Association of D-RNA with Polyribosomes in the Soybean Root[1, 2]

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Summary. Ribosomes from soybean root tips were shown to consist of about 75 % polyribosomes based on size distribution on sucrose gradients. Electronmicrographs showed the presence of ribosome clusters in normal preparations and only monomers and dimers in ribonuclease-treated preparations. The polyribosome preparations, but not single ribosomes, showed good in vitro amino acid incorporating activity.

D-RNA was associated with the ribosome preparations, and preferentially in the polyribosome structure. It is suggested that at least a part of the soybean D-RNA represents a class of messenger RNA.

During studies on RNA metabolism in excised soybean hypocotyl, a fraction of RNA, namely DNA-like RNA (D-RNA), was characterized (7) which possessed many of the properties ascribed to bacterial messenger RNA (19). The synthesis of D-RNA was shown to be essential for growth of excised plant tissues (9). Subsequently, the synthesis of D-RNA was demonstrated in the intact root (8). Although the properties of D-RNA (7, 9) were consistent with it being messenger RNA, additional information was needed to support this view.

Since polyribosomes, which are the active sites of protein biosynthesis (5, 6, 15, 21), are held in association by messenger RNA (17, 18), experiments were performed to see if D-RNA was associated with the polyribosome structure. In this communication we report the isolation and characterization of a polyribosome fraction from the soybean root. The ribosomes isolated from pulse-labeled roots showed a preferential association of D-RNA with the polyribosomes relative to the monomer unit.

Materials and Methods

Soybean seeds were germinated in rolls of moist paper as described previously (8). The 5-mm root tips were excised from either 2- or 3-day-old seedlings onto pulverized dry ice and ground in dry ice by mortar and pestle. The powder was gently homogenized in a loose-fitting ground glass conical hand homogenizer (usually 2 strokes with 8 to 10 quarter turns each) in a 0.25 M sucrose solution containing 0.05 M Tris (pH 7.4), 0.015 M KCl, and 0.02 M $MgCl_2$.

The homogenate was filtered through Mira cloth, and the filtrate centrifuged at 13,500 × *g* for 10 minutes. Ribosomes were prepared from the supernatant solution by layering the sample over successive layers of 0.5 and 1.8 M sucrose (each contained 0.05 M Tris, 0.015 M KCl and 0.005 M $MgCl_2$) followed by centrifugation at 105,000 × *g* for 4 hours in the No. 40 Spinco rotor (21). The ribosome pellets were dissolved in Tris containing 0.015 M KCl and 0.005 M $MgCl_2$. All steps were carried out at about 0°.

The ribosome preparations were either used at this stage in studies of amino acid incorporation, following the methods of Mans and Novelli (12, 13) and Williams and Novelli (22), or characterized by the following methods. A sample containing about 1 mg of ribosomes (1 ml or less) was layered on linear sucrose gradients (10–34 %) and centrifuged at 23,000 rpm in the SW 25 rotor for 2 hours. Distribution of ribosomes on the gradient was measured by obtaining 10 drop fractions (made to 2 ml) by

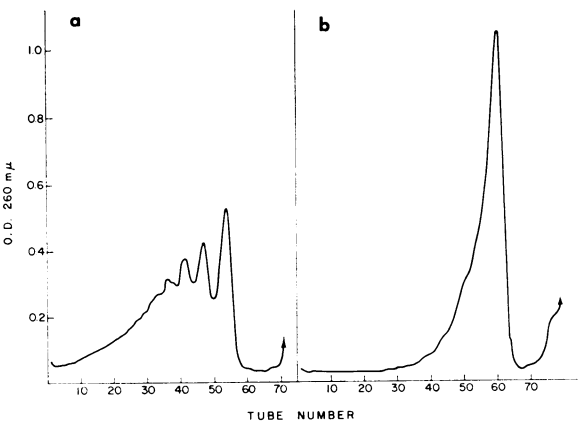

FIG. 1. Zone centrifugation analysis of ribosomes. a) Control. b) 0.5 μg of ribonuclease at 37° for 5 minutes. Tube puncture collection with OD profile at 260 mμ. Top of gradients to the right.

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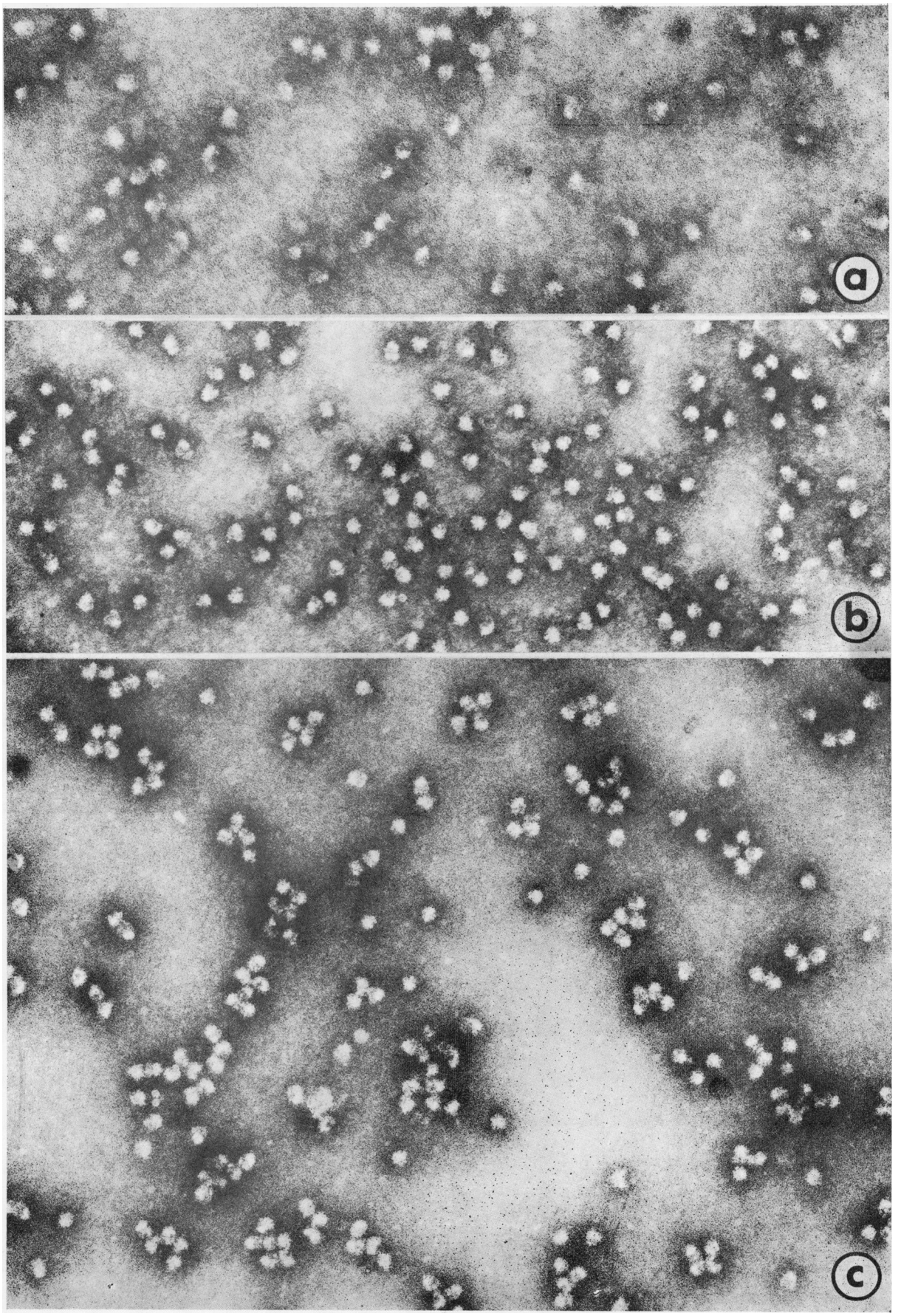

FIG. 2. Electron micrographs of isolated ribosomes negatively stained with phosphotungstic acid. a) Polyribosome preparation after treatment with ribonuclease. b) Ribosome monomers (isolated at room temperature). c) Polyribosome preparation without ribonuclease treatment showing monomer, dimer, trimer, tetramer and other polymeric forms of associated ribosomes. Magnification: ×107,000.

hypodermic needle puncture of the bottom of the centrifuge tube followed by reading at 260 mμ or by collection in an ISCO Model D density gradient fractionator. Collection in the ISCO fractionator was made at a syringe speed of 3 and one-third ml per minute with the OD profile being made at a wavelength at 254 mμ with a 1-cm light path. Sedimentation coefficients ($S_{20,w}$) were obtained by analytical centrifugation using Schlieren optics and broadbean mosaic virus (91S) as a standard. For electron microscopy drops of ribosome preparations were placed on carbon-stabilized collodion coated grids. They were then fixed by floating the grids on a 2.5 % glutaraldehyde solution (charcoal treated) for 5 minutes, rinsed with water, and negatively stained with 1 % phosphotungstic acid containing 5×10^{-3} $MgCl_2$ (pH 5.4). These specimens were examined and photographed with a Philips EM/200.

RNA was extracted from whole tissue or from ribosome pellets as described previously (7). Where RNA was purified from sucrose gradient-fractionated ribosomes, the monomer ribosome and the polyribosomes were collected separately and pelleted by centrifugation. The purified RNA from whole tissue or from the ribosomes was hydrolyzed in 0.3 N KOH and fractionated on Dowex-1 columns for base composition analyses or fractionated on the MAK column of Mandel and Hershey (11) as described previously (8). Absorption profiles were made at 260 mμ, and radioactivity was measured by scintillation counting of samples precipitated and collected on nitrocellulose membrane filters (type B-6 S & S).

Results

Demonstration of Polyribosomes in the Soybean Root. The distribution of ribosomes following centrifugation on a sucrose gradient is shown in figure 1a. About 75 % of the preparation was larger than the monomer unit. S values obtained by analytical centrifugation agree closely with those reported by Bayley (1) for pea ribosomes and by Wettstein et al. (21) for rat liver ribosomes (i.e. 75–80 S, 112–115 S, 148–150 S, and 172–175 S for the major components, presumably the monomer, dimer, trimer, and tetramer, respectively). As would be expected for polyribosomes, the preparation was degraded to the monomer unit and leading shoulder during a brief treatment with the enzyme ribonuclease (fig 1b). Electron micrographs of ribosome preparations corresponding to figure 1a revealed clusters of ribosomes in the untreated preparation (fig 2c) and only monomer and dimer units in the ribonuclease-treated sample (fig 2a). A preparation of ribosomes made at room temperature and corresponding to the gradient profile shown in fig 3a showed only the monomer unit following fixation and electron microscopic examination (fig 2b). Thus, the sucrose gradient profiles and the electron micrographs show particles isolated from the soybean root which are similar to the polyribosomes in other systems (1, 2, 3, 15, 20, 21). In addition, the ribosome preparations (fig 1a, 3c) were active in amino acid incorporation (fig 4, table I). After a few minutes incubation in ^{14}C-amino acid, the rate of incorporation decreased considerably coinciding with the decrease in heavy ribosomes and the corresponding increase in the monomer and leading shoulder components (fig 3b). In addition, the ribosome preparations made at room temperature consisted of only the monomer unit (fig 3a) and were relatively inactive in amino acid incorporation (fig 4). The requirements for amino acid incorporation by the active preparations are shown in table I, and are similar to those of other plant systems (13, 14.)

Table I. *Requirement for in vitro Amino Acid Incorporation by Ribosome Preparations from Soybean Root Tips*

Incubation time was 30 minutes at 37°. Complete system consisted of 50 μmoles of Tris (pH 7.4), 5 μmoles of $MgCl_2$, 8 μmoles of KCl, 0.5 μmole of ATP, 0.15 μmole of GTP, 6.4 μmoles of P-enolpyruvate, 0.05 mg of pyruvic kinase, corn supernatant, (0.5–1.0 mg protein), 0.2 ml of ribosomes (0.5–1.0 mg protein), and 0.5 μc of ^{14}C-leucine (240 mc/mmole) in a final volume of 0.5 ml. Average cpm of ^{14}C-leucine incorporated into protein from 5 experiments was 19,920 in the complete system.

Complete system (19,920 cpm incorporated)	100 %
Complete + ribonuclease (0.5 μg)	5 %
— Ribosomes	1 %
— Supernatant	4 %
— ATP	98 %
— GTP	82 %
— ATP, GTP, P-enolpyruvate, and pyruvate kinase	4 %
+ Amino acid mixture*	78 %

* A mixture of 19 L-amino acids (0.1 μmole each) excluding leucine (21).

The ribosome preparations consisted of 37 to 40 % RNA and 60 to 63 % protein.

Association of D-RNA with the Polyribosomes. In studying the association of D-RNA with ribo-

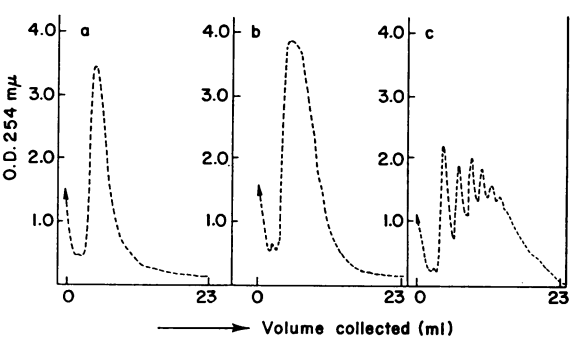

FIG. 3. Sucrose gradient profiles of ribosome preparations. OD profile at 254 mμ was obtained by ISCO gradient fractionator. Tops of gradients to the left. a) Ribosomes prepared at room temperature. b) Ribosomes at the end of 30-minutes incubation at 37°. c) Ribosomes before incubation.

somes, soybean seedlings were pulse-labeled with ^{32}P-orthophosphate. Ribosome preparations from the pulse-labeled roots were centrifuged on sucrose gradients yielding the OD 260 mμ and radioactivity profiles shown in figure 5. The polyribosome regions of the gradient showed considerably higher specific activity than the monomer region. Following longer labeling periods, there was less difference in specific activity of the polyribosome and monomer regions.

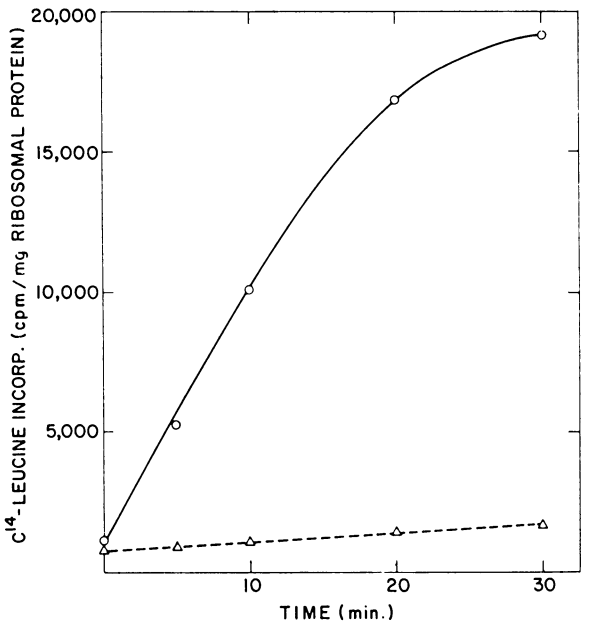

FIG. 4. Kinetics of ^{14}C-leucine incorporation into protein by ribosome preparations. The incubation medium consisted of 50 μmoles of Tris (pH 7.4), 5 μmoles of $MgCl_2$, 8 μmoles of KCl, 0.5 μmole of ATP, 0.15 μmole of GTP, 6.4 μmoles of P-enolpyruvate, 0.05 mg of pyruvic kinase, corn supernatant (0.5-1.0 mg protein), 0.2 ml of ribosomes (0.5–1.0 mg protein), and 0.5 μc of ^{14}C-leucine (240 mc/mmole) in a final volume of 0.5 ml. ———, Ribosome preparation corresponding to figure 3c. - - - - - , Ribosome preparation corresponding to figure 3a.

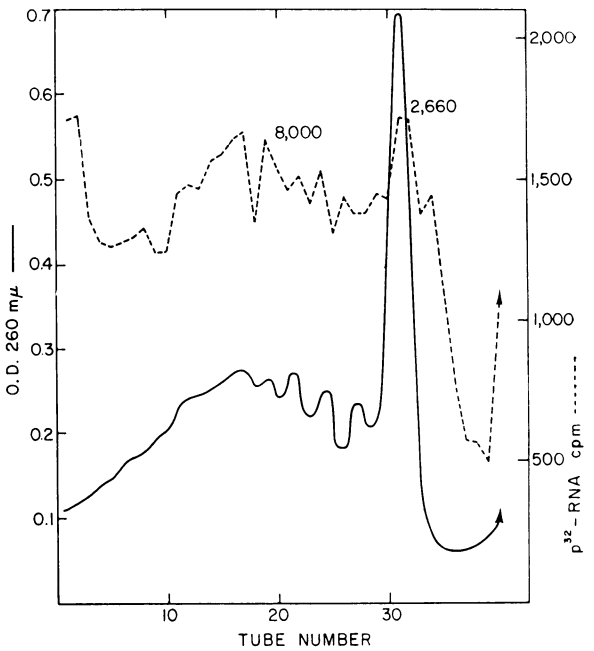

FIG. 5. Sucrose gradient profile of ribosomes from ^{32}P pulse labeled roots. 300 seedlings were preincubated in shake culture for 1.5 hours followed by a 10-minute pulse with 4 mc of ^{32}P-orthophosphate prior to isolation of ribosomes. Specific activities (cpm/OD) for monomer and polyribosome units are given.

Most of the newly synthesized RNA (^{32}P-labeled) in the ribosome preparation was D-RNA as shown by base composition analyses (table II) (see ref 7 and 8 for details of RNA base compositions). Furthermore, the base compositions revealed an enrichment of D-RNA in the polyribosomes relative to the monomer (table II). Separation of purified RNA on MAK columns confirmed the presence of D-RNA (based on ^{32}P-RNA distribution) in the ribosome preparation (fig 7). It should be pointed out that both the base composition analyses (table II) and the

Table II. *Base Composition of Newly Synthesized ^{32}P-RNA of Intact Soybean Root Tips*

Base composition analyses were performed as previously described (7) on ^{32}P-RNA purified from soybean root tips which were pulsed for 10 minutes with ^{32}P-orthophosphate. The monomer and polyribosomes were collected separately from sucrose gradients prior to extraction and hydrolysis of the RNA in 0.3 N KOH and separation of the nucleotides on Dowex-1 columns. The reported compositions are based on the distribution of ^{32}P in the nucleoside-2′-3′-phosphates.

RNA Fraction	Mole %				GMP
	CMP	AMP	GMP	UMP(TMP)	AMP
Total ^{32}P-RNA	21.6	30.1	24.7	23.5	0.82
Monomer ribosomal ^{32}P-RNA	22.8	27.7	29.4	20.1	1.06
Polyribosomes ^{32}P-RNA	21.3	30.7	24.8	23.2	0.81
DNA*	19.5	30.5	19.5	30.5	0.64
^{32}P-D-RNA*	22.1	32.9	20.6	24.4	0.63
Total RNA*	23.9	23.2	31.9	21.1	1.37

* Data taken from Ingle, Key, and Holm (7).

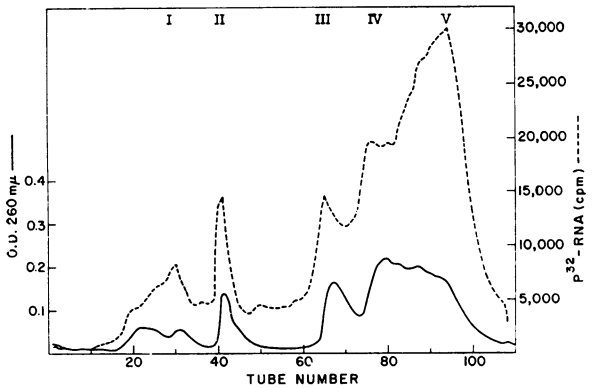

FIG. 6. MAK fractionation of total root RNA. 120 seedlings were labeled with 1.2 mc of ^{32}P-orthophosphate for 20 minutes and the apical 0.5 cm of the roots combined with similar tissue from 120 unlabeled seedlings prior to isolation of the RNA. ———, OD 260 mμ; - - - - -, radioactivity. Fractions I, II, III, IV, and V generally delineate soluble RNA, DNA, l.r.-RNA, h.r.-RNA, and D-RNA, respectively (7).

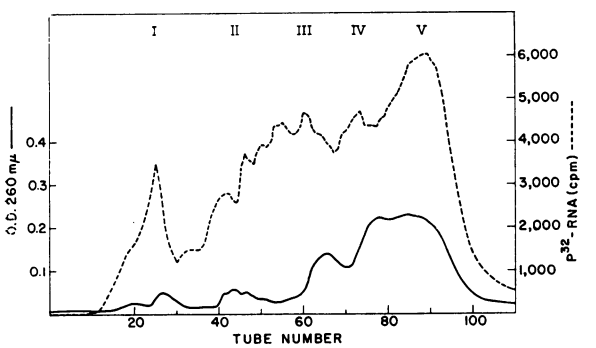

FIG. 7. MAK fractionation of RNA prepared from isolated ribosomes. Ribosomes, corresponding to figure 3c, were isolated separately from 0.5 cm root tips of seedlings, 360 of which were labeled for 20 minutes in ^{32}P-orthophosphate and 480 unlabeled seedlings. ———, OD 260 mμ; - - - - -, radioactivity.

MAK profiles show the presence of a mixture of ^{32}P-D-RNA and ^{32}P-ribosomal RNA in the polyribosome preparation, but clearly a relatively larger proportion of D-RNA. The radioactivity profiles of the MAK separations suggest some degradation of the D-RNA during ribosome preparation since areas of high radioactivity (especially between the DNA and light ribosomal RNA) were present where only a relatively small amount of newly synthesized RNA was usually found in total RNA preparations (fig 6). Such degradation of D-RNA might be expected since the base composition of newly synthesized RNA in the monomeric ribosome showed the presence of some D-RNA, presumably a result of polyribosome destruction by endogenous nuclease with fragments of the D-RNA remaining attached to the monomer.

Discussion

The results provide evidence of polyribosomes in the soybean root tip with properties similar to polyribosomes from other systems. Although the proportion of ribosomes present in the form of polyribosomes (70–75 %) was reproducible, there was some variation in the size distribution of the ribosomes on sucrose gradients (cf. fig 1, 3, 5). There is no evidence that the size distribution of the isolated ribosomes reflects the native state, and this would seem unlikely since the polyribosome structure is so sensitive to both endogenous and exogenous ribonuclease. The preparations are comparable, however, to polyribosomes described in many plant and animal systems (1, 2, 3, 6, 14, 15, 21) both in terms of physical properties and amino acid incorporating activity.

Of more interest is the apparent specific association of D-RNA with the polyribosomes of the soybean root. Loening (10) recently reported the association of a fraction of RNA (designated messenger RNA) with a microsome preparation from pea roots which is similar in many respects to soybean D-RNA. Thus, the association of a DNA-like fraction of RNA with ribosomes may be general in plants.

Although there appears to be specificity in the association of D-RNA with ribosomes (i.e. in the polyribosomes), the D-RNA in the ribosome fraction represents at most 10 to 20 % of the total D-RNA in the tissue. Such a distribution might be expected since the conditions required for extraction of D-RNA (7) suggest considerable localization within the nucleus. When intact tissue is homogenized with phenol, about 20 % of the D-RNA is extracted under conditions where the bulk of ribosomal and soluble RNAs is extracted, with the remainder of the D-RNA being extracted under conditions that extract the DNA (7).

Taken together with the general properties of D-RNA (7, 9), namely A) its similarity in base composition to DNA, especially in relation to AMP and GMP contents, B) a rapid rate of labeling relative to ribosomal RNA, C) a shorter mean life than ribosomal RNA (in the range of 2–3 hours in excised soybean hypocotyl), D) heterogeneity in molecular size, and E) general biological activity as exemplified by the requirement for D-RNA synthesis for growth and enzyme induction in excised plant tissues; the association of D-RNA with ribosomes in an active polyribosome structure lends strong support to the view that at least a part of the D-RNA is messenger RNA.

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Literature Cited

1. BAYLEY, S. T. 1964. Physical studies on ribosomes from pea seedlings. J. Mol. Biol. 8: 231–38.
2. CLARK, M. F., R. E. F. MATTHEWS, AND R. K. RALPH. 1964. Ribosomes and polyribosomes in *Brassica pekinensis*. Biochim. Biophys. Acta 91: 289–304.
3. DURE, L. AND L. WATERS. 1965. Long-lived messenger RNA: Evidence from cotton seed germination. Science 147: 410–12.
4. GIERER, A. 1963. Function of aggregated reticulocyte ribosomes in protein synthesis. J. Mol. Biol. 6: 148–57.
5. GILBERT, W. 1963. Polypeptide synthesis in *Escherichia coli*. 1. Ribosomes and the active complex. J. Mol. Biol. 6: 374–88.
6. HARDESTY, B., R. MILLER, AND R. SCHWEET. 1963. Polyribosome breakdown and hemoglobin synthesis. Proc. Natl. Acad. Sci. Wash. 50: 924–31.
7. INGLE, J., J. L. KEY, AND R. E. HOLM. 1965. The demonstration and characterization of a DNA-like RNA in excised plant tissue. J. Mol. Biol. 11: 730–46.
8. INGLE, J. AND J. L. KEY. 1965. A comparative evaluation of the synthesis of DNA-like RNA in excised and intact plant tissues. Plant Physiol. 40: 1212–19.
9. KEY, J. L. AND J. INGLE. 1964. Requirement for the synthesis of DNA-like RNA for growth of excised plant tissue. Proc. Natl. Acad. Sci. Wash. 52: 1382–88.
10. LOENING, U. E. 1965. The synthesis of messenger and ribosomal RNA in pea seedlings as detected by electrophoresis. Proc. Roy. Soc. (B) 162: 121–36.
11. MANDELL, J. D. AND A. D. HERSHEY. 1960. A fractionating column for analysis of nucleic acids. Anal. Biochem. 1: 66–77.
12. MANS, R. J. AND G. D. NOVELLI. 1961. Measurement of the incorporation of radioactive amino acids into protein by a filter paper disk method. Arch. Biochem. Biophys. 94: 48–53.
13. MANS, R. J. AND G. D. NOVELLI. 1964. Stabilization of the maize seedling amino acid incorporating system. Biochim. Biophys. Acta 80: 127–36.
14. MARCUS, A. AND J. FEELEY. 1965. Protein synthesis in imbibed seeds. II. Polysome formation during imbibition. J. Biol. Chem. 240: 1675–80.
15. MARCUS, L., R. BRETTHAUER, H. HALVORSON, AND R. BOCK. 1965. Polysomes from yeast: Distribution of messenger RNA and capacity to support protein synthesis in vitro. Science 147: 615–17.
16. MARKS, P. A., E. R. BURKA, AND D. SCHLESSINGER. 1962. Protein synthesis in erythroid cells. I. Reticulocyte ribosomes active in stimulating amino acid incorporation. Proc. Natl. Acad. Sci. Wash. 48: 2163–71.
17. PENMAN, S., K. SCHERRER, Y. BECKER, AND J. E. DARNELL. 1963. Polyribosomes in normal and poliovirus-infected HeLa cells and their relationship to messenger-RNA. Proc. Natl. Acad. Sci. Wash. 49: 654–62.
18. RISEBROUGH, R. W., A. TISSIERES, AND J. D. WATSON. 1962. Messenger-RNA attachment to active ribosomes. Proc. Natl. Acad. Sci. Wash. 48: 430–36.
19. SPIEGELMAN, S. AND M. HAYASHI. 1963. The present status of the transfer of genetic information and its control. Cold Spring Harbor Symp. Quant. Biol. XXVIII: 161–81.
20. WARNER, J. R., A. RICH, AND C. E. HALL. 1962. Electron microscope studies of ribosomal clusters synthesizing hemoglobin. Science 138: 1399–1403.
21. WETTSTEIN, F. O., T. STAEHELIN, AND H. NOLL. 1963. Ribosomal aggregate engaged in protein synthesis: Characterization of the ergosome. Nature 197: 430–35.
22. WILLIAMS, G. AND G. D. NOVELLI. 1964. Stimulation of an in vitro amino acid incorporating system by illumination of dark-grown plants. Biochem. Biophys. Res. Commun. 17: 23–27.