), and knowing that oat globulin contains about 1% (w/w) methionine

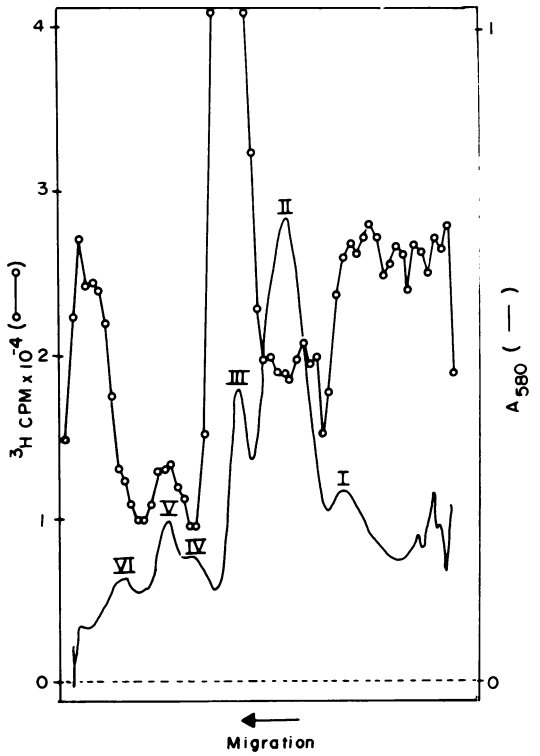

FIG. 5. Analysis of cyanogen bromide peptides from authentic and *in vitro* synthesized oat globulin on 12.5% polyacrylamide-SDS gels using the procedure of Laemmli (9). Both proteins were cleaved with CNBr and electrophoresed. An aliquot (200 µl) of radioactive peptides was placed on the gel. An identical gel containing 500 µg of authentic globulin was stained with Coomassie blue and scanned at 580 nm.

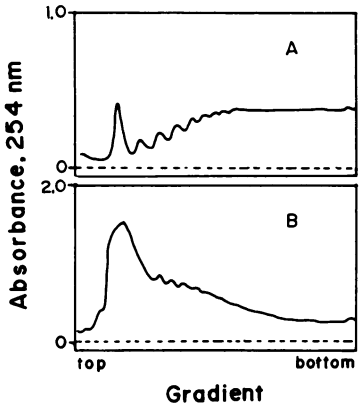

FIG. 6. Sucrose density gradient profiles of membrane-bound (A) and free (B) polyribosomes. Aliquots containing 2.0 A_{260} units of membrane-bound polysomes and 5.0 A_{260} units of free polysomes were placed on each gradient.

(D. M. Peterson, unpublished data), then one can estimate that there are four methionine residues distributed between the two polypeptides. Consequently, six CNBr peptides would be expected from globulin if there were no terminal methionine residues. Figure 5 shows the A_{580} scan of CNBr peptides electrophoresed on 12.5% (w/v) polyacrylamide-SDS gels and stained with Coomassie blue. The stained material which remained at the top of the gel may have been globulin that did not react with CNBr. Lower in the gel, as expected, there were six polypeptide peaks (Fig. 5, I-VI). When CNBr peptides from the *in vitro* product were electrophoresed on an identical gel, radioactive peaks corresponded to peaks III and V, and possibly to peaks I and VI as

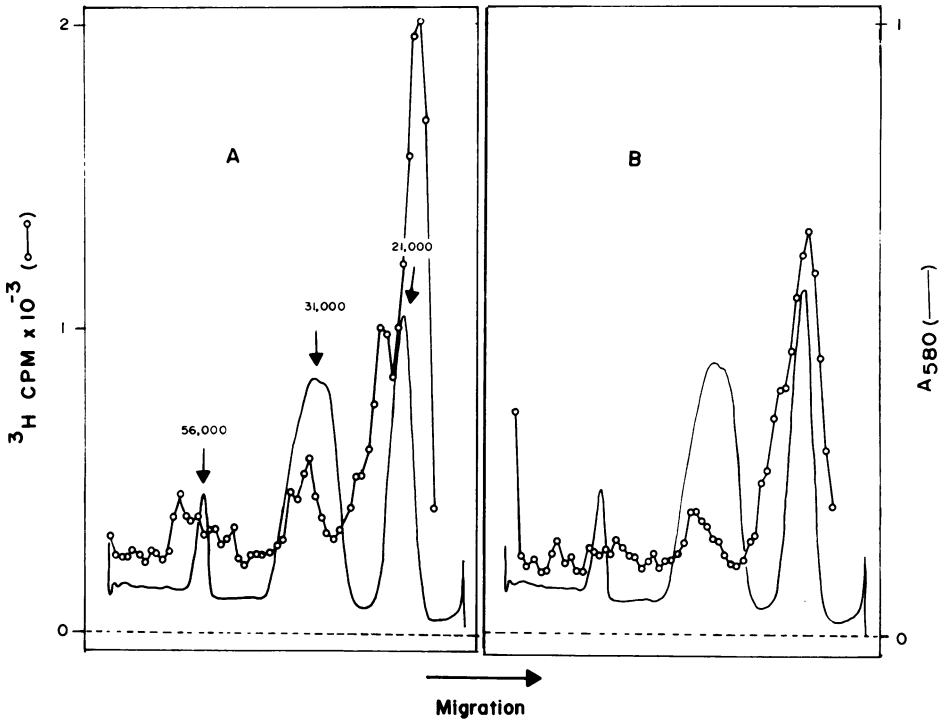

FIG. 7. Analysis on 12.5% polyacrylamide-SDS gels (9) of products formed on membrane-bound (A) and free (B) polysomes isolated from developing oat seeds. Radioactive products and authentic globulin were prepared for electrophoresis as described in "Materials and Methods." Aliquots (200 μl) of products from membrane-bound and free polysomes were placed on the gels. An identical gel contained 300 μg of authentic globulin, which was stained with Coomassie blue and scanned at 580 nm.

Table III. *Amino Acid Incorporation Directed by Membrane-Bound and Free Polyribosomes*

The standard reaction mixture is described in "Materials and Methods." Each reaction volume (50 µl) contained 2.8 A_{260} units (10 µl) of membrane-bound, free, or control (total polysome population) polysomes and wheat germ S23 (10 µl). The incorporation values for 10 µl samples are corrected for zero time and endogenous control values. Results are the averages of 2 experiments.

Cell-Free Extract Used	Incorporation
	cpm
Membrane-bound polysomes	83,394
Free polysomes	44,917
Total polysomes	98,759

well. The relatively different magnitudes of the peptides obtained from the *in vitro* product and authentic globulin may have resulted from incomplete cleavage with CNBr, due to methionine oxidation (22) or inaccessibility of methionine residues to the reagent, or because of relatively different proportions of labeled (leucine, phenylalanine, valine) *versus* unlabeled amino acid residues in the various peptides. These data suggest similarity between CNBr peptides of the *in vitro* product and authentic globulin, and support the hypothesis that oat globulin is the primary synthesis product *in vitro*.

Membrane-bound and Free Polysomes from Oats. In order to determine if there were a differential site of synthesis for oat globulin, both free and membrane-bound polysomes were isolated from oat groats and the protein product from each type was analyzed. The membrane-bound polysome population (Fig. 6A) had a much larger proportion of polysomes (9-mer was the largest polysome resolved) than did the free polysome population (Fig. 6B). Free polysomes had a much larger monosome peak. Since both classes of ribosomes were extracted simultaneously in the same buffers, it is doubtful that degradation of free polysomes occurred (because membrane-bound polysomes were not degraded). Amino acid incorporation by membrane-bound polysomes was double that of free polysomes (Table III). These results compare favorably with those of Larkins *et al.* (10) who found that membrane-bound polysomes were 2- to 3-fold more active in protein synthesis than free polysomes. The relatively lower population of large polysome classes in the free polysomes, as compared to the membrane-bound polysomes, can explain the lower amount of protein synthesized on the free polysomes.

Figure 7 shows the electrophoretic analysis of the products formed *in vitro* by membrane-bound and free polysomes. Both products have peaks corresponding to the 31,000 and 21,000 dalton peaks of authentic globulin (electrophoresed on an identical gel, stained with Coomassie blue, and scanned at 580 nm). These data suggest that there are no qualitative differences in the products formed by membrane-bound and free polysomes.

Early work by Morton and Raison (14) and Opik (16) suggested that plant storage proteins may be synthesized by membrane-bound polysomes. Recently, Burr and Burr (3) found that polysomes isolated from the protein bodies of maize synthesized zein *in vitro*. Larkins and co-workers (10, 11) have demonstrated the *in vitro* synthesis of zein by membrane-bound polysomes of maize. Free polysomes also synthesized zein but in smaller quantities than membrane-bound polysomes (10, 11). We found that both membrane-bound and free polysomes from developing oat groats synthesized the 21,000 and 31,000 dalton peptides of globulin.

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