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PROTEIN SYNTHESIS BY 80S RIBOSOMES DURING PLANT DEVELOPMENT*

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Key Word Index—*Pisum sativum*; Leguminosae; pea; *Zea mays*; Gramineae; corn; 80S ribosomes; protein synthesis; development; magnesium activation.

Abstract—Protein synthesis by ribosomes from the meristematic region of pea roots (0–0·3 cm) and 2-day-old corn shoots (young tissues) relative to ribosomes from matured regions of pea roots (2·0–2·5 cm) and 10-day-old corn leaves (aged tissues) was compared in the poly U-phenylalanine system. With normal polyribosome preparations, ribosomes from young tissues required approx. 16 mM Mg^{2+} while ribosomes from aged tissues required 20–22 mM Mg^{2+} for optimal activity. With monomeric ribosome preparations induced by anaerobic treatment of the seedlings, the Mg^{2+} optimum was 20–22 mM for ribosomes from both young and aged tissues. A higher level of peptidyl-*t*RNA in ribosomes from young tissues accounts, at least in part, for the differences in Mg^{2+} optima between ribosomes from young and aged tissues. Monomeric ribosomes were used for assaying the activity of ribosomes *per se*. Ribosomes from young pea root tips and ribosomes from 2-day-old corn shoots were 25–30% and 100–150% more active, respectively, than the corresponding ribosomes from aged tissues. Differences in ribosomal proteins revealed by gel electrophoresis correlated with the change in ribosomal activity. Reduced activity in the aged ribosomes was not due to RNase activity or inhibitors.

INTRODUCTION

IT HAS previously been shown that both the rate of protein synthesis and the net accumulation of protein decreases during development of seedlings.[1,2] A decline in the level of polyribosomes in the ribosome population apparently relates to the decrease in protein synthetic activity.[3,4] *In vitro* phenylalanine incorporation by ribosome preparations in the presence of polyuridylic acid (poly U) is also related to the polyribosome level. In developing seeds of *Vicia faba* higher levels of phenylalanine incorporation occur with ribosome preparations containing a higher proportion of polyribosomes in the population;[5] however, opposite results were reported with ribosome preparations from liver of diabetic rats[6] and mammary gland of guinea-pigs.[7]

To date, studies on changes in protein synthetic activity of ribosomes during development have utilized the ribosomal complex, i.e. ribosomes associated with endogenous *m*RNA and peptidyl-*t*RNA. We have previously reported that the ribosome complex (polyribosomes) can be dissociated under anaerobiosis into single 80S ribosomes resulting from

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completion and release of nascent protein.[8] This conclusion was subsequently substantiated by the ease of dissociation of N_2 gas-derived single ribosomes into ribosomal subunits[9] relative to normal ribosomes.

In this communication we compare the change in protein synthetic activity of single 80*S* ribosomes, as measured *in vitro* with synthetic *m*RNA (poly U), during maturation of pea roots and aging of corn seedlings in darkness. Our results indicate that the decrease in activity of single ribosomes in these tissues relates to alteration of the ribosomal protein complement. Magnesium optima differences of the normal ribosomes relates in part to the level of endogenous peptidyl-*t*RNA.

RESULTS

Sucrose density gradient profiles of ribosomes isolated from pea root-tip segments, mature pea root segments, 2-day-old corn shoots and 10-day-old dark-grown corn leaves are shown in Fig. 1. In the following discussion pea root-tips and 2-day-old corn shoots are referred to as young tissues. The more mature pea root segments and leaves of 10-day-old dark-grown corn seedlings are referred to as aged tissues. In young tissues the level of polyribosomes in the ribosome population exceeded 80%. As the tissues aged the proportion of polyribosomes decreased with a concomitant increase in monoribosomes. This aging response was more significant in corn as polyribosomes were nearly non-existent after 10 days growth in darkness. The age differential between cells was greater in corn than in the tissues used from the pea root where the polyribosomes differential was much less.

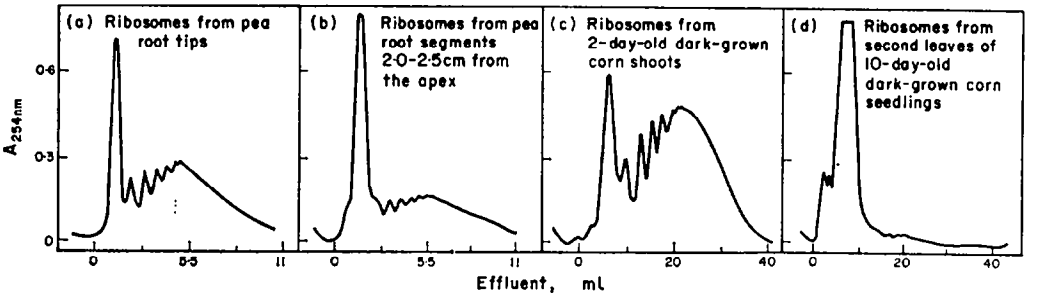

Fig. 1. Sucrose density gradient profiles of cytoplasmic ribosomes.
For pea 4–5 A_{260} units of ribosomes were layered on 11 ml 10–34% linear sucrose gradients. Centrifugation was for 70 min at 39 000 *g* (Spinco SW 41 rotor). For corn 12–15 A_{260} units of ribosomes were layered on 35 ml 10–34% linear sucrose gradients. Centrifugation was for 85 min at 25 000 *g* (Spinco SW 27 rotor).

Poly U-directed *in vitro* phenylalanine incorporation by pea root and corn polyribosomes at varying Mg^{2+} concentrations is shown in Fig. 2. Polyribosomes isolated from young tissues attained maximum incorporation activity at 16–17·5 mM Mg^{2+} whereas polyribosomes isolated from the aged tissues required 20–22·5 mM Mg^{2+} for optimum activity. Additionally, polyribosomes from young tissues were more active in phenylalanine incorporation than polyribosomes from aged tissue at all Mg^{2+} levels tested over the 30-min incubation period.

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In an earlier communication we reported that polyribosomes could be dissociated into single (monomeric) ribosomes by subjecting the tissue to an anaerobic environment (N_2 gas) prior to ribosome isolation.[8] In the present study the effect of N_2 on Mg^{2+} optima and ribosome activity was most pronounced with ribosomes from young tissues. The optimal Mg^{2+} concentration for N_2 gas-derived single ribosomes isolated from young tissues increased to 20–22·5 mM (Fig. 2), and at optimal Mg^{2+} levels the single ribosomes from both species were even more active than were polyribosomes. At low Mg^{2+} levels (10–15 mM) N_2 treatment greatly decreased the incorporation activity of single ribosomes from young tissues. While this response was greater with corn ribosomes a similar but less pronounced effect also occurred with pea ribosomes. The optimal Mg^{2+} requirement for single ribosomes isolated from aged tissues remained at 20–22·5 mM.

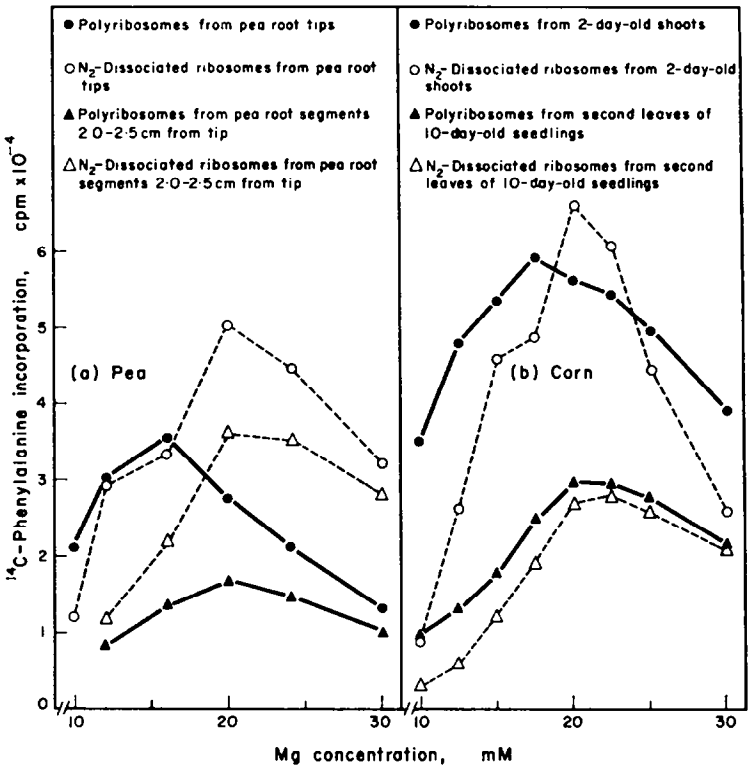

FIG. 2. INFLUENCE OF N_2 GAS DISSOCIATION ON ^{14}C-PHENYLALANINE INCORPORATION BY CYTOPLASMIC RIBOSOMES AT VARYING Mg^{2+} LEVELS.

Intact pea or 2-day-old corn seedlings and detached second leaves of 10-day-old dark-grown corn seedlings were N_2 gas-treated for 1·5 hr prior to ribosome isolation. Pea or corn ribosomes (1·8 A_{260} units) were incubated with 150 μg poly U and 0·5 μCi ^{14}C-phenylalanine (455 μCi/μmol) for 30 min at 37°. Other reaction conditions are described in Experimental.

There was a major difference in activity of N_2 gas-derived single ribosomes from aged pea and corn tissues. Single ribosomes from aged pea tissue were much more active at all Mg^{2+} levels tested than were polyribosomes. In corn, however, single ribosomes from 10-day leaves were slightly less active than polyribosomes at all Mg^{2+} levels. The protein synthetic

activity of N_2 gas-derived single ribosomes from pea root-tips was 25–30% greater at the optimal Mg^{2+} concentration than that of similar ribosomes isolated from the more mature segments. In corn the corresponding difference between single ribosomes from young and older tissues was 100–150%.

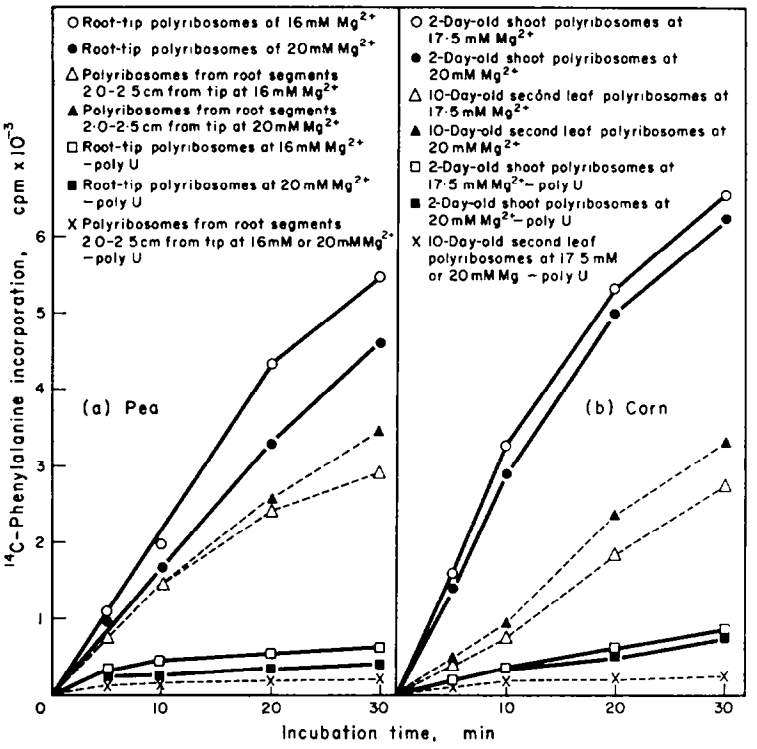

Fig. 3. Kinetics of ^{14}C-phenylalanine incorporation by cytoplasmic polyribosomes.
The reaction conditions are described in the legend for Fig. 2. Aliquots containing 0·36 A_{260} units ribosomes were removed at various intervals.

The kinetic data for phenylalanine incorporation by the polyribosome preparations at their respective optimal Mg^{2+} concentrations are shown in Fig. 3. These data further indicate the obvious differences in the level of phenylalanine incorporation by ribosomes isolated from young and aged tissues. Incorporation activity of ribosomes isolated from either tissue was essentially linear over a 20-min assay period. In addition there was a significant level of phenylalanine incorporation in the absence of exogenously supplied synthetic messenger RNA.

The kinetics for phenylalanine incorporation by N_2 gas-derived single ribosomes are shown in Fig. 4. Again incorporation was linear over a 20-min incubation period for corn and nearly so for pea. However, less activity was found with the single ribosomes than with the polyribosomes in the absence of exogeneously supplied poly U (Fig. 3).

Ribosome preparations were incubated with ^{3}H-poly U to test whether ribonuclease activity associated with the ribosomes might be responsible for the decline in activity with aging. Since the greatest aging response occurred with the corn system these ribosomes were

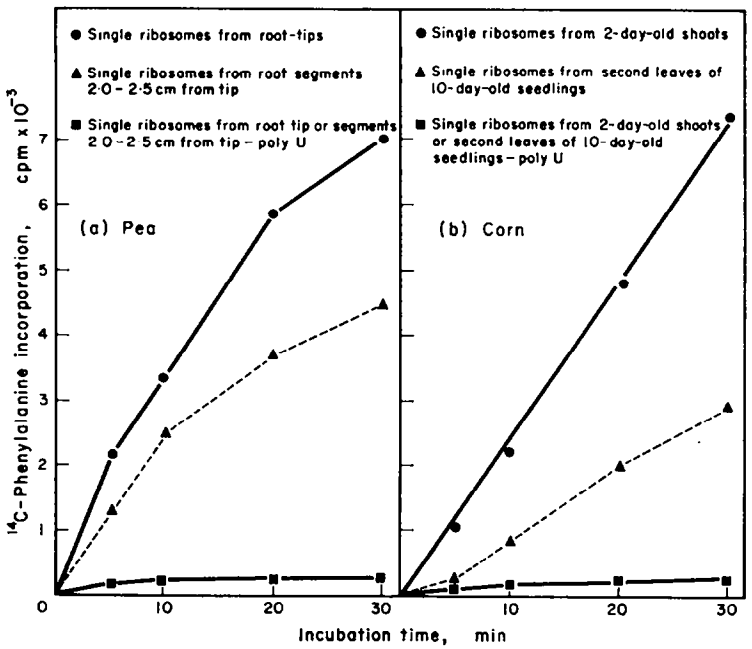

FIG. 4. KINETICS OF ^{14}C-PHENYLALANINE INCORPORATION BY N_2 GAS-DISSOCIATED CYTOPLASMIC RIBOSOMES AT 20 mM Mg^{2+}.

Reaction conditions are described in the legends for Figs. 2 and 3.

selected for this experiment. More than 30% of the ^{3}H-poly U was hydrolyzed by ribosomes from the aged tissue during the first 5 min of the incubation period. No further hydrolysis occurred during the remainder of the 30-min period. While the initial rate of hydrolysis by ribosomes isolated from young tissue was negligible, the level of hydrolysis also reached 30% by 15 min. After 15 min there was no further hydrolysis of poly U in either preparation.

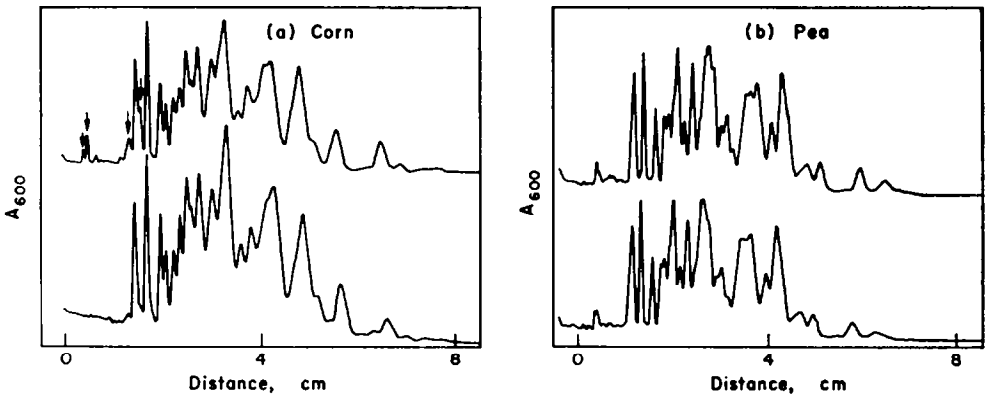

FIG. 5. POLYACRYLAMIDE GEL ELECTROPHORESIS OF RIBOSOMAL PROTEINS, OF YOUNG (above) AND AGED (below) CORN AND PEA TISSUES.

Ribosomal proteins were extracted with 67% HOAc Protein (50–60 g) were layered on 15% acrylamide gels and electrophoresis was for 4 hr 45 min at 1 mA/gel. Gels were stained with amidoblack destained with acetic acid and scanned at 600 nm.

Mixing experiments, again with corn ribosome preparations, were performed to eliminate the possibility that an inhibitor(s) might be responsible for the decreased activity of ribosomes isolated from aged tissue. There was an additive effect of mixing, indicating that inhibitors were not responsible for the decline in ribosome activity associated with aging. Also, the decrease in *in vitro* activity of ribosomes from aged tissues was not affected by the addition of 10^{-3} M dithiothreitol during the isolation procedure (data not included).

The electrophoretic profiles of ribosomal proteins extracted from N_2 gas-derived single corn ribosomes are shown in Fig. 5(a). Significant differences in several peaks were detected in the ribosomal protein complement of ribosomes from young and aged tissues (see arrows). Significant differences in these bands were not observed in the pea root system (Fig. 5b).

DISCUSSION

Ribosomal preparations from pea root-tip segments and 2-day-old corn shoots contained over 80% polyribosomes. The percentage of polyribosomes, however, decreased significantly with increasing tissue age. The level of poly U-directed phenylalanine incorporation by ribosome preparations was greater when the relative level of polyribosomes was high. Similar results have been obtained with ribosome preparations from developing seeds of *Vicia faba*.[5] These results relate to the presence of peptidyl-*t*RNA which is associated to a greater extent with polyribosomes and which increases the level of incorporation of phenylalanine into polypeptides.[10-12] Under these conditions phenylalanine is incorporated into peptidyl–phenylalanine rather than polyphenylalanine, and the protein synthetic activity assayed *in vitro* is that of the ribosomal complex (i.e. ribosome-endogenous *m*RNA-peptidyl-*t*RNA) rather than of single ribosomes *per se*. Conversely ribosome preparations from the mammary glands of pregnant guinea-pigs were stimulated more by poly U than were similar preparations from lactating animals even though the former contained a higher level of polyribosomes in the total ribosome population.[7] Similar results were reported by Pilkis and Korner[6] for ribosome preparations from liver of normal and diabetic rats. Additionally we observed that ribosome preparations from young tissues required lower Mg^{2+} levels for optimal incorporation than similar preparations from aged tissues.

Poly U-dependent phenylalanine incorporation was studied with single ribosomes derived by subjecting the tissues to an anaerobic environment prior to ribosome isolation. Under these conditions translation of *m*RNA is apparently completed and nascent proteins released, while further chain initiation is prevented.[8] When single ribosomes isolated from young tissues were produced in this manner the optimal Mg^{2+} concentration for phenylalanine incorporation increased to the same level as that required by ribosomes isolated from aged tissues. Similar treatment of aged tissue prior to ribosome extraction had no effect on Mg^{2+} requirements for phenylalanine incorporation. This Mg^{2+} shift also occurred in the light-activation of ribosomes from dark-grown corn seedlings[11] and with carrot ribosomes during tissue aging (unpublished data of Lin, Travis and Key). It has been reported that similar Mg^{2+} shifts in mammalian[10,12] and plant[13] systems are dependent upon the presence of *t*RNA or peptidyl-*t*RNA in the ribosomal *P*-site. In those experiments ribosomes required a lower Mg^{2+} concentration for optimal incorporation activity when the *P*-site was occupied. The removal of peptidyl-*t*RNA from the *P*-site also greatly decreased

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the incorporation activity of the ribosomes at low Mg^{2+} levels. Thus one explanation for the decrease in plant ribosome activity and the increase in Mg^{2+} requirements associated with aging may be that the number of ribosomes with peptidyl-*t*RNA at the *P*-site decreases. This possibility is substantiated by the Mg^{2+} shift which was induced by anaerobiosis.

One question that remains unanswered is why there was no shift in Mg^{2+} requirements of ribosomes from aged pea root tissue in response to anaerobiosis. It is reasonable that un-treated ribosomes would require less Mg^{2+} for optimal activity since there are more polyribosomes in the ribosome population. However, there was a relatively high level of polyribosomes in the older tissue as well (compared to aged corn leaves), thus an even higher Mg^{2+} requirement might be expected for ribosomes isolated from aged pea root segments after anerobic treatment.

The level of polyribosomes in the various preparations may represent the *in vivo* state of ribosome populations. The formation of polyribosomes *in vivo* may be controlled by the relative proportion of ribosomes and *m*RNA, and by relative rates of initiation, elongation and termination of polypeptide synthesis. For example, the low rate of protein synthesis and high level of monoribosomes in mature pea cotyledons is apparently related at least in part to a deficiency of *m*RNA.[14] It is difficult at present to assess the nature and availability of *m*RNA in the young and aged tissues. From *in vitro* tests performed here it seems unlikely that the low protein synthetic activity of ribosomes isolated from aged tissues is a reflection of *in vivo* *m*RNA limitation. If, however, we assume that the binding of *m*RNA to single ribosomes (small subunits) is a prerequisite for the initiation of protein synthesis,[15,16] then the higher *in vitro* level of phenylalanine incorporation by ribosomes from young tissues might also reflect their higher affinity for *m*RNA.

The decrease in *in vitro* protein synthetic activity associated with aging was more distinct with ribosomes prepared from corn leaves than pea roots. This obviously relates to the degree of aging that the two tissues were subjected to. The mature pea root ribosomes were isolated from root segments only 2·0–2·5 cm from the tip. Mature corn ribosomes, conversely, were isolated from second leaves of seedlings after 10 days growth in darkness. Thus, while both tissue sources were used for isolation of aged ribosomes, the pea root segments represented a relatively younger group of cells than 10-day-old corn leaves and produced the more active aged ribosome preparation.

Gel electrophoretic studies indicated that the change in *in vitro* protein synthetic activity of single ribosomes from corn leaves might relate to quantitative changes of several ribosomal proteins. Thus the decrease in protein synthetic activity associated with aging apparently involves the loss of specific ribosomal proteins. There was no obvious correlation between the change in protein synthetic activity and possible changes in ribosomal proteins in pea root. Since the change in protein synthetic activity was not as great as in corn leaves, it is possible that the electrophoretic technique used was not sensitive enough to detect a qualitative or quantitative change in ribosomal proteins of the magnitude which might relate to the relatively smaller differences in protein synthetic activity. Studies are in progress to determine which step(s) in protein synthesis (i.e. initiation, elongation, or termination) that these proteins might be involved in. The possibility that initiation factors might be involved appears most promising since a decrease in the rate of chain initiation

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would account for a decrease in the level of peptidyl-*t*RNA associated with the ribosomes and perhaps also to a lower relative level of polyribosomes. The mechanism might include detachment of specific proteins, prevention of binding of newly formed proteins, or changes in turnover rate of ribosomal proteins.

EXPERIMENTAL

Plant materials. Peas (var. Alaska) for 4-day-old root tissue, and corn (WF9 X M14) for 3-day-old shoots were germinated in rolls of moist absorbent paper in darkness.[17] 10-day-old corn seedlings were grown in vermiculite as previously described.[18] Apical (tip 0·3 cm) and maturing (2·0–2·5 cm back of the apex) sections were excised from 4-day-old pea roots. For 3-day-old corn tissue the entire shoot (*ca.* 0·5 cm in length) was used. For 10-day-old corn tissue the second leaves from dark-grown seedlings were cut to 10 cm.

Preparation of polyribosomes. All tissues were cut onto dry ice. Polyribosomes were prepared from frozen tissue as previously described.[19]

Preparation of monomeric ribosomes. Intact 4-day-old pea seedlings, 3-day-old corn seedlings or detached leaves from 10-day-old dark-grown corn seedlings were immersed in dist.H_2O at room temp. N_2 was bubbled through the system for 1–1·5 hr to dissociate polyribosomes into monomeric ribosomes.[8] Preparation of monomeric ribosomes from anaerobically treated tissues was as previously described.[19]

Amino acid incorporation studies. Amino acid incorporation was studied by the methods of Mans and Novelli[20] and Williams and Novelli.[21] Soluble post-ribosomal supernatant factors for all assays were prepared from 2-day-old dark-grown corn shoots.

Assay for ribonuclease activity. Ribonuclease activity associated with the ribosome preparations was assayed in a 0·5-ml reaction mixture similar to that used in amino acid incorporation studies. In the ribonuclease assay the soluble post-ribosomal supernatant fraction was omitted and 0·5 μCi ^{3}H-Poly U was included. At various time intervals 0·1 ml aliquots were removed from the reaction mixture. Trichloroacetic acid (TCA) was added to each fraction (final conc. 5%) to precipitate non-hydrolyzed polynucleotides. Cold TCA-insoluble radioactivity (i.e. non-hydrolyzed polynucleotides) was collected on Whatman GFA glass fiber discs. Radioactivity was determined in a Packard Liquid Scintillation Spectrometer.

Acrylamide gel electrophoretic separation of ribosomal proteins. Ribosomal proteins were extracted from ribosome pellets with 67% HOAc. Gel electrophoresis was as described by Panyim and Chalkley.[22] Acrylamide gels (0·45 × 9 cm, containing 6 M urea) were overlayed with approximately 50 μg protein and electrophoresed at room temp. for 4·75 hr at 1 mA/tube. Gels were then stained for 2 hr with 0·1% amidoblack in 7% HOAc, destained with 7% HOAc and scanned at 600 nm with a Gilford model 2400 spectrophotometer.

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