Plant Physiol. (1978) 61, 918-923

Protein Synthesis and Accumulation in Bean Cotyledons during Growth[1]

Received for publication December 5, 1977 and in revised form January 31, 1978

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ABSTRACT

Analysis of total protein, of specific proteins by gel electrophoresis and immunoelectrophoresis, and of protein synthetic activity *in vitro* confirmed that intense protein synthesis and accumulation occurred as the French bean (*Phaseolus vulgaris* L). seed grew from 12 to 20 millimeters. These techniques showed that there was no globulin-1 (G1) fraction (requiring high salt for solubility) present in 6-millimeter seeds, and only very small amounts were synthesized in seeds less than 9 millimeters long. The 7- to 9-millimeter stages represent a 2-day transition period over which genetic information for the G1 protein becomes actively expressed, accounting for at least 50% of all protein synthesized in this tissue during the following 14 days. At maturity, the electrophoretic analysis confirmed that G1 globulin was the major storage protein, representing some 50% of the dry seed protein. Cell-free protein synthesis assays, including immunoprecipitation of the *in vitro* products, clearly showed G1 polypeptides to be among the polysome-directed products.

The genetic information for French bean (*Phaseolus vulgaris* L. cv. Tendergreen) storage proteins appears to be repressed in all tissues except for certain stages in the development of the cotyledon, when it is actively expressed. At maturity, a dry seed (450 mg) contains approximately 90 mg of protein (20%); about 50% of this is globulin. Because of the active synthesis and accumulation of relatively few molecular species over a short and well defined period, the bean cotyledon is an excellent tissue for studies toward understanding the molecular basis for regulation of specific gene expression.

Aspects of the changes in biochemistry, morphology, and fine structure of *P. vulgaris* seeds during development have been studied extensively (2, 6, 24, 31). Currently, there is much interest in the synthesis and regulation of storage proteins in developing legume seeds (4, 21), and since the detection of the onset of storage protein synthesis was of special interest, both SDS gel analysis and immunochemical techniques were used. In this article we describe the *in vivo* and *in vitro* synthesis of G1[2] and G2 fraction storage protein polypeptides (29) in growing cotyledons.

MATERIALS AND METHODS

Plant Materials. Dry seeds of *P. vulgaris* L. cv. Tendergreen were purchased from Olds Seeds Co., Madison, Wisconsin and grown in the University of Wisconsin Biotron. The seeds were germinated in moist Vermiculite and then transferred to pots and grown in Vermiculite irrigated with Hoagland solution. Under these conditions, flowering occurred in 30 days from sowing the seeds. The day of full bloom was recorded for individual flowers and used as zero date (DAF) in developmental studies. At intervals after flowering, the fruits were harvested and the seeds removed from the pods. The seeds were pooled according to the length of their long axis, and the testa and embryonic axis were removed from the cotyledons. The length of cotyledons from each size of seeds was measured and recorded. The pooled cotyledons were frozen immediately in liquid N_2 and stored at −85 C for further use. Cotyledons from field-grown (summer, 1977) beans were also used in some of the experiments. The time course of G1 protein accumulation and the content of protein in field-grown cotyledons were found to be similar to those for Biotron-grown plants.

Protein Extraction. Cotyledons were ground in a prechilled mortar together with a freshly broken Pasteur pipette in 0.05 M Na phosphate (pH 7.2) containing 0.5 M NaCl at a ratio of 1 g fresh wt of material to 5 ml of buffer. The homogenate was centrifuged at 50,000*g* for 20 min, and the supernatant removed. The pellet was reextracted once. Protein in the combined supernatant was determined by the method of Lowry *et al.* (18) using BSA as standard and designated as total extractable protein. Purified G1 and G2 proteins were prepared as described previously (29). For immunochemical studies, the protein samples were prepared by grinding 0.1 g of cotyledons with 1 ml of medium of 0.5 M NaCl in 0.5 M glycine (pH 2) in a polycarbonate tube with a glass rod. An additional 1 or 2 ml of medium was used subsequently for rinsing. After stirring for 1 hr at 5 C, the homogenate was centrifuged for 30 min at 8,000 rpm (Beckman J21) and the supernatant fraction used for analysis.

Production of Antibody. Four injections of 2 mg of purified G1 protein were given to rabbits subcutaneously during a period of 2 weeks. One month after the last injection, an additional 2 mg of protein was given. The first injection used Freund's complete adjuvant, subsequent injections used the incomplete adjuvant. One week later a serum of high titer was collected. This serum was monovalently specific for G1 protein since there was no precipitation reaction between G1 and antiserum prepared against G2 + albumin, or between G2 + albumin and antiserum against G1 protein. For immunodiffusion and rocket gel studies, the serum was purified by ammonium sulfate precipitation (45% saturation). After washing with 1.75 M $(NH_4)_2SO_4$, the precipitate was suspended in 0.5 M NaCl containing 15 mM NaN_3, and stored at −20 C (8). For immunoprecipitation of protein synthesized *in vitro*, the rabbit IgG fraction was isolated as described by Livingston (16).

Electrophoresis. The protein sample was dissociated into its polypeptide subunits by adding an equal volume of a solution containing 2% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 2 mM EDTA, 40% (w/v) sucrose, 0.01% (w/v) bromophenol blue in 62.5 mM Tris-HCl (pH 6.8), heating for 2 to 3 min in a boiling water bath. Different dissociation techniques are noted below in connection with immunoelectrophoresis and for immunoprecipitated products. The subunits were electrophoretically separated in an

[1] This research was supported by grants from the National Science Foundation (PCM 74-21675), the Herman Frasch Foundation, the U.W. Graduate School, and the College of Agricultural and Life Sciences.

[2] Abbreviations: G1: globulin-1 fraction (requiring high salt for solubility); G2: globulin-2 (soluble in weaker saline solutions than G1); DAF: days after flowering.

SDS-acrylamide slab gel (11), and stained with Coomassie brilliant blue (32). After destaining, the gel was dried onto a sheet of filter paper (27) and autoradiographed using Kodak no-screen x-ray film.

G1 Protein Quantitation. The G1 content of total extracts was determined by rocket immunoelectrophoresis (14, 15, 33) using 1% agarose (Bio-Rad) in a buffer containing 74 mM Tris, 24 mM barbital, 0.34 mM Ca lactate, and 3 mM NaN_3 (pH 8.6). To prepare gels, 40 ml of buffered agarose was melted, the temperature lowered to 47 C, and about 0.7 ml of rabbit anti-G1 serum added. The mixture was injected into a mold to give a gel of 18 × 10 × 0.15 cm. Sample wells (32, each 2.4 mm in diameter and spaced 3 mm apart) were punched out at least 1.5 cm distant from gel edges. Lyophilized purified G1 protein was dissolved (5 mg/ml) in the glycine buffer as described above for sample extraction. The samples and standard were carbamylated by heating to 50 C for 20 min with an equal volume of 2 M KOCN. An aliquot (4 μl) of sample or of dilutions of a standard (containing from 2.5 to 0.25 mg of G1/ml) was applied to the wells, the standards being distributed across the gel to minimize any errors due to position. The gel was run at 90 v (25–15 mamp) for about 15 hr using the Tris-barbital-lactate buffer as electrolyte, then pressed, dried, and stained with Coomassie brilliant blue. The heights of the resulting rockets were measured, and the G1 content calculated from a standard curve obtained from the reference samples. Values given are the mean of at least three extracts, with replication within extracts.

Polysome Preparation and Cell-free Protein Synthesis. The preparation of total polysomes from developing cotyledons was as described previously (28). Free and membrane-bound polysomes were prepared by the differential centrifugation method of Larkins and Davies (13) using the media of Verma *et al.* (30). The wheat germ S23 extract was prepared by a procedure similar to that described by Davies and Kaesberg (3). The extract was passed through a Sephadex G-25 (fine) column (1.6 × 25 cm) equilibrated with a buffer containing 2 mM Mg acetate, 50 mM K acetate, 5 mM DTT, and 1 mM GSH in 10 mM HEPES (pH 7.4 with KOH) before using as the cell-free protein-synthesizing system. The standard reaction mixture (50 μl) for the incorporation of amino acids into protein contained 2.5 mM ATP, 0.25 mM GTP, 4 mM Mg acetate, 5 mM P-enolpyruvate, 20 mM HEPES (pH 7.4 with KOH), 70 mM K acetate, 1 mM DTT, 25 μCi of [^{35}S]Met, 25 μM each of the other 19 amino acids, 10 μl of S23 extract, and 0.25 A_{260} units of polysomes. The reaction mixture was incubated at 25 C for 60 min. The paper disc procedure of McLeester and Hall (19) was used to measure total amino acid incorporation into trichloroacetic acid-insoluble material.

Immunoprecipitation of Cell-free Protein Synthesis Products. After incubation, 2 μl of 20 mM unlabeled methionine and 10 μg of purified G1 protein were added to 100 μl of reaction mixture. Following centrifugation for 1 hr at 100,000g, the supernatant was removed and 12 μl of a solution containing 1.5 M NaCl, 10% (v/v) Triton X-100, 10% (w/v) sodium deoxycholate, and 0.1 M Na phosphate (pH 7.2) was added. Purified IgG (20 μl, containing about 300 μg of protein) was added and incubated at 37 C for 1 hr, then at 4 C overnight. The precipitate was recovered by centrifugation at 800*g* for 15 min. After three cycles of washing with a solution of 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 0.15 M NaCl in 0.01 M Na phosphate (pH 7.2), the precipitate was dissociated with a medium containing 0.1 M Tris, 1.5% (w/v) DTT, 1% (w/v) SDS, 10% (v/v) glycerol, and 10% (w/v) bromophenol blue by heating for 2 to 3 min in a water bath. The dissociated sample was then subjected to SDS gel electrophoresis and autoradiography as described above.

RESULTS

Total Protein Changes during Cotyledon Growth. The most dramatic changes in protein accumulation occurred during cotyledon growth from 6 to 21 mm (Fig. 1), which corresponds to the late heart to maturation phase of development (31). Over this time, elongation of the seed was proportional to the increase in fresh wt (specific data not shown, but see abscissa to Fig. 1). Other studies on the development of bean seeds have used measurement of the long axis of seeds (6) or cotyledons (22) or the number of days after flowering (DAF; 10, 23) as physical reference parameters. For the cultivar Tendergreen, these criteria were in good accord up to the 21-mm stage (31 DAF), but thereafter there was little increase in length although the weight continued to increase. Also, the length of very young cotyledons (less than 3 mm) correlated poorly with either DAF or seed length since during this period the liquid endosperm was utilized and the cotyledons expanded rapidly. Thus, although seed length has been used as the basic parameter of growth in this study, these complications, plus the fact that seeds in the same pod (hence of the same DAF) often varied in size, made it necessary to cite both cotyledon lengths and DAV values for very early and late stages of cotyledon growth.

Only low levels of protein were extractable in cotyledons of young seeds (smaller than 9 mm), but after 13 mm, a dramatic increase occurred. In cotyledons of 20-mm (37 DAF) seeds, the protein content was 75 times that for 9-mm (14 DAF) seeds. The seeds increased in length by about 0.5 mm/day and in protein by about 3.3 mg/day for a cotyledon pair over the active phase of protein accumulation. On a dry wt basis, cotyledons contained 16% protein at the 20-mm (37 DAF) seed stage and 20% at maturity. As the seeds dried out (more than 45 DAF), they decreased in length from about 21 to 13 mm.

Qualitative Protein Changes during Growth. After dissociation, total protein extracts from cotyledons at various stages of growth were examined by SDS-acrylamide gel electrophoresis. Figure 1 shows that the polypeptide profiles of 6-, 7-, and 8-mm seeds were very similar. Thereafter, prominent changes occurred. These included the appearance, disappearance, and variations in the relative amount of individual polypeptides at specific stages. Mature, dry seeds of the cultivar Tendergreen contain 20% protein, of which some 50% is globulin (26). G1 requires stronger saline solutions for solubility (29) and is about four times more abundant than G2. In the cultivar Tendergreen, G1 consists of three subunits of 43,000, 47,000, and 53,000 daltons (5, 20). These can be clearly identified in the 9-mm stage in Figure 1 but are not detectable in extracts from 8-mm seeds. This electrophoretogram shows that a sharp increase in the synthesis of G1 protein occurs between the 11- and 12-mm stages; thereafter, the G1 protein polypeptides are predominant. Polypeptides of the G2 fraction are also detectable in the 9-mm seeds. Extracts from even younger cotyledons appear to contain trace amounts of these components, but the higher resolution of two-dimensional gels is needed to confirm their identity.

The ratio of the three G1 polypeptides remained unchanged throughout the growth period studied, indicating that the synthesis of these subunits was closely coordinated. Three polypeptides, mol wt 34,000, 32,000, and 30,000, can be detected in the G2 fraction, but the smallest only became visible in the electrophoretogram of 19-mm seeds. At this stage, the 32,000 dalton band stained more intensely, while the amount of the largest polypeptide remained fairly constant throughout growth. These changes in individual polypeptides support our previous belief that the G2 fraction contains several different proteins (20).

Immunochemical Measurement of G1 Protein Accumulation during Development. Using large quantities of material (30 cotyledons/ml of extract) for an immunodiffusion assay (25), G1 globulin was first detected in 7-mm seeds (3-mm cotyledons). Seeds of 4, 5, and 6 mm contained no detectable G1 protein in their less than 2-mm cotyledons (data not shown).

The technique of rocket immunoelectrophoresis (14, 15, 33) permits accurate quantitative measurement of the amount of a specific protein present in a mixture of proteins. The lengths of

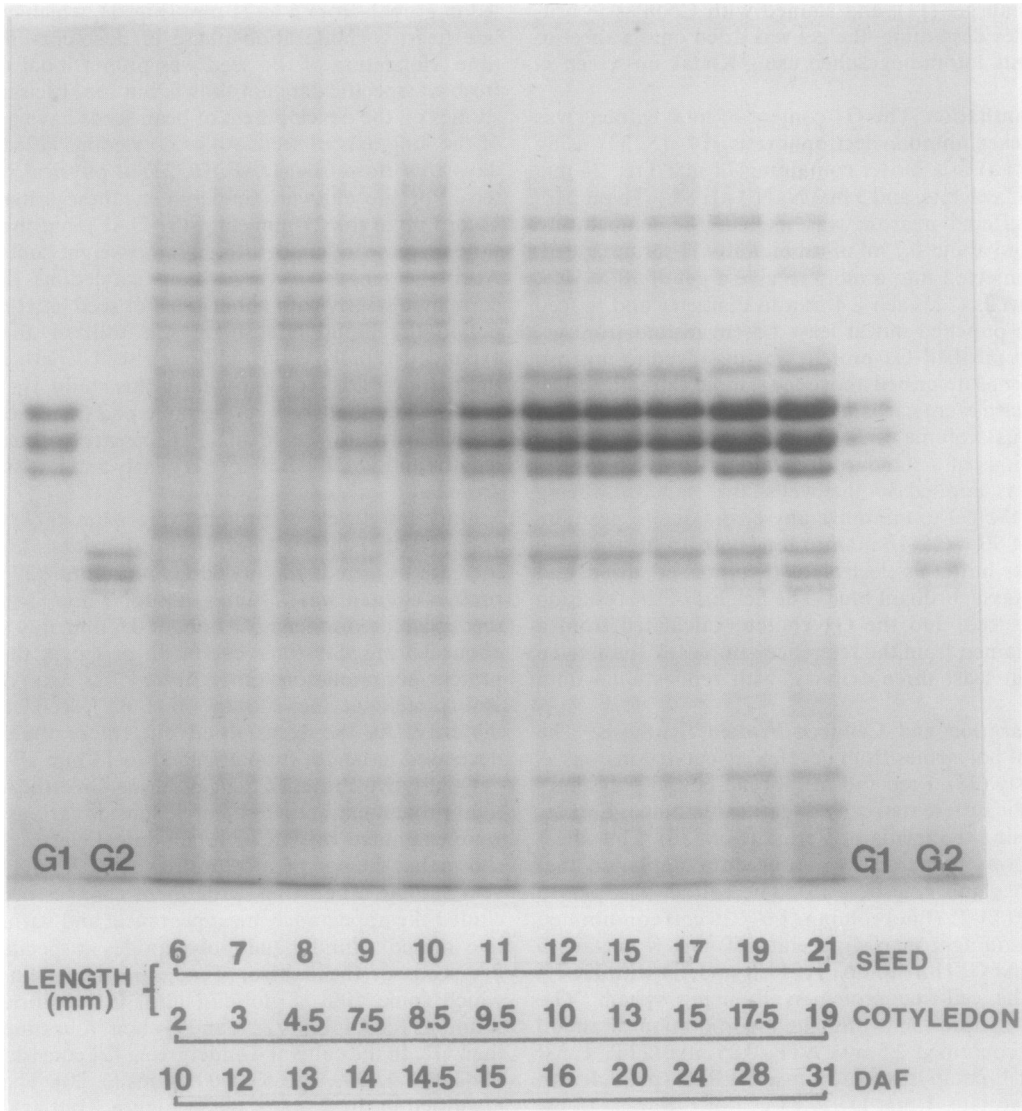

FIG. 1. Electrophoretic separation of bean cotyledon proteins during growth. Numbers under each lane of this 16% acrylamide gel denote the seed and cotyledon lengths and DAF. Standard G1 and G2 proteins were dissociated in the same way as the samples and run as markers in the outer lanes.

the well defined rockets obtained using monovalent antisera prepared against G1 protein (Fig. 2A) were proportional to G1 concentration (Fig. 2B). Using this sensitive immunoassay, a trace of G1 protein was observed at the 7-mm seed stage, and the increase in G1 protein during seed growth from 6 to 20 mm was assayed (Fig. 3).

G1 protein content increased 41-fold (from 0.5 to 21.5 mg/cotyledon pair) over the 12 days in which the seeds grew from 12 to 19 mm (Fig. 3, curve a). This is an average rate of 1.75 mg of G1 protein/day for a cotyledon pair, representing some 50% of total protein synthesis. Over the period from 10 to 19 mm, the contribution of G1 in the total protein increased from 10% to 50% (Fig. 3, curve b), and some 50% of the G1 present in a mature dry seed was synthesized.

Synthesis of Protein by Cotyledon Polysomes. Convincing evidence has been advanced for the synthesis of zein (the storage protein in *Zea mays* kernels) on membrane-bound polysomes surrounding the protein bodies (1, 12). Rapid proliferation of RER has been shown to occur in bean cotyledon cells during the period of active protein accumulation (24). Free and detached membrane-bound polysomes were isolated, and their *in vitro* products examined. Panel A of Figure 4 shows that although their protein products were similar to those of the total extractable polysomes, the membrane-bound polysomes appear to synthesize slightly higher amounts of G1. Although there were about 18 times as many free as membrane-bound polysomes in extracts from 15- to 18-mm cotyledons, it is possible that bean polysomes synthesizing G1 peptides are more readily dislodged from membranes than are zein-synthesizing polysomes. It is not yet clear if the synthesis of G1 polypeptides is restricted to membrane-bound polysomes.

A comparison of the autoradiographic profile of products synthesized in the wheat germ cell-free system dependent on polysomes isolated from various stages of growth (Fig. 4, panel B) with electrophoretograms of seed polypeptide changes *in vivo* (lanes 7p, 18p, G1p, G2p, and also Fig. 1) reveals that there are many bands in common. The autoradiograph of products synthesized by polysomes from 18-mm seeds (Fig. 4, panel B) clearly shows bands which migrate in positions corresponding to those of the 47,000 and 43,000 dalton subunits of G1 protein. Decreasing amounts of these bands are present in products of polysomes from younger seeds. However, trace quantities are present in products of polysomes from 7-mm seeds. This suggests that small amounts of G1 (and possibly G2, since bands corresponding to G2 polypeptides can also be seen) are synthesized at the 7-mm stage, as was seen immunochemically for the G1 protein (Fig. 2B).

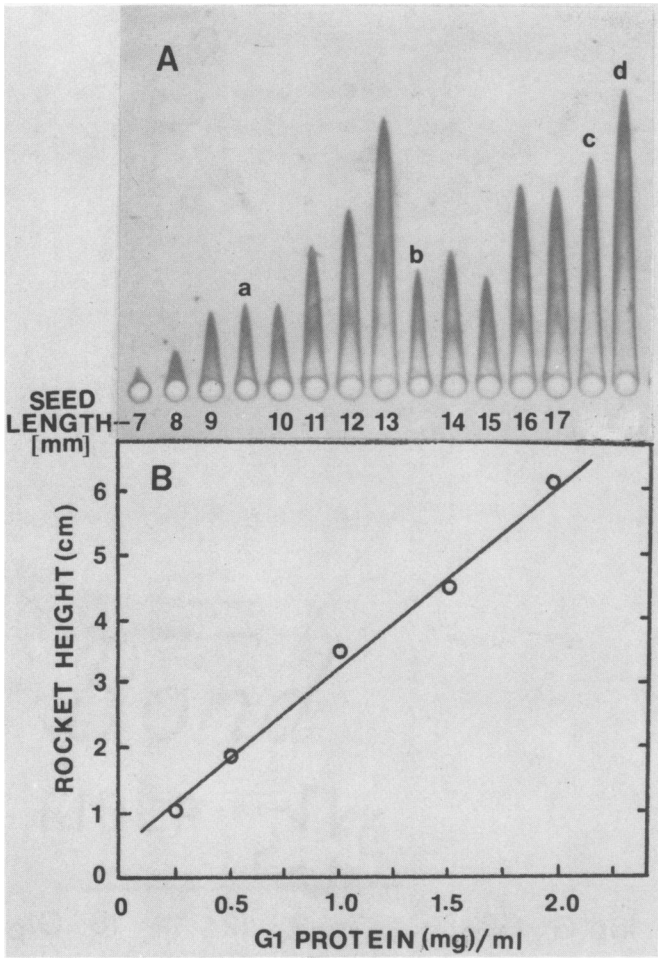

FIG. 2. Determination of G1 protein content by rocket immunoelectrophoresis. A: protein samples from cotyledons of each seed size (7–17 mm) were run in wells as indicated by the numbers below the rockets. These protein samples were obtained by extraction of 30, 20, 20, 20, 15, 10, 7, 2, 1, 1, and 1 cotyledons (with 2 ml of buffer) for 7- to 17-mm seeds, respectively. Equal volumes (4 μl) were applied to each well. Samples of standard G1 solutions of 0.25, 0.5, 1.0, and 1.5 mg/ml were run in wells marked a, b, c, and d, respectively. B: typical standard curve for G1 protein determination.

Immunoprecipitation of products obtained from reactions containing polysomes from seeds 9 mm and larger (Fig. 4, panel C) confirmed that considerable amounts of the G1 polypeptides were synthesized *in vitro*. Although no G1 polypeptides can be seen for the *in vitro* products from 7-mm seeds in the comparative series of Figure 4, panel C, their presence was detected in a separate experiment in which a large volume of sample was loaded onto the gel.

In agreement with our previous observations using coelectrophoresis of polysome-directed products with authentic G1 polypeptides in tube gels (28), the autoradiographs of Figure 4 indicate that lower amounts of the 53,000 mol wt subunit were synthesized than the 47,000 subunit. This is despite the apparently equal amounts of these two subunits present in native G1 protein, as judged by staining.

DISCUSSION

Because G1 protein represents such a large part of the total protein of the dry bean seed, we were interested in following its synthesis and accumulation during the growth of cotyledons. The cotyledons were less than 2 mm long when the seed attained 6 mm (10 DAF), and analytical gel electrophoresis, immunoelectrophoresis, and polysome-directed protein synthesis failed to detect any G1 protein at this stage. Over the 48-hr period between 13 and 15 DAF, the cotyledons more than doubled in length and small amounts of G1 protein subunits could then be detected (Figs. 1, 2, and 4). These changes coincide with the onset of high O_2 uptake found previously for black valentine bean (17), and although it is difficult to make absolute correlation between seeds of different varieties, this would be in accord with the involvement of respiratory metabolism in the accumulation of storage protein.

The major burst of G1 synthesis started abruptly at 16 DAF. This is when the cotyledon attained 10 mm in length (Figs. 1 and 3), having just filled the testa. Presumably, the genetic information for the G1 polypeptide subunits is derepressed at this stage, although the biochemistry of regulatory events leading to the expression of this storage protein remains to be elucidated.

Using polysomes from developing peas, Higgins and Spencer (9) obtained only tentative evidence for the cell-free synthesis of polypeptides similar to those of legumin and vicilin. They suggested that the newly synthesized protein subunits may lack antigenic determinants present in the holoprotein of mature seed. The results shown in Figure 4 provide convincing evidence for the synthesis of the 47,000 and 43,000 dalton subunits of G1 storage protein. Satisfactory immunoprecipitation was obtained, even though it is unlikely that the sugar residues known to be present in each of the G1 subunits (7) are added *in vitro* under the conditions used. Nevertheless, the very small amount of 53,000 dalton subunit detected among the translation products shows that the efficiency of translation (and, perhaps, immunoprecipitation) of individual storage protein polypeptides is variable. At present, it is not known if this reflects technical problems, such as antibody specificity and conditions used for synthesis *in vitro*, or if the variation is associated with regulatory events which also occur *in vivo*.

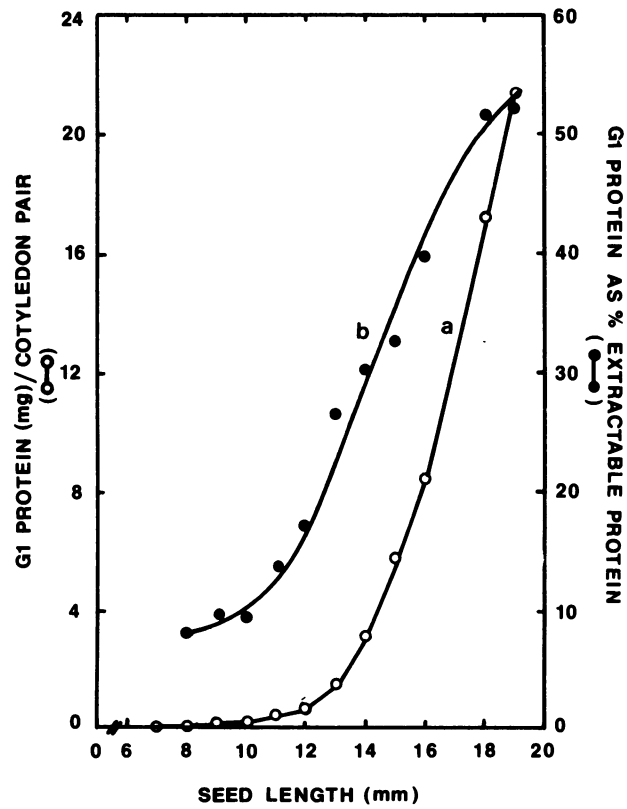

FIG. 3. Changes in G1 protein content of cotyledons during growth. a: G1 protein content in developing cotyledons (○—○) was determined by rocket immunoelectrophoretic techniques, as detailed in Figure 2 and under "Materials and Methods." b: G1 protein content is also shown as a percentage of extractable protein (●—●).

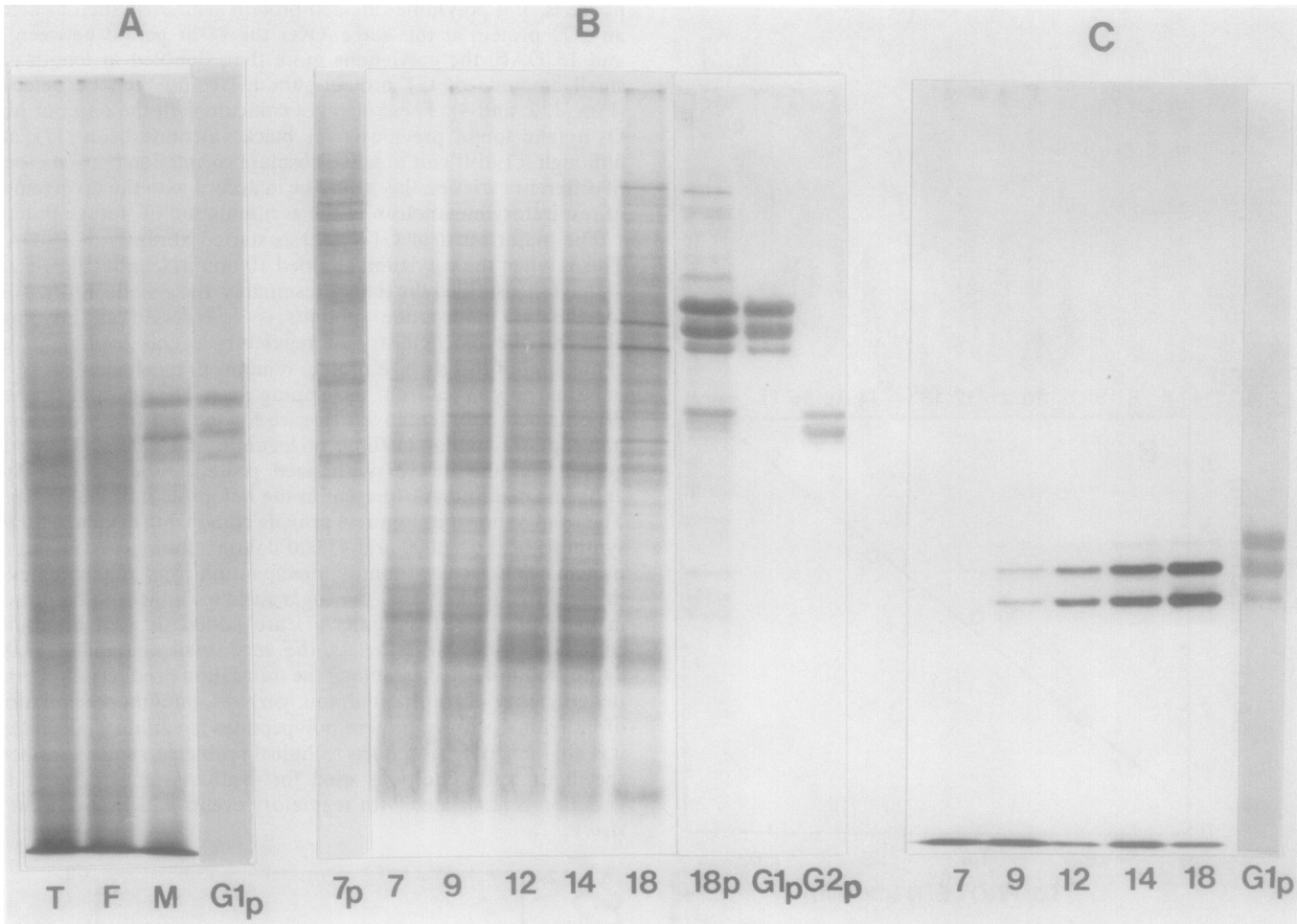

FIG. 4. Electrophoretic analyses of products synthesized by bean cotyledon polysomes *in vitro*. Numbers below each lane indicate the seed length (mm) from which polysomes were extracted. Lanes marked with a suffix p were visualized by Coomassie brilliant blue staining (G1 and G2 were authentic reference samples); all of the other lanes are autoradiographs of [^{35}S]Met-labeled products. Panel A shows products (on a 13% gel) synthesized in the wheat germ system dependent on: total polysomes extracted in the presence of Nonidet P40 detergent (lane T); free polysomes extracted without detergent (lane F); and by polysomes detached from membrane-bound polysomes by detergent treatment (lane M). In Panel B, products directed by total polysomes isolated from seeds of different ages are compared. Samples of the reactions were taken and assayed for trichloroacetic acid-insoluble products by disc counting, and the results used as the basis for adding equal amounts of radioactive product to the lanes of a 16% gel. Panel C shows the changing proportion of G1 polypeptides present among the products directed by total polysomes isolated from seeds of increasing age. Portions of the cell-free reactions were chosen so as to contain similar amounts of total radioactive products, the G1 polypeptides selectively immunoprecipitated, and then separated on a 10% gel.

Acknowledgment—We thank General Mills, Inc. for a generous supply of wheat germ.

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