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Ribosomal Ribonucleic Acids of Chloroplastic and Mitochondrial Preparations

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Summary. RNA prepared from fractions of chloroplasts and mitochondria sedimented at rates characteristic of ribosomal RNA. A predominance of the 18S species was frequently observed in preparations from chloroplasts from romaine lettuce and endive. The usual distribution, a preponderance of the 28S species, was observed in studies on tomato and spinach chloroplasts and mitochondria from mushroom and cauliflower. Comparisons of the base composition of RNA from organelles with their cytoplasmic ribosomal counterparts revealed that the 18S component from romaine lettuce chloroplast was different. A marginally significant difference was observed in the 28S particle from mushroom mitochondria preparations whereas distinct differences, reflected in all the bases, were seen when the 18S component of cauliflower mitochondria preparations was compared with cytoplasmic RNA.

It has become evident in recent years that cellular organelles such as chloroplasts and mitochondria possess genetic information. This is based on traditional genetic studies and is supported by demonstrations of unique species of DNA in these particles. Evidence from both approaches has been reviewed recently (17). The existence of independent structures for the biosynthesis of protein derived from these organelles would give further support to theories concerning the autonomous nature of the organelles. At present, information on the nature of the nucleic acids of the protein-synthesizing apparatus of subcellular particles, especially with respect to mitochondria, is fragmentary. Thus Brawerman (3) has reported on the uniqueness of whole ribosomal ribonucleic acids from *Euglena* chloroplasts. On the other hand, Odintsova, et al. (15) could find no major differences in base composition between whole cytoplasmic and whole chloroplastic ribosomal ribonucleic acids from higher plants.

The ability of isolated mitochondria from various sources to incorporate labelled amino acids into protein has been amply documented (6, 18, 21). The latter 2 reports have emphasized that the small amounts of RNA invariably found associated with mitochondria are probably indigenous to the mitochondria. In a preliminary report Truman and Korner (23) suggested that mitochondrial RNA may be unusual since extraction of rat-liver mitochondria yielded a ribonucleic acid with an S value of 8.39; this is intermediate between that of ribosomal and soluble RNA. To our knowledge no other investigations on the nature of mitochondrial ribonucleic acids have been reported.

In this paper we report techniques for the isolation of the 18S and 28S species of ribosomal ribonucleic acids from chloroplasts and from mitochondria. In addition, a comparison of the base composition of the cytoplasmic ribonucleic acid components with those from chloroplasts and from mitochondria is given.

Materials and Methods

Preparation of Subcellular Particles. Green leaf blades, usually 200 to 400 g portions, torn from romaine lettuce (*Lactuca sativa*, var. *longifolia*) and endive (*Cichorium endivia*) were used to prepare chloroplast fractions. The shredded tissue, chilled to 5°, was homogenized in a Waring Blendor with 3 to 5 volumes of solution A (0.25 M sucrose, 0.01 M $MgCl_2$, 0.05 M Tris pH 7.8) at one-third to one-half line voltage for 90 seconds. The resulting slurry was filtered through 4 layers of facial tissue previously wetted with solution A. Centrifugation of the filtrate at 1,000 × g for 10 minutes resulted in the sedimentation of chloroplasts as well as large nuclear particles and a few

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intact nuclei. The residue was resuspended in solution A by thorough shaking. Nuclei, nuclear particles and heavy chloroplasts were then sedimented by centrifugation at 50 to 100 × *g* for 5 minutes. Chloroplasts, essentially free of nuclear particles as judged by the absence of particles staining with acetocarmine, were then sedimented at 1,000 × *g*. These were washed once by resuspending in solution A and recentrifuging at 1,000 × *g* for 5 minutes.

Solution A was also used in the preparation of mitochondrial fractions. The fruiting bodies of the fungus (*Psalliota campestris*) or the entire cauliflower head (*Brassica oleracea*, var. *botrytis*) was first chilled in ice water. The mushroom caps were then trimmed so that the outer surface and the gills were discarded. Florets void of chlorophyll were trimmed from cauliflower and used as starting material in the preparation of mitochondrial fractions from cauliflower. The chilled tissue was homogenized with solution A and the slurry filtered as in the preparation of the chloroplast fractions. The filtrate was centrifuged at 1,500 × *g* for 15 minutes and the residue discarded. The supernatant solution was then centrifuged at 15,000 × *g* for 15 minutes to sediment the mitochondrial fraction. The pellet was resuspended in solution A and collected by recentrifugation at 15,000 × *g* for 15 minutes. After 3 or 4 such washings the resulting pellet was used. Approximately 400 g of tissue were used for the preparation of mitochondrial fractions from mushrooms whereas 150 to 200 g of trimmed floret tissue from cauliflower were employed.

Conditions for the isolation of cytoplasmic ribosomes were outlined in a previous communication (16). The only essential modification employed in the present experiments was the use of solution A as the homogenizing medium. Briefly, a microsomal fraction was prepared by centrifuging post-mitochondrial supernatant solutions at 105,000 × *g*. After deoxycholate treatment, ribosomes were collected by recentrifugation at 105,000 × *g*. The ribosomal subunits extracted from chloroplast fractions with deoxycholate were separated on 5 to 20 % sucrose density gradients prepared with a solution of 1×10^{-4} M magnesium chloride and 5×10^{-3} M Tris buffer pH 7.4. A Spinco 25.1 rotor centrifuged at 23,000 rpm for 4 hours was used to effect the separation. It is important to note that midribs, trimmed of all chlorophyll-containing areas except vascular bundles, were used in the preparation of cytoplasmic ribosomes from romaine lettuce.

Extraction, Separation and Hydrolysis of Ribonucleic Acid. Cytoplasmic ribosomal RNA was extracted by homogenizing ribosomes in the cold with 2 % sodium dodecyl sulfate (SDS) and equilibrating the solution with an equal volume of 80 % phenol. The aqueous phase was removed after centrifugation and the nucleic acid precipitated in the cold by the addition of an equal volume of absolute ethanol. The entire procedure was repeated to effect further purification.

RNA from chloroplast fractions and mitochondrial fractions from cauliflower was extracted by a combination of 2 techniques. The cellular fractions were first homogenized with a solution (10–15 ml) containing 0.28 M Li_2SO_4, 2×10^{-3} M acetate buffer, pH 5.0, 2×10^{-3} M $MgCl_2$, 1 % SDS and 100 ug/ml bentonite (13). The mixture was then equilibrated with an equal volume of 80 % phenol by shaking at room temperature for 5 minutes. After cooling to 5° the phases were separated by centrifugation at 10,000 × *g* in the cold. The nucleic acids were precipitated from the cold aqueous phase by the addition of an equal volume of ethanol; they were then collected by centrifugation. The second stage of purification consisted of dissolution of the nucleic acids isolated from the first step in a solution of 4 % phenolphthalein diphosphate and 1 % SDS adjusted to pH 6.0 (5–10 ml). The solution was shaken with an equal volume of 80 % phenol, chilled and the phases separated by centrifugation. Nucleic acids were precipitated from the aqueous layer by the addition of an equal volume of ethanol. The precipitate was dissolved in cold 3 M sodium acetate pH 6.0 (5–8 ml) and allowed to stand in an ice bath for at least 10 minutes. The insoluble RNA was then collected by centrifugation. This part of the isolation was based on the work of Kirby (9). The resulting RNA was of high apparent optical purity (OD 260/280 = 2.0–2.15) and was virtually free of DNA contamination as indicated by the absence of a reaction with diphenylamine. It was not feasible to employ the techniques outlined above for the extraction of RNA from mushroom mitochondrial fractions since impurities which interferred with the sedimentation of RNA on sucrose density gradients contaminated the RNA thus prepared. Accordingly, a ribosomal fraction was first extracted from mitochondria with deoxycholate as is usually done with the microsomal fraction (16). The RNA was then extracted by the same technique employed with cytoplasmic RNA.

Separation of the ribonucleic acids was effected by sucrose density gradient centrifugation. Linear gradients (5–20 % sucrose) were prepared in 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0 and centrifuged for 13 to 16 hours at 23,000 rpm with a Spinco 25.1 rotor. Hydrolysis, chromatography and estimation of bases were performed as given by Bendich (2). The base analyses were done on pooled samples taken from areas of the sucrose gradients containing the purified RNA species. Each analysis represents 4 or more isolations of the organelle and of the RNA species in question. Protein was determined with the biuret reagent (11).

Results

Chloroplasts. Since the validity of these studies rests on the demonstration that relatively pure or-

ganelles were being studied we would like first, in the case of both organelles, to cite experimental evidence to support this contention: A) Cytological examination with the light microscope revealed that whole chloroplasts and chloroplast fragments comprised the bulk of the material used in these studies. Particles of the size of mitochondria were virtually absent. Nuclei and nuclear fragments were not present as judged by the absence of particles staining with acetocarmine.

B) It was not possible to use biochemical markers such as enzymes of the electron transport chain to assess mitochondrial contamination of romaine lettuce chloroplasts since activity of these enzymes could not be detected even in mitochondrial preparations from romaine lettuce. It may be possible, however, to extrapolate from our findings with spinach preparations. In these studies fractions of chloroplasts and mitochondria were prepared from spinach leaves with solution A. The mitochondrial fraction was a 10,000 × *g* pellet of the post-chloroplast supernatant solution. Spinach mitochondria had the ability to oxidize malate with the concomitant reduction of diphosphopyridine nucleotide coupled to the reduction of 2, 6 dichlorophenol indophenol in the presence of cyanide. Cytochrome C could also serve as a final electron acceptor. Spinach chloroplasts, on the other hand, were void of these enzymatic activities. That the absence of enzymatic activity was not due to the inhibitors in chloroplast was shown by mixing experiments. Chloroplasts added to the reaction mixture did not inhibit the oxidation of malate by the mitochondrial preparations. These experiments suggested that there was no contamination of spinach chloroplasts by mitochondria.

C) Our final argument, based on our experimental data, rests on the nature of RNA extracted from romaine lettuce chloroplast fractions. In the many published papers on cytoplasmic ribosomal RNA and in our own experiments, a predominance of the 28S species was usually noted. With romaine lettuce chloroplast fractions in only 3 out of more than 30 experiments has this relationship held. In the majority of the cases, the amount of the 18S species exceeded that of the 28S species. In some cases, such as the one shown in figure 1, the RNA species from romaine lettuce chloroplast fractions were present in approximately equal amounts. The curves in figure 1 represent RNA extracted from cellular fractions isolated from the same leaf tissue. Note that the pattern of distribution of RNA from the mitochondrial fraction was similar to cytoplasmic ribosomal RNA. Conceivably this is indicative of the uniqueness of romaine chloroplastic RNA. Unexpectedly, the bulk of the RNA of romaine lettuce chloroplasts was not extracted by a combination of freezing and thawing and homogenization in the presence of sodium deoxycholate. We have routinely employed a solution containing 0.001 M magnesium chloride, 0.01 M sodium acetate and 0.5 % sodium deoxycholate to

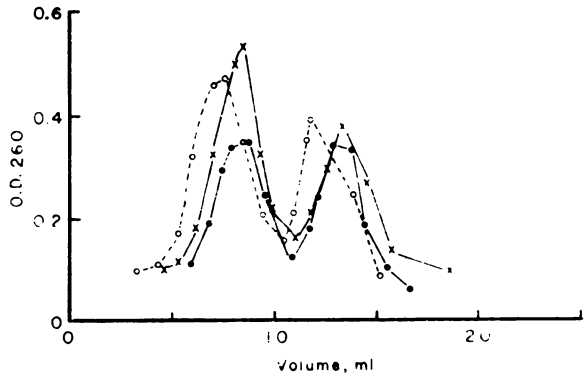

FIG. 1. Sedimentation profiles of ribosomal RNA from romaine lettuce leaf cellular fractions. 20 to 5 % Linear sucrose gradients. Centrifugation for 16 hours with Spinco 25.1 rotor. Five drop fractions. x - - - x - - - x - - - x, Cytoplasmic; o - - - o - - - o - - - o, mitochondrial; ● - - - ● - - - ●, chloroplastic.

purify cytoplasmic ribosomes and in some instances, to extract ribosomes from mitochondrial fractions. Whether the homogenization was done with a Tenbroeck all-glass hand homogenizer or with alumina and sand in a Waring blendor did not appear to make any difference since only a small amount of ribosomes was set free from romaine chloroplast fractions. The quantity of ribosomes released in this manner varied from virtually none to 8 % of the total RNA of the chloroplast fraction. The released ribosomes proved to have a sedimentation pattern resembling that of cytoplasmic ribosomes. This is illustrated in figure 2. We shall refer to the high molecular weight peak and the low molecular weight peak as the 60S and the 40S subunits, respectively, although the sedimentation constants have not been determined. No significance is at-

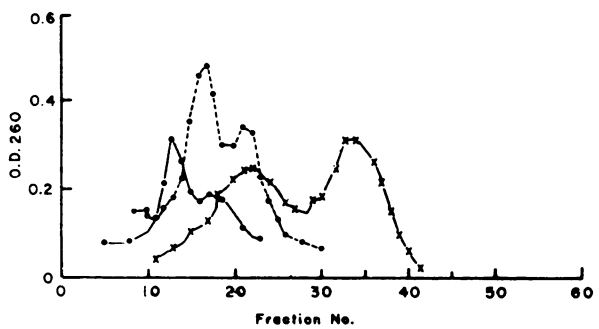

FIG. 2. Sedimentation patterns of ribosomes and ribosomal RNA from romaine lettuce. o - - - o - - - o - - - o, Cytoplasmic ribosomes; ● - - - ● - - - ● - - - ●, ribosomes extracted from chloroplasts by deoxycholate; x - - - x - - - x - - - x, ribosomal RNA extracted from chloroplasts after deoxycholate treatment. 20 to 5% sucrose gradients. Ribosomes centrifuged for 4 hours; ribosomal RNA centrifuged for 16 hours 25.1 rotor at 23,000 rpm in each instance. Eight drop fractions for ribosomes. Five drop fractions for ribosomal RNA.

tached to the comparative rates of sedimentation since the experiments were done at different times. Attention is directed to the fact that in each instance the 60S subunit predominates. Such a distribution of ribosomes would be expected to give rise to RNA whose profile would be similar to that given by cytoplasmic ribosomal RNA or the RNA from the mitochondrial fraction illustrated in figure 1. That is, 60S ribosomes give rise to 28S RNA and 40S ribosomes give rise to 18S RNA (8).

Upon extraction of the chloroplast residue remaining after deoxycholate treatment with sodium dodecyl sulfate-phenol solutions, ribosomal RNA sedimenting with the profile shown in figure 2 was obtained. Observe that this profile is characteristic of romaine lettuce chloroplastic ribosomal RNA. We interpret these data to mean that deoxycholate treatment removed contaminating ribosomes only. Assuming this interpretation is correct, then a quantitative assessment can be made of the degree of contamination of romaine chloroplasts. Since, in our experiments, deoxycholate removed from virtually none to 8 % of the total RNA, then our preparations of chloroplastic RNA are at least 92 % pure.

The inability to extract ribosomes from romaine lettuce chloroplasts and the inability to detect activity of enzymes of the respiratory chain in lettuce mitochondrial preparations are probably related and, though singular, are not atypical of the kinds of results that may be obtained with materials from higher plants. It is possible that polyphenols and latex, perennial anathemas to plant biochemists and substances that are known to be present in lettuce, combined with proteins during the isolations. In the case of mitochondria, this could have led to an inactivation of the enzymes. In the case of chloroplasts, a protective coating impermeable to deoxycholate could have been formed. Romaine lettuce chloroplast fractions contained 100 to 118 micrograms of ribosomal RNA per milligram of total chlorophyll. Substances interfering with the biuret reaction in lipid-extracted preparations made it impossible to do protein determinations. Therefore, the RNA values could not be expressed in terms of protein content.

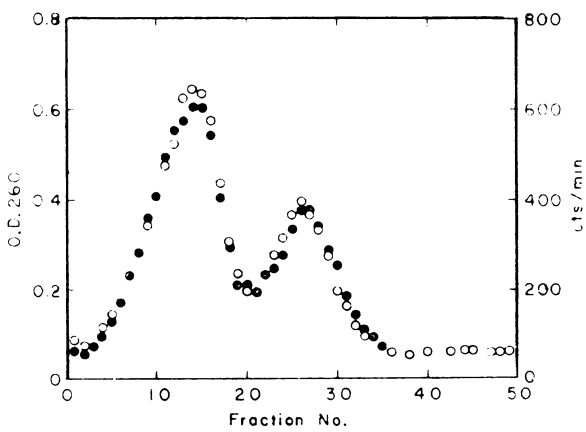

FIG. 3. Co-sedimentation of ^{32}P-labelled pea ribosomal RNA with romaine lettuce chloroplastic ribosomal RNA. 20 to 5 % linear sucrose gradient centrifuged at 23,000 rpm for 16 hours; Spinco 25.1 rotor. Five drop fractions collected. • • •, Radioactivity; o o o, OD.

Figure 3 demonstrates results obtained when approximately equal quantities of RNA from romaine lettuce chloroplast fraction and ^{32}P labelled pea ribosomal RNA were sedimented together on a sucrose gradient. Optical measurements were made on the fractions and they were then dried and counted in a liquid scintillation counter. Quantitative recovery of ultraviolet absorbing material and of radioactivity from the gradient was obtained. Since it has been shown by Ts'o and Squires (24) that pea ribosomal RNA consists of an 18S and a 28S particle, we have employed ^{32}P labelled pea ribosomal RNA as markers in determining the sedimentation values of the RNA species reported in this paper. Our concern for determining the sedimentation value was based on reports that chloroplast ribosomes have somewhat lower sedimentation values than microsomal particles (1, 3, 4, 12). Figure 3 reveals that ribosomal RNA of romaine lettuce chloroplast fractions have sedimentation values similar, if not identical, to pea ribosomal RNA. In general, sedimentation profiles of RNA of chloroplast preparations from certain plants such as spinach, tomatoes and water cress have resembled the usual pattern observed with cytoplasmic ribosomal RNA. However there was a predominance of the 18S RNA component in the majority of preparations from chloroplasts from romaine lettuce, endive and Canada thistle. This pattern was also observed by Yudkin and Davis (26) in the RNA extracted from membranes of *Bacillus megaterium* KM. They suggested that degradative enzymes convert 23S RNA to 16S RNA. A possibly more reasonable explanation would be the preferential destruction of the larger RNA component during preparation since the RNA species differ in base composition and are coded from different loci on the DNA molecule (25). Although not seen in the experiment illustrated by figure 1, RNA of 4 to 5S presumably corresponding to transfer RNA was present in a number of preparations of chloroplastic RNA. It usually comprised less than 2 % of the total RNA.

As shown in table I, the base composition of the 28S RNA component from the cytoplasm of romaine lettuce resembled the chloroplastic counterpart although a possibly significant difference was observed in the guanine content. Note, however, that the standard deviation was fairly high for guanine in the cytoplasmic species. A greater difference was manifest in the uracil content of the 18S species. It should be pointed out that these differences were of the magnitude of differences that may be found between the 18S and 28S RNA from a given organism (16).

Mitochondria. In the present studies, the prob-

Table I. *Base Composition of RNA from Romaine Lettuce Subcellular Fractions*

RNA species	Number of analyses	Moles percent* Guanine	Adenine	Cytosine	Uracil
28S Cytoplasmic	3	35.6 ± 1.1	21.8 ± 0.4	23.5 ± 2.1	18.9 ± 0.2
28S Chloroplastic	3	33.0 ± 0.4	22.3 ± 1.2	25.5 ± 0.5	19.2 ± 0.2
18S Cytoplasmic	3	30.8 ± 0.3	23.0 ± 0.8	22.2 ± 0.7	23.8 ± 0.6
18S Chloroplastic	3	31.5 ± 1.0	24.6 ± 0.6	23.3 ± 0.9	20.5 ± 0.0

* Values are means and standard deviations.

Table II. *Effect of Washing on the RNA and Protein Content of Cauliflower Mitochondria*

Times washed	Total protein, mg	Total RNA, μg	RNA/Protein*
1X	31.5	1220	31.4
4X	25.4	540	21.2
5X	22.6	500	22.1
6X	22.6	470	20.8

* Micrograms RNA per milligram protein.

Table III. *Base Composition of RNA of* Psalliota campestris *Subcellular Fractions*

RNA species	Number of analyses	Moles percent* Guanine	Adenine	Cytosine	Uracil
28S Cytoplasmic	3	30.3 ± 0.1	24.9 ± 0.0	21.4 ± 1.1	23.2 ± 0.4
28S Mitochondrial	4	32.3 ± 0.9	24.3 ± 1.3	21.3 ± 1.0	22.8 ± 0.7
18S Cytoplasmic	4	29.4 ± 0.6	24.6 ± 0.9	22.2 ± 1.1	23.8 ± 1.3
18S Mitochondrial	3	29.7 ± 0.0	25.4 ± 0.9	21.4 ± 1.0	23.5 ± 0.4

* Values are means and standard deviations.

lem of contamination by cytoplasmic ribosomes was difficult to assess. The use of glucose-6-phosphatase (5) as a marker for microsomal contamination was not possible since the activity of the enzyme was low even in the microsomal fractions from cauliflower and mushroom.

We submit 2 lines of evidence to support our contention that the RNA extracted from mitochondria was contained in mitochondria. A) It has been demonstrated in our laboratory that it is virtually impossible to isolate undegraded cytoplasmic ribosomal RNA from beef and pork liver obtained from local markets. Yet the mitochondrial pellet isolated from such tissue readily yielded a ribosomal pellet after treatment with deoxycholate. This pellet in turn yielded 28S and 18S RNA. We interpret this to indicate that compartmentation of mitochondrial ribosomal RNA serves to protect it from autolytic degradation by cytoplasmic ribonucleases. B) The purification of mitochondria was accomplished by washing and recentrifuging the particles with the homogenizing mixture. Nuclear contamination, as assessed by staining with acetocarmine, was virtually nil. The mitochondrial particles showed succinic dehydrogenase activity and were found to contain coenzyme Q, criteria which established their identity. As has been shown by Truman and Korner (23) and confirmed by the data in table II, repeated washing of mitochondria results in particles with a constant RNA: protein ratio. Note that 4 to 6 washings resulted in a loss of only 38 % protein but about 62 % of the RNA was lost, as compared with the first washing. Values of 28 to 32 μg of RNA per mg protein were obtained with other washed preparations of cauliflower mitochondria. By way of comparison, beef-liver and mushroom mitochondria gave values of 18 and 15, respectively. Truman and Korner (22) reported that rat-liver mitochondria contained 13.8 μg RNA per mg protein, a value very similar to those reported by others for rat-liver mitochondrial RNA. When co-sedimented with marker ^{32}P labelled pea ribosomal RNA, our preparations of mitochondrial RNA gave patterns similar to that shown in figure 3. This is our justification for designating RNA from mitochondrial preparations as 28S and 18S particles. The earlier report of the existence of mitochondrial ribosomal RNA of *S* value of 8.39 (23) can probably be explained as due to lack of adequate magnesium in the isolation medium. In most experiments small quantities of transfer RNA (4–5S) were also observed.

A small and perhaps marginally significant difference was found in the guanine content of the 28S RNA components from the mushroom (table III). Since the higher value for guanine in the

Table IV. *Base Composition of RNA from Cauliflower Subcellular Fractions*

RNA species	Number of analyses	Moles percent* Guanine	Adenine	Cytosine	Uracil
28S Cytoplasmic	4	33.1 ± 0.4	24.1 ± 1.5	23.1 ± 1.4	19.6 ± 0.8
28S Mitochondrial	4	33.3 ± 0.9	23.4 ± 0.8	24.4 ± 1.4	18.6 ± 0.7
18S Cytoplasmic	3	32.6 ± 0.5	25.2 ± 0.7	20.5 ± 0.3	21.3 ± 1.4
18S Mitochondrial	3	29.9 ± 0.0	22.9 ± 0.4	23.8 ± 0.5	23.4 ± 0.6

* Values are means and standard deviations.

mitochondrial component was not reflected in a difference in any other base and since the standard deviations were rather high, this is a dubious distinction. No differences were seen in the 18S components. For some unexplained reason, the base composition of the cytoplasmic ribosomal ribonucleic acids from mushrooms differ significantly from values reported earlier (16). This discrepancy is probably not attributable to a lack of precision in the estimation of the base composition of RNA since the values for cauliflower cytoplasmic RNA agree with values reported earlier. This discrepancy does not affect the comparison made in table III, since comparisons were made between cytoplasmic RNA and RNA from mitochondrial fractions from the same source. It is possible that fungi resemble bacteria in that mutation can result in detectable changes in the base composition of the DNA (7) which in turn would be reflected in the base composition of the ribosomal RNA.

The 28S components from cauliflower exhibited no differences in base composition (table IV). The base compositions of the 18S components are unequivocally different, this difference being reflected in all of the bases.

Discussion

Recently, ribosome-like particles were demonstrated in chloroplasts and mitochondria of swiss chard (*Beta vulgaris* var. *cicla*) by electron microscopy (10, 20). In many mitochondria the particles were sparse, poorly defined or absent. In short, the accumulated evidence supports the existence of ribosomes in these particles. That chloroplast ribosomes are capable of protein synthesis was demonstrated recently by Spencer (19).

We have indicated in certain instances that the base composition of the ribosomal RNA from the subcellular particles was significantly different from cytoplasmic ribosomal RNA thereby inferring that they were unique. Two bases underlie these claims: an appreciation for the degree of precision of the method of estimation of bases as performed under our conditions and congnizance of the magnitude of differences that may be found between the 2 cytoplasmic ribosomal RNA species of an organism. These are known to be distinctly different from each other. Buttressing the differences found in base composition were certain trends seen in this study that were also seen in a previous study on plant ribosomal RNA (16). Usually the guanine content, predominant in both components, was higher in the 28S species than in the 18S species. Also there was often an increased amount of uracil in the 18S component.

The experiments in the present paper were performed with the belief that findings of differences in base composition between cytoplasmic RNA and RNA of the organelles would further affirm the autonomous nature of the organelles. The differences found probably reflect differences in the DNA cistrons from whence they were coded (25).

After the completion of this manuscript, E. Baltus and J. Quertier (Biochim. Biophys. Acta 119, 192 (1966) reported several findings on *Acetabularia* chloroplastic RNA similar to the results reported here. Chloroplast ribosomal RNA had sedimentation constants of 18S and 28S. There was also a preponderance of the 18S RNA subunit in chloroplasts whereas the subunits occurred in equal amounts in non-chloroplastic RNA.

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