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*CHARACTERIZATION OF CYTOPLASMIC AND CHLOROPLAST POLYSOMES IN PLANTS: EVIDENCE FOR THREE CLASSES OF RIBOSOMAL RNA IN NATURE**

BY ERHARD STUTZ† AND HANS NOLL

DEPARTMENT OF BIOLOGICAL SCIENCES, NORTHWESTERN UNIVERSITY, EVANSTON, ILLINOIS

Recent work, reviewed by Gibor and Granick,[1] established that chloroplasts are endowed with their own DNA complement and thus suggested that these organelles with highly specialized functions and an intriguing life cycle are semi-autonomous systems, capable of self-replication, and useful as models for the study of differentiation. In addition to their own DNA, chloroplasts were also reported to contain a unique class of 70*S* ribosomes that are distinct from the 80*S* particles of the surrounding cytoplasm.[2–4] This rather unusual situation invites closer examination, since it suggests a separate origin for these organelles and hence might throw light on the evolution of the photosynthetic apparatus. The coexistence of two ribosome classes within the same cell suggests the presence of two protein-synthesizing systems, each capable of forming polysomes and subject to different mechanisms of control. In addition, it poses the question of how the two classes of ribosomes are related, both structurally and functionally, to the ribosomes of the main phyla of the living order.

Available evidence indicates that ribosomes from all sources studied so far fall into one of two classes: 70*S* ribosomes occurring in bacteria and 80*S* ribosomes characteristic of nucleated cells of animals and plants.[5] Although the evidence is somewhat less clear-cut, it is generally believed that the two kinds of ribosomes have their correspondence in two types of ribosomal RNA. Thus, rRNA from 70*S* ribosomes of bacteria has been found to consist of a 23*S* and 16*S* component, whereas the corresponding rRNA components of the 80*S* particles of nucleated cells have been characterized by *S*-values ranging from 16 to 18*S* for the small, and 25 to 30*S* for the larger subunit.[6]

In this report we shall show by the use of high-resolution zone-velocity sedimentation analysis that plant cells contain cytoplasmic polysomes made up of 80*S* monomers and chloroplast polysomes consisting of 70*S* ribosomes. In addition, evidence will be presented that, contrary to general belief, there are not two, but at least *three* classes of ribosomes: (1) 80*S* ribosomes of animal origin characterized by 29/18*S* rRNA; (2) 80*S* ribosomes of plant cells containing 25/16*S* rRNA; and (3) 70*S* bacterial ribosomes made up of 23/16*S* rRNA. Finally, we have found rRNA from 70*S* chloroplast ribosomes to be indistinguishable from bacterial rRNA when compared under rigorously standardized conditions.

Methods.—Isolation of chloroplast polysomes: The method described is a modification of several published procedures.[2–4] Primary leaves of 5- to 6-day-old pinto beans (300–500 gm fresh weight) were washed in cold distilled water and homogenized in a Waring Blendor for 30 sec with 3 ml of cold buffer I (0.7 *M* sucrose, 0.1 *M* tris-HCl, pH 7.5, 0.005 *M* $MgCl_2$, 0.05 *M* KCl, 0.005 *M* 2-mercaptoethanol) per gram leaf weight. All operations were performed at +4°C. The homogenate was filtered through several layers of gauze, and the filtrate centrifuged for 2 min at 600 × *g* to remove nuclei and cell debris. Centrifugation at 1100 × *g* for 12 min yielded pellets of crude chloroplasts and a supernatant containing the cytoplasmic ribosomes. The crude chloro-

plasts were washed by resuspension in buffer I and the previous centrifugation was repeated. To lyse the chloroplasts and solubilize the polysomes, the pellet (1x chloroplasts) was taken up in a small volume ($^1/_{10}$ of original leaf weight) of hypotonic buffer II (0.01 *M* tris-HCl, pH 7.5, 0.005 *M* $MgCl_2$, 0.05 *M* KCl, 0.005 *M* 2-mercaptoethanol), and a 20% solution of Triton X-100 was added to a final concentration of 5%. The dark green lysate was centrifuged at 26,000 × *g* for 30 min to remove starch granules and insoluble green material. The green supernatant was layered over 2 ml of 1 *M* sucrose in buffer II and centrifuged in a Spinco 40 rotor at 40k rpm for $2^1/_2$ hr at 2°C. The yellowish ribosome pellets and the walls of the tubes were repeatedly and carefully rinsed with cold distilled water. The pellets were then resuspended by gentle stirring with a glass rod in buffer III (0.001 *M* tris-HCl, pH 7.5, 0.004 *M* $MgCl_2$). After a clarifying spin, the strongly light-scattering ribosome suspension was decanted and stored at −60°C. In a representative run the procedure yielded 1.2 mg chloroplast RNA per 100 gm fresh weight of leaves.

Isolation of cytoplasmic polysomes: The supernatant remaining after spinning down the crude chloroplasts was centrifuged at 26,000 × *g* for 30 min to sediment the mitochondria. The post-mitochondrial supernatant was made 0.5% with respect to Triton X-100 by adding a 20% solution of the detergent and centrifuged as before. The supernatant, containing ribosomes and polysomes that had been released from membranes, was layered over 1 *M* sucrose and the ribosome fraction isolated by high-speed centrifugation as described for chloroplast ribosomes. The yield was about 19 mg RNA per 100 gm fresh weight of leaves.

Zone velocity centrifugation and calibration of sucrose gradients: Convex exponential gradients (0.3–1.0 *M*) were prepared with a burette and mixing chamber as described by Wettstein and Noll.[7] By adding 12 ml of 1.4 *M* sucrose to the mixing chamber containing 10 ml of 0.3 *M* sucrose, an equivalent volume was displaced into the centrifuge tubes. Centrifugations were carried out with the IEC 6 × 12 ml swinging-bucket rotor no. 269 in a B-35 ultracentrifuge. The gradient tubes were punctured and the absorbancy of the effluent at 260 mμ was scanned and recorded automatically with the IEC-Gilford high-resolution-gradient analyzing system. The design and components of this system will be described in detail.[8] Sedimentation constants were determined by calibrating the gradient with a standard of the same density as the unknown sample. This is done by measuring the distance traveled by the standard (29*S* rRNA or 80*S* ribosomes of rat liver) as a function of centrifugation time, using sufficient different time intervals to cover the entire length of the tube. The calibration curve, obtained by plotting the distance (*d*) traveled (or the effluent volume corresponding to the band center) against the product (*st*) obtained by multiplying the known sedimentation constant (*s*) with the experimentally chosen centrifugation time (*t*), is characteristic for a given temperature, gradient, rotor performance, and for molecules of the same density. The sedimentation constant of the unknown is obtained from the graph by finding the *st*-value corresponding to its path length *d*. Since *d* is not a linear function of *st*, the linear interpolation method of Martin and Ames[9] is not applicable under our experimental conditions A detailed discussion of this method and its theoretical basis will be published.[8]

Following convention we have used *S*-values in a generic sense to identify classes of ribosomes and rRNA molecules rather than as an absolute measure of sedimentation rate.

Results.—The strip chart recordings of the sedimentation patterns of cytoplasmic (*A*) and crude chloroplast (*B*) polysome preparations are compared in Figure 1. In the pattern of the cytoplasmic preparation (*A*), the strongest peak (*a*), the 80*S* monomer, is followed by corresponding polysome peaks ranging from dimer to nonamer (*b*, *c*, . . . *i*). By contrast, in the chloroplast preparation (*B*), the heavy polysome region is less clearly resolved and a number of strong peaks not present in (*A*) appear in addition to those (principally the 80*S* monomer and 120*S* dimer) recognizable in the cytoplasmic extract. Particularly prominent are peaks corresponding to sedimentation values of 30*S* (μ), 50*S* (π), 70*S* (α), and 100*S* (β), all of which are detectable only as trace components in the cytoplasmic extract. Treatment of the crude chloroplast fraction with RNase produced a dramatic and preferential increase of the 70*S* peak accompanied by the disappearance of the larger polysomes (Fig. 1*C*). This suggests that the crude chloroplast fraction is a mixture

of two polysome populations differing in the size of the monomer unit: (*a*) chloroplast polysomes made up of a 70*S* ribosome species and (*b*) a contaminating popula-

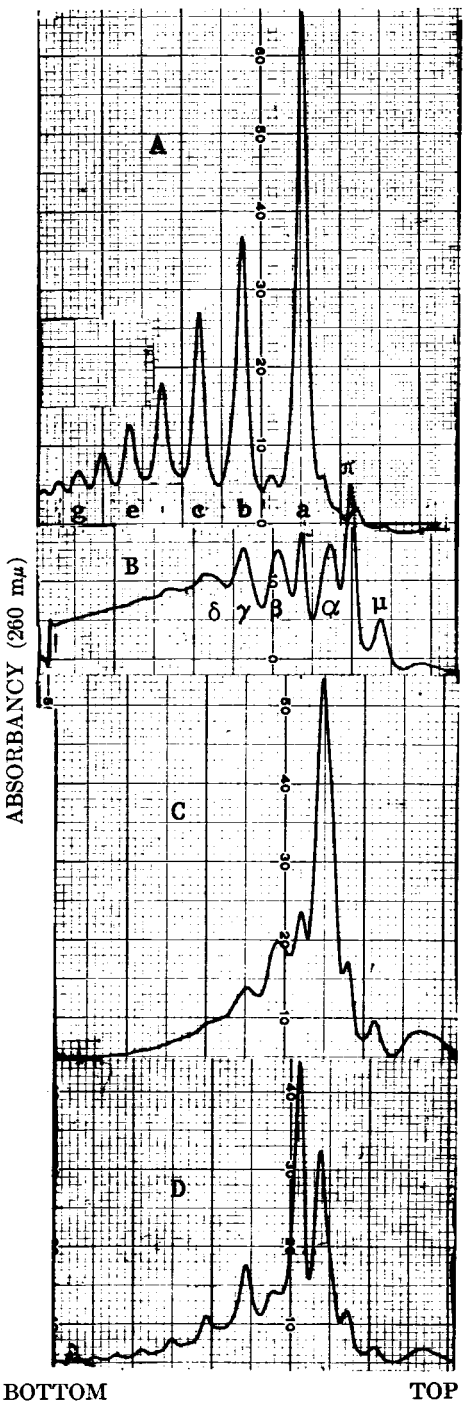

FIG. 1.—Sedimentation patterns of polysomes from bean cytoplasm and chloroplasts. Polysomes from (*A*) cytoplasm, (*B*) crude chloroplast fraction, (*C*) crude chloroplast fraction treated with 0.1 μg/ml crystalline bovine ribonuclease for 5 min at 0°C, (*D*) mixture of equal parts (total input 0.18 mg RNA) of preparations (*A*) and (*C*). The ordinate has been calibrated to correspond at full scale (= 100 partitions on chart paper) to 5.0 (*A*, *B*) or 10.0 (*C*, *D*) A_{260} units. Peaks corresponding to the 80*S* polysome series are lettered *a*, *b*, *c*, ...; 70*S* polysome peaks α, β, γ, ... Input: 3.6 A_{260} units equivalent to 0.18 mg RNA in 0.2 ml of 0.001 *M* tris-HCl, pH 7.5, 0.004 *M* $MgCl_2$ layered over a 12-ml convex sucrose gradient in the same buffer. Centrifugation for 2.5 hr at 35k rpm at 2°C.

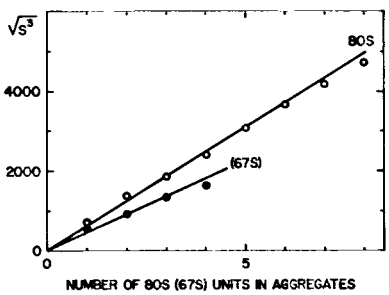

FIG. 2.—Relationship between sedimentation rate and particle mass of polysomes from cytoplasm (80*S* monomers) and from chloroplasts (67*S* monomers). The value of 67*S* for chloroplast monomers was obtained by comparison with 80*S* monomers from the cytoplasm as calibrating standard.

tion of cytoplasmic polysomes composed of 80*S* ribosomes. This interpretation is further supported by the following evidence: (1) Addition of cytoplasmic polysomes to the chloroplast preparation (*A* + *C*) enhances the 80*S* and derivative polysome peaks in the pattern of the mixture (*D*), thus confirming sedimentation rate and cytoplasmic origin of the 80*S* contaminant in the chloroplast preparation. At the same time, the presence of two clearly resolved peaks corresponding to 70*S* and 80*S* strikingly illustrates the distinct nature of the two ribosome species. (2) Confirming earlier reports by other investigators,[2-4] we found that further purification of the chloroplast fraction resulted in a decrease of the 80*S* contaminant relative to the 70*S* component. (3) The spacing of the polysome peaks (*b*, *c*, . . .) derived from the 80*S* monomer is wider than the spacing of the peaks (β, γ, δ, . . .) belonging to polysomes of the 70*S* series. Consequently, a mixture of the two populations should show both series of peaks, except for the regions in which the phase difference results in an overlapping of positions and hence enhancement of peak intensities. This situation is illustrated in the chloroplast polysome pattern (Fig. 1*B*). Here contamination of chloroplast with cytoplasmic polysomes produces a mixed pattern in which peaks belonging to either of the two different series alternate or overlap with each other according to the sequence 70*S*, 80*S*, β, $\frac{\gamma}{b}$, δ, *c* . . .. This identification of the peak sequence in Figure 1*B* is again confirmed in pattern (*D*) where addition to the chloroplast preparation of relatively pure cytoplasmic polysomes increases only the cognate 80*S* series of peaks (Fig. 1*D*). (4) Another distinctive feature of the chloroplast ribosome population is the presence of a measurable fraction in 50*S* and 30*S* subunits or precursor particles (peaks μ and π in Fig. 1*B*).

It has been shown previously[11, 12] that the *S*-value of a polysome containing *n* ribosomes is closely approximated by the expression $S^{3/2} = kn$ in which *k* is a constant related to the mass of the monomer unit. A plot of $S^{3/2}$ against *n* gives a straight line with a different slope for each series (Fig. 2). The ratio of the two slopes corresponds to a mass ratio of 1.4 for the two monomer units. Because of contamination with cytoplasmic polysomes, chloroplast polysomes have, so far, only been resolved up to the tetramer peak. The existence of larger chloroplast polysomes is indicated by the presence of heavier material which disappears after treatment with traces of RNase with concomitant increase of the 70*S* monomer (Fig. 1*B* and *C*).

The assignment of approximate *S* values of 80*S* for the cytoplasmic and 70*S* for the chloroplast ribosomes is based on a comparison with 80*S* rat liver ribosomes which, in turn, had been calibrated against 70*S* bacterial ribosomes. Thus, only one 80*S* peak was observed when rat liver and cytoplasmic bean ribosomes were mixed (Fig. 3*A*), and one 70*S* peak when chloroplast and *E. coli* monomers were mixed (Fig. 3*B*), whereas a mixture of cytoplasmic bean polysomes and *E. coli* monomers was clearly resolved into an 80*S* and 70*S* component (Fig. 3*C*).

A second series of experiments was carried out to determine whether 70*S* chloroplast ribosomes resembled those of bacteria also with respect to ribosomal RNA. Sedimentation patterns (Fig. 4) obtained after treating ribosomes with sodium dodecyl sulfate (SDS) to release RNA show that 80*S* ribosomes from bean cytoplasm are made up of a 25*S* and 16*S*r RNA component (*A*), whereas the cor-

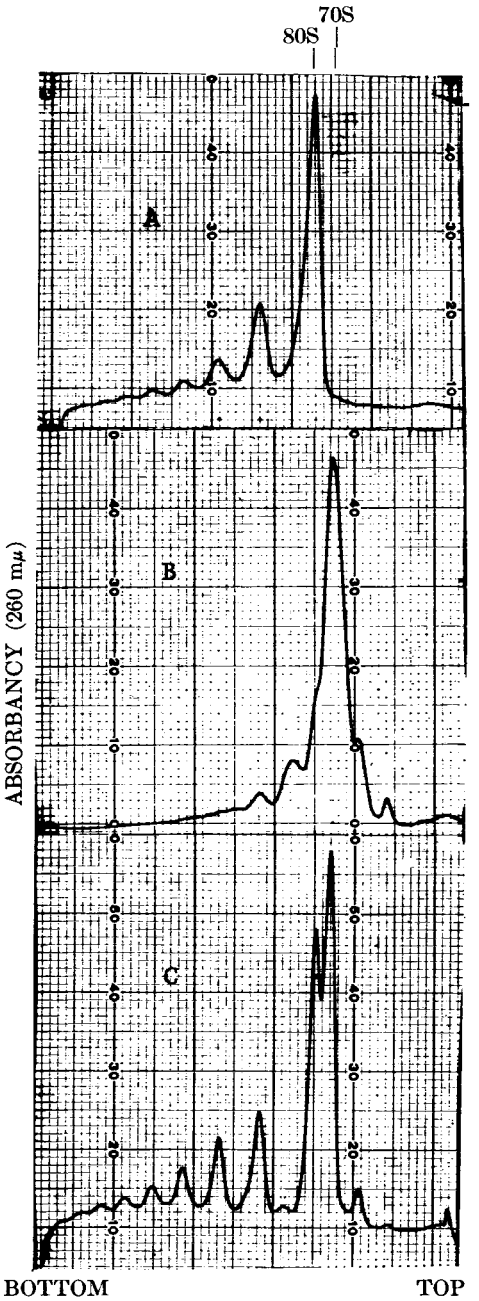

FIG. 3.—Calibration of sedimentation rate of ribosome monomers and polysomes from different origin. Equal parts (equivalent to 0.09 mg RNA) of the indicated polysome preparations were mixed in the following combinations: (*A*) rat liver + bean cytoplasm, (*B*) *E. coli* + bean chloroplast, (*C*) bean cytoplasm + *E. coli*. Ordinate at fulls cale = 10.0 (*A*) or 5.0 (*B*, *C*) A_{260} units. Experimental conditions as in Fig. 1.

responding values for rRNA of 70*S* chloroplast ribosomes are 23*S* and 16*S* (*B*). Again, calibration errors are ruled out by the demonstration that the sedimentation pattern (*C*) of a mixture of the two samples shows three peaks (25*S*, 23*S*, and 16*S*), the positions of which coincide exactly with those of its components. The calibration of 23/16*S* chloroplast rRNA was independently confirmed by comparison with *E. coli* rRNA. Thus, the sedimentation pattern of a mixture of the two rRNA samples shows only two peaks (*D*) which coincide with the original 23/16*S* positions of chloroplast rRNA (*B*) before the addition of *E. coli* rRNA. As a further control, 25/16*S* rRNA from cytoplasmic ribosomes of beans was mixed with 23/16*S* rRNA of *E. coli* (Fig. 5). The resulting sedimentation pattern (*A*) exhibits the expected similarity with the pattern (*C*) of Figure 4, showing the three peaks (25*S*, 23*S*, 16*S*) corresponding to each of its components.

So far we have established that rRNA from 70*S* chloroplast ribosomes is clearly different from rRNA of cytoplasmic 80*S* ribosomes, but indistinguishable from that of bacteria under the conditions of sedimentation analysis employed. The remaining question, whether or not cytoplasmic rRNA of plants is identical with that of mammalian cells, was settled by the experiments described in Figure 5. As evident from the sedimentation pattern (*B*), a mixture of rat liver and cytoplasmic bean rRNA is resolved into three peaks (29*S*, 25*S*, 16*S*) when sedimented under exactly the same conditions that were used to separate bean cytoplasmic rRNA from *E. coli* rRNA (*A*). In the presence of 0.001 *M* Mg^{++} all four components of the mixture could be separated into well-defined peaks corresponding to the 29/18*S* rat liver and 25/16*S* bean cytoplasmic rRNA pairs.[10]

Discussion.—The results reported in this paper confirm and extend earlier work

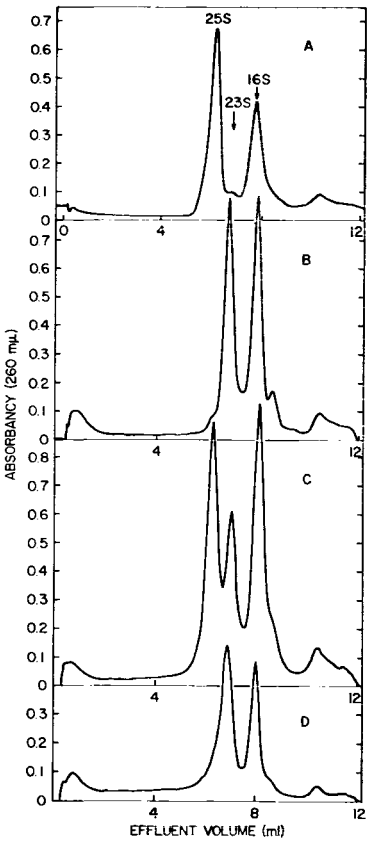

FIG. 4.—Comparison of sedimentation rates of rRNA from chloroplast ribosomes with rRNA from bean cytoplasm and bacterial ribosomes. Polysomes equivalent to 0.9 mg RNA are dissolved in 1 ml of 0.005 *M* tris-HCl, pH 7.5, 0.01 *M* NaCl, and a 10% solution of SDS is added to a final concentration of 2%. The mixture is incubated for 10 sec at 37°C. After chilling, 0.2 ml of the mixture is immediately layered on the convex sucrose gradient made up in the same buffer. Centrifugation was for 12 hr at 35k rpm at 4°C. (*A*) Cytoplasm, (*B*) 1x chloroplasts, (*C*) mixture of 0.1 ml (*A*) + 0.1 ml (*B*). (*D*) Mixture of 0.1 ml 1x chloroplast + 0.1 ml *E. coli* rRNA. In (*B*) the ratio of the integrated peaks 23/16*S* is about 1:1 instead of 2:1. The factors responsible for the incomplete recovery of the 23*S* component will be discussed in a forthcoming paper.[10]

by Lyttleton and by Clark *et al.* Lyttleton first demonstrated that chloroplasts contained a smaller 66*S* species of ribosomes that is clearly distinct from the ribosomes of the cytoplasm.[2] Clark *et al.* first reported the presence of polysomes of 83*S* monomers in the cytoplasm of higher plants.[3] Clark further concluded that polysomes also occurred in chloroplasts, since upon treatment of his fractions with RNase he observed an increase of 68*S* monomers which subsequently[13] was found to be related to the disappearance of a more rapidly sedimenting, broad, unresolved band in sucrose gradients. The direct demonstration of chloroplast polysomes given here was made possible by the use of high-resolution zone centrifugation techniques which permitted the detection of chloroplast polysome peaks in the presence of contaminating cytoplasmic polysomes. The yields of purified chloroplast polysomes, however, are still small because of the increased exposure to nucleases during the additional procedures required to eliminate cytoplasmic contamination. RNase action presumably also accounts for the failure of Boardman *et al.* to detect polysomes in chloroplast extracts capable of amino acid incorporation.[4]

Of greatest interest is our finding that chloroplast ribosomes resemble bacterial ribosomes not only in their over-all size as reflected in the closely similar sedimentation rate, but also in the nearly identical sedimentation behavior of the ribosomal RNA. These observations complement and reinforce the results of Boardman *et al.*

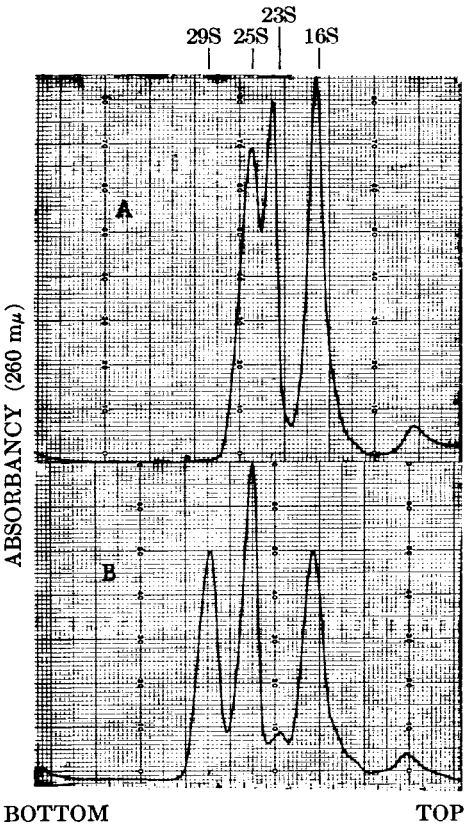

FIG. 5.—Heterogeneity of rRNA of different origin. Equal parts of rRNA from different sources were mixed as follows: *E. coli* + bean cytoplasm (*A*), rat liver + bean cytoplasm (*B*). Experimental conditions for (*A*) and (*B*) are as described under Fig. 4. Ordinate at full scale = 5.0 A_{260} units.

who recently found that chloroplast and bacterial ribosomes require the same Mg^{++} concentrations for optimal amino acid incorporation and for reversible dissociation into subunits.[4] This similarity appears to extend even further and includes the subtle properties responsible for sensitivity to those antibiotics that are known to specifically inhibit bacterial protein synthesis by interaction with the ribosomal surface, e.g., chloramphenicol and members of the tetracycline and streptomycin families. Thus, inhibition of chloroplast but not of cytoplasmic protein synthesis would explain the hitherto enigmatic bleaching effect of streptomycin.[14, 15] Conversely, agents known to interfere with translation on 80*S* animal and plant but not on 70*S* bacterial ribosomes (e.g., cycloheximide) should permit selective inhibition of protein synthesis in the cytoplasm without directly affecting that in chloroplasts. Hence, it should be possible by the proper choice of inhibitors to sort out the proteins with respect to their origin in cytoplasm or chloroplasts. Consistent with this hypothesis are observations indicating that chloramphenicol selectively inhibits the light-induced formation of certain photosynthetic enzymes[16] and the light-dependent incorporation of amino acids into the chloroplast fraction.[17] Analogous findings of a preferential inhibition of mitochondrial protein synthesis by chloramphenicol[18] suggest that mitochondria also possess their own ribosomes with properties closely related to those of chloroplasts and bacteria.

Finally, our finding that cytoplasmic ribosomes from plants form a separate class contributes new information to the problem of ribosome heterogeneity and evolution. The significance of previous reports[19, 20] indicating somewhat lower *S* values for plant than for animal rRNA was difficult to evaluate because the determinations were either not carried out under strictly comparable conditions or involved samples from only a few species. In our experiments the claim of nonidentity is based on the demonstration that a mixture of two rRNA samples from different origin can be resolved under proper conditions. It should be pointed out that while positive results in this test are conclusive evidence for nonidentity, negative results do not prove identity. Our conclusion that cytoplasmic ribosomes of plants belong to a common class is based upon the identical sedimentation behavior of ribosomal RNA from organisms covering a wide range of the evolutionary spectrum (yeast,

Chlamydomonas, maize, and bean).[10] Evidence for three major classes of ribosomes has also been obtained from base composition studies.[20] Because of the paucity of data on rRNA from invertebrate and especially unicellular animals, we do not as yet know whether all ribosomes from the animal kingdom fall into a single class. The only exception so far are the results obtained by Brawerman and Eisenstadt who reported that *Euglena* contained only 19*S* rRNA in the cytoplasm and 19/14*S* rRNA in chloroplasts.[21] However, since their sedimentation patterns show evidence of degradation, the significance of these findings remains in doubt. Most intriguing is the finding that with the emergence of the bigger and presumably more complex 80*S* ribosome in nucleated cells, the more primitive bacterial or 70*S* type is preserved in organelles entrusted with photosynthesis and respiration. Evidently, already at this level of early morphogenesis, the specialization characteristic of differentiation is achieved by the preservation and integration of more primitive functions.

This paper is dedicated to the 80th birthday of ARTHUR STOLL who in his pioneering studies on the structure of chlorophyll and CO_2-assimilation half a century ago initiated the chemical approach to an understanding of chloroplast function.

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