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Polyacrylamide Gel Analysis of High Molecular Weight Ribonucleic Acid from Etiolated and Green Cucumber Cotyledons

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ABSTRACT

Cucumis sativus L. **seeds and 5-day-old dark-grown cotyledons contain 25 and 18 S cytoplasmic ribosomal RNAs as main components. The major increase in nucleic acid content in both green and etiolated cotyledons occurs between days 5 and 7 of germination. This increase is characterized by an important synthesis of 23 and 16 S plastid (chloroplast and proplastid) ribosomal RNAs. Proplastid RNA synthesis appears to continue for a longer period in the dark-grown cotyledons, despite a total RNA content considerably less than in the light-grown cotyledons.**

The nonribosomal distribution of the chloroplast and proplastid ribosomal RNAs observed in all cases (after extraction and fractionation) results from the lability of the 23 S component. This degradation increases if the chloroplasts and proplastids are isolated prior to extraction of their nucleic acid.

The presence of low and high molecular weight ribosomal RNA components of chloroplastic origin has been established for many higher plant species. Namely, four high molecular weight RNAs (25, 23, 18, and 16 S) could be fractionated from green cotyledons and leaves (18), by the high resolving technique of polyacrylamide gel electrophoresis.

More recently, it has been found that bacteria, *Actinomycetes*, blue-green algae, and higher plant chloroplasts all have 23 and 16 S ribosomal RNAs (molecular weights 1.1×10^6 and 0.56×10^6), whereas 25 and 18 S ribosomal RNAs (molecular weights 1.3×10^6 and 0.7×10^6) are characteristic of the cytoplasm of higher plants, ferns, algae, fungi, and some protozoa (17).

The chloroplast ribosomal RNAs are distinct from cytoplasmic ribosomal RNAs not only in size, but also apparently in stability. Thus, after extraction and fractionation, the expected 2:1 ratio for the 23 to 16 S ribosomal RNA is not observed. However, incorporation of radioactive precursors has shown that these two components are in fact synthesized in a 2:1 ratio (12).

Ribosomal RNA components different in size from those of the cytoplasm have also been demonstrated in proplastids obtained from dark-grown leaves and cotyledons. Ribonucleic acid particles about 170 A in diameter have been observed in both proplastids and chloroplasts of maize (15). More recently it has been shown that dark-grown cucumber and radish cotyledons possessed the four high molecular weight RNA components obtained from green tissues (13, 24). These findings suggest that the 23 and 16 S components of dark-grown cotyledons originate from the proplastids.

The results presented in this paper compare the synthesis of ribosomal RNA during germination and early growth of cucumber cotyledons both with and without light. The fractionation and stability of the plastid RNAs are also examined in relation to three different organelle isolation buffers.

MATERIALS AND METHODS

Plant Material. Cucumber seeds (*Cucumis sativus* L. var. Marketmore) were washed in 1% sodium hypochlorite, rinsed with distilled water, and germinated for 5 days in the dark (20 C, 65% relative humidity) (Fig. 1), in vermiculite maintained moist by daily addition of standard Arnon and Hoagland nutritive solution (1). Following this germination period, growth was continued under the same conditions of temperature and humidity either in the dark, or with a 16-hr photoperiod of 4000 ft-c.

Subcellular Fractionation. Chloroplasts and proplastids were isolated from light- and dark-grown cotyledons of 14-day-old cucumber seedlings. All operations were performed at 4 C. Plant tissue was thoroughly rinsed with cold distilled water and homogenized in a chilled mortar and pestle, under a dim green safe light, in three times their weight of one of the three following buffer systems: (*a*) 0.5 M sucrose, 0.1 M tris-HCl, 0.05 M KCl, 0.005 M $MgCl_2$, 0.005 M mercaptoethanol, pH 7.5 (22); (*b*) 0.5 M sucrose, 0.02 M tris-HCl, 0.01 M NaCl, 0.005 M EDTA, 0.005 M mercaptoethanol, pH 8.0 (3); (*c*) 0.5 M sucrose, 0.1 M glycine, 0.1 M NaCl, 0.01 M EDTA, pH 9.5 (7).

The homogenate was filtered through 12 layers of gauze, and the filtrate was centrifuged for 5 min at 100*g* to remove nuclei and cell debris. Plastids were isolated from the supernatant by sedimentation at 1000*g* for 12 min. Plastid pellets were then washed once by resuspension in the homogenization buffer and sedimented as before.

Isolation of RNA. Total nucleic acid was prepared from seedling tissues by SDS[1]-phenol extraction at 4 C. Tissues were homogenized for 1 min in a Virtis blender, in presence of glycine buffer (0.1 M glycine, 0.1 M NaCl, 0.01 M EDTA, pH 9.5), phenol, SDS, and bentonite (7).

Homogenate was filtered through four layers of gauze, and the filtrate was stirred for 15 min; after centrifugation (5000*g* for 10 min) the aqueous layer was removed and the phenol extraction was repeated once. Nucleic acids were precipitated from the final supernatant solution by addition of sodium acetate (2% final concentration) and 2.5 volumes of 95% (v/v) cold ethanol. The precipitate was then dissolved in glycine buffer and reprecipitated as before with ethanol in order to purify further the RNA and to remove phenol; this step was repeated twice. Final precipitates were stored in 95% (v/v) ethanol at −20 C.

Total nucleic acid at different developmental stages was obtained by measuring the absorbance, at 260 nm, of the purified RNA.

[1] Abbreviation: SDS: sodium dodecylsulfate.

FIG. 1. Initial germination stages of *Cucumis sativus* L. seedlings 1, 2, 3, 4, 5, and 6 days old, grown in the dark, are shown from left to right.

RNA isolation from subcellular fractions was performed as above, except that Virtis homogenization and gauze filtration were omitted.

Gel Electrophoresis. Polyacrylamide gels were prepared by a modification of the procedure described by Loening (16). Acrylamide and bisacrylamide (Eastman Organic Chemicals) were recrystallized from chloroform and acetone, respectively. Polyacrylamide 2.4% (w/v) gels were prepared by mixing 4.8 ml of an aqueous stock solution of 15% (w/v) acrylamide and 0.75% (w/v) bisacrylamide with 10 ml of 3E buffer (0.12 M tris, 0.06 M sodium acetate, 0.003 M sodium EDTA, pH 7.8 with glacial acetic acid) and 14.98 ml of distilled water. The mixture was degassed at room temperature *in vacuo*, and 0.02 ml of N,N,N^1,N^1-tetramethylethylenediamine and 0.2 ml of fresh aqueous 10% (w/v) ammonium persulfate solution were added. After swirling, the mixture was transferred to quartz tubes, (0.55 cm internal diameter and 7.5 cm length) and allowed to polymerize for 20 min in an upright position. The gel length was made exactly 7 cm. The electrophoresis buffer was buffer E added with 0.1% (w/v) SDS. The gels were submitted to a pre-electrophoresis run with 10 v/cm, 5 ma per gel, for about 30 min at room temperature.

The RNA samples were dissolved in buffer E containing 10% (w/v) RNase-free sucrose (Schwartz BioResearch) and 0.1% SDS. Twenty-five, 50, or 75 μl of the solution, containing approximately 1 mg of RNA per ml, were layered over the gels. Electrophoresis was performed at room temperature (20 C) at 10 v/cm, 5 ma per gel, for 60 min or more, as indicated. By this time, the soluble RNA had migrated out of gel, as indicated by the migration of the pyronine dye (0.02% pyronine in buffer E containing 10% sucrose), since soluble RNA and pyronine have about the same migration rate in the gels.

After electrophoresis, the gels containing quartz tubes were removed and scanned directly at 260 nm with a Schoeffel densitometer.

RESULTS

Changes in Nucleic Acid Content during Development of Light- and Dark-grown Cucumber Cotyledons. These changes are shown in Figure 2. In both light- and dark-grown cotyledons, the major increase in total RNA occurs over a relatively short period, between days 5 and 7, at which time 90% of the total nucleic acid has been accumulated in the etiolated cotyledons compared to 80% in the green cotyledons. Etiolated cotyledons accumulate only 60% as much nucleic acid as the green cotyledons, and this maximum is reached earlier (day 9) than with the green cotyledons (day 14). The decline in nucleic acid content is more pronounced in the dark-grown cotyledons and occurs 5 days earlier than in the light-grown cotyledons.

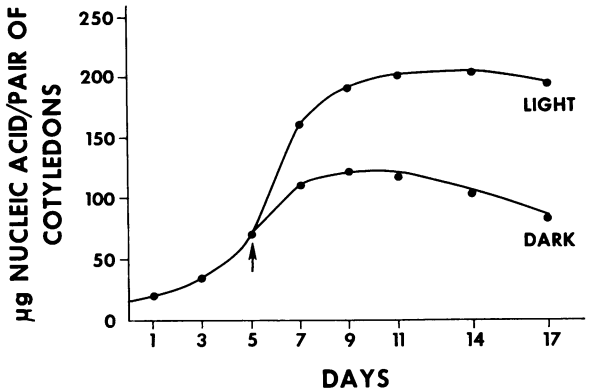

FIG. 2. Change in total nucleic acid content during the development of the cucumber cotyledons, in both presence and absence of light. Light-grown seedlings were illuminated from day 5, as indicated by arrow.

Polyacrylamide Gel Analysis of High Molecular Weight RNA. Figure 3 shows the electrophoretic gel fractionation of RNA prepared from dry seeds (A), and from 3- and 5-day-old seedling cotyledons (B and C). In all three cases, the main components are the 25 and 18 S cytoplasmic RNAs. However, in the 3- and 5-day cotyledons, two minor components appear as shoulders of about 23 and 16 S, probably from proplastic origin. Barely detectable amounts of these minor fractions are also present in dry seeds; however, it has not been possible to show that the heaviest has a lower molecular weight than 23 S, as recently suggested for radish seeds (12).

Isolation of RNA from cucumber dry seeds is noteworthy, because the mortar and pestle homogenization must be performed in the presence of sand. This might partly explain the larger DNA peak in dry seeds as compared with cotyledons.

After 5 days of germination in the dark, the cotyledons just emerge from the vermiculite and are separating from one another (*cf.* Fig. 1). One day later (day 6) the dark-grown cotyledons contain an appreciable amount of proplastic 23 and 16 S RNA, and also a minor fast migrating RNA fraction (Fig. 4B). Measure of the sedimentation coefficient for this last RNA peak, using the linear relation between relative electrophoretic mobility and log molecular weight (4), gives a value of 13 S. The 13 S RNA has recently been attributed to the degradation of 23 S proplastic RNA during tissue homogenization (12).

The important relative increases in 23, 16, and 13 S RNAs in

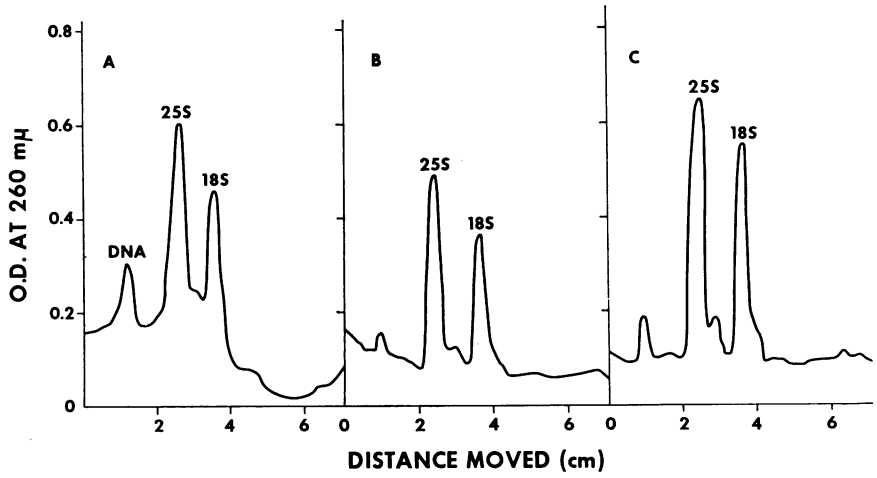

FIG. 3. Fractionation of cucumber nucleic acids prepared from dry seeds (A); 3-day-old cotyledons (B); 5-day-old cotyledons (C). The gel electrophoresis were run for 75 min.

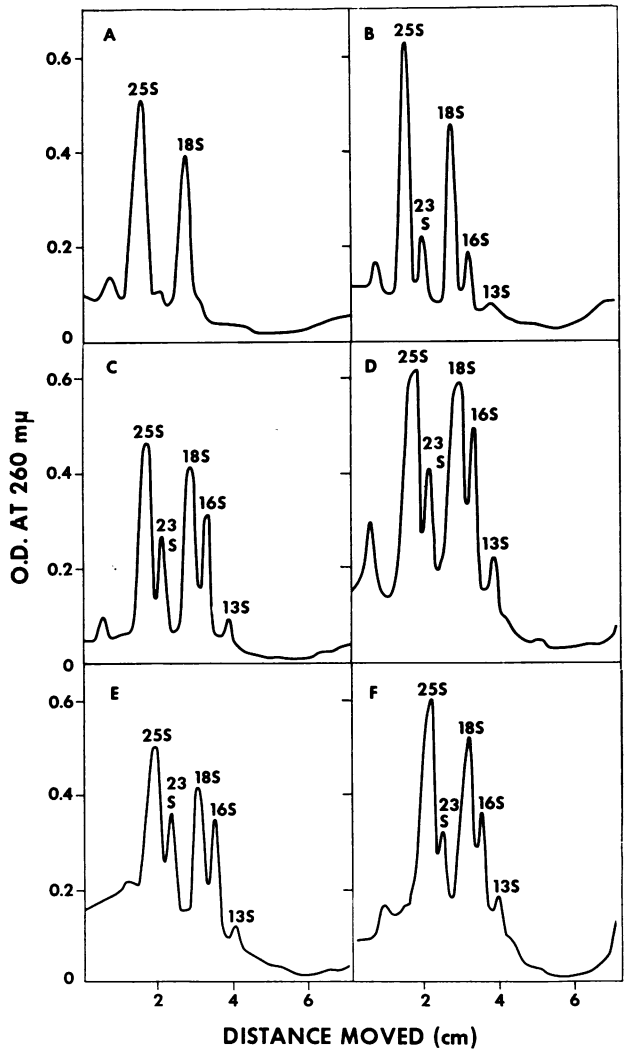

FIG. 4. Fractionation of nucleic acids prepared from light- and dark-grown cucumber seedlings. A: 6-day-old dark-grown hypocotyls and roots; B: etiolated cotyledons at day 6; C and D: etiolated cotyledons at days 10 and 16, respectively; E and F: 10- and 21-day-old light-grown cotyledons. The gel electrophoreses were run for 60 min.

Table I. *Chloroplastic and Cytoplasmic Ribosomal RNA Ratios of the Cucumber Cotyledon*

The amounts of the individual RNAs were determined from the fractionation data (Figs. 3 and 4). Corrected ratios were calculated assuming a breakdown of the 23 S RNA into 18 and 13 S pieces. The percentage breakdown of the 23 S RNA was determined from the observed and corrected values for this component.

Tissue	Age of Seedling	Condition	Observed Ratios		Corrected 23 S:16 S Ratio		Estimated 23 S Breakdown
			25 S:18 S	23 S:16 S	23 S + 13 S:16 S	23 S + 13 S + 18 S piece[1]: 16 S	
	days						%
Cotyledons	3		1.63	...	...	...	...
	5		1.65	...	...	...	...
	6	Dark	1.65	1.40	1.95	1.95	29
	10	Dark	1.24	0.87	1.18	1.63	46
	16	Dark	1.04	0.83	1.16	1.92	57
	10	Light	1.52	1.22	1.40	1.61	24
	21	Light	1.29	0.92	1.25	1.75	47
Hypocotyls and roots	6	Dark	1.65	...	...	...	...

[1] The 18S piece from 23S breakdown was measured assuming an average 25 S:18 S ratio of 1.65. Correction according to Ingle (12) gives unreliable values with cucumber cotyledon RNAs.

etiolated cotyledons between days 5 and 6 notably coincide with the period of major increase in total RNA (Fig. 2). Electrophoretic analysis of RNA prepared from the hypocotyls and roots of 6-day-old dark-grown seedlings is shown in Figure 4A. The separation pattern is similar to that obtained after 3 days of germination (Fig. 3B), showing slight shoulders for the minor RNAs and being characterized by a 25 S:18 S ratio of 1.65 (Table I). It is of interest that the 25 S:18 S normal ribosomal ratio of about 2, observed recently with cucumber seedling after centrifugal fractionation (25), is never obtained in these data. This appears to be due to nonlinearity of scanning for the conditions of loading and scanning in this type of experiment (12).

Planimetric measurements give a 25 S:18 S ratio of 1.65 for the dark-grown 6-day cotyledons, whereas the 23 S:16 S ratio is only

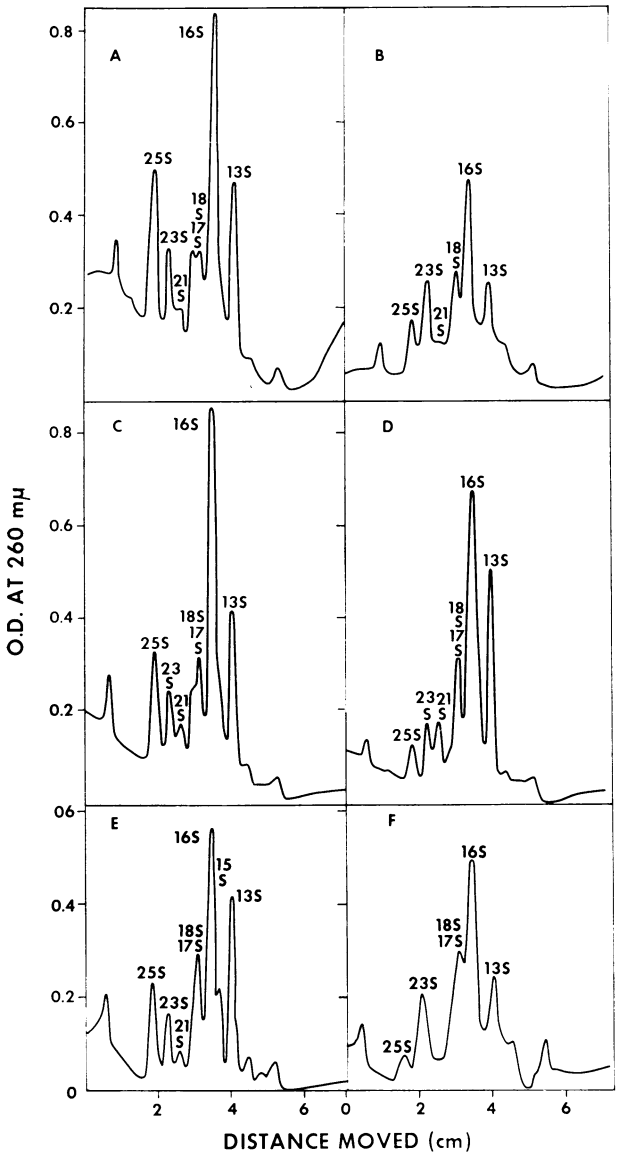

FIG. 5. Fractionation of nucleic acids prepared from cucumber plastids. A and B: Chloroplasts and proplastids, respectively, isolated with tris-HCl buffer, pH 7.5; C and D: chloroplasts and proplastids, respectively, isolated with glycine buffer, pH 7.5; E and F: chloroplasts and proplastids, respectively, isolated with tris-HCl buffer, pH 8.0. The gel electrophoreses were run for 75 min.

1.40. Correcting this value on the assumption that the 13 S fraction is a 23 S degradation product gives the value of 1.95 (Table I), which is near normal ribosomal distribution.

Figures 4C and 4D show the fractionation obtained with the 10- and 16-day dark-grown cotyledons. The three proplastic components 23, 16, and 13 S are present, but a significant 23 S degradation is suggested with a concomitant increase in 13 S. For both stages of growth, the planimetrically measured 25 S:18 S ratio is close to 1, whereas the 23 S:16 S ratio is less than 1. If the area measured for the 18 S peak is decreased so as to give a 25 S:18 S ratio of 1.65 (ratio obtained for days 5 and 6), the ratio 23 S + "excess 18 S" + 13 S:16 S can be calculated, greatly improving the values obtained. For example, the ratio obtained for the day 16 fractionation is very close to 2. It would therefore appear that the 23 S proplastic RNA breaks down into 18 and 13 S subunits. The fractionations obtained with the 10- and 21-day light-grown cotyledons (Fig. 4, E and F) are consistent with the above explanation.

Table II. *Proplastic and Chloroplastic Ribosomal RNA Ratios of Etiolated and Green Cucumber Cotyledons*

The amounts of the individuals RNAs were determined from the fractionation data of Figure 5. Corrected ratios were calculated assuming a breakdown of the 23 S RNA into 18 and 13 S pieces.

Isolation Medium	Subcellular Fraction	Observed 23 S:16 S Ratio	Corrected[1] 23 S:16 S Ratio
Sucrose, tris-HCl, KCl, $MgCl_2$, mercaptoethanol, pH 7.5	Chloroplast	0.18	0.70
	Proplastid	0.49	1.40
Sucrose, glycine, NaCl, EDTA, pH 9.5	Chloroplast	0.26	0.93
	Proplastid	0.17	0.85
Sucrose, tris-HCl, NaCl, EDTA, mercaptoethanol, pH 8.0	Chloroplast	0.23	1.10
	Proplastid	0.44	1.50

[1] The 18 S piece from 23 S breakdown was measured assuming an average 25 S:18 S ratio of 1.65.

Planimetric measurements further suggest that degradation of 23 S RNA (proplastic or chloroplastic) is lesser at early than at older stages of germination and is more important in etiolated than in green cotyledons at the same stage.

Effect of Plastid Isolation Medium on RNA Stability. Figure 5 shows the gel electrophoretic fractionation of the nucleic acids extracted from chloroplasts and proplastids prepared with the three different homogenizing buffers. Despite contamination with 25 and 18 S cytoplasmic RNA, there is in each case a significant degradation of the 23 S fraction; this degradation appears much greater than that observed with total nucleic acid fractionation. Minor 21, 17, 15 S, and some smaller than 13 S components are present in nearly all diagrams, while 16 and 13 S appear as major peaks in a number of instances.

Table II gives the observed 23 S:16 S ratios, and the ratios corrected on the basis of a breakdown of the 23 S fraction into 18 and 13 S pieces. The corrected ratios obtained are always smaller than 1.65. It is therefore suggested that the 21 S fraction, the smaller than 13 S components, and possibly a fraction of the 16 S RNA appearing as shoulders in Figure 5, C and E, might all be 23 S degradation products. Degradation of the 23 S chloroplastic RNA thus appears to be roughly similar with the three buffer systems. In comparison, stability of proplastic 23 S RNA appears greater with tris-HCl buffers than with glycine buffers (Fig. 5, B and F). It is, however, clearly apparent that cellular fractionation prior to RNA extraction, as opposed to total nucleic acid extraction, increases the degradation of the 23 S fraction.

DISCUSSION

The presence of ribosomes of proplastid origin in dark-grown cotyledons and leaves has been demonstrated in several studies. Electron microscopy shows that proplastids containing protochlorophyllide and a crystal lattice-like body (the prolamellar body) are elaborated in higher plants grown in darkness (6). These organelles contain ribosome-like particles smaller than cytoplasmic ribosomes (80 S), but similar in size (70 S) to those formed in the mature chloroplast (15, 5). Two RNAs comparable in size to the chloroplast RNA components have been shown in etiolated radish and cucumber cotyledons by sucrose gradient centrifugation (24) and polyacrylamide gel electrophoresis (13).

Upon exposure to light of dark-grown tissues, chlorophyll synthesis and development of chloroplasts from proplastids are induced. This last phenomenon has been extensively studied in bean leaves and subsequently divided into three main steps: (*a*) tube transformation, (*b*) vesicle dispersal, and (*c*) grana formation (27, 26, 10). Doubling of the plastid-associated RNA has been observed just prior to the synthesis of the grana-containing lamellar system (10). It has been assumed that this change, occuring over an illumination period of 45 hr, implies a strong increase in the plastid ribosomes at this developmental stage.

More recently, comparison of light- and dark-grown radish cotyledons has shown that accumulation of chloroplast RNA is stimulated by light (13). This observation is supported by the present data. However, it appears that light is not an absolute requirement for proplastid RNA synthesis, a slight accumulation being observed in the dark (Fig. 2). Moreover, after day 7, the proportions of proplastic RNA in etiolated cotyledons and of chloroplastic RNA in green cotyledons are similar, amounting to about 30% of the total.

With total nucleic acid extraction, the 23 S proplastic RNA appears as unstable as the 23 S chloroplastic RNA, with production of 18 and 13 S pieces. If a correction is made on the basis of such a breakdown, the 23 S:16 S ratio is close to that expected for ribosomal RNAs (Table I). On the basis of the above correction, Table I shows a 57% breakdown of the 23 S RNA in the etiolated cotyledons on day 16, compared to only 29% on day 6. Similarly, in the green cotyledons, 23 S chloroplastic RNA degradation increases with age. On the other hand, for a given stage of development, 23 S RNA breakdown is greater in etiolated than in green tissues.

These results suggest that stability of the 23 S proplastic or chloroplastic RNAs changes with aging of the 70 S ribosomes and also with external factors such as lighting. Ingle (12) has recently mentioned three possible causes of increased instability with aging of 70 S ribosomes in radish: (*a*) increase in ribosomal ribonuclease activity, (*b*) structural changes in the RNA molecule, and (*c*) occurrence *in vivo* of breaks in the RNA molecules. That ribonuclease activity is a determinant factor is strongly suggested in a study by Hsiao (11), who has shown a sharp increase, with aging, in the ribonuclease activity associated with ribosomes. On the other hand, increase in 23 S breakdown in darkness is consistent with the observation of the increase in RNAse activity associated with stress conditions such as plant bacterial infection (20) and water stress (2); it is also consistent with the hypothesis that accelerated aging can be regarded as a consequence of general stress (9).

It can be assumed that one of the conditions for more specific investigations of the 23 and 16 S proplastic (and chloroplastic) RNAs is the obtainment of these components free or relatively free from cytoplasmic ribosomal RNAs. In this study, proplastids and chloroplasts were rapidly isolated from cotyledons, prior to RNA extraction, with media belonging to the common tris-HCl and glycinate systems. This procedure considerably decreases the amount of cytoplasmic RNA, but in all cases complex gel electrophoretic separations are obtained with an important 23 S degradation. This breakdown is characterized not only by increased 18 and 13 S fractions relative to total RNA extraction, but also by the presence of 21, 17, 15 S, and smaller than 13 S pieces. It appears, therefore, that subcellular fractionation increases 23 S RNA degradation. Further purification of proplastid (or chloroplast) preparation, as suggested by Jacobson (14), would undoubtedly lead to an increased breakdown. Ribonucleic acid preparation from ribosomes prepared according to Tewari *et al.* (23) from isolated proplastids (or chloroplasts) leads to an important 23 S degradation (F. Vedel, unpublished results).

The 23 S degradation during subcellular fractionation can be explained by recent data (11) suggesting that most of the ribonuclease activity associated with corn ribosomes may not be meaningful *in vivo* and occurs only once the cells are ruptured. However, the fact that cytoplasmic ribosomes and their RNA constituents remain stable, during isolation, suggests a particular RNAse-sensitive structure for proplastid and chloroplast ribosomes.

The extensive breakdown of 23 S chloroplastic RNA constitutes for the moment the major abnormality relative to bacterial RNA. Other characteristics such as sedimentation behavior, ease of dissociation in low magnesium ion concentration, and inhibition of amino acid-incorporating ability by chloramphenicol appear to be common for both chloroplastic and bacterial ribosomes (19, 21, 8).

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