Phytochemistry, 1973, Vol. 12, pp. 2801 to 2807. Pergamon Press. Printed in England.

CHANGES IN RIBOSOMAL ACTIVITY DURING INCUBATION OF *DAUCUS CAROTA* ROOT DISKS*

CHU-YUNG LIN, ROBERT L. TRAVIS, LI-LI S. Y. CHIA and JOE L. KEY
Department of Botany, University of Georgia, Athens, GA 30602, U.S.A.

(*Received* 11 *April* 1973. *Accepted* 1 *June* 1973)

Key Word Index—*Daucus carota*; Umbelliferae; carrot; ribosomal activity; change; incubation.

Abstract—Cytoplasmic monoribosomes from freshly cut and 'aged' carrot root disks were characterized relative to the Mg^{2+} optima for poly U (polyuridylic acid)-directed phenylalanine incorporation, the ease of dissociation by KCl in the presence of Mg^{2+}, the ability to bind ^{3}H-poly U, and acrylamide gel fractionation of the ribosomal proteins. The differences in *in vitro* amino acid incorporation by ribosomes and supernatant from fresh and 'aged' disks were confined to the ribosome fraction. The Mg^{2+} optima for poly U-directed ^{14}C-phenylalanine incorporation was 16 mM for ribosomes from 'aged' disks compared to 20 mM for ribosomes from fresh disks. Monoribosomes from the fresh disks were easily dissociated into subunits (0·2 M KCl in 5 mM Mg^{2+}) while the ribosomes from 'aged' disks were not completely dissociated even in 0·5 M KCl. Ribosomes from 'aged' disks were more effective in binding ^{3}H-poly U than ribosomes from fresh disks. When the disks were subjected to an anaerobic environment prior to ribosome extraction (to strip monoribosomes of peptidyl-*t*RNA) the above effects of 'aging' were reversed. These results suggest that increased monoribosome activity associated with 'aging' may be related in part to an increase in the level of peptidyl-*t*RNA associated with the ribosomes. Acrylamide gel electrophoresis profiles of ribosomal proteins extracted from ribosomes of fresh and 'aged' tissue suggest that a change in the protein complement may also be important to the observed changes in ribosomal activity. The ribosomes from 'aged' disks contained at least two components not associated with ribosomes from fresh disks.

INTRODUCTION

WE PREVIOUSLY reported that a rapid and dramatic transition from monoribosomes to polyribosomes occurs during the 'aging' (tissue excised and incubated in water for 5 hr) of carrot root disks.[1] This transition required the synthesis of an RNA fraction having the properties of messenger RNA. Ribosomal RNA synthesis, and hence new ribosome formation, were not required. Similar ribosome transitions occur in other plant systems in response to light,[2,3] gibberellin[4,5] and auxin.[6,6a] The transition from monoribosomes to polyribosomes in corn leaves in response to light[2] and basal soybean hypocotyl in response to auxin[6a] involves activation of the 80*S* monoribosome in addition to a requirement for *m*RNA synthesis. To determine whether a similar situation exists in 'aging' carrot root disks, ribosome preparations from fresh and 'aged' disks were examined relative to Mg^{2+} optima for poly U-directed phenylalanine incorporation, the ability to dissociate 80*S* ribosomes into subunits with KCl, the ability to bind synthetic mRNA (poly U) and acrylamide gell fractionation of ribosomal proteins.

* Research supported by AEC Contract AT(38-1)-643.

[1] LEAVER, C. J. and KEY, J. L. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 1338.
[2] WILLIAMS, G. R. and NOVELLI, G. D. (1968). *Biochim. Biophys. Acta* **155**, 183.
[3] TRAVIS, R. L., HUFFAKER, R. C. and KEY, J. L. (1970) *Plant Physiol.* **46**, 800.
[4] EVINS, W. H. (1971) *Biochemistry* **10**, 4295.
[5] EVINS, W. H. and VARNER, J. E. (1972) *Plant Physiol.* **49**, 348.
[6] TREWAVAS, A. (1968) *Phytochemistry* **7**, 673.
[6a] TRAVIS, R. L., *et al.* (1973) *Plant Physiol. In press.*

PHYTO 12/12—A

The data indicate that the increase in polyribosome level in response to 'aging' relates (in addition to *m*RNA synthesis) to activation of the 80*S* ribosome resulting in increased ability to participate in protein synthesis.

RESULTS

Relative Efficiency of Supernatant Fractions from Fresh and 'Aged' Disks

In the experiments discussed below monoribosome activity was measured as poly U-directed phenylalanine incorporation into polyphenylalanine. In addition to ribosomes, poly U and the standard reaction ingredients (see Experimental), incorporation requires a soluble or supernatant fraction. This fraction, prepared from a post 100 000 *g* supernatant, contains the various protein factors necessary for peptide synthesis, transfer RNA and the appropriate aminoacyl-*t*RNA synthetases. It is possible that 'aging' may affect the activity of this supernatant fraction as well as the ribosomal fraction. Thus, to identify the fraction of the system responsible for the enhanced incorporation, the following experiment was performed. Polyribosomes (entire ribosome population) and supernatant were recombined for assay as indicated in Fig. 1. The results show that the enhanced incorporation associated with 'aging' was a feature of the ribosomes and was independent of the supernatant source. Similar results were shown by Williams and Novelli[2] for light-enhanced leucine incorporation from maize shoots. We have previously shown that a supernatant fraction prepared from shoots of 48-hr dark-germinated corn seedlings is an effective source of soluble components for the *in vitro* amino acid incorporation system.[7] In these experiments the corn supernatant was much more active than carrot supernatant and was used in all subsequent comparisons of ribosomal activities.

Effect of Mg^{2+} *Concentration on* ^{14}C*-Phenylalanine Incorporation by Monoribosomes*

At low Mg^{2+} levels (10–12 mM) monoribosomes from 'aged' disks were approximately twice as active at phenylalanine incorporation as ribosomes from fresh disks (Fig. 2). The amino acid incorporating activity of ribosomes from 'aged' disks increased to a maximum at 16 mM then declined rapidly. Incorporation by ribosomes from fresh disks increased to a maximum at 20 mM, again followed by a sharp decline as Mg^{2+} concentration was increased. Ribosomes from fresh disks were approximately twice as active as ribosomes from 'aged' disks at 20–24 mM Mg^{2+}. The lower Mg^{2+} requirement for optimal activity of poly U-directed phenylalanine incorporation, the increased activity at low Mg^{2+} levels and the decreased activity at high Mg^{2+} levels by monoribosomes from 'aged' disks may relate to the level of peptidyl-*t*RNA associated with the ribosomes. Ribosome preparations from light-treated corn leaves have a higher level of peptidyl-*t*RNA relative to ribosome preparations from dark-control leaves and respond to in a similar way Mg^{2+} concentration.[8] Similar results have been reported for ribosomes from lactating versus pregnant[9] and diabetic versus normal[10] mammals.

Ribosomes from unfertilized sea urchin eggs are less active at poly U-directed phenylalanine incorporation than are ribosomes from fertilized eggs.[11] These results have been attributed to the association of inhibitory proteins with the ribosomes of unfertilized eggs.

[7] LIN, C. Y., KEY, J. L. and BRACKER, C. E. (1966) *Plant Physiol.* **41**, 976.
[8] TRAVIS, R. L., LIN, C. Y. and KEY, J. L. (1972) *Biochim. Biophys. Acta* **277**, 606.
[9] FAIRHURST, E., MCILREAVY, D. and CAMPBELL, P. N. (1971) *Biochem. J.* **123**, 865.
[10] PILKIS, S. J. and KOENER, A. (1971) *Biochim. Biophys. Acta* **247**, 597.
[11] METAFORA, S., FELICATTI, L. and GAMBINO, R. (1971) *Proc. Nat. Acad. Sci. U.S.* **68**, 600.

In our experiments the high level of phenylalanine incorporation at 20 mM Mg^{2+} by ribosomes from fresh disks suggests that the low level of incorporation at low Mg^{2+} by these same ribosomes is not due to the association of inhibitory protens.

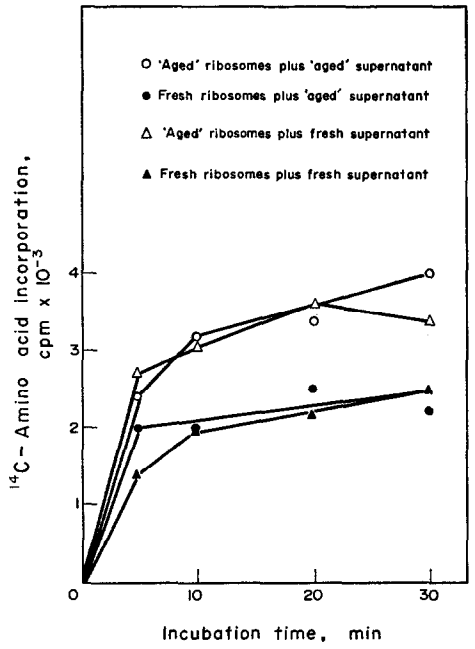

FIG. 1. KINETICS OF ^{14}C-AMINO ACID INCORPORATION BY RIBOSOMES FROM FRESH AND 'AGED' DISKS. Supernatant factors for this experiment were prepared from similar fresh and 'aged' disks. The reaction mixture contained 1·35 A_{260} units ribosomes and 0·25 μCi of ^{14}C-amino acid mixture. Aliquots containing 0·25 A_{260} units ribosomes were removed at various time intervals.

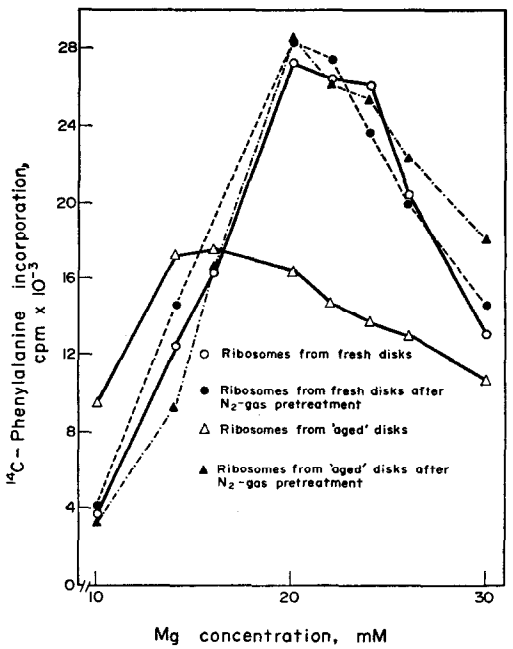

FIG. 2. INFLUENCE OF N_2-GAS PRETREATMENT ON ^{14}C-PHENYLALANINE POLYMERIZATION BY RIBOSOMES PREPARED FROM FRESH AND 'AGED' DISKS. 1·0 A_{260} unit ribosomes was incubated with 100 μg poly U and 0·5 μCi ^{14}C-phenylalanine (455 μCi/mol) for 30 min at 37°. Other reaction conditions are cited in the Experimental.

If the lower Mg^{2+} optimum and increased activity at low Mg^{2+} levels associated with ribosomes from 'aged' disks are related to the presence of peptidyl-*t*RNA, the 'aging' response might be reduced or abolished by treating the ribosomes in such a manner as to remove peptidyl-*t*RNA. Castles *et al.*[12] have shown that incubation with puromycin effectively strips mammalian ribosomes of peptidyl-*t*RNA. Additionally, we have shown that subjecting plant tissue to an anaerobic environment (tissue placed in water in air-tight container with nitrogen gas bubbled through the system) strips ribosomes of peptidyl-*t*RNA as effectively as puromycin does.[8] In the following experiments anaerobiosis was used to prepare stripped ribosomes.

A 1-hr nitrogen pretreatment of the tissue prior to ribosome preparation completely reversed the 'aging' response (Fig. 2). The nitrogen treatment decreased the incorporating activity of ribosomes from 'aged' disks at low Mg^{2+} levels and increased their activity at high Mg^{2+} levels, effectively equalizing the activity of both preparations at all Mg^{2+} levels

[12] CASTLES, J. J., ROLLESTON, F. S. and WOOL, I. G. (1971) *J. Biol. Chem.* **246**, 1799.

tested. Nitrogen treatment had no effect on ribosomes prepared from fresh disks, indicating that such ribosomes contain little or no peptidyl-*t*RNA.

KCl *Dissociation of Monoribosomes*

The purpose of monoribosome dissociation studies is 2-fold. First, the process of peptide chain initiation involves the binding of ribosomal subunits to *m*RNA in bacterial,[13] mammalian,[14] and plant[15] systems. Thus, a possible mechanism underlying the quiescent state of fresh, non-aged carrot disks might be a stabilization of ribosomal subunit association which could be released during 'aging'. Secondly, the ability to dissociate monoribosomes into ribosomal subunits on sucrose gradients containing KCl can be considered as a further indication of the presence of peptidyl-*t*RNA associated with ribosomes.[8,16] Ribosomes lacking peptidyl-*t*RNA readily dissociate into subunits at low KCl (0·1–0·2 M) concentration whereas ribosomes containing peptidyl-*t*RNA resist dissociation even at relatively high

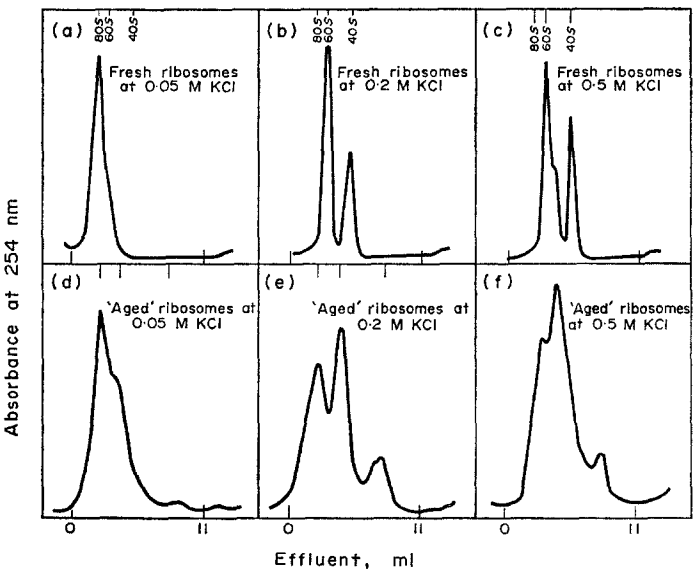

FIG. 3. SUCROSE GRADIENT PROFILES OF RIBOSOMES DISSOCIATED BY KCl IN THE PRESENCE OF 5 mM $MgCl_2$.

KCl (0·5–0·8 M). Profiles of ribosomes from fresh and 'aged' disks following KCl treatment are presented in Fig. 3. Ribosomes isolated from fresh disks were easily dissociated into ribosomal subunits by a moderately low concentration of KCl (0·2 M). Similar results were reported for ribosomes isolated from unfertilized sea urchin eggs.[17] In contrast, ribosomes isolated from 'aged' disks were only partially dissociated into ribosomal subunits even at 0·5 M KCl. In an earlier communication we reported that N_2-induced monoribosomes

[13] NOMURA, M. and LOWRY, C. V. (1967) *Proc. Nat. Acad. Sci. U.S.* **58,** 946.
[14] SHAFRITZ, D. A., LAYCOCK, D. G., CRYSTAL, R. G. and ANDERSON, W. F. (1971) *Proc. Nat. Acad. Sci. U.S.* **68,** 2246.
[15] WEEKS, D. P., VERMA, D. P. S., SEAL, S. N. and MARCUS, A. (1972) *Nature* **236,** 167.
[16] STURANI, E., ALBERGHINA, F. A. M. and CASACCI, F. (1971) *Biochim. Biophys. Acta* **254,** 296.
[17] INFANTE, A. A. and GRAVES, P. N. (1971) *Biochim. Biophys. Acta* **246,** 100.

(run-off ribosomes) from pea root were also easily dissociated into ribosomal subunits.[18] Results obtained in these experiments indicate that since ribosomes from fresh carrot disks are easily dissociated into subunits they probably represent free ribosomes and lack peptidyl-*t*RNA. The ease of dissociation of ribosomes from fresh disks also suggests that the quiescent state of protein synthesis is not likely to be due to a limitation of ribosomal subunits (through monoribosome stabilization) required for peptide chain initiation.

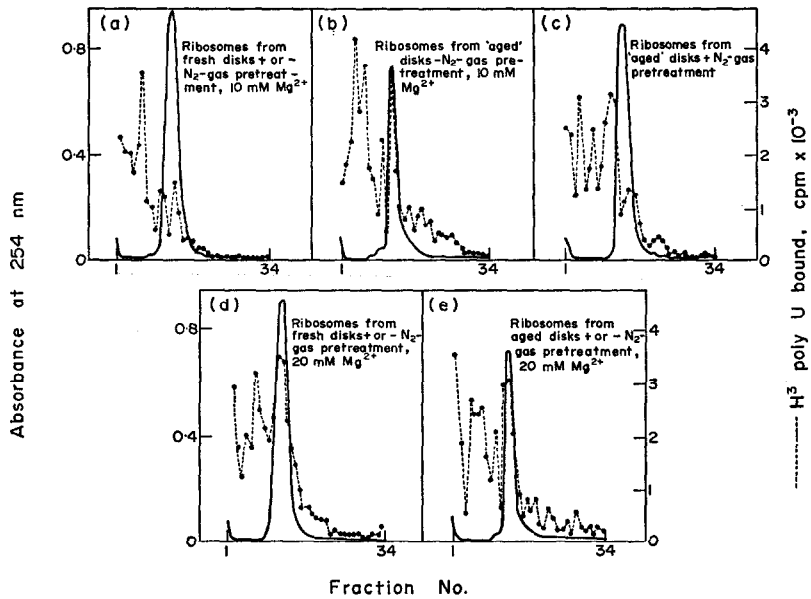

FIG. 4. INFLUENCE OF N_2-GAS PRETREATMENT AND Mg^{2+} CONCENTRATION ON ^{3}H-POLY U BINDING BY MONORIBOSOMES PREPARED FROM FRESH AND 'AGED' DISKS.
Reaction conditions are described in the Experimental.

Binding of ^{3}H-*Poly U to Monoribosomes*

To further substantiate that 'aging' enhanced the activity of 80*S* monoribosomes their ability to bind synthetic messenger RNA was evaluated (Fig. 4). Binding experiments were performed by incubating the monoribosome preparations with ^{3}H-poly U then determining the radioactivity associated with the monoribosome fraction recovered from sucrose gradients (see Experimental). At low Mg^{2+} (10 mM) ribosomes from 'aged' disks (Fig. 4b) bound approximately four times as much poly U as did ribosomes from fresh disks (Fig. 4a). Treating the disks with N_2 prior to ribosome preparation again completely reversed the 'aging' response (Fig. 4c). At high Mg^{2+} (20 mM) binding of poly U was similar for ribosomes from both fresh and 'aged' disks and was not affected by N_2 treatment (Fig. 4d,e).

Gel Electrophoresis of Ribosomal Proteins

The enhanced ability of the monoribosomes to participate in protein synthesis and the subsequent increase in peptidyl-*t*RNA might be expected to relate to changes in some

[18] LIN, C. Y. and KEY, J. L. (1971) *Plant Physiol.* **48**, 547.

ribosome-associated protein(s) required for optimal protein synthetic acitvity. This possibility was explored by comparing the proteins associated with fresh and 'aged' ribosomes. Ribosomal proteins were extracted with 67% acetic acid and fractionated by acrylamide gel electrophoresis as described by Panyim and Chalkley.[19] Ribosome preparations from 'aged' disks contained at least two protein bands which were not detected in ribosome preparations from fresh disks (Fig. 5). That there is a modification of the ribosomes during 'aging' is further substantiated by this change in the ribosomal protein complement.

It is generally believed that peptide chain initiation begins by the binding of *m*RNA to the small ribosomal subunit.[20] This initial step in protein chain initiation is catalyzed by initiation factors which are apparently associated with the subunit. Thus, it is reasonable to assume that the continuous modification of at least the small subunit is essential for the recognition of *m*RNA for initiation. The change in the ribosomal protein complement during 'aging' may well reflect this requirement. That such modification of the small subunit does occur, at least with *E. coli* ribosomes, is indicated by the heterogeneity of the ribosomal protein complement.[21]

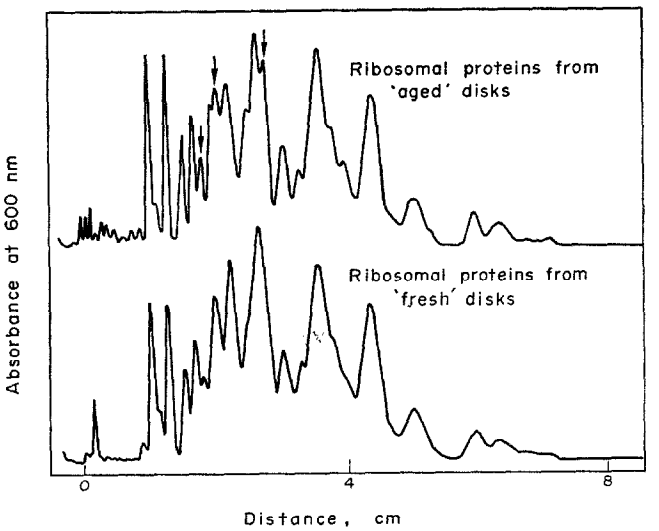

FIG. 5. POLYACRYLAMIDE GEL ELECTROPHORESIS OF RIBOSOMAL PROTEINS.
Ribosomal proteins were extracted with 67% acetic acid. 50–60 μg of protein were layered on 15% acrylamide gels, and electrophoresis was for 4·75 hr at 1 mA per gel. Gels were stained with amidoblack, destained with acetic acid and scanned at 600 nm.

DISCUSSION

These results support the hypothesis that the increase in polyribosome level in response to 'aging' relates, at least in part, to some changes in the free 80*S* ribosome resulting in an increased ability of the ribosome to participate in protein synthesis. The ribosomal protein and poly U-binding experiments suggest in fact that a basic change in the ribosome occurs in response to 'aging'. If this alteration led to an increase in ribosome activity then an increase in the level of peptidyl-*t*RNA associated with 'aged' ribosomes would be expected. The

[19] PANYIM, S. and CHALKLEY, R. (1969) *Arch. Biochem. Biophys.* **130,** 337.
[20] MILLER, M., ZASLOFF, M. and OCHOA, S. (1969) *FEBS Letters* **3,** 50.
[21] BURKA, E. R. and BULOVA, S. I. (1971) *Biochem. Biophys. Res. Commun.* **42,** 801.

increase in peptidyl-*t*RNA as demonstrated by Mg^{2+} requirements for poly U-directed phenylalanine incorporation and ease of KCl-dissociation, is likely a secondary effect (i.e. a result of rather than mechanism for enhancement of protein synthesis) of 'aging'. Further experiments should establish if the observed changes in ribosomal proteins with 'aging' relate possibly to changes in initiation factors or some other protein(s) essential to protein synthesis.

EXPERIMENTAL

Plant materials. Preparation and 'aging' of carrot tissue were as previously described.[1] For 'aging' 10 g of fresh carrot disks (*ca.* 0·5 × 5 mm) were incubated with continuous shaking in 40 ml dist. H_2O containing chloramphenicol (50 μg/ml) for 5 hr at 30°.

Preparation of ribosomes. Isolation and characterization of ribosomes were as previously described[7] with minor modifications. In these experiments the tissue was not frozen prior to ribosome isolation. Fresh or 'aged' disks were homogenized with a Willems Polytron PT 20 st. at high speed for 8 sec in Tris-K^+-Mg^{2+} buffer (50 mM Tris, pH 7·5; 15 mM KCl; and 20 mM $MgCl_2$) containing 0·25 M sucrose. The homogonate was filtered through Miracloth, and the resulting filtrate was centrifuged at 20000 *g* for 20 min. In order to separate monoribosomes from polyribosomes *ca.* 10 ml of the crude supernatant were layered on a 3-ml discontinuous sucrose gradient consisting of 2 ml of 1 M sucrose in Tris-K^+-Mg^{2+} buffer layered over 1 ml of 2 M sucrose. After centrifugation at 159 000 *g* for 30 min (Spinco type 65 rotor) the monoribosomes were recovered from the upper layer of the gradient (1 M sucrose) and re-layered over 4 ml 1·5 M sucrose containing Tris-K^+-Mg^{2+} buffer. The polyribosome fraction (2 M sucrose) was discarded. Monoribosomes were pelleted at 221 000 *g* for 85 min, then resuspended in Tris-K^+-Mg^{2+} buffer. All steps were carried out at 0–3°. Dissociation of 80*S* monomeric ribosomes into subunits was done as previously described.[8]

Poly U-directed phenylalanine incorporation and ^{3}*H-poly U binding.* The monoribosome preparations were used for *in vitro* poly U-directed ^{14}C-phenylalanine incorporation following the methods of Mans and Novelli[22] and Williams and Novelli.[23] Supernatant factors (protein) were prepared from fresh and 'aged' tissue or from shoots of 48-hr dark-germinated corn seedlings as previously described.[7] For *m*RNA binding studies 2–3 A_{260} units of ribosome suspension were incubated with 0·5 μCi ^{3}H-poly U for 3 min at 37°.

Sucrose density gradient analysis of ribosomes. A 0·3 ml aliquot containing 3–5 A_{260} units of ribosomes was layered over a linear 10–34% sucrose gradient and centrifuged at 39 000 rpm (Spinco, SW 41 rotor) for 70 min at 3°. The distribution of ribosomes in the gradient was monitored with a continuous recording ISCO model D density gradient fractionator. For ^{3}H-poly U-associated ribosomes, 10-drop fractions were collected. The samples were precipitated with an equal volume of 10% trichloroacetic acid, collected on Whatman GFA glass fibre disks, washed with 5% trichloroacetic acid and dried. Radioactivity was determined in a Packard Liquid Scintillation Spectrometer.

Extraction of ribosomal proteins. Ribosomal proteins were extracted with 67% acetic acid; the proteins were resolved by acrylamide gel electrophoresis essentially as described by Panyim and Chalkley.[19]

Source of materials. ^{14}C-Phenylalanine (455 μCi/μmol) and ^{14}C-amino acid mixture (Algal profile) were obtained from Schwarz Radio Bio Research; ^{3}H-poly U from Miles Laboratories; and poly U, ATP, GTP, phosphoenol-pyruvate, and pyruvate kinase from Cal Biochem.

[22] MANS, R. J. and NOVELLIA, G. D. (1961) *Arch. Biochem. Biophys.* **94**, 48.
[23] WILLIAMS, G. R. and NOVELLI, G. D. (1964) *Biochem. Biophys. Res. Commun.* **17**, 23.