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Characterization of Rapidly Labeled Ribonucleic Acid from Dwarf Peas

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M. M. JOHRI[1] AND J. E. VARNER
Michigan State University-Atomic Energy Commission Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48823

ABSTRACT

The ribonucleic acid synthesized by excised shoots of dwarf pea (*Pisum sativum* L. cv. Progress No. 9) during short labeling periods has been characterized. Thirty per cent of the total $^{32}P_i$ incorporated in 1 hour is found in the ribosomal fraction. This labeled RNA was polydisperse (6-18 Svedberg units) and after chromatography on a methylated albumin-kieselguhr column about 80% of the radioactivity appeared in two peaks. One of these appeared on the shoulder of heavy ribosomal RNA ("mRNA") while the other was tenaciously bound to the column (TB-RNA). In the presence of high NaCl concentration, about half of the polydisperse RNA interacted with ribosomal RNA and eluted as "mRNA" while the remainder eluted as TB-RNA. This interaction in the presence of salt seems to result in the alteration of secondary structure because the "mRNA" fraction had a high sedimentation coefficient (45-50 Svedberg units). The polydisperse RNA approaches DNA in low cytidylate and guanylate content. After short periods of labeling TB-RNA showed higher adenylate content than "mRNA." The radioactivity from the "mRNA" peak can be chased, and these counts may represent a class of short-lived messenger RNA molecules with an average half-life of 10 to 15 minutes. The other component, TB-RNA, could not be chased and accumulated radioactivity during the chase period.

In bacteria and viruses the information for the sequence of amino acids in a polypeptide chain is encoded into messenger RNA. Presence of a similar fraction has been suggested in some higher plants (5, 6, 21, 26, 39). All of our knowledge about messenger RNA from higher plants is presumptive and based on analogy to microorganisms; in no case has this fraction been purified or characterized, nor has its rate of synthesis or its stability been studied.

We chose a dwarf pea as the experimental material because we have been investigating the RNA synthesized by nuclei isolated from shoots of this plant (19), and the knowledge obtained from the system *in vivo* may be applicable to the results obtained with isolated nuclei.

[1] Present address: Molecular Biology Unit, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 5, India.

MATERIALS AND METHODS

Uptake and Incorporation of Labeled Precursors. Dwarf pea seeds (*Pisum sativum* L., cv. Progress No. 9) were surface-sterilized for 7 to 10 min in 1% sodium hypochlorite, implanted in 500-ml Erlenmeyer flasks containing moist, sterile vermiculite, and allowed to germinate at 23 C in continuous light. At the end of 5 or 6 days, shoots 0.8 to 1 cm long were excised and 10 or 15 shoots were incubated in 25-ml Erlenmeyer flasks under aseptic conditions with labeled uridine or $^{32}P_i$ (carrier-free $H_3{}^{32}PO_4$, neutralized) in 2 ml of water containing 100 μg of chloramphenicol. Flasks were transferred to a shaker, and, after incubation for various times, the shoots were washed thoroughly in ice-cold 0.05 M KH_2PO_4 and deionized water and were then extracted for nucleic acids.

In experiments designed to study the relationship between uptake and incorporation, the labeled shoots were homogenized in ethanol, 80% (v/v,) and the nucleic acids were determined (33). The residue obtained after centrifuging at 1500*g* for 5 min was extracted three times with 5% trichloroacetic acid (w/v) containing KH_2PO_4 to remove the acid-soluble nucleotides. The pigments and lipid materials were removed by extracting the residue three times with ethanol-ether (3:1). The supernatants at all steps were saved and the radioactivity was determined to calculate the total uptake of labeled precursors. The residue was hydrolyzed in 0.3 N KOH for 20 hr at 37 C, and after hydrolysis DNA was precipitated by the addition of perchloric acid. The radioactivity in perchloric acid-soluble and -insoluble fractions was determined to measure the incorporation into RNA and DNA, respectively. The ribonucleotide fraction in experiments with labeling for less than 3 hr contained considerable amounts of radioactivity as inorganic phosphate (carried over from the soluble pools). Incorporation into the RNA fraction in these experiments was taken as the total radioactivity present in 2'-3'-AMP,[2] CMP, GMP, and UMP only.

Extraction of Nucleic Acids. To prepare total nucleic acids, the SAS and phenol method of Kirby (22) was used after slight modification. The shoots (1-2 g) were homogenized in a porcelain mortar with 1 ml of bentonite (50 μg/ml, purified), 2 ml each of SAS (pH 7.0, 6%, w/v) and phenol-cresol, and a little sand. The mortar was rinsed twice with 2 ml each of SAS and phenol-cresol, and the combined homogenate was vigorously mixed for 2 min followed by centrifugation at 3,500*g* for 10 min. The aqueous

[2] Abbreviations: AMP: adenylate or adenylic acid; CMP: cytidylic acid; DNA-RNA: DNA and the RNA associated with it; FU: 5-fluorouracil; GMP: guanylic acid; hrRNA: heavy ribosomal RNA; lrRNA: light ribosomal RNA; MAK: methylated albumin-kieselguhr; "mRNA": rapidly labeled RNA as described in the text; SAS: sodium 4-amino-salicylate; SLS: sodium lauryl sulfate; sRNA: transfer RNA; TB-RNA: tenaciously bound RNA; UMP: uridylic acid or uridylate.

phase was removed carefully, and the phenol phase was extracted twice with 3 ml of SAS. To the combined aqueous phase, solid NaCl (30 mg/ml) and 0.5 volume of phenol-cresol were added. The emulsion was mixed for 2 min. After centrifugation at 10,000*g* for 10 min, the aqueous phase was removed, potassium acetate was added to 1% final concentration, and nucleic acids were precipitated overnight at −20 C by adding 2 to 3 volumes of ethanol-cresol. Usually, 75 to 90% of RNA and DNA present in the tissue were recovered.

Isolation of Polyribosomes and Sucrose Density Gradient Centrifugation. Ribosomes were isolated from about 3 g of pea shoots (24). A sample containing 0.5 to 0.75 mg of ribosomes was layered over a 24-ml linear gradient of sucrose (10–34%, w/v, sucrose, prepared in 0.05 M tris, pH 7.4; 0.015 M KCl and 0.005 M $MgCl_2$) and centrifuged at 24,000 rpm in the SW-25.1 Spinco rotor for 2 hr at −2 C. The gradient tubes were punctured, and the absorbance of the effluent was scanned at 260 mμ with a continuous Gilford recorder and the ISCO model 180 density gradient fractionator. When radioactivity determinations were done, the fractions (20 drops each, 60–70 fractions/24 ml) were collected from the top of the gradient.

The ribosomal subunits and ribosomal RNA were characterized by zonal centrifugation with linear gradients of sucrose from 4 to 20%, 4 to 40%, or 17 to 34% in 0.05 M NaCl and 0.05 M sodium acetate (pH 5.3). After centrifugation fractions were collected either manually by piercing the tube from the bottom or mechanically using the ISCO density gradient fractionator as described above. The concentration of monovalent cations was kept around 0.1 M and no further additions were made, in order to avoid aggregation of RNA. In gradients containing less than 0.1 M monovalent cations, the heavy ribosomal RNA was degraded. The samples were precipitated by the addition of equal volume of 10% trichloroacetic acid, and the precipitate was collected by filtration on membrane filters that were washed with five 5-ml portions of 5% cold trichloroacetic acid. The filters were air-dried, and the radioactivity was determined after suspending filters in an appropriate scintillator. The sedimentation coefficients of rapidly labeled RNA fractions were determined as described by Martin and Ames (30), using the coefficients of light and heavy ribosomal RNA as 18 and 25 S (27).

Fractionation of Nucleic Acids. The crude nucleic acid pellet was washed three times with 66% ethanol containing 1% potassium acetate, dissolved in 0.3 M NaCl in 0.05 M KH_2PO_4 (pH 6.7), and fractionated on MAK columns (28). After addition of the sample, the column was washed with 200 ml of 0.3 M NaCl in 0.05 M KH_2PO_4 (pH 6.7), and elution was done at 37 C with a 300- or 400-ml linear gradient of NaCl from 0.3 M to 1.6 M in 0.05 M KH_2PO_4, pH 6.7. The fraction of RNA not eluted by the salt (tenaciously bound RNA) was eluted with 1% SLS or with 1.5 M NH_4OH after the termination of the salt elution (9). Most of the TB-RNA was found to be eluted at 37 C. Fractions of 2.5 to 5.0 ml each were collected, absorbance at 260 mμ was measured, and radioactivity was determined after filtering the acid-insoluble material on the membranes as described above.

When TB-RNA was to be purified for rechromatography, centrifuge tubes containing this fraction were cooled in ice for 4 to 6 hr, and the precipitated SLS (also containing the methylated albumin from column) was separated by centrifugation. The RNA from the supernatant was recovered after addition of NaCl (30 mg/ml), phenol deproteinization, and ethanol precipitation. A considerable amount of TB-RNA (about 40%) remained in the pellet which contained SLS and protein. These counts were recovered by suspending the pellet in 5 ml of SAS, addition of NaCl, and phenol deproteinization. About 70% of the radioactivity was recovered with this procedure.

Determination of Nucleotide Composition. The nucleotide composition of $^{32}P_i$-labeled RNA samples was determined by paper electrophoresis (34). The tubes containing the sample were precipitated with 2 volumes of ethanol at −20 C overnight in the presence of 250 μg of carrier pea ribosomal RNA. The pellet was dissolved in a small volume of water, and perchloric acid was added to a final concentration of 0.6 N. After 4 hr in an ice bath, the pellet was washed twice with 70% ethanol to remove the residual perchloric acid. The pellets were hydrolyzed in 0.3 N KOH at 37 C for 18 to 20 hr, neutralized with perchloric acid, and subjected to paper electrophoresis. The distribution of radioactivity in the 2′–3′-riboside monophosphates was determined.

RESULTS

The present study deals with the characterization of rapidly labeled RNA from the shoots of dwarf pea. We felt that it was desirable to have a detailed knowledge of: (*a*) the relationship between the uptake and the incorporation of labeled precursors; (*b*) the half-time of formation of this rapidly labeled fraction; (*c*) its chromatographic behavior during MAK chromatography; and (*d*) its subcellular localization.

Kinetics of $^{32}P_i$ Uptake Incorporation into Nucleic Acids. As shown in Figure 1, $^{32}P_i$ is taken up by pea shoots at a constant rate up to 10 to 12 hr when 19% of the input radioactivity was found in the shoots. After an initial lag of about 1 hr, the incorporation into nucleic acids was linear up to 24 hr in spite of the fact that the uptake leveled off after 10 hr. At the end of 24 hr the radioactivity incorporated into nucleic acids amounted to only 12 to 15% of that taken up by the shoots. During 1 hr of incubation less than 3% of the radioactivity accumulated was found in the nucleic acids.

The nucleic acids were resolved into seven fractions by MAK chromatography (Fig. 2). The over-all pattern of elution obtained was similar to that described in bacteria and in higher plants (5, 16, 37, 41). Following the absorbance and radioactivity profiles as a function of time, it was found that the two patperns did not coincide up to 6 hr of labeling. After shorter labeling periods, the radioactivity in the light and heavy ribosomal RNA region always appeared 1 or 2 tubes earlier than the bulk of the RNA. In these experiments (15-, 30-, and 60-min labeling), 65 to 70% of the radioactivity was distributed into two prominent peaks. One of these peaks, "mRNA" is identifiable as a separate peak up to 60 min of labeling; with longer times it becomes masked by the trailing edge of ribosomal RNA. After 12 hr of incorporation the radioactivity and absorbance profiles match one another.

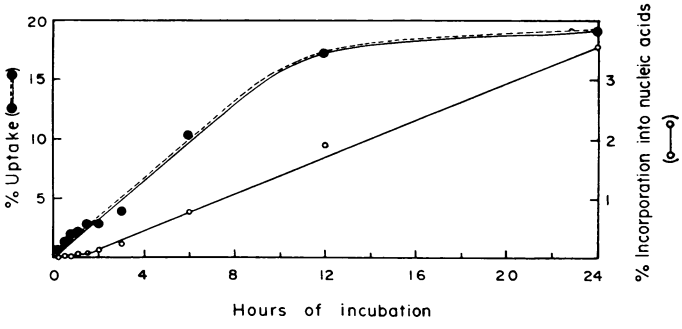

FIG. 1. Kinetics of $^{32}P_i$ uptake and incorporation into nucleic acids. Ten excised shoots were used for each treatment, and uptake and incorporation into nucleic acids were determined as described in "Materials and Methods" (1 mc/flask for 15-, 45-, and 60-min incorporations; 500 μc/flask for 1.5-, 2-, 3-, and 6-hr incorporations; and 250 μc/flask for 12- and 24-hr incorporations). All points represent the average of two separate incubations and have been corrected for the same amount of input isotope.

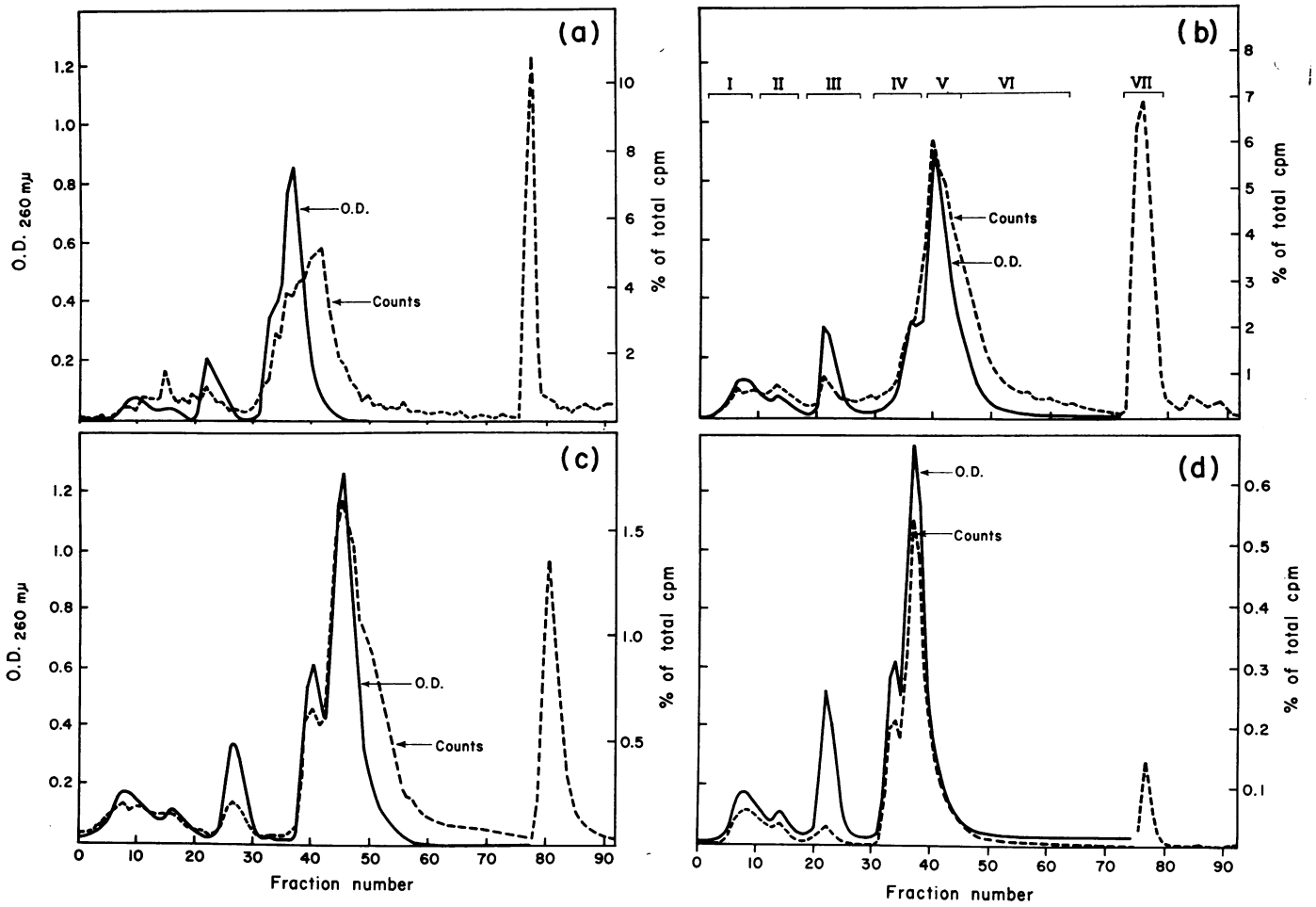

FIG. 2. MAK chromatography of nucleic acids synthesized by pea shoots labeled with $^{32}P_i$ for 15 min (a), 90 min (b), 3 hr (c), and 12 hr (d). Fifteen pea shoots were used for each treatment, and the amount of isotope used is the same as described in Figure 1. The radioactivity has been expressed as a percentage of total incorporation per tube. The numbers I through VII refer to transfer RNA, 5 S ribosomal RNA, DNA and the RNA associated with it, light ribosomal RNA, heavy ribosomal RNA, "mRNA," and tenaciously bound RNA.

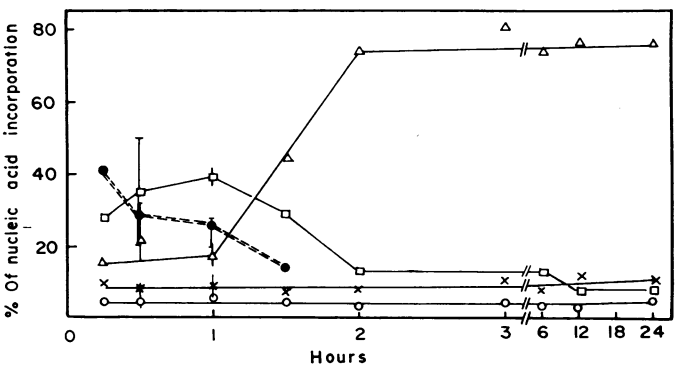

FIG. 3. Distribution of radioactivity in the different nucleic acid fractions. Nucleic acids were extracted from pea shoots labeled with $^{32}P_i$ for various periods of time and were analyzed by chromatography on MAK columns. The vertical bars represent the scatter, and the points are means of several determinations. ○: DNA-RNA; ×: transfer and 5 S ribosomal RNA; △: light and heavy ribosomal RNA; ●: "mRNA"; □: TB-RNA.

The relative distribution of $^{32}P_i$ in the different fractions after various periods of labeling is shown in Figure 3. The sRNA and DNA (also containing RNA associated with it) each contained about 4 to 6% of the total counts, and this proportion remained constant throughout the course of incorporation (15 min to 24 hr). The fractions "mRNA" and TB-RNA accounted for more than two-thirds of the radioactivity incorporated during 1 hr into nucleic acids. The appearance of radioactivity in ribosomal RNA was slow during the 1st hr of incorporation, but later the percentage of counts increased 4-fold. After a 2-hr labeling period steady state levels were observed, and under these conditions ribosomal RNA contained 75 to 80% of the total radioactivity incorporated into nucleic acids.

The nucleotide compositions of various nucleic acid fractions are shown in Table I. In the DNA peak isolated from shoots labeled for 16 hr, 40 to 60% of the total radioactivity was alkali-hydrolyzable, and on electrophoresis all four 2′–3′-riboside monophosphates were found to be labeled. This peak thus contains both RNA and DNA as indicated by alkali-hydrolyzable and -nonhydrolyzable counts. The composition of 1rRNA is different from that of hrRNA, and both of these are rich in AMP + GMP (54–56%), not in GMP + CMP as is usually the case. The only fractions that approach DNA in low GMP + CMP are "mRNA" and TB-RNA. Since these two fractions accumulated the maximum radioactivity during 1-hr labeling, this incorporation period was used in all the subsequent experiments to characterize the rapidly labeled RNA in detail.

Sedimentation Characteristics and Purification of the Two Rapidly Labeled Fractions. The RNA eluting with the trailing edge of hrRNA has been variously described as D-RNA (DNA-

like RNA) or messenger RNA (5, 18). In yeast and mammalian cells this peak has been designated as Q_1-RNA; it sediments around 45 S and has been shown to be a precursor for ribosomal RNA (9, 32, 40, 42).

The sedimentation pattern of "mRNA" from pea shoots (1-hr labeling) indicated that it was a fairly large molecule sedimenting around 45 to 50 S (Fig. 4a). Its nucleotide composition, however, was very different from either lrRNA or hrRNA; it is, therefore, probably not a ribosomal precursor. The nucleotide composition was more like pea DNA and also somewhat similar to TB-RNA. Because of the high sedimentation constant it was suspected that it might be an aggregate. Interaction between ribosomal ribonucleic acids themselves or between ribosomal RNA and rapidly labeled RNA occurs in the presence of a high concentration of cations (12–15, 17, 31). The concentration of NaCl used for MAK chromatography provides the right conditions for formation of such an aggregate. The high sedimentation constant of "mRNA" from pea shoots did in fact turn out to be due to aggregate formation (results presented later). The aggregate was not dissociated with SLS or EDTA.

The other rapidly labeled fraction, TB-RNA, was very heterogeneous, ranging in sedimentation from 4 to 32 S. After short periods of labeling (up to 2 hr) this fraction was very rich in AMP (48%, Table II), and the counts in the peak region showed a sedimentation coefficient around 13 to 15 S (Fig. 4b). After longer periods of labeling (more than 2 hr) the AMP content gradually decreased to 26 to 31%, and a relatively larger contamination with ribosomal RNA was observed. On further chromatography, more than 95% of the counts showed a nucleotide composition similar to ribosomal RNA. On rechromatography of TB-RNA on a MAK column (16-hr labeling, TB-RNA-1), two fractions were obtained: about 65% of the radioactivity appeared at the same place as ribosomal RNA and the rest as TB-RNA-2 (Fig. 5a). On further chromatography each of these two behaved in an identical manner, and three components were observed (Fig. 5b): (*a*) 35% of the counts appeared in a peak preceding hrRNA, and in sucrose gradients it sedimented between light and heavy ribosomal RNA (Fig. 6a); (*b*) 35% of the counts appeared on the shoulder of hrRNA and sedimented around 45 to 50 S in sucrose gradient (Fig. 6b); (*c*) 30% appeared as TB-RNA-3 with a slight enrichment of AMP content over the TB-RNA-1 or TB-RNA-2 (Table III). The nucleotide compositions of the first two components are almost identical and like that of hrRNA. Fraction b seems to arise because of aggregation, since no heavy moving component was demonstrable in TB-RNA-2 in sucrose gradients prior to MAK chromatography (Fig. 7).

On rechromatography of the "mRNA" on a MAK column (1-hr labeling), about 15% of the counts appeared with carrier hrRNA, 65% as "mRNA-2," and the rest (20%) as tenaciously bound RNA (Table IV). The fraction "mRNA-2" behaved in much the same way on further chromatography. In short label-

Table I. *Nucleotide Composition of Various Nucleic Acid Fractions*

The nucleotide composition of "mRNA" was determined after labeling the pea shoots for 1 hr. All other fractions were isolated from shoots labeled for 24 hr with $^{32}P_i$. The nucleic acids were separated by MAK chromatography, and the nucleotide composition of each fraction was determined as described under "Materials and Methods."

Fraction	Percentage of Total Counts				Nucleotide Ratio, AMP/GMP
	CMP	AMP	GMP	UMP	
1. Transfer RNA	33.5	17.9	27.8	20.8	0.64
2. RNA associated with DNA	26.8	25.9	18.3	27.4	1.41
3. 5 S ribosomal RNA	30.2	24.2	14.6	28.0	1.65
4. Light ribosomal RNA	20.7	25.3	28.7	25.3	0.88
5. Heavy ribosomal RNA	21.4	25.0	32.1	21.5	0.77
6. "mRNA"	20.7	32.5	20.2	26.6	1.60
7. Rapidly labeled RNA[1]	23.3	30.8	20.7	25.4	1.48
8. Tenaciously bound RNA	22.4	28.2	21.0	26.6	1.34
9. DNA[2]	18.5	30.8	19.2	30.5	1.60

[1] After Loening (26). The rapidly labeled RNA was isolated from roots labeled for 10 min.

[2] From J. Bonner (3). Cytidine also includes 5-methyldeoxycytidine. The value under the column of UMP refers to thymidine.

Table II. *Nucleotide Composition of Tenaciously Bound RNA*

The pea shoots were labeled with $^{32}P_i$, and nucleic acids were extracted and separated by MAK chromatography. TB-RNA fractions were purified and the nucleotide composition was determined as described under "Materials and Methods."

$^{32}P_i$ Incorporation	Percentage of Total Counts				Nucleotide Ratio, AMP/GMP
	CMP	AMP	GMP	UMP	
hr					
1	16.0	47.8	21.4	18.9	2.23
16					
TB-RNA-1	23.2	26.6	22.8	25.7	1.16
TB-RNA-2	23.0	28.2	21.7	26.2	1.29
TB-RNA-3	20.4	31.6	19.7	28.3	1.59
24	22.4	28.2	21.0	26.6	1.34

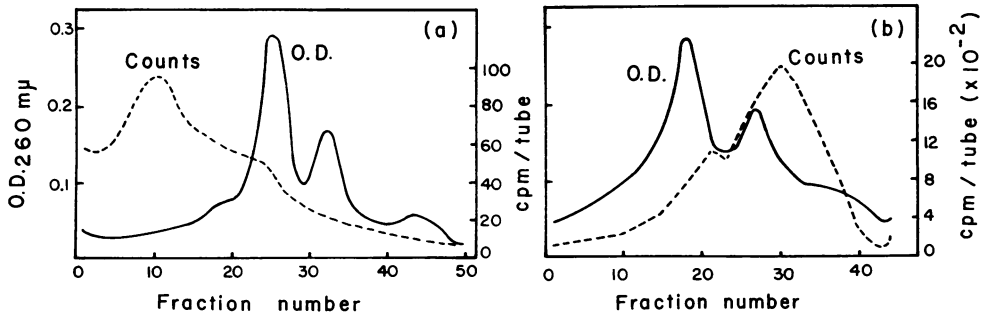

FIG. 4. Sedimentation profiles of "mRNA," shoots labeled with $^{32}P_i$ for 1 hr (a), and TB-RNA from shoots labeled with $^{32}P_i$ for 2 hr (b). The nucleic acids were first separated on a MAK column, and the tubes corresponding to "mRNA" or TB-RNA were pooled and analyzed on sucrose gradients from 5 to 40% (a), or from 5 to 20% (b). Centrifugation at 23,000 rpm at −2 C for 18 hr in an SW 25.1 rotor. Fractions were collected from bottom.

ing periods the "mRNA" fraction was much less contaminated by ribosomal RNA than was TB-RNA-1. This would be expected because the steady state level of $^{32}P_i$ in ribosomal RNA is reached only after 2 hr.

Effect of 5-Fluorouracil and Actinomycin D on RNA Synthesis. The purpose of these experiments was to determine whether it is possible to inhibit selectively the synthesis of either the rapidly turning over RNA with actinomycin D, or of ribosomal RNA and transfer RNA with FU, and then to use these inhibitors as tools for characterizing the messenger RNA. FU inhibited the synthesis of nucleic acids at relatively high concentrations (10^{-3} to 10^{-2} M). Pretreating the shoots for 2 hr inhibited the incorporation of $^{32}P_i$ into nucleic acids about 35% (Table V). The accumulation of radioactivity was reduced in all classes of RNA, and the inhibition of ribosomal RNA and DNA-RNA was very marked at a concentration of 10^{-2} M. The decrease of counts in the "mRNA" and TB-RNA peaks may be due to the absence of ribosomal RNA which is no longer contaminating these peaks. In pea shoots, the synthesis of "mRNA" and TB-RNA is affected by FU less than that of transfer RNA and ribosomal RNA. Similar partially selective effects of FU have been

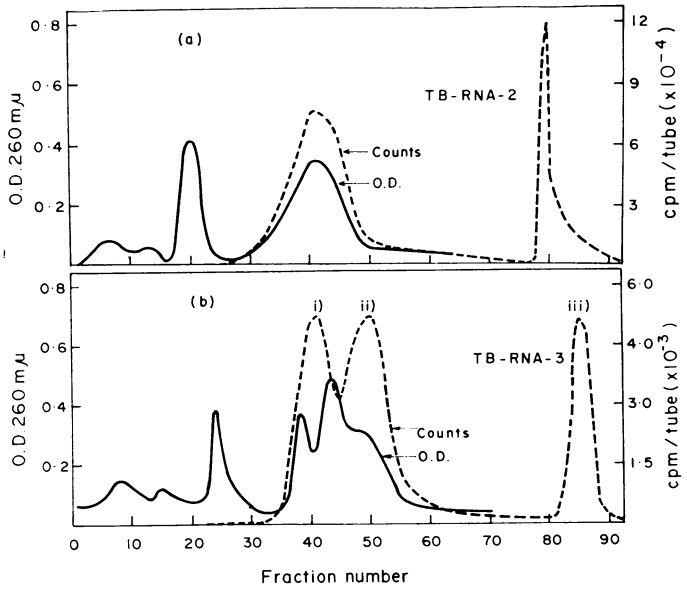

FIG. 5. Rechromatography of TB-RNA. Twenty pea shoots were incubated with 500 μc of $^{32}P_i$ for 16 hr, and nucleic acids were extracted and chromatographed on a MAK column. The TB-RNA-1 obtained was purified and applied to a second column. The transfer RNA, 5 S ribosomal RNA, and DNA were added as carrier (a). The TB-RNA-2 was again purified and chromatographed on a third column together with carrier pea nucleic acids (b).

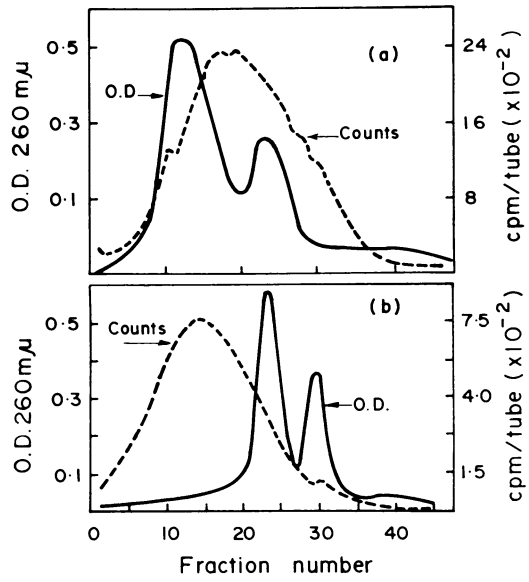

FIG. 6. Sedimentation pattern of the fractions obtained on the rechromatography of TB-RNA-2. The tubes containing peaks i and ii (marked in Fig. 5b) were pooled and analyzed on sucrose gradients (5–20% in a, 5–40% in b) after ethanol precipitation and removal of NaCl by dialysis. Centrifugation at 23,000 rpm for 20 hr in an SW 25.1 rotor. Ribosomal RNA was added as marker in each case.

Table III. *MAK Column Chromatographic Behavior of Tenaciously Bound RNA*

Pea shoots were incubated for 16 hr with $^{32}P_i$, and nucleic acids were extracted and separated by MAK chromatography. TB-RNA-1, containing 6 to 8% of total radioactivity, was purified and applied to a second column. The radioactivity eluting in the region of ribosomal RNA and as TB-RNA-2 were again chromatographed on two separate columns. The nucleotide composition of various fractions was determined as described in Table I.

TB-RNA-1

CMP	AMP	GMP	UMP
23.2	26.6	22.8	25.7

Rechromatographed on MAK column

	Ribosomal RNA (65%)				TB-RNA-2 (35%)			
	CMP	AMP	GMP	UMP	CMP	AMP	GMP	UMP
	21.4	25.6	29.8	22.2	23.0	28.2	21.7	26.2

Rechromatographed on MAK column

	Ribosomal RNA				TB-RNA-2			
	CMP	AMP	GMP	UMP	CMP	AMP	GMP	UMP
i)	35% 21.5	25.0	27.5	22.2	35% 21.6	25.5	31.3	20.9
ii)	35% 21.8	25.8	29.0	27.8	40% 20.8	26.5	27.1	24.4
iii)	30% 21.8	26.9	27.0	23.4	25% 20.4	31.6	19.7	28.3
							TB-RNA-3	

reported in soybean hypocotyl tissue (20), bacteria (1), and yeast (7).

To investigate the effect of actinomycin D, shoots treated with this inhibitor for 2 hr were incubated with uridine 2-^{14}C for 1 hr, and the nucleic acids were extracted. At 10 μg/ml the synthesis of nucleic acids was inhibited by approximately 41%, while 50 μg/ml caused 63% inhibition. The synthesis of all classes of RNA was affected by actinomycin D (Table V), and a preferential inhibition of a specific fraction was not observed. Since in peas actinomycin D inhibited the formation of all classes of nucleic acids, it was thought possible to find the half-life of the presumed messenger RNA by following the decay of this fraction directly in the presence of actinomycin D.

Decay of Rapidly Labeled RNA Fractions. To follow the decay of these fractions, shoots labeled with $^{32}P_i$ for 1 hr were rinsed three times in unlabeled KH_2PO_4 (0.001 M, pH 5.5) and then incubated in KH_2PO_4 (0.001 M, pH 5.5). The changes in radioactivity as a function of time were followed. No chase of rapidly labeled RNA was observed unless KH_2PO_4 was vacuum-infiltrated. In such shoots the incorporation into nucleic acids continued for 1 hr after transferring of the shoots to KH_2PO_4, but during the second hr about 70% of radioactivity from "mRNA" disappeared (Fig. 8). Only 50% of the radioactivity from TB-RNA was lost. A clearer and better defined chase of the "mRNA" peak was observed in the presence of actinomycin D (Fig. 9). The shoulder of counts on the trailing edge of hrRNA characteristic of "mRNA" disappeared, and the absorbance profiles and counts matched in the ribosomal region. The TB-RNA, in contrast, accumulated more counts during chase in the presence of actinomycin D, and the reason for this discrepancy is not clear.

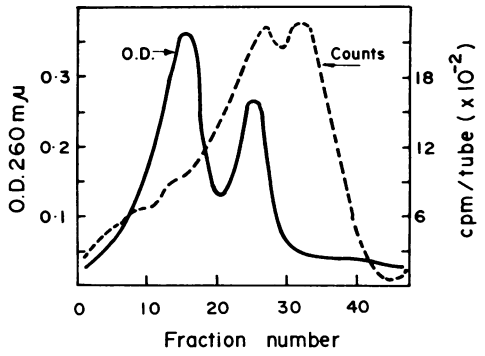

FIG. 7. Sedimentation pattern of TB-RNA-2 (marked in Fig. 5a). The tubes containing this fraction were pooled, and RNA was purified and analyzed on sucrose gradient (5–20%) in the presence of carrier ribosomal RNA.

The profiles presented in Figures 9 and 10 suggest also a chase of radioactivity from the transfer RNA and ribosomal RNA. The decrease of counts from transfer RNA can be explained if most of the radioactivity in this fraction during 1 hr is due to labeling of —CCA end groups which are turning over rapidly. In case of ribosomal RNA the decline of counts is perhaps due to a lesser overlapping by the "mRNA" peak. The chase of radioactivity from the "mRNA" peak would also cause an apparent lowering of the counts in the ribosomal region.

The objection might be raised that the disappearance of counts from the "mRNA" peak was an artifact caused by partial anaerobic conditions produced by the vacuum infiltration. However, this explanation is rendered improbable by the fact that infiltrating water alone does not result in a chase of counts from the "mRNA" (Fig. 10). The lack of chase in the absence of vacuum infiltration of KH_2PO_4 could be due to a high concentration of endogenous unincorporated $^{32}P_i$. As mentioned earlier, less than 3% of the $^{32}P_i$ taken up by shoots in 1 hr was incorporated into nucleic acids. The application of vacuum enhances the entry of KH_2PO_4, and it takes 1 hr for the endogenous pool to get diluted. Since most of the "mRNA" fraction can be chased in 1 hr, it must have gone through four to five half-lives; this would indicate a half-life of 10 to 15 min.

Isolation of "mRNA" from Polyribosomes. Evidence that the "mRNA" fraction is in fact messenger RNA derives from the fact that we can isolate this fraction from polyribosomes. About 30% of the total $^{32}P_i$ incorporated in 1 hr was associated with cytoplasmic ribosomes while the rest was present in the pellet containing unbroken cells, nuclei, chloroplasts, mitochondria, and the post ribosomal supernatant. The profiles indicated an equal proportion of polyribosomes and monoribosomes after 1 hr of labeling. The polyribosomes observed are not an artifact due to aggregation of ribosomes in the presence of high Mg^{2+} concentration because polyribosomal peaks were eliminated if the preparations were treated with pancreatic ribonuclease for 5 min at 37 C before separation (Fig. 11). Messenger RNA associated with ribosomes has been shown to be much more sensitive to ribonuclease than the ribosomal RNA in ribosomes (11, 29, 35, 38).

The specific radioactivity of labeled RNA in the polyribosomes was at least 3 times higher than the RNA in the monoribosomes after 1 hr of labeling. Chromatography on a MAK column

Table IV. *MAK Column Chromatographic Behavior of "mRNA"*

Nucleic acids were extracted from pea shoots labeled with $^{32}P_i$ for 1 hr and separated by MAK chromatography. The tubes containing "mRNA" fraction (41% of the total radioactivity incorporated) were pooled, and this fraction ("mRNA-1") was applied to a second column after purification. The fraction "mRNA-2" was likewise chromatographed on a third column.

4 S + 5 S RNA	DNA-RNA	Ribosomal RNA	"mRNA-1"	TB-RNA
9.2%	7%	18.7%	41%	24.1%

Rechromatographed on MAK column

15%	64.5% "mRNA-2"	20.5%

Rechromatographed on MAK column

9.2%	55% "mRNA-3"	35%

Table V. *Inhibition of Nucleic Acid Synthesis by 5-Fluorouracil and Actinomycin D*

Ten shoots were incubated aseptically in 2 ml of water containing FU or actinomycin D for 2 hr and were then allowed to incorporate 500 μc of $^{32}P_i$ (FU experiment) or 5 μc of uridine 2-^{14}C (actinomycin D experiment) for 1 hr. Nucleic acids were extracted and analyzed by MAK chromatography.

Inhibitor	Percentage Inhibition of Ribosomal RNA						
	SRNA	DNA-RNA	5 S	Light + heavy	"mRNA"	TB-RNA	Total nucleic acids
5-Fluorouracil							
10^{-3} M	20.5	54.7	11.3	47.9	24.7	33.9	33.4
10^{-2} M	30.9	62.7	28.9	71.6	17.7	32.4	37.3
Actinomycin D							
10 μg/ml	42.9	23.2	48.5	41.4	39.8		40.6
50 μg/ml	53.0	48.4	55.0	73.3	64.2		62.7

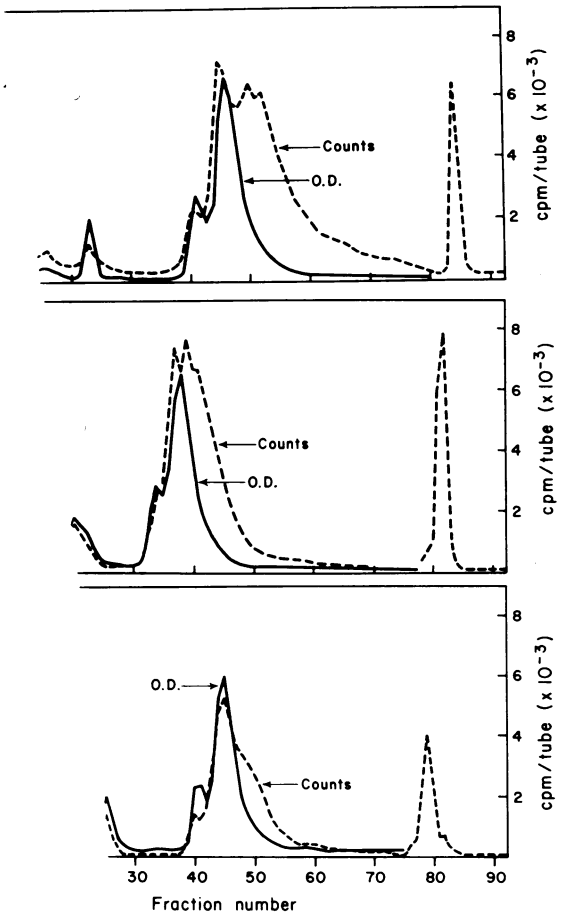

FIG. 8. Decay of "mRNA" fraction. Thirty pea shoots were incubated in a 50-ml Erlenmeyer flask (in 6 ml of sterile water containing 1 mc of $^{32}P_i$ and 300 μg of chloroamphenicol) for 1 hr, and nucleic acids from 10 shoots were washed three times with ice-cold 0.0001 KH_2PO_4 (pH 5.5) and subjected to vacuum for 2 min to infiltrate KH_2PO_4. Ten shoots were removed after every hour, and nucleic acids were extracted and analyzed. Nucleic acids from shoots labeled for 1 hr immediately extracted (top), extracted 1 hr after transfer to KH_2PO_4 (middle), and 2 hr after transfer to KH_2PO_4 (bottom).

indicated that the radioactivity associated with ribosomes represented the same rapidly labeled RNA fractions as observed in the total nucleic acids from shoots after a short labeling period.

The labeled RNA associated with ribosomes was released either by removing Mg^{2+} (by treating ribosomes with EDTA or sodium pyrophosphate) (Fig. 12) or by treatment with SLS, and separated on sucrose gradients. This labeled RNA was polydis-

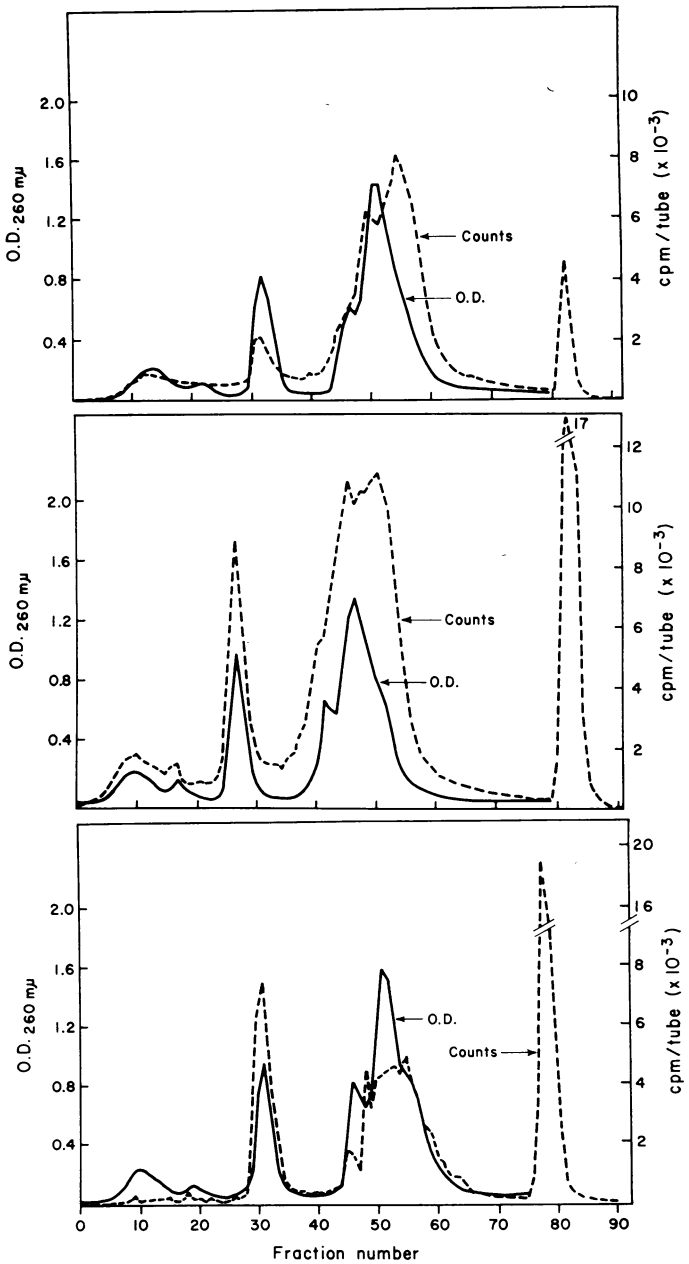

FIG. 9. Decay of "mRNA" fraction during chase with 0.001 M KH_2PO_4 in the presence of actinomycin D. All experimental details as described for Figure 8. During vacuum infiltration of KH_2PO_4, actinomycin D (50 μg/ml) was also included.

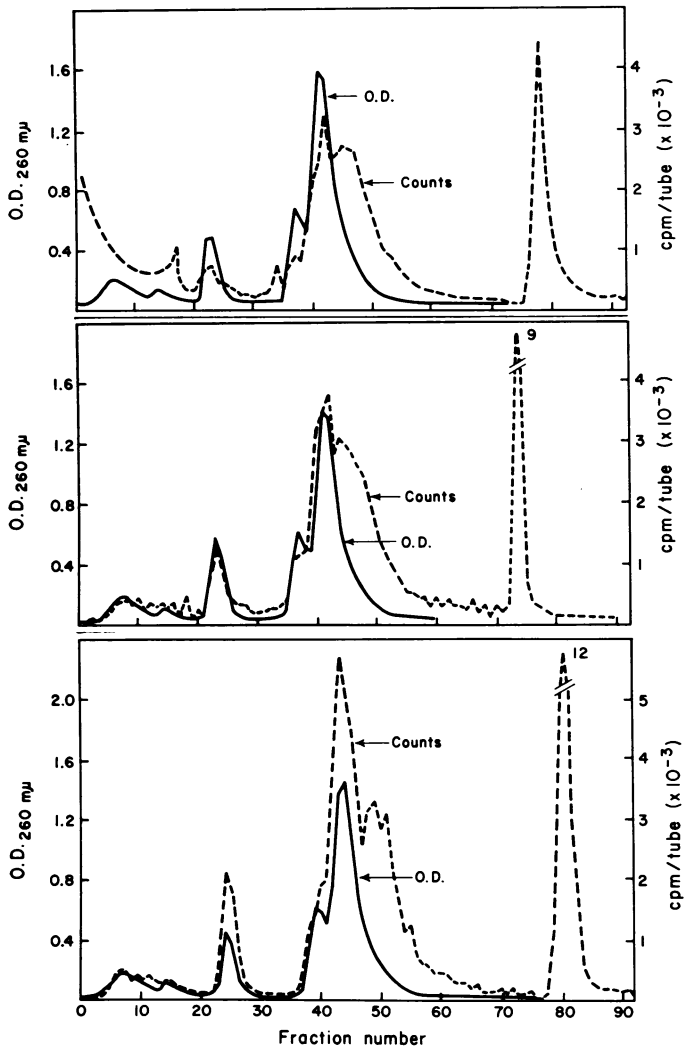

FIG. 10. Effect of infiltrating water under vacuum on the decay of "mRNA" fraction. All experimental details as described for Figure 8. The shoots labeled for 1 hr with $^{32}P_i$ were washed three times with ice-cold 0.001 M KH_2PO_4, and water was infiltrated. Note the lack of chase of "mRNA" fraction.

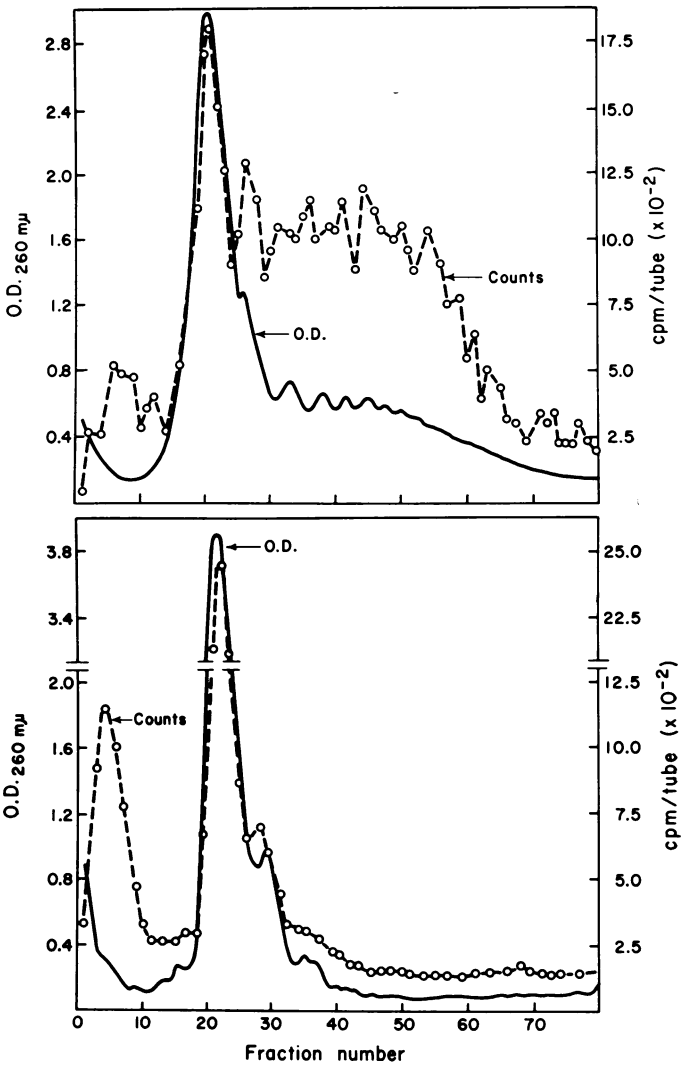

FIG. 11. Sedimentation profiles of polyribosomes from pea shoots labeled with $^{32}P_i$ for 1 hr. Polyribosomes equivalent to 0.8 mg of RNA were either layered directly (top) or were treated with 0.1 μg of pancreatic ribonuclease for 5 min at 37 C and then layered (bottom) over a 24-ml gradient of sucrose from 10 to 34%. Centrifugation for 2 hr at −2 C at 23,000 rpm in an SW 25.1 rotor.

perse (4–18 S) and about 30 to 40% of the counts were associated with a small ribosomal subunit; these counts may represent the RNA still bound to it. The SLS-released RNA separated on sucrose gradient was divided into four fractions, and the nucleotide composition of each was determined (Fig. 13). The AMP content was different in each of these, but the over-all composition of the first three fractions was the same as the "mRNA" peak. The RNA associated with 1rRNA (fraction 4) had the higher GMP content characteristic of ribosomal RNA. The SLS-released RNA was also separated on a MAK column (Fig. 14, without deproteinization to avoid degradation and also to minimize loss). The fractions "mRNA" and TB-RNA each contained 41% of the counts, the rest being in transfer RNA and ribosomal RNA. The D-RNA (RNA eluting on the shoulder of hrRNA) from soybean is also associated with ribosomes (25).

DISCUSSION

The rapidly labeled RNA after short labeling of pea shoots has been characterized by MAK chromatography and sucrose density gradient centrifugation. Whereas transfer RNA, DNA, and ribosomal RNA (all of which possess highly ordered secondary structure) are eluted on the basis of size, base composition, and extent of hydrogen bonding (36), the rapidly labeled RNA behaves differently. In several cases it has been reported that RNA aggregates because of high salt concentration and further that a large percentage of rapidly labeled RNA cannot be eluted with salt (9, 10, 23).

When the "mRNA" fraction from pea shoots was analyzed on sucrose gradients after separation by MAK chromatography, a sedimentation coefficient of 45 to 50 S was noticed. An analysis of nucleotide composition eliminated the possibility of this fraction being the 45 S ribosomal precursor. No such heavy sedimenting fraction was found if the rapidly labeled RNA released by SLS from ribosomes was analyzed. This RNA was polydisperse (6–18 S), and on analysis on a MAK column 41% of the radioactivity appeared in the "mRNA" peak. It is conceivable that labeled RNA may have suffered some degradation during the preparation of ribosomes, but our results argue against this and degradation alone cannot account for the lack of a heavy moving component from the rapidly labeled RNA prepared without the use of high salt concentrations. On rechromatography on MAK column of TB-RNA-2 the radioactivity eluting as "mRNA" also

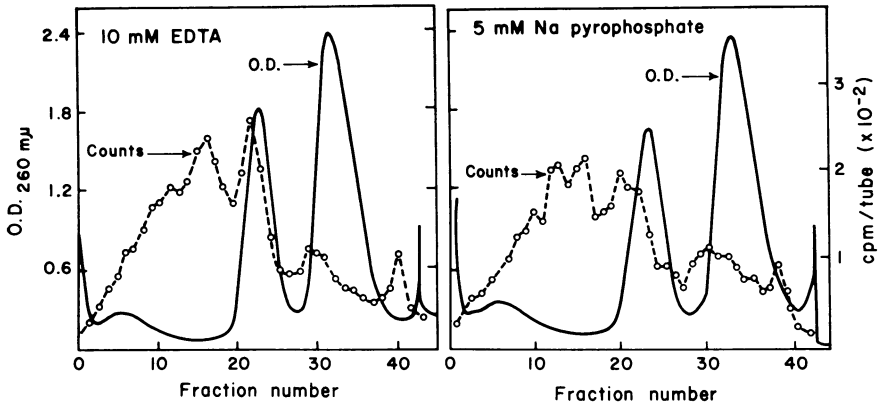

FIG. 12. Sedimentation pattern of labeled RNA released on dissociating the ribosomes. Pea shoots were labeled with $^{32}P_i$ for 1 hr, and the ribosomes were prepared as described under "Materials and Methods." Polyribosomes equivalent to 1 mg of RNA were suspended in 0.2 ml of water, and a solution of EDTA or sodium pyrophosphate was added to the indicated final concentration. The mixture was layered over a 24-ml gradient of sucrose from 17 to 34% and centrifuged for 16 hr at 23,000 rpm in an SW 25.1 rotor. Fractions were collected from the top.

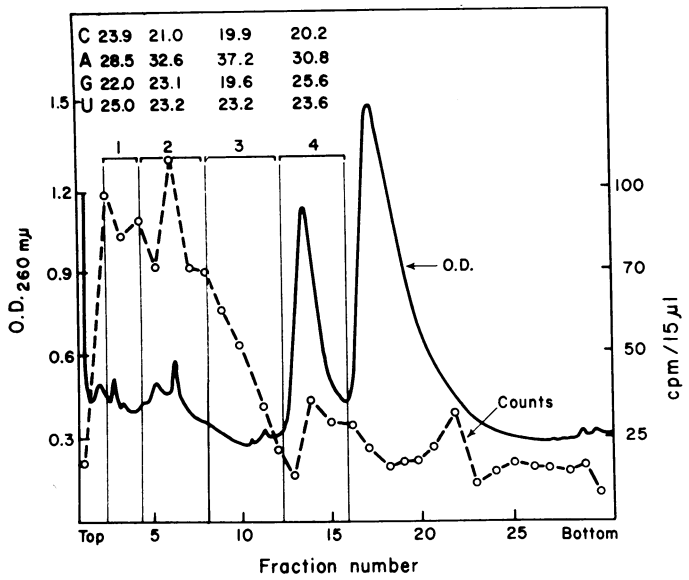

FIG. 13. Sedimentation pattern of the labeled RNA released by treating the ribosomes with SLS. Polyribosomes equivalent to 1 mg of RNA in 0.2 ml of water were treated with SLS (final concentration 1%) prior to layering over a 24-ml gradient of sucrose from 17 to 34%. Centrifugation for 20 hr at 23,000 rpm in an SW 25.1 rotor. The nucleotide composition of the four fractions has been indicated at top left.

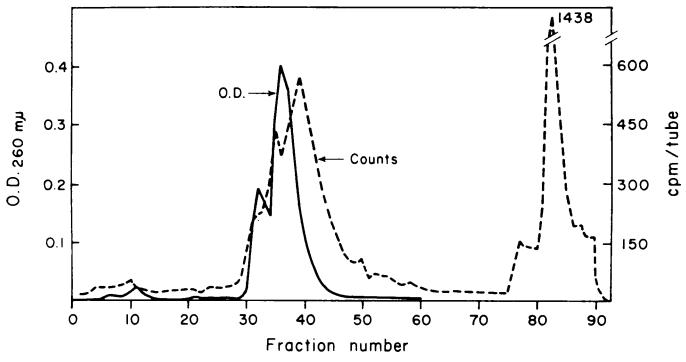

FIG. 14. MAK chromatography of the SLS-released RNA from ribosomes. To polyribosomes (equivalent of 1 mg of RNA) in 1 ml of water, a solution of SLS was added to a final concentration of 1%, and the mixture was applied to the MAK column. The column was washed with 200 ml of 0.3 M NaCl in 0.05 M KH_2PO_4 (pH 6.7) before the elution of nucleic acids with salt.

sedimented around 45 to 50 s and resembled ribosomal RNA in nucleotide composition. This heavy sedimenting component was not observed in TB-RNA-2 prior to MAK chromatography.

These results also indicate that entirely dissimilar RNA fractions can elute on the shoulder of hrRNA. After a short labeling period the radioactivity appearing as "mRNA" had properties similar to messenger RNA whereas on rechromatography of TB-RNA (prepared after a 16-hr labeling period) the radioactivity appearing in this peak was entirely different and resembled ribosomal RNA in composition. If it is assumed that in peas whatever type of RNA elutes on the shoulder of hrRNA is aggregated, the high sedimentation coefficient values can be explained.

The tenaciously bound RNA from mammalian and plant sources has been suggested to be DNA-like RNA and classed with messenger RNA (9, 10, 37, J. L. Key, personal communication). The MAK column separated the various species of rapidly labeled RNA into two major fractions based on AMP content, because TB-RNA always had a higher percentage of AMP than "mRNA" and on rechromatography of TB-RNA the subsequent tenaciously bound RNA fractions became more and more enriched in AMP. Why a certain proportion of TB-RNA elutes as "mRNA" or vice versa on rechromatography of either fraction could be related to the secondary structure. The TB-RNA and single-stranded DNA resemble each other in the tenacious binding property to MAK columns, and a more open structure for messenger RNA and TB-RNA has been proposed (2, 4, 10).

Table VI. *Estimated Half-life of Presumed Messenger RNA Fraction from Higher Plants*

Material	Estimated Half-life	Reference
1. Cotton embryo (from germinating seeds)	16 hr	39
2. Soybean hypocotyl	2 hr[1]	21
3. Potato tuber slices	1.6 hr	6
4. Peanut cotyledons	1.5 hr	5
5. Pea roots (var. Meteor)	10 min	26
6. Pea shoots (var. Progress No. 9)	10–15 min	Present work

[1] Refers to mean life of D-RNA fraction.

If all the rapidly labeled RNA has an open structure, then those species of this RNA which have a high adenine content (and consequently a lesser guanine + cytosine content, *i.e.*, very little secondary structure) will be expected to interact very strongly with the methylated albumin and finally could appear at TB-RNA. The rest of the rapidly labeled RNA having a lower AMP content seems to interact with ribosomal RNA and elute with salt as "mRNA" under our experimental conditions. In *Escherichia coli* a relationship between the secondary structure of ribosomal RNA synthesized in the presence of chloramphenicoland its affinity to MAK column has also been reported (8). This will also explain the lack of coincidence of radioactivity (which always precedes the absorbance profile by one or two tubes) in the light and heavy ribosomal RNA observed in the nucleic acids from peas after short labeling periods. The ribosomal RNA synthesized during short labeling periods may have a greater degree of ordered helical structure (owing to the deficiency of methylatable bases which would tend to interfere with normal base pairing), and such ribosomal RNA will in turn show a lesser affinity for the column (8).

Our estimated half-life of the "mRNA" fraction based on the chase data after vacuum infiltration of precursors is in excellent agreement with that reported for the messenger RNA from pea roots (26). Of all the higher plants investigated so far, the "mRNA" fraction from dwarf peas has the shortest half-life (Table VI).

The finding that TB-RNA cannot be chased remains unexplained, and in fact this fraction accumulated more radioactivity during chase. Loening (26), using electrophoresis on cellulose acetate paper, also observed two rapidly labeled fractions in the RNA from microsomes prepared from pea roots. One of these became labeled with a 10-min pulse, while the other became labeled only after a chase. It is possible that the second fraction described by Loening may be similar to the TB-RNA from pea shoots (present work).

The fraction "mRNA" resolved by MAK chromatography after short labeling periods has many properties ascribed to messenger RNA isolated from microorganisms. Our evidence, which is based on the short half-life and the nucleotide composition of this fraction and its localization in the polyribosomes, strongly suggests that the "mRNA" fraction represents a class of short-lived messenger RNA molecules. The fact that radioactivity does not appear in the ribosomal RNA after the chase of "mRNA" eliminates the possibility of this fraction being a ribosomal precursor. The hybridization behavior of rapidly labled RNA with DNA and the identification of the protein (or proteins) for which it may carry the message are obvious lines for further investigation.

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