Synthesis of Messenger Ribonucleic Acid in Excised Pea-Seedling Root Segments

SEPARATION OF THE MESSENGER FROM MICROSOMES BY ELECTROPHORESIS

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1. Electrophoresis on cellulose acetate membrane in a tris–pyrophosphate buffer was used to separate microsomal fractions into three components: (1) the lipoprotein; (2) the nucleoprotein (termed the β-band); (3) traces of free RNA (termed the α-band). In tris buffer containing Mg^{2+} the α-band was not obtained. 2. The incorporation of uridine and phosphate into RNA by excised pea-seedling root segments was studied by using this electrophoretic technique. 3. It was shown that after a short ('pulse') incubation in the radioactive precursor and a longer ('chase') incubation in the non-radioactive precursor most of the incorporation was into the RNA of the α-band and little into that of the β-band. Previous work showed that in roots of whole seedlings the incorporation is mostly into the ribosomal RNA, corresponding to the material in the β-band. 4. A pulse-labelled RNA has also been found; this seems to be a cell fraction distinct from the microsomes or ribosomes. 5. The apparent base compositions of labelled RNA in the α-band and small amounts of labelled RNA in the β-band and of unfractionated RNA were very different from the composition of ribosomal or transfer RNA, and somewhat like that of DNA. 6. It is suggested that the excised root segment synthesizes a messenger-RNA fraction labelled after a pulse incubation and a distinct messenger RNA labelled after a pulse and chase incubation, but no ribosomal or transfer RNA. The system is thus similar to the 'step-down' culture conditions in bacteria.

The synthesis of a number of RNA fractions in the root tips of pea seedlings has been described by Loening (1965). A method of electrophoresis on a cellulose acetate membrane was used to separate the microsomal fractions into essentially three components: (1) the lipoprotein membranous material, which remained on the origin of the electrophoresis strip; (2) the nucleoprotein (the β-band), which contained the bulk of the ribosomal RNA; (3) a trace of RNA (the α-band), which dissociated from the microsomes in the presence of citrate but not of Mg^{2+}. The appearance of the strips under u.v. light is shown in Fig. 1 and described more fully below. It was suggested that the RNA of the α-band was a mixture of transfer RNA and messenger RNA.

The RNA was labelled by incubation of the seedlings in [^{32}P]phosphate for a 10–15min. 'pulse' followed by a 'chase' in unlabelled phosphate for various times from 15 to 70min. RNA labelled by this procedure is referred to below as 'chase-labelled'. It was found that the α-band RNA and the β-band ribosomal RNA become labelled 40–60min. after the entry of [^{32}P]phosphate into the cell. A messenger-like RNA labelled within a 10min. pulse incubation in [^{32}P]phosphate has also been found (Loening, 1962*a*). It was suggested that the pulse-labelled RNA was in the nucleus (which is damaged on homogenization of this tissue) and that the chase-labelled α-band RNA was cytoplasmic.

It has now been found that pyrophosphate is much more effective than citrate in separating the α-band RNA from the microsomes. The present paper describes experiments on the synthesis of RNA by excised cultured segments of the pea-seedling roots by using the electrophoretic technique with pyrophosphate.

The root segments used were 1·8mm. long, extending from 1·6 to 3·4mm. from the tip of the root. This zone includes small cells that in the intact root are expanding rapidly, and that continue to expand at a decreased rate when the cut segment is incubated in 2% sucrose. There is less cell division than in the meristem at the tip, and the effect of excision is to stop division, and net protein and RNA synthesis, completely. The growth of these segments, and similar segments from 2·0 to 4·0mm. from the

tip, has been studied in some detail in this Laboratory (Vaughan, 1964; Yeoman, 1962), and some further features of this are given in the Discussion section.

It is shown below that the cultured segments no longer incorporate RNA precursors into ribosomal or transfer RNA. This provides an opportunity to investigate the distribution of messenger RNA in the cell, and the extent to which pyrophosphate causes the dissociation of the labelled messenger from unlabelled ribosomes or microsomes.

EXPERIMENTAL

Materials. [^{32}P]Phosphate in dilute HCl and [^{3}H]uridine (1·2c/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. The phosphate was dried *in vacuo* and dissolved in the required volume of 2% (w/v) sucrose, pH 6–7.

Guanidinium chloride was prepared from AnalaR guanidinium carbonate (British Drug Houses Ltd., Poole, Dorset) by adding conc. HCl. The solution was adjusted to 6 M at pH 7·0 ± 0·2 and sodium EDTA (final concn. 0·1 M) was added.

Tris buffer was prepared from British Drug Houses Ltd. tris and HCl. The British Drug Houses Ltd. 'specially purified grade' of tris was occasionally used, without any noticeable differences.

Yeast RNA and bentonite were obtained from British Drug Houses Ltd. The bentonite was washed by the method of Singer & Fraenkel-Conrat (1961).

Ondina Oil 17 was obtained as a sample from Scottish Oils and Shell Mex Ltd.

Other chemicals were reagent grade.

Cellulose acetate membrane (5 cm. × 20 cm.) was obtained from Oxoid division of Oxo Ltd., London, S.E.1.

Pea seeds, *Pisum sativum* var. Meteor, were obtained from Dobbie and Co., Edinburgh.

Growth of peas and incubation of root segments. Pea seeds were soaked for a few hours in water, sown in vermiculite–water mixture (3:1, v/v) and incubated at 22·5° in the dark for 2 days. The seedling roots were then 3–4cm. long. The harvesting and cutting of the roots was carried out at 23–25°. The root segments, extending from 1·6 to 3·4 mm. from the root tips, were cut in batches by using drilled Perspex sheets 1·6 mm., 1·8 mm. and thicker to hold the roots. New razor blades were used; poor cutting or other damage resulted in reduced cell expansion of the cultured segments. Batches of 70–80 segments were incubated in 7 ml. of 2% sucrose in 50 ml. conical flasks shaken on a rotary shaker at 100 oscillations/min. The temperature of incubation was 25·0–25·5° throughout. The root segments were incubated for 2 hr. before the beginning of the radioactive incubation.

For the radioactive pulse incubation, the sucrose solution was removed with a pipette, and 2 ml. of the [^{32}P]phosphate or [^{3}H]uridine solution in 2% sucrose added. Shaking was continued and after a suitable time, usually 8–16 min., the pulse medium was replaced by 7 ml. of the chase medium. For the [^{32}P]phosphate experiments this was unlabelled 20 μM-potassium phosphate buffer, pH 6·5, in 2% sucrose, and for the [^{3}H]uridine experiments it was unlabelled 20 μM-uridine in 2% sucrose. The chase medium was again replaced by a fresh chase medium after 5–10 min. At the end of the chase incubation, the root segments were chilled in ice-cold 2% sucrose. All subsequent operations were at 0–4°.

Homogenization and cell fractionation. Batches of 70–80 segments were homogenized in 1·2 ml. of homogenizing medium in a glass centrifuge tube with a loose-fitting Teflon pestle driven at up to 1000 rev./min. In most cases the medium contained: sucrose, 0·5 M; KCl, 50 mM; tris, 35 mM; magnesium acetate, 1 mM; adjusted to pH 7·4–7·5 at 0° with HCl. In a few experiments the KCl was omitted and the Mg^{2+} concentration raised to 5 mM. This latter medium was used in previous experiments (Loening, 1965) and gives a different distribution of pulse-labelled RNA among the cell components.

The following cell fractions were obtained by centrifugation; the cell walls, broken nuclear fragments and other cell debris were sedimented at 1000*g* for 5 min.; the mitochondria and other cell particles, fraction M, were sedimented at 10000*g* for 15 min. in the MSE Superspeed 50 centrifuge; fraction X, consisting of a mixture of vesicular bodies containing some nucleoprotein, was sedimented at 25000*g* for 30 min.; the microsomes were sedimented at 125000*g* for 90 min. The microsomal pellet was separated into two fractions by inverting the centrifuge tube after removal of the supernatant, and centrifuging the inverted tube (inside a larger glass tube) at about 200*g* for 2–3 min. The vesicular fraction V slides down into the glass tube, leaving the ribosomal fraction R as a firm pellet in the ultracentrifuge tube (Loening, 1961). Traces of fraction V left behind in streaks were suspended in a drop of electrophoresis buffer and the tube again centrifuged inverted. A sample of the supernatant remaining from the 125000*g* centrifugation was counted for ^{32}P and ^{3}H content as a measure of the total uptake of the precursor by the cell. The bulk of the supernatant was acidified at 0° with acetic acid to pH 4·5–5·0 and the precipitate sedimented at 25000*g* for 20 min. RNA was purified from the resulting pellet as described below, and is referred to below as s-RNA, although it may contain RNA in addition to aminoacyl transfer RNA.

Electrophoresis of the cell fractions. 0·1 M-Tris–HCl buffers were used for electrophoresis on cellulose acetate membrane (Kohn, 1958) as described by Loening (1962*b*). The tris–PP_i buffer contained sodium pyrophosphate (5 mM), pH 8·0. The tris–Mg^{2+} buffer contained magnesium acetate (0·1 mM), pH 7·5. The pH was adjusted and the electrophoresis carried out at 0–5°. For suspending the sedimented cell fractions and for soaking the acetate membranes, the buffer also contained washed bentonite, at a concentration of about 0·01 mg./ml. in the tris–Mg^{2+} buffer and 0·1 mg./ml. in the tris–PP_i buffer. Electrophoresis was continued for up to 2 hr. on 10 cm. long × 5 cm. membranes at 20 v/cm. and 1·5–2·0 mA/strip. The membranes were then dried cold *in vacuo*, washed for 15 min. in 5% (w/v) trichloroacetic acid at 0°, rinsed twice in water and again dried. They were cleared for scanning at 260 mμ in liquid paraffin or in Ondina Oil 17. With a 1·5 mm. × 1 cm. slit for scanning, 1 extinction unit was approximately equivalent to 4·8 μg. of RNA. The strips were scanned for radioactivity by using an attachment to the Ekco scintillation counter, or by cutting into segments and counting on a Packard Tri-Carb scintillation counter. Usually a 1 cm.-wide band from the middle of the electrophoresis strip was scanned for radioactivity and for extinction. With weak samples, however, a wider band was scanned for radioactivity.

The attachment on the Ekco scintillation counter was

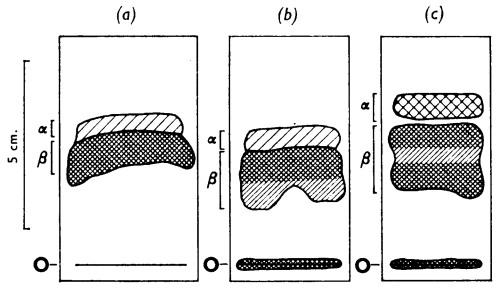

Fig. 1. Electrophoresis of ribosomes and microsomes in tris–PP_i buffer. The samples were suspended in buffer (0·1 M-tris–5 mM-pyrophosphate, pH 8) and applied to the cellulose acetate strips on the origin, O. Electrophoresis was continued for up to 2 hr. at 20 v/cm. and approx. 2 mA/5 cm.-wide strip. The strips were washed and dried as described in the text. (*a*) Ribosome fraction R. The bulk of the ribosomes move as a single band, marked β, which can be stained for protein (e.g., with Nigrosine or Amido Black). A trace of free RNA, marked α, separates from the ribosomes. The scan of this strip is shown in Fig. 4(*a*). (*b*) Microsome fraction V. The vesicular material remains on the origin and its sharp outline indicates that it cannot enter the pores of the acetate membrane. Ribosomal material (β-band) and RNA (α-band) are seen as in the ribosome R fraction. The scan of this strip is shown in Fig. 5(*b*). (*c*) Fraction X. The proportion of RNA in the α-band appears to be much greater than in fraction R or V, and the β-band shows evidence of two components. The scan of this strip is shown in Fig. 6(*a*). The apparent mobilities of the fractions in these three examples are not exactly comparable, since the samples were run in separate electrophoresis tanks for slightly different times.

used with a Nuclear Enterprises NA 102 plastic scintillator, so that the strips were counted dry, with an efficiency for ^{32}P of approx. 40% and a background counting rate of approx. 30 counts/min. For counting in the Packard counter, samples were placed in counting bottles containing liquid scintillator [5 g. of 2,5-diphenyloxazole and 0·3 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. of AnalaR toluene]. The counting efficiency for ^{32}P was 75% with a background of 18 counts/min., and for ^{3}H on cellulose acetate 7% with a background of 23 counts/min. Samples of the incubation medium were diluted with non-radioactive carrier and dried on Whatman no. 1 filter paper (for ^{32}P) or on cellulose acetate membrane (for ^{3}H). The dried pieces were counted on the same instrument as the electrophoresis strips for each experiment, so that results could be expressed as counts/min. of sample per unit counts/min. of the medium, without corrections for efficiencies.

The separations obtained by the electrophoresis are shown in Fig. 1. In tris–PP_i buffer, most of the material of the ribosomes (R fraction) moved as a single component, the β-band. It is to be expected that the ribosomes will be dissociated into sub-units by the tris–PP_i buffer (Lamfrom & Glowacki, 1962). Evidently these sub-units have closely similar electrophoretic mobilities. The small amount of RNA released from the ribosomes by pyrophosphate has a higher mobility than the nucleoprotein and appears as the α-band. The microsome fraction V shows similar α- and β-bands to the ribosomes. The lipid membranous material cannot enter the pores of the cellulose acetate membrane and remains on the origin. In fraction X the proportion of α-band RNA is greater than in fraction R or V, and the β-band tends to split into two components. RNA eluted and purified from the α- and β-bands is referred to below as α-RNA and β-RNA respectively. In tris–Mg^{2+} buffer, the α-band was not obtained, and the mobility of the nucleoprotein was lower than in tris–PP_i buffer and showed considerable trailing in fractions X and V.

The pH 5 precipitate from the supernatant cell sap was separated by electrophoresis in tris–PP_i buffer into an RNA component with a mobility similar to that of the α-RNA and a protein component of low mobility.

Purification and hydrolysis of RNA, and separation of the nucleotides. RNA was extracted and purified from the cell-fraction pellets and from the electrophoresis strips by a slight modification of the method of Cox & Arnstein (1963). The sample containing less than 100 μg. of RNA was dissolved in 1 ml. of guanidinium chloride–EDTA solution. To aid solution sedimented cell fractions were homogenized and sometimes warmed to room temperature or 30° (H. V. R. Arnstein, personal communication), and any insoluble residues were removed by centrifugation. RNA from the electrophoresis strips (1 μg. for α-band RNA) was eluted overnight at 0°. Then 0·5–1·0 mg. of yeast RNA was added as carrier, and the RNA precipitated with 1 ml. of ethanol. Several hours at 0° was allowed for complete precipitation. This procedure was repeated twice. The precipitated RNA was then washed twice in aq. 80% (v/v) ethanol, once in ethanol and once in ethanol–ether (1:1, v/v), and dried. The RNA was hydrolysed in 0·1–0·15 ml. of 0·33 N-KOH (protected from air by a layer of heptane) at 37° for 14–18 hr. The digest was acidified to about pH 3 with 0·5 N-$HClO_4$ at 0° and centrifuged for 10 min. The supernatant was dried on polythene strips after the addition of a small amount of 50 mM-potassium citrate buffer, pH 3·5 ± 0·05. The dried samples could be stored desiccated at −20° without change. The nucleotides were separated by electrophoresis on Whatman no. 3MM paper in the potassium citrate buffer, cooled in carbon tetrachloride. Satisfactory separations were obtained in 40 min. at 40 v/cm. The nucleotide bands and adjacent blanks were cut out under u.v. light. Radioactivity was counted with a Packard Tri-Carb scintillation counter until at least 1000 and usually 2000 counts above background were obtained. The cut paper pieces were laid flat in the glass vials containing the liquid scintillator; several pieces could be over-laid without loss of counting efficiency. In most cases the radioactivity on the blank regions adjacent to the nucleotides was less than 5% of that of the nucleotides. Any samples in which it was higher than 10% were rejected.

RESULTS

Composition of DNA and RNA of pea seedlings. Table 1 shows the composition of DNA and total RNA of pea seedlings as determined by Heyes (1960) and the distribution of radioactivity in β-band RNA after a pulse and long chase incubation of intact seedling roots (Loening, 1965). These values are given for comparison with the results obtained from the cultured root segments, presented below.

Table 1. *Composition of DNA and RNA of pea-seedling root tips*

The composition of pea-seedling DNA and bulk RNA is quoted from Heyes (1960), and that of s-RNA from Brown (1963). The composition of ^{32}P-labelled ribosomal RNA was obtained by incubation of whole seedlings in [^{32}P]phosphate for 15min. and in unlabelled phosphate for 50–75min. The ribosomal RNA was isolated from the β-band after electrophoresis (Loening, 1965).

	Percentage composition (±S.E.M.)			
	Adenylic acid	Cytidylic acid	Guanylic acid	Uridylic acid (or thymidylic acid)
DNA	31·7	19·6	20·6	28·4
Bulk RNA	25·6	22·4	28·3	23·8
s-RNA	19·3	30·1	29·7	18·4
Ribosomal ^{32}P-labelled RNA	25·0±0·18	22·1±0·50	30·7±0·83	22·2±0·74

RNA labelled during short incubation times. As described by Loening (1962*a*), labelled RNA can be detected in the microsomal fractions after a few minutes' incubation of intact seedling roots in [^{32}P]phosphate. This RNA has a lower mobility than the β-band in tris–Mg^{2+} buffer, and it is spread out and difficult to detect in the tris–PP_i buffer. Similar results were obtained in preliminary experiments with the cultured root segments (Fig. 2). It has now been found that the yield of this pulse-labelled RNA varies with the type of homogenizing medium used. RNA was purified from the cell fractions M, X, V, R and S after a 10min. incubation of the root segments in [^{32}P]phosphate. The specific radioactivity of the RNA obtained from each cell fraction was at least twice as great when the tissue was homogenized in a medium containing 5mM-Mg^{2+} as in that containing 1mM-Mg^{2+} and 50mM-potassium chloride. After a further 10min. incubation in unlabelled phosphate, the specific activities of fractions from the medium containing 1mM-Mg^{2+} and 50mM-potassium chloride increased at least twofold, whereas those from the medium containing 5mM-Mg^{2+} remained constant. Electrophoresis of fraction R in tris–Mg^{2+} buffer showed that the radioactivity peak was then almost coincident with the β-band.

This result indicates that the 10min.-pulse-labelled RNA loses its radioactivity during the 10min. chase, as in the roots of intact seedlings (Loening, 1962*a*). A second RNA component, detected in cell fractions prepared with either homogenizing medium, becomes labelled during this chase. There is therefore no net loss of activity from any cell fraction during the chase, and in this respect the root segments differ from the intact seedlings.

The apparent composition of the labelled RNA from these fractions has been determined. No significant differences were found in composition after 10 or 20min. incubation with either homogenizing medium. The mean composition of labelled

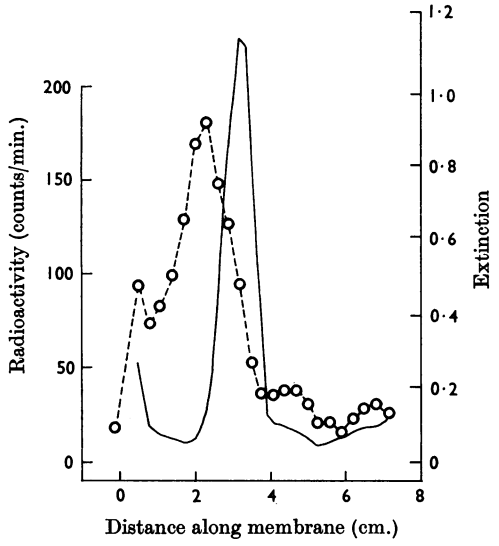

Fig. 2. Pulse incubation of root segments, and electrophoresis of the ribosome fraction R in tris–Mg^{2+} buffer. Root segments were preincubated for 45min. and then incubated in [^{32}P]phosphate ($3·6\times10^7$ counts/min./ml.) for 15min. Homogenization was in the medium containing 5mM-$MgCl_2$ and no KCl, and the ribosomes were isolated and fractionated by electrophoresis in the tris–Mg^{2+} buffer as described in the text. The cellulose membrane strips were scanned for radioactivity at 3mm. intervals. O--O, Radioactivity; ——, extinction.

RNA from fractions X, V and R was: adenylic acid, 30·8±0·83; cytidylic acid, 23·3±0·64; guanylic acid, 20·7±0·67; uridylic acid, 25·4±0·81 (ten determinations). This resembles the composition of DNA given in Table 1 more closely than that of the bulk RNA. RNA from the mitochondrial and debris fractions was difficult to purify after a pulse incubation; radioactivity was found in some blanks adjacent to the nucleotides. It is probable that the

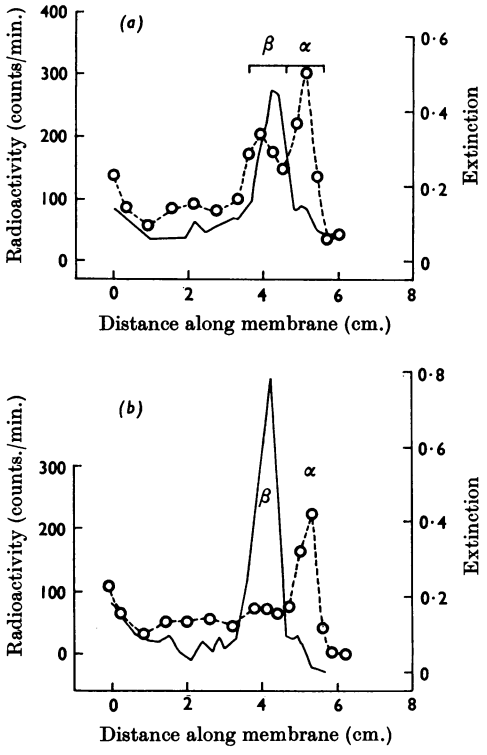

Fig. 3. Chase incubations of the root segments and electrophoresis of the ribosome fraction R in tris–PP_i buffer. The segments were preincubated for 1½–2 hr., and then incubated in [^{32}P]phosphate ($8{\cdot}0 \times 10^7$ counts/min./ml.) for 10 min. The chase incubation in unlabelled 20 μM-phosphate was for 65 min. in (*a*) and for 25 min. in (*b*). The roots were homogenized in the medium containing 1 mM-$MgCl_2$ and 50 mM-KCl and the ribosomes were isolated as described in the text. The electrophoresis was in the tris–PP_i buffer and the strips were scanned for radioactivity at 3 mm. intervals. O--O, Radioactivity; ——, extinction.

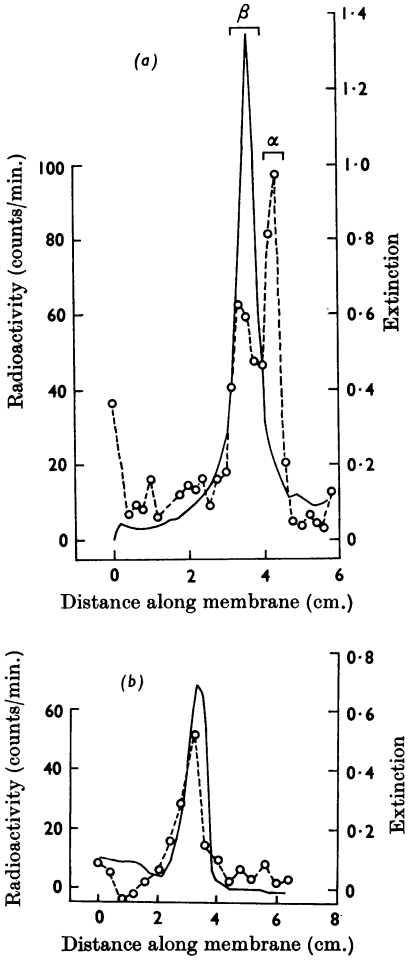

Fig. 4. Chase incubation of root segments and electrophoresis of the ribosome fraction R. The segments were preincubated for 2 hr. and then incubated in [^{3}H]uridine ($3{\cdot}2 \times 10^6$ counts/min./ml.) for 12 min. The chase incubation was in unlabelled 20 μM-uridine for 32 min. Homogenization was as described in Fig. 3. In (*a*), electrophoresis was in tris–PP_i buffer; the ribosomes were applied as a 3 cm.-wide band, and the strip was scanned by cutting into 2 mm. sections and counting the sections in the Packard scintillation counter. In (*b*), electrophoresis was in tris–Mg^{2+} buffer on a band 1·2 cm. wide, and cut into 4 mm. sections for counting. The wider band in (*a*), and greater section size in (*b*), were used to give sufficiently high counting rates. The u.v. scan in both cases was on a 1 cm.-wide band as usual. O--O, Radioactivity; ——, extinction.

mitochondrial fraction RNA had a slightly higher content of guanylic acid plus cytidylic acid.

RNA components labelled after a pulse and long chase incubation. After a total pulse and chase incubation time of 20 min. or more, labelled RNA appears coincident or very nearly coincident with the β-bands on electrophoresis in tris–Mg^{2+} buffer. In tris–PP_i buffer, however, most of the radioactivity is in the α-band RNA and the β-band is almost unlabelled. This suggests that the structural RNA of the ribosomes does not become labelled.

Fig. 3 shows the label in fraction R after incubation for totals of 35 min. and 75 min. The relatively small amount of label in the β-band at 35 min. increases slowly after 50 min. A similar result was obtained when [^{3}H]uridine was used as the RNA precursor (Fig. 4*a*). In tris–Mg^{2+} buffer the label was in the β-band (Fig. 4*b*), although not exactly coincident with it. Separation of the α-RNA in fraction V is shown in Fig. 5. The large ^{32}P-radioactive peak of lower mobility than the β-band was

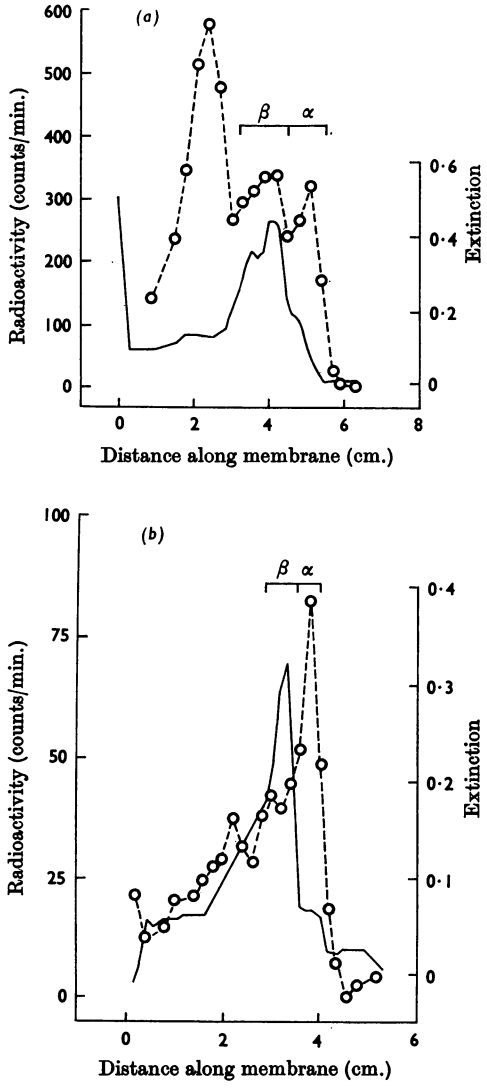

Fig. 5. Chase incubations of root segments and electrophoresis in tris–PP_i buffer of the microsome fraction V: (*a*) labelled with [^{32}P]phosphate, with details as given for Fig. 3(*a*); (*b*) labelled with [^{3}H]uridine, with details as given for Fig. 4(*a*).

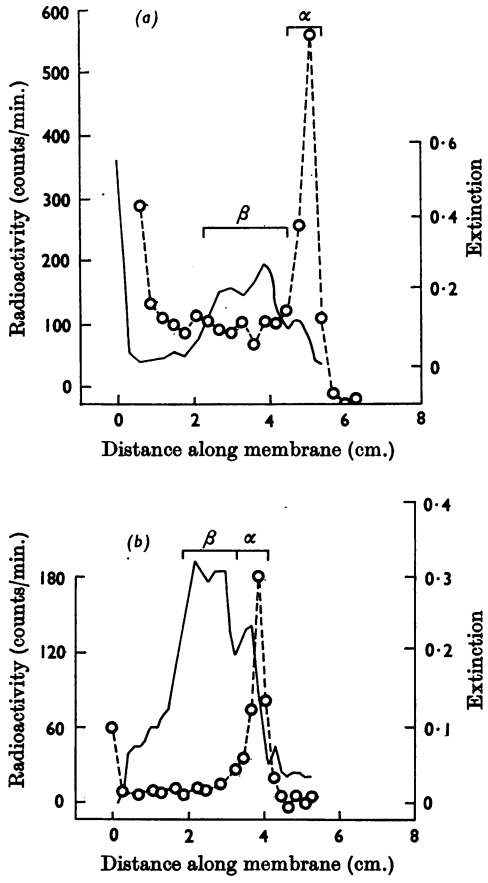

Fig. 6. Chase incubations of root segments and electrophoresis of cell fraction X: (*a*) labelled with [^{32}P]phosphate with details as given for Fig. 3(*a*); (*b*) labelled with [^{3}H]-uridine, with details as given for Fig. 4(*a*).

difficult to purify and probably not RNA. It was not found when uridine was used as the precursor (Fig. 5*b*). Fig. 6 shows the separations obtained with fraction X. The extinction profile shows that the β-band is double, and it was entirely unlabelled. The α-band was relatively more highly labelled than that of fractions R and V.

The distribution of ^{32}P in the labelled RNA of the α- and β-bands of fractions R, V and X and that of the mitochondrial fraction M is given in Table 2. In no case does the composition resemble that of ribosomal RNA labelled in the intact root (Table 1). The relatively small amount of labelled RNA remaining in the β-band has a similar composition to that in the α-band, and both show some resemblance to the DNA. The α-band of fraction X shows a high apparent content of cytidylic acid, as was found in incubations of intact roots (Loening, 1965). This is thought to be due to terminal labelling of s-RNA present in the α-band.

The apparent composition of s-RNA labelled by incubation of the root segments is compared with that of intact roots in Table 3. Though the terminal –CpCpA group of the s-RNA is labelled by exchange in both cases, the low content of labelled guanylic acid suggests that the cultured root segment does not incorporate ^{32}P into s-RNA as a whole.

Table 2. *Composition of RNA of cell fractions labelled after a long incubation*

Root segments were incubated for a 12 min. pulse (approx. 50 μc of [^{32}P]phosphate/ml.) followed by 41 min. chase. After homogenization and cell fractionation, α- and β-components were obtained by electrophoresis of fractions X, V and R, as in Figs. 3, 5 and 6. These and the mitochondrial fraction M were purified and hydrolysed, and the nucleotides counted, as described in the text.

Cell fraction	Percentage composition (± S.E.M.) Adenylic acid	Cytidylic acid	Guanylic acid	Uridylic acid	No. of determinations
Rα	32·3±1·0	22·0±1·8	23·3±1·0	22·2±0·2	3
Rβ	28·6	25·0	23·3	23·1	2
Vα	28·7	25·4	23·1	23·2	2
Vβ	29·3±1·5	23·0±1·5	24·1±0·5	23·5±0·5	3
Xα	27·0±0·6	29·5±0·9	20·5±1·0	23·1±1·0	3
Xβ	35·5	22·1	20·0	22·4	1
M	28·5±0·5	22·1±1·1	24·2±1·9	24·9±0·9	3

Table 3. *Composition of s-RNA labelled after a long incubation in root segments and in root tips of whole seedlings*

Whole seedlings were incubated for a 10–16 min. pulse and 50 and 75 min. chase, dipping the roots only into the solutions. Root segments were incubated for 16 min. pulse and 60 min. chase. s-RNA was isolated by electrophoresis of the pH 5 precipitate and purified as described in the text.

	Percentage composition (± S.E.M.) Adenylic acid	Cytidylic acid	Guanylic acid	Uridylic acid	No. of determinations
s-RNA from whole seedlings	19·6±0·54	34·1±0·72	28·9±0·97	17·3±0·82	8
s-RNA from root segments	24·9	33·8	21·3	20·2	2

Table 4. *Composition of unfractionated RNA labelled after a long incubation*

Root segments were incubated for a 10 min. pulse and 30 min. chase. They were homogenized and the cell debris was removed by centrifugation at 1000*g* for 5 min. RNA from the debris and from the supernatant was then purified and hydrolysed, and the nucleotides were counted, as described in the text.

	Percentage composition (± S.E.M.) Adenylic acid	Cytidylic acid	Guanylic acid	Uridylic acid	No. of determinations
RNA from debris	28·2	21·5	25·8	24·9	2
RNA from supernatant	32·2±0·22	19·7±0·17	21·1±0·64	27·0±0·43	4

Apparent composition of labelled unfractionated RNA. The results described above suggest that, up to the maximum incubation time used, no labelled RNA with a composition of ribosomal or s-RNA can be detected. The composition of labelled RNA prepared from the whole tissue without fractionation would therefore be expected to have a composition similar to that given in Table 2. Since RNA from the whole homogenate frequently contained impurities, the cell debris was removed from a homogenate by centrifugation at 1000*g*, and RNA was purified separately from the debris and the supernatant. The RNA from the debris was more labelled than that from the supernatant. The composition of the RNA from the supernatant was closely similar to that of the DNA (Table 4). RNA from the debris had a slightly higher guanylic acid content, which may indicate a small amount of labelling of ribosomal or s-RNA.

DISCUSSION

These and previous experiments have shown the existence of pulse-labelled and chase-labelled RNA fractions that are distinct from ribosomal or s-RNA. The evidence that these fractions include messenger RNA rests at present on the base-composition data. These show a low and equal proportion of labelled guanylic acid and cytidylic acid, as in DNA, but the content of uridylic acid is lower than that of adenylic

acid. If only one of the two DNA chains is used in transcription and if only a part of the genome is being transcribed in this system, then there is no reason why the composition of messenger RNA should parallel that of DNA. A comparison of the composition of messenger RNA in several higher organisms may be instructive; a direct comparison is possible since the DNA of all these is similar in composition.

Hotta & Stern (1963) found an RNA composition closely similar to that of DNA by short pulse-labelling of *Trillium microsporophytes* during meiosis and at low temperature and low metabolic activity. Georgiev & Mantieva (1962) isolated a DNA-like RNA from the 'nucleolochromosomal' fraction of rat liver and of Ehrlich ascites-tumour cells. By fractionation with hot phenol they obtained an RNA of very similar composition to that of DNA. When labelled with [^{32}P]phosphate in 'aurantin'-treated rats, however, this RNA and a cytoplasmic RNA fraction showed a lower proportion of labelled uridylic acid than of adenylic acid (Georgiev, Samarina, Lerman, Smirnov & Severtzov, 1963). Brawerman (1963) used a pH-dependent phenol fractionation of rat-liver nuclei. The RNA fraction most closely resembling DNA was obtained at pH 8·5, and had a lower proportion of uridylic acid than of the other nucleotides. Korner & Munro (1964) found a rapidly labelled RNA in rat liver with approximately equal proportions of the four nucleotides. If this is correctly interpreted on the basis of the composition as a mixture of 40% ribosomal and 60% messenger RNA, then the proportion of uridylic acid in the messenger component equals or exceeds that of adenylic acid. Harel, Harel, Boer & Imbenotte (1964) found in a liver fraction from rats treated with actinomycin D, and in a fraction separated by gradient centrifugation a labelled RNA of almost exactly the same composition as that reported in the present work for pea seedlings.

There is therefore general agreement that the guanylic acid plus cytidylic acid content of messenger RNA is low, but an excess of adenylic acid over uridylic acid was frequently found. In the absence of further evidence it will be assumed provisionally that the pulse-labelled RNA and some of the α-band RNA described in the present paper is messenger.

The use of the cultured root segment, which does not seem to incorporate precursors into ribosomal RNA, has provided an opportunity to test the method of electrophoresis and the effectiveness of the tris–PP_i buffer in dissociating messenger RNA from the ribosomes. After the shorter chase incubations up to 1 hr., the labelled RNA was entirely dissociated from the ribosomes by 5 mM-pyrophosphate. A proportion of RNA synthesized during the 75 min. incubation remained associated with ribosomes of slightly lower mobility than the bulk of the ribosomes, suggesting perhaps a larger structure such as polysomes. The degree of separation of the α-RNA from the ribosomes was much greater than when citrate was used (Loening, 1965). This agrees with Lamfrom & Glowacki (1962), who found that, of several chelating agents tried, only pyrophosphate caused complete dissociation of reticulocyte ribosomes. The mechanism of this dissociation, whether it involves ribonuclease action and its relation to the structure of polysomes are not known.

The converse question, whether the α-RNA is contaminated by ribosomal RNA, is better studied in the whole growing root. The previous experiments with citrate, and preliminary ones with pyrophosphate, show that the composition of α-band RNA is similar to that reported in the present paper, and has a much lower proportion of guanylic acid than the ribosomal β-RNA. In fraction X some unlabelled RNA appears to be released slowly, since the mobility of the labelled RNA is always slightly greater than the extinction profile suggests.

The cultured root segments provide an opportunity to investigate the distribution of messenger RNA in the cell, since the only contaminating labelled RNA is the terminal sequence of s-RNA. The pulse-labelled RNA and chase-labelled RNA seem to occur in distinct cell fractions. The pulse-labelled RNA does not seem to be bound to the bulk of the microsomes or ribosomes, since it has a lower mobility than the ribosomes in tris–Mg^{2+} buffer, and the yield obtained varied with the composition of the homogenizing medium. Its low mobility suggests that it is bound to protein and does not occur as a free RNA in the homogenate. In many tissues it has been found that the pulse-labelled RNA is confined to the nucleus (see, e.g., Harris, 1963). The present experiments do not contradict this finding, since the nuclei are broken during homogenization, and the labelled RNA could be released. The extent of release seems to depend on the composition of the homogenizing medium.

The chase-labelled RNA was obtained in the microsome and ribosome fractions in either of the two homogenizing media. Previous work with the intact growing root (Loening, 1965) suggested that the chase-labelled messenger RNA was bound to the ribosomes in the tris–Mg^{2+} buffer. In the excised root segments, however, the ribosomal RNA is not labelled and it was then found that the messenger RNA has a slightly lower mobility than the ribosomes in tris–Mg^{2+} buffer (Fig. 4*b*). This is to be expected if those ribosomes that are attached to messenger RNA form very much larger particles than single ribosomes, but it means that the association of the label with a cytoplasmic component has not been confirmed.

Whether the pulse-labelled RNA is a precursor of the chase-labelled α-RNA is not yet known. However, it is clear from the present experiments that messenger RNA is labelled after chase incubations and that its stability is greater than suggested by pulse experiments alone.

The effect of excising the segment from the whole root seems to be similar to the 'step-down' conditions in bacteria. The synthesis of messenger RNA continues but that of ribosomal RNA and s-RNA is stopped. The following other properties of these segments (and similar segments 1 to 3mm. and 2 to 4mm. from the root tip) may be noted (J. K. Heyes, D. Vaughan, U. E. Loening & M. H. Williams, unpublished work). (1) Cell expansion continues at about one-third of the rate of expansion in the whole root. Ribonuclease (Yeoman, 1962) and 8-azaguanine and thiouracil (Vaughan, 1964) increase the cell expansion over a 18–48hr. incubation. It is suggested that these substances interfere with a control mechanism involving RNA. (2) Cell division ceases abruptly; no mitotic figures are visible a few hours after excision. (3) There is no net protein or RNA synthesis. The total protein per segment remains approximately constant and there is some loss of RNA. [^{14}C]Leucine is rapidly incorporated into protein, suggesting an active protein turnover. (4) The activities of some enzymes change during culture. In most cases there is an increase in enzyme activities during the first 12hr. of culture, followed by a decrease. Chloramphenicol reduces the increase in activity, but those analogues of RNA precursors that increase cell expansion also increase the enzyme activities above the control. (5) In the absence of sucrose there is a rapid loss of protein and RNA, and little cell expansion.

These properties of the root segment suggest that protein synthesis continues after excision and is to some extent controlled by RNA synthesis.

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