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A Detailed Evaluation of the Possible Contribution of Bacteria to Radioactive Precursor Incorporation into Nucleic Acids of Plant Tissues[1, 2]

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Abstract. **An investigation of the possible contribution of bacteria to the labeling patterns of soybean seedling nucleic acid was made. The results using sucrose gradient, MAK column, and acrylamide gel electrophoretic fractionation together with base composition analyses of nucleic acid preparations show that contaminating bacteria do not contribute to the incorporation of ^{32}P-orthophosphate into the RNA of excised hypocotyl or soybean root tip. Sterile, non-sterile, and CM-treated soybean hypocotyl synthesize D-RNA to the same extent. The contaminating bacteria do not synthesize an AMP-rich RNA. The G-C rich ^{32}P-DNA component of the soybean tissues used in these studied results, at least primarily, from the incorporation by contaminating bacteria. CM can be used successfully to eliminate the contribution of bacteria to the labeling of nucleic acids by etiolated plant tissues. Bacterial counts, although valuable, are not sufficient to determine if contaminating bacteria will significantly contribute to nucleic acid labeling in plants.**

The pattern of labeling of nucleic acids following exposure of plant tissues to some radioactive precursor (usually ^{32}P-orthophosphate) has been described using several systems (2, 3, 7, 8, 17, 21, 23). Recently Lonberg-Holm (12) and Hock (6) suggested that bacterial contaminants of plant tissues contribute significantly to the nucleic acid labeling patterns observed by several workers (2, 3, 7, 8, 17, 18, 23). Because of this we undertook an investigation of the contribution of bacteria to nucleic acid synthesis in plant tissues used extensively in this laboratory for the study of nucleic acid metabolism (7, 8). Sucrose gradient centrifugation, MAK column fractionation, and base composition analyses of nucleic acid preparations were used in these studies since they were the methods frequently used and also used by Lonberg-Holm (12). In addition we used polyacrylamide gel electrophoretic fractionation of nucleic acid preparations as this method gives complete separation of bacterial r-RNAs (23 and 16S) from plant cytoplasmic r-RNAs (25 and 18S) (11). Nucleic acids were extracted from ^{32}P-labeled sterile and non-sterile plant tissues labeled both in the absence and presence of 50 μg/ml chloramphenicol. Chloramphenicol was the bacteriastat of choice based on the work of Leaver and Edelman (9) and Wilson (24). In brief, the results show that bacteria do not contribute to the results of RNA studies which have been reported from this laboratory (7, 8); the newly synthesized G-C rich DNA component (or leading peak DNA of MAK columns) which has been described in several plant systems (2, 8, 17, 18, 23) appears to result primarily from bacterial contamination in soybean tissues, in agreement with the results of Tester and Dure using *Avena* coleoptiles (21) and of Lonberg-Holm (12).

Materials and Methods

Soybean seeds (*Glycine max*, var. Hawkeye 63) were routinely planted in moist vermiculite and germinated at about 29° in the dark for 3 days. Sterile plants were grown from seeds which were rinsed in sterile water and then ethanol, followed by a 20-minute wash in 1 % sodium hypochlorite. Finally the seeds were washed 5 times with sterile distilled water and planted in 1 % Bacto-agar in covered sterile glass trays. Germination was for 3 days at 29° in the dark. When intact seedlings were used, germination was accomplished in Kimpac as described (8). Two-g samples of hypocotyl taken one-half to 1 and one-half cm below the cotyledons were incubated with shaking at 30° in 50 ml Erlenmeyer flasks containing 5 ml of medium (1 % sucrose, 5×10^{-4} M ammonium citrate, pH 6.0, ± 50 μg/ml chloramphenicol). Sterile hypocotyl was excised under aseptic conditions and incubated in sterile medium as described above. Tissue was preincubated for 2 hours prior to the addition of carrier-free ^{32}P-orthophosphate. Intact seedlings were incubated with shaking at 30° in 250-ml beakers containing 30 ml distilled water ± 50 μg/ml chloramphenicol. The roots were submerged to about 3 cm. The apical 0.5 cm section and a section taken from 1.5 to 3.5 cm from the tip were excised from the root following a 2-hour incubation in ^{32}P-ortho-

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phosphate. Following the 2-hour labeling period tissue was washed extensively with 0.05 M Na_3PO_4 followed by cold deionized water.

One-g samples of hypocotyl or root tissue were homogenized in 5 ml of sterile Ringer's solution, and serial dilutions were made for bacterial counts. Aliquots from each dilution were plated onto nutrient agar. The plates were incubated at 25° for 48 hours at which time colony counts were made.

Bacteria were isolated by scraping colonies from the nutrient agar plates. The colonies were transferred to a liquid nutrient medium and cultured for 24 hours at 30° with continuous shaking. One ml of this culture was then transferred to 9 ml of nutrient media containing either ^{32}P-orthophosphate or ^{3}H-thymidine. Incubation was continued for 2 hours.

Nucleic acids were extracted as described by Ingle *et al.* (7). Methylated albumin, kieselguhr column (MAK) chromatography (13) was done as described by Ingle *et al.* (7) with the following modifications. A 2-stage linear gradient of potassium chloride in 0.05 M potassium phosphate buffer (pH 6.7) was used to elute the nucleic acids. The first stage was 60 ml each of 0.45 and 0.85 M KCl, followed by 120 ml each of 0.85 and 1.35 M KCl.

Sucrose gradient centrifugation was carried out using 5 to 20 % gradients containing 0.01 M sodium acetate pH 6.0 for 18 hours at 23,000 rpm (−12°) in the SW 25 rotor of a Spinco Model L2 centrifuge.

Polyacrylamide gel electrophoresis was performed essentially in the manner of Loening (10) and Loening and Ingle (11). Gels containing 2.4 % acrylamide and 0.12 % bisacrylamide were prepared in 7.5 × 0.63 cm 'plexiglas' tubes, and electrophoresis was for 3 or 4 hours at 5 ma/gel, at which time the low molecular weight RNA's had run off the gel.

Results

Bacterial Studies. Immediately following excision, the 1-cm sections of routinely grown soybean hypocotyl used in these experiments contained from 1.4 to 1.7 × 10^4 viable bacteria per g fresh weight. The number of bacteria increased to about 3.7 × 10^4 to 5.8 × 10^4 per g fresh weight after a standard incubation of 2 and 4 hours, respectively. The inclusion of 50 μg/ml chloramphenicol (CM) in the incubation medium prevented any increase in contaminating bacteria over a 4-hour incubation. Soybean roots used in these experiments contained from 3.1 to 4.6 × 10^7 viable bacteria per g fresh weight. When soybean seedlings were grown under aseptic conditions, there were no detectable bacteria in the tissue used (the lower limit of detection was 10^2 bacteria per g fr wt). Examination of the isolated bacteria showed them to be gram-negative rods of the Pseudomonad type, in agreement with other workers (6, 9).

Table I. *Influence of Chloramphenicol on Growth of Excised Soybean Hypocotyl*

CM	Increase in fr wt: Control	2,4 D
	4-Hr incubation	
	g	*g*
0	1.12	1.29
50	1.12	1.27
100	1.11	1.28
	8-Hr incubation	
0	1.25	1.56
50	1.24	1.58
100	1.25	1.58

The data presented in table I show that CM, even in excess of that required to prevent bacterial growth, did not affect elongation of the soybean hypocotyl.

Nucleic Acid Characterization. In order to assess critically the contribution of bacteria to the newly synthesized nucleic acids of excised soybean hypocotyl, both routinely grown (non-sterile) and sterile tissues were labeled with ^{32}P-orthophosphate for 2 hours with and without 50 μg/ml CM. A culture of bacteria, which was isolated from the routinely-grown hypocotyls, was also labeled for 2 hours with either ^{32}P-orthophosphate or ^{3}H-thymidine. Purified preparations of nucleic acids from these sources were characterized by 1) sucrose gradient centrifugation, 2) MAK column fractionation, 3) acrylamide gel electrophoretic separation, and 4) base composition analyses. Figure 1 shows the distribution of ^{32}P-RNA from bacteria together with carrier soybean RNA on a 5 to 20 % linear sucrose gradient. As expected based on the sizes of bacterial and plant ribosomal RNAs (11), the

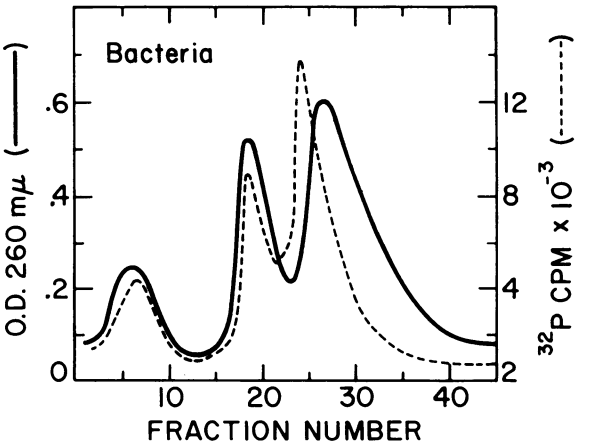

FIG. 1. Sucrose gradient fractionation of ^{32}P-bacterial RNA and carrier soybean hypocotyl RNA. Five μg bacterial RNA were cofractionated with 1 mg soybean RNA on 5 to 20 % sucrose gradients. Centrifugation was for 18 hours at 23 K rpm in a SW 25 rotor. Bacteria were labeled for 2 hours with ^{32}P.

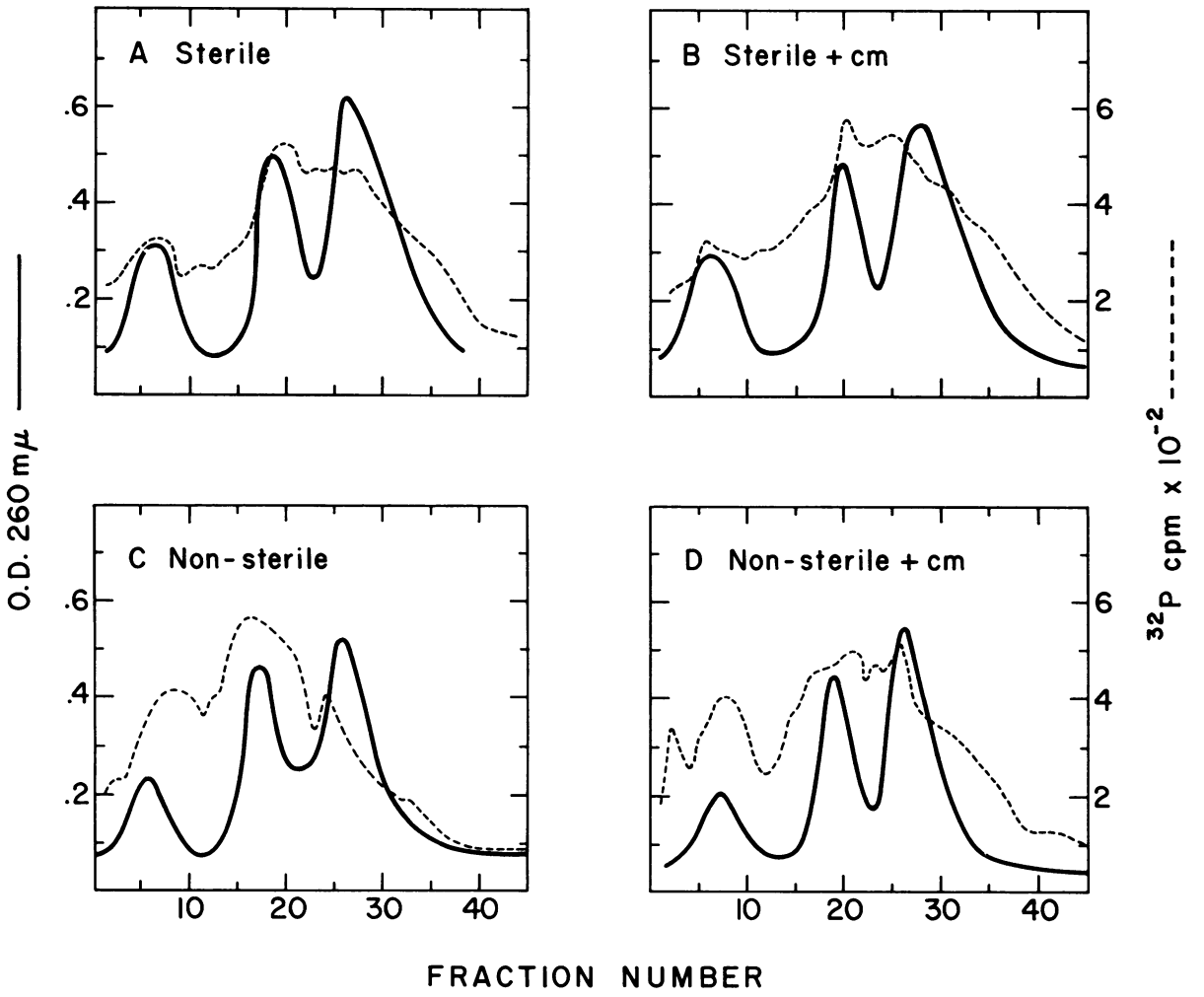

FIG. 2. Sucrose gradient fractionation of soybean RNA. Sterile and non-sterile soybean hypocotyl were labeled with ^{32}P for 2 hours ± 50 μg/ml CM. Fractionation was accomplished as described in figure 1.

bacterial r-RNA sedimented to the light side of the plant r-RNA with the effect being more pronounced in the case of the heavier component (4). There was no polydisperse bacterial RNA following a 2-hour label. In contrast the ^{32}P-RNA from the soybean hypocotyl treatments described above showed a heterogeneous but rather uniform distribution over the gradient (fig 2, A to D). There were no marked differences between profiles of RNA from sterile and non-sterile tissue except in the case of figure 2C. The reason for this difference is not immediately obvious in view of the data presented in figures 3 and 4. CM had no apparent effect either on the distribution of newly synthesized RNA on the gradients or on the level of ^{32}P incorporation into the RNA.

MAK profiles of ^{32}P-labeled nucleic acids prepared from sterile and non-sterile soybean hypocotyl along with ^{3}H-labeled DNA from bacteria are shown in figure 3. There are no discernible differences in the ^{32}P-labeling pattern of sol-RNA, r-RNA, or D-RNA from sterile and non-sterile tissue. Furthermore, CM had no effect on the labeling pattern or the extent of labeling of RNA. In the case of DNA there were marked differences. The ^{3}H-labeled bacterial DNA clearly eluted earlier than the bulk soybean DNA. Likewise the ^{32}P-DNA from non-sterile hypocotyl eluted as a leading peak relative to the bulk soybean DNA, but coincident with the ^{3}H-bacterial DNA. Sterile tissue and CM-treated tissue incorporated much less ^{32}P into DNA, and the ^{32}P-DNA from these tissues eluted coincident with the bulk soybean DNA.

Because of its high resolving power (10), acrylamide gel electrophoresis was used to fractionate nucleic acids from sterile and non-sterile soybean hypocotyl (fig 4). This method completely separates bacterial r-RNAs (16 and 23S) from plant r-RNAs (18 and 25S) (ref 11 and fig 4F). There are clearly no differences in RNA labeling patterns between sterile and non-sterile hypocotyl (fig 4A and C) or between CM-treated (fig 4B and D) and

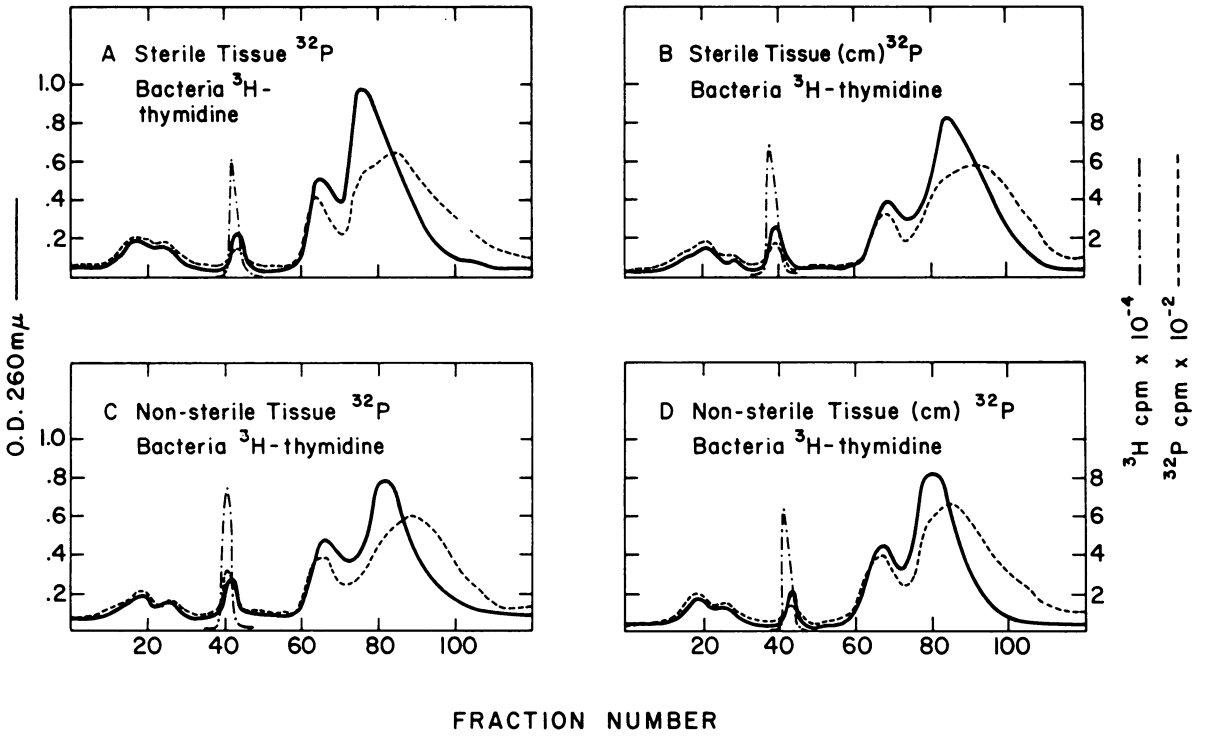

FIG. 3. MAK column fractionation of ^{32}P soybean hypocotyl nucleic acids with ^{3}H bacterial DNA. Sterile and non-sterile tissue were labeled with ^{32}P for 2 hours ± 50 μg/ml CM and cofractionated with ^{3}H bacterial DNA.

untreated (fig 4A and C) hypocotyl. There was no evidence for incorporation into 16 and 23S r-RNAs. The incorporation of ^{32}P into DNA (fig 4A to D) was too low to be detected by this method since the polydisperse, AMP-rich RNA (D-RNA) overlaps the DNA region as well as the r-RNA region of the gel just as on sucrose gradients (7).

Base composition analyses of total ^{32}P-RNA from the soybean hypocotyl and from bacteria are given in table II. There were no significant differences in the composition of newly synthesized RNA from sterile and non-sterile tissue or from CM-treated tissue with the GMP/AMP ratio of the ^{32}P-RNA ranging from 0.86 to 0.88 in all treatments. The composition of ^{32}P-bacterial RNA is clearly different from ^{32}P-hypocotyl RNA, giving essentially a r-RNA composition after the 2-hour label, in agreement with published values for Pseudomonad RNA (14). In addition, the pulse-labeled RNA of a Pseudomonad is even lower in AMP than long-time labeled RNA (14).

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

Source of RNA	Mole % CMP	AMP	GMP	UMP	GMP/AMP
Bacteria	22.1	24.6	32.6	20.7	1.36
Non sterile[1]	22.7	28.8	25.3	22.6	0.88
Non sterile[1] (+ CM)	22.6	30.4	26.4	21.4	0.86
Sterile[1]	22.1	29.1	26.3	23.4	0.86
Sterile[1] (+ CM)	23.4	28.2	24.6	23.6	0.86

[1] Elongating soybean hypocotyl.

Since the evidence presented above indicates that synthesis of the G-C-rich DNA component by non-sterile soybean hypocotyl (total counts in the DNA component represent about 1 % of the total ^{32}P-incorporated into total nucleic acid) results from bacterial contamination, an investigation was made of nucleic acid synthesis in the intact soybean seedling root. The mature region of the root was shown earlier to incorporate considerable ^{32}P into a fraction of DNA eluting at a lower salt concentration than the bulk soybean DNA (8). The base composition analysis of this ^{32}P-DNA showed it to be 64 % GMP + CMP (8), precisely the composition of Pseudomonad DNA (14). Three-day old soybean seedlings were grown for 2 hours with the roots immersed in distilled water ± 50 μg/ml CM followed by an additional 2 hours in ^{32}P-orthophosphate. Nucleic acids were prepared from the apical 0.5 cm and the 1.5 to 3.5 cm zones of the root and fractionated by acrylamide gel electrophoresis (fig 5). In addition ^{32}P-labeled nucleic acids from bacteria were fractionated separately and with nucleic acids from the 1.5 to 3.5 cm zone of the root. Nucleic acids from the 0.5 cm root tip (fig 5A) showed considerable incorporation into 18 and

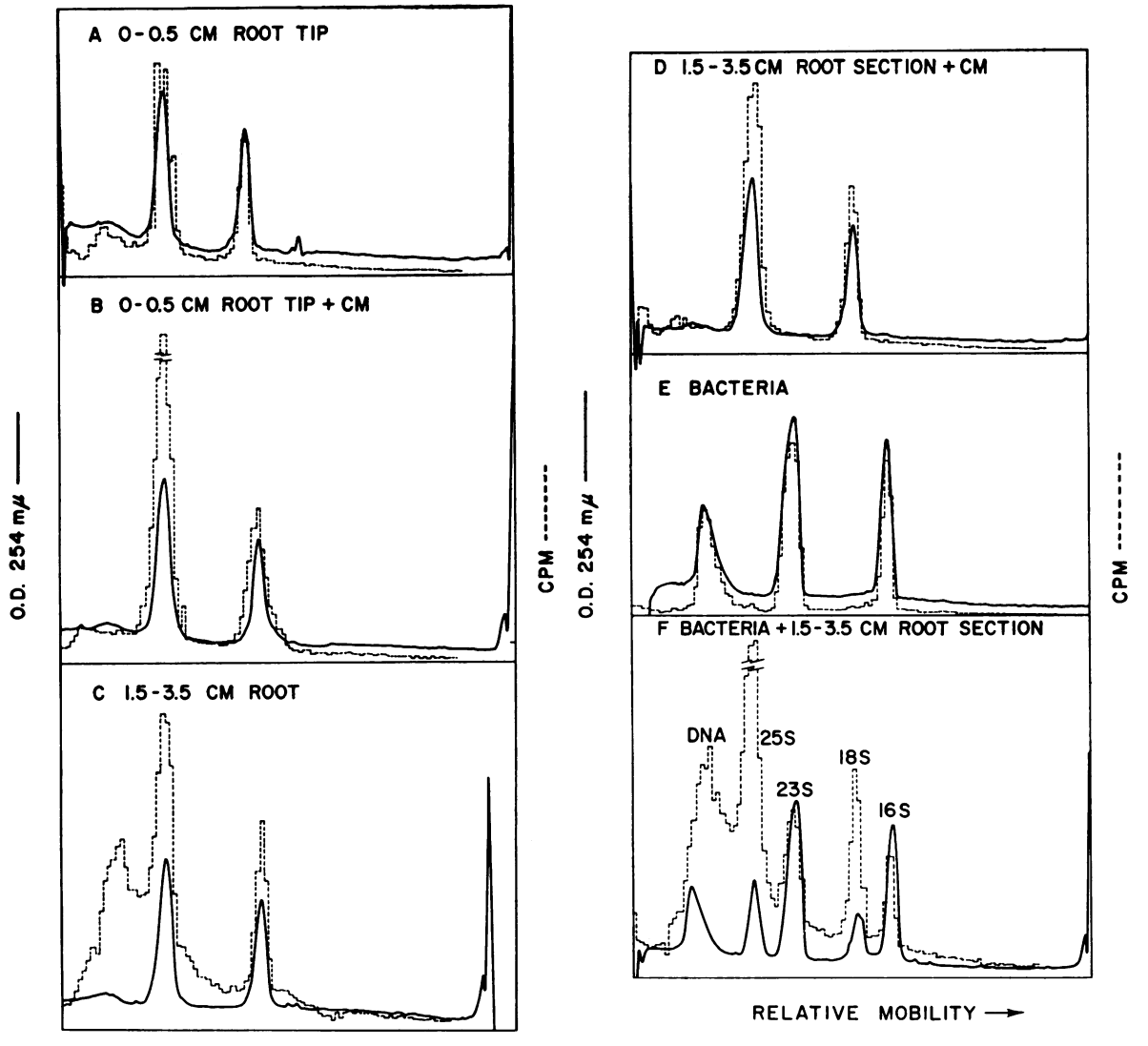

FIG. 5. Fractionation of soybean root and bacterial nucleic acids on polyacrylamide gels. RNA was prepared and fractionated as in figure 4 except that electrophoresis was for 3 hours.

25S r-RNA, with no evidence for incorporation into the 16 and 23S regions of the gel which would correspond to plant organelle or bacterial r-RNA (11, 20). CM did not alter the pattern of incorporation into nucleic acids of this tissue (fig 5B). Most of the ^{32}P-RNA of the 1.5 to 3.5 cm zone of the root corresponded to 18 and 25S r-RNA (fig 5C). There was, however, a slight indication of ^{32}P-RNA in the 16 and 23S regions of the gel. Furthermore, there was considerable incorporation of ^{32}P into DNA of this tissue as reported earlier (8). Treatment of the roots with CM virtually eliminated ^{32}P incorporation into the DNA as well as the trace of incorporation in the 16 and 23S regions of the gel (fig 5D). The bacterial nucleic acid profile is shown in figure 5E, and the co-electrophoresed root and bacterial nucleic acid in figure 5F.

Discussion

The data presented here clearly show that bacterial contamination of the soybean tissues with which we have worked does not contribute to the reported labeling patterns of RNA (7, 8). This conclusion is supported by A) the essentially identical labeling pattern and extent of ^{32}P-incorporation into RNA of sterile and non-sterile soybean hypocotyl using 3 methods of RNA fractionation, B) the absence of any appreciable ^{32}P incorporation into 16 and 23S RNA which corresponds to bacterial r-RNA, and C) the identical base composition analyses of RNA from sterile and non-sterile soybean hypocotyl, compositions which differ grossly from either pulse-labeled or uniformly-labeled Pseudomonad RNA (14).

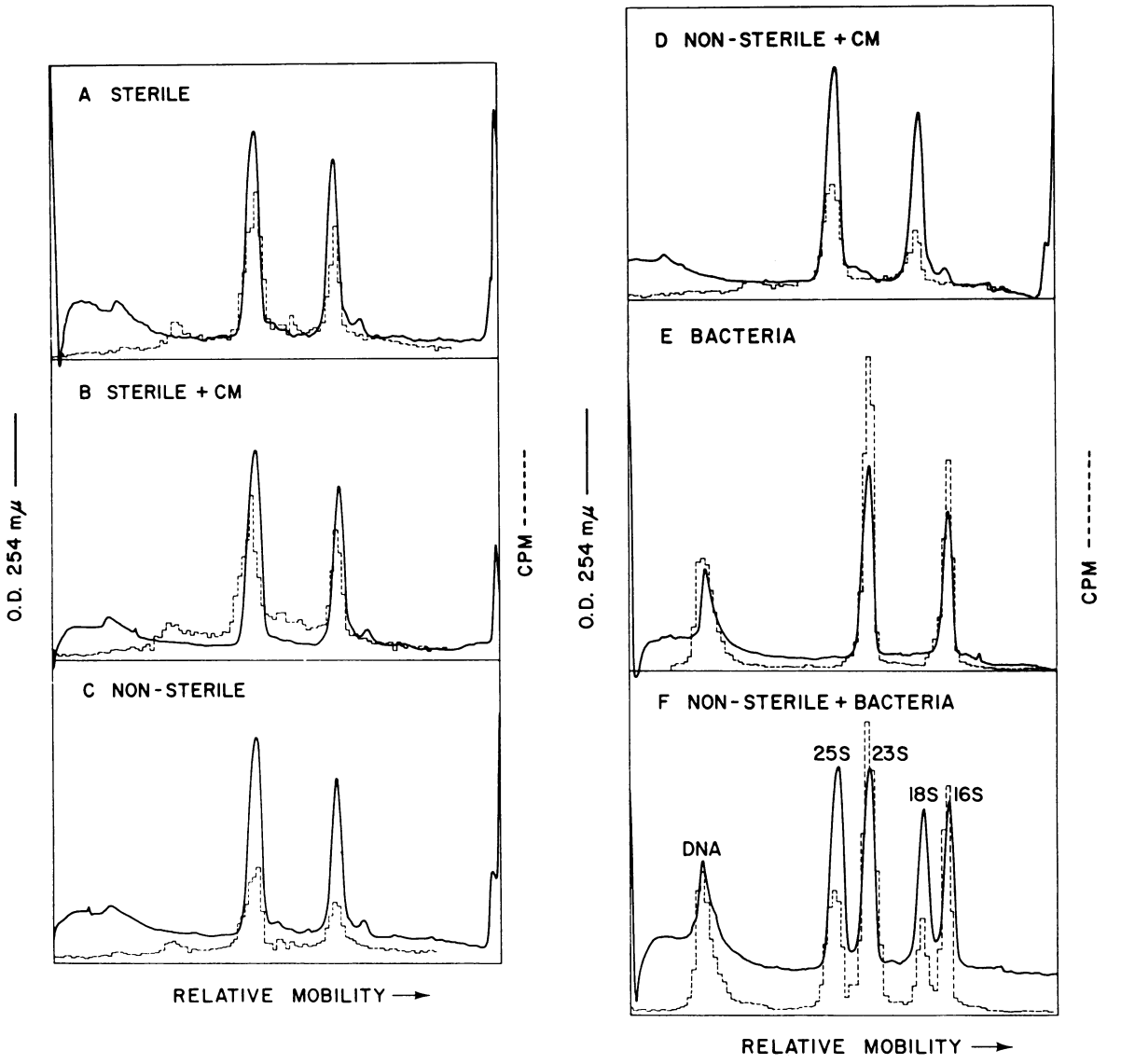

FIG. 4. Fractionation of soybean hypocotyl and bacterial nucleic acids on polyacrylamide gels. Sterile and non-sterile hypocotyls ± 50 μg/ml CM and bacteria were labeled separately with ^{32}P for 2 hours. Electrophoresis at 5 ma/gel was for 4 hours. The gels were scanned with a Joyce, Loebl Chromoscan. The gels were then sectioned into 0.67 mm slices and placed on strips of filter paper. The strips were dried, cut, and each gel section was placed in a scintillation vial and counted.

These data also show that the G-C-rich DNA component (*i.e.* the fraction eluting at a somewhat lower salt concentration from the MAK column than the A-T-rich plant DNA) of the soybean hypocotyl and mature region of the soybean root (7, 8) probably can be attributed to the contaminating bacteria. This conclusion is supported by A) the absence of measurable G-C-rich DNA synthesis by sterile soybean hypocotyl, B) the elimination of this DNA synthesis by chloramphenicol at a concentration which prevented an increase in bacteria and C) the work of Lonberg-Holm (12) and Tester and Dure (21). The high G-C content of this DNA, and therefore its greater buoyant density (19), could readily account for the result of Cherry (2) which was interpreted to show the presence of a DNA-RNA hybrid. These results should not be interpreted to mean that sterile plant tissues never synthesize a G-C-rich DNA, because such synthesis would be expected (1).

The absence of appreciably labeled bacterial RNA from these preparations of nucleic acids even under conditions that the DNA of contaminating bacteria became very highly labeled (mature soybean root) may at first seem a paradox. However, this would be expected to occur under the incubation conditions which were used here. It is known that bacteria will increase their DNA by as much as

40 to 50 % without increasing their RNA when growing on a minimal or "step-down" medium (15, 16).

These results show that chloramphenicol can be used successfully during the incubation of etiolated plant tissues to prevent bacterial growth without affecting growth of the plant tissue or the extent and pattern of labeling of plant nucleic acids. This might not be true for green tissues where chloroplasts might make a major contribution to nucleic acid metabolism (1, 11, 20).

It should be emphasized here that sterile soybean hypocotyl, as well as all other plant tissues which we have studied, synthesizes D-RNA (7). Hock's failure to detect this fraction of RNA in sterile watermelon cotyledons (6) probably relates to the method of nucleic acid extraction which he used, namely the method of Gierer and Schramm (5) which makes use of phenol in the absence of a detergent. A detergent is required in the extraction medium to effect appreciable extraction of the D-RNA (7). Even so, the specific activity of the watermelon RNA is higher in the D-RNA region than, for example, in the light ribosomal RNA region after a 3-hour label (6).

These data, along with those of Venis (22), serve to point out that a level of bacteria as high as 10^6 to 10^8 per g may not contribute to the labeling pattern of RNA and protein. As an example, about 10^7 bacteria per g in the 0.5 cm soybean root tip do not contribute even to the labeling of DNA (8) or RNA as seen here, while comparable contamination in the 1.5 to 3.5 cm zone of the soybean root leads to a marked contribution of DNA labeling but not significantly to RNA labeling. Up to 10^6 bacteria per 200 mg of pea or cucumber tissue did not contribute significantly to incorporation of precursor into RNA and protein (22). Thus, a measure of bacterial contamination is not sufficient to make conclusions about their contribution to labeling patterns of nucleic acids and protein in plant tissues. A direct determination using MAK fractionation for DNA, acrylamide gel fractionation for RNA, and the methods of Venis for protein (22) should prove sufficient and probably necessary.

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