the slide and the presence of only globulin on the other. After radioautography there was no line of exposure corresponding to the position of the normal rabbit γ-globulin in the side where L-chain had been applied, whereas in the other part of the slide a line could be seen which was due to the antibody (3 μg/ml) which had been added to the normal rabbit γ-globulin on that part of the slide. This means that there was less than 3 μg of intact antibody per milliliter in the preparation containing 1.2 mg of L-chain per milliliter.

Although there was a line on the radioautograph corresponding to the L-chain region, this may not be due to specific binding of the $^{125}$I-labeled insulin-4-azonaphthalene-1-sulfonate antigen, but may be due to nonspecific binding of this antigen by L-chain in general.

The L-chain preparation was also free of serum albumin because the addition of small amounts of 9-chloro-2-methoxy-acridinylbenzenesulfonate, a fluorescent probe analytical reagent for albumin (*17*), showed no fluorescence enhancement.

The equilibrium constant ($K_0$) for the binding of L-chain to ligand or ligand to L-chain was calculated to be about $10^{-2}$ liter/mole. This was done by using the data of Fig. 1 and the value for the fluorescence enhancement of the ligand by binding with L-chains. The latter value was obtained by measuring the fluorescence enhancement of the ligand at a low concentration upon the addition of increasing amounts of L-chain (Fig. 4). From this fluorescence-enhancement value, the free and bound concentrations of ligand on the point in Fig. 1 were calculated, and, by use of the Sips (*18*) equation, an average value for $K_0$ was found.

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19. Supported by NIH grant AI3962. We thank Prof. G. Weber, University of Illinois, for providing a sample of acridinyl compound; and also Dr. A. L. Grossberg, Roswell Park Memorial Institute, for the preparation of antiserum.

17 April 1967; revised 24 May 1967

## Cytoplasmic and Chloroplast Ribosomes of Chlamydomonas: Ultracentrifugal Characterization

Abstract. *Ribosomes isolated from the cytoplasmic and chloroplast fractions of* Chlamydomonas *were characterized in the ultracentrifuge. The cytoplasmic ribosomes belong to the 80S class of ribosomes, and, like animal ribosomes, dissociate to 60, 50, and 40S subunits. However, like the ribosomes of microorganisms, they contain smaller RNA's, 24 and 16S, and require 0.01 mole of magnesium ions per liter for stability. Chloroplast ribosomes are 70S like those of higher plants but are very unstable. A stable 50S subunit has been observed.*

The comparative biochemistry of ribosomes is relevant both in the study of evolution and in the analysis of ribosomal function. By 1963, bacterial ribosomes were generally accepted (*1, 2*) to have sedimentation coefficients of 70*S* with RNA components of 23 and 16*S*, while the ribosomes of higher plants and animals are 80*S* with correspondingly larger RNA components (for example, 28 and 18*S* in mammals). Since that time, several investigators have reported further distinctions among ribosomes from different classes of organisms.

Taylor and Storck (*3*) showed that the sedimentation coefficients of ribosomes of yeast and fungi are about 80*S*, and they proposed that 70*S* ribosomes are characteristic of procaryotic organisms and 80*S* ribosomes of eucaryotes, from yeast to man. More recently, distinctions among ribosomes have been proposed on the basis of the size of the RNA (*4, 5*) and on ribosomal sensitivity to low concentrations of $Mg^{2+}$ (*6*). Furthermore, higher plants have been shown to contain two classes of ribosomes: 80*S* in the cytoplasm and 70*S* in the chloroplasts (*5, 7, 8*).

Electron microscopic studies (*9*) of *Chlamydomonas* show that the chloroplasts of normal green cells are packed with ribosome-like particles lying free between the lamellae. If these particles are ribosomes, their number may be estimated as at least 25 percent that of the cytoplasmic ones, since the chloroplast represents 50 percent of the cell volume. Thus they should be seen readily in the ultracentrifugal analysis of crude lysates.

We now report ultracentrifugal studies of ribosomes extracted from the cytoplasm and chloroplast of the green alga *Chlamydomonas*, a phytoflagellate considered to be on the main evolutionary line of both the higher plants and the animals (*10*). We have found that the cytoplasmic ribosomes of *Chlamydomonas*, like those of animals and higher plants, are approximately 80*S*, but that, like bacterial ribosomes, they require high concentrations of $Mg^{2+}$ (0.01*M*) for monosome stability. The larger RNA component has a sedimentaion coefficient of 24*S*, closer to that of higher plants (*5*), fungi (*4*), and bacteria (*2*) than to the 28*S* typical of animal ribsomes (*2*). *Chlamydomonas* ribosomes contain about 40 percent RNA (*11*); in this respect they are more like plant and animal ribosomes than they are like those of bacteria.

The chloroplast ribosomes apparently are 70*S*, but they are extremely unstable. They have only been observed as such in homogenates of whole cells. After fractionation only a stable 50*S* subunit has been observed.

Sonic oscillation was used to prepare homogenates of *Chlamydomonas* for immediate examination in the ultra-

centrifuge. This method breaks the chloroplasts and frees their ribosomes into the supernatant, which also contains the cytoplasmic ribosomes. Preparations so obtained are highly active in polypeptide synthesis in vitro (*12*, *13*). The upper schlieren pattern of Fig. 1 shows the ribosomal components found in such a preparation. When analyzed at a low concentration with ultraviolet absorption optics, the sedimentation coefficients of the two peaks were 79*S* and 69*S*. Further studies designed to characterize these components were carried out with fractionated preparations.

Cytoplasmic and chloroplast ribosomes were prepared in a single fractionation procedure. Cultures of *Chlamydomonas reinhardi* strain 21*gr* were grown, harvested, and broken in a French pressure cell in the cold under conditions that maintain chloroplast integrity, as previously described (*14*). Preparations of broken cells were fractionated in sucrose density gradients that yielded a major band containing over 50 percent of the chloroplasts in a relatively intact (unswollen) condition. This band was the source of chloroplast ribosomes. Most of the discarded chloroplasts were also unbroken, but they were aggregated and contaminated with other cell fractions.

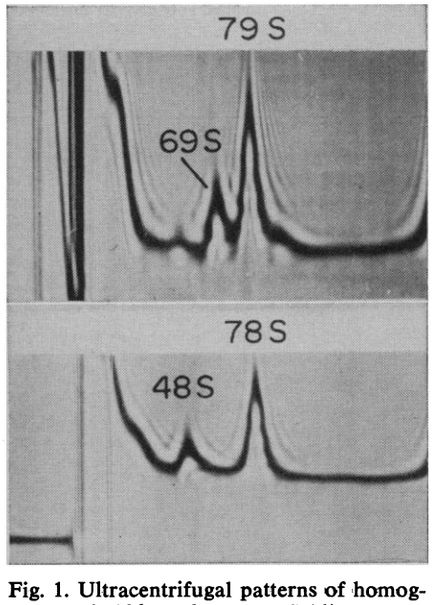


Fig. 1. Ultracentrifugal patterns of homogenates of *Chlamydomonas*. Schlieren patterns obtained after 20 minutes at 40,000 rev/min at 6°C. Upper pattern: Lysate obtained by sonic oscillation. Lower pattern: The same solution after the addition of sodium deoxycholate to a final concentration of 0.3 percent. The sedimentation coefficients given were calculated from separate analyses at lower concentrations with ultraviolet absorption optics. The solvent was 0.01*M* tris (*p*H 7.8), 0.01*M* Mg acetate, 0.06*M* KCl, and 0.006*M* mercaptoethanol. Sedimentation is to the right.

Cytoplasmic ribosomes were collected from the top of the gradient, diluted 1:1 with double-strength standard buffer [SB: 0.01*M* tris(hydroxymethyl)-aminomethane (tris)-HCl, *p*H 7.8, at room temperature; 0.01*M* magnesium acetate; 0.06*M* KCl; 0.006*M* mercaptoethanol] and sedimented for 90 minutes at 50,000 rev/min. The pellets were resuspended either in SB or in RSB (standard buffer with 0.001*M* $Mg^{2+}$) to dissociate and purify the subunits (*13*). After 30 minutes, the $Mg^{2+}$ concentration was readjusted to 0.01*M* to reconstitute the monosomes. Subsequently, the suspensions were centrifuged at low speed to remove aggregates, frozen in SB containing 0.25*M* sucrose, and stored at −70°C. [Both procedures gave preparations with good polypeptide synthesizing activity in the in vitro system (*12*, *14*)].

Chloroplast ribosomes were prepared by diluting one part of isolated chloroplast suspension with one part of double-strength SB and centrifuging the suspension for 15 minutes at 45,000 rev/min. The pellet obtained was resuspended in SB containing 0.1 percent sodium deoxycholate, treated with sonic oscillation for 1 minute to fragment chloroplasts, and centrifuged for 10 minutes at 30,000 rev/min to remove debris. The supernatant fluid was then centrifuged for 90 minutes at 50,000 rev/min, and the pellets thus formed were treated in the same manner as the cytoplasmic ones.

The RNA was extracted from ribosomes by the phenol-bentonite procedure of Petermann and Pavlovec (*15*), except that only one phenol extraction was carried out, and additional bentonite was added to the alcohol precipitate. The precipitate was dialyzed into solution against a several hundredfold excess of 0.01*M* sodium phosphate buffer (*p*H 6.8) and 0.01*M* sodium citrate.

A Spinco model E ultracentrifuge equipped with schlieren and ultraviolet absorption optics was used for the ultracentrifugal analyses, all of which were carried out at temperatures between 5° and 10°C.

Ultracentrifugal analysis of purified cytoplasmic ribosomes at a concentration of 6 mg/ml in SB showed a major component of 74*S* and minor components of 66 and 47*S*. Analyses at 3 mg/ml and at 0.05 mg/ml, the latter by ultraviolet absorption optics (Fig. 2A), gave values of 76 and 83*S* for the sedimentation coefficient of the main component. From a plot of $1/s$ against concentration, the value at infinite dilution, $s^{\circ}_{20,\,w}$, was 83*S*.

Figure 2A shows the dissociation of cytoplasmic ribosomes with a decrease of the $Mg^{2+}$ concentration. Samples of ribosomes in SB were diluted a hundredfold into the same buffer containing 0.01*M*, 0.001*M*, or no added $MgCl_2$ and analyzed immediately. Most of the 83*S* component present in 0.01*M* $Mg^{2+}$ dissociated into 61, 53, and 41*S* subunits in 0.001*M* $Mg^{2+}$ (Fig. 2A). At very low concentrations of $Mg^{2+}$ no intact particles remained; only the 60, 50, and 40*S* components were present. Analysis of another sample (not shown) that had been dialyzed overnight against the buffer containing no $MgCl_2$ showed only two boundaries of 45 and 25*S*.

The pattern of dissociation resembles that reported for liver ribosomes (*16*), except that the $Mg^{2+}$ requirement of *Chlamydomonas* ribosomes is closer to that reported for bacterial ribosomes than to that reported for mammalian ones. The RNA components of cytoplasmic ribosomes of *Chlyamydomonas*

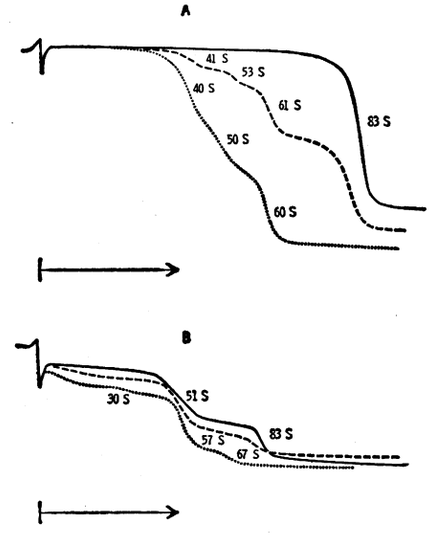


Fig. 2. The effect of changes in the concentration of $Mg^{2+}$ on the ultracentrifugal patterns of cytoplasmic and chloroplast ribosomes. Densitometer traces of ultraviolet absorption patterns after 14 minutes (A) or 10 minutes (B) at 44,000 rev/min at 8° to 10°C. All solutions contained 0.06*M* KCl, 0.01*M* tris (*p*H 7.8), and $MgCl_2$ as follows: solid line, 0.01*M*; dashed line, 0.001*M*; and dotted line, no added $MgCl_2$. (A) Cytoplasmic ribosomes. (B) Chloroplast ribosomes.

were analyzed with ultraviolet absorption optics. The sedimentation patterns of the RNA extracted from three samples of cytoplasmic ribosomes were essentially the same: two main components of 24 and 16*S*, with some 12*S* and slower components also present. When the solutions were reanalyzed at room temperature in the presence of added NaCl (0.1*M* $Na^+$), the conditions used by Taylor *et al.* (*4*), the sedimentation coefficients of the two main components were 20 and 15*S*, values very similar to the ones those authors found for the RNA's of bacteria and different from their values for the RNA's of fungi.

Characterization of the chloroplast ribosomes proved to be difficult. Ultracentrifugal analysis of ribosomes isolated from the chloroplast fraction did not reveal the 70*S* component expected from the sedimentation studies of crude homogenates (Fig. 1, upper pattern). On the contrary, as shown in Fig. 2B, the components present had sedimentation coefficients of 83 and 51*S* when examined in SB. At very low concentrations of $Mg^{2+}$, 67, 57 and 30*S* components appeared, but the 51*S* component remained unchanged in amount. These results suggested that the 51*S* component is a stable subunit. The 83*S* component probably is a cytoplasmic contaminant.

Evidence that the 51*S* component is probably a subunit of the 70*S* ribosome comes from a comparison of the two schlieren patterns of Fig. 1. The upper pattern shows two components which are about 80 and 70*S* at infinite dilution. The lower pattern was obtained by deoxycholate treatment of the material shown in the upper pattern. The 80*S* component was unchanged after deoxycholate was added, but the 70*S* component disappeared and a similar amount of 48*S* component appeared. Since deoxycholate was used in the preparation of ribosomes from the chloroplast fraction, it seems likely that the stable 51*S* component found in the chloroplast preparations (Fig. 2B) is also derived from 70*S* ribosomes. It should be noted that the stable 50*S* component was not seen in the cytoplasmic ribosomal preparations (Fig. 2A). Because of their instability, we have not so far been able to isolate intact 70*S* ribosomes for further characterization.

In discussing these results, we shall first consider the cytoplasmic ribosomes. The 80*S* ribosomes of *Chlamydomonas* resemble the 70*S* ribosomes of bacteria in their sensitivity to low concentrations of $Mg^{2+}$ and in the size of their RNA. They differ from bacterial ribosomes in their low ratio of RNA to protein and in their sedimentation coefficient. The 80*S* ribosomes of *Chlamydomonas* differ from those of higher plants in their $Mg^{2+}$ requirement, and in this respect they differ also from the ribosomes of yeast and fungi (*6*).

These ribosomal variations could result from differences in the RNA or in one or more of the proteins. It has been suggested (*4*) that the size of the RNA accounts for the different sedimentation coefficients of 70*S* bacterial and 80*S* fungal ribosomes; however, this suggestion does not account for the constancy of sedimentation coefficient of 80*S* ribosomes with RNA's of different lengths. Further, the relative constancy of the base composition of RNA (*17*) in the face of great evolutionary variation in DNA suggests that it has a functional significance. It seems more likely that most of the variation we have noted results from differences in the proteins.

Variation in the $Mg^{2+}$ requirement is interesting. Hsiao (*6*) has pointed out that decreasing sensitivity to low concentrations of $Mg^{2+}$ correlates well with increasing complexity of the organism. The functional importance of $Mg^{2+}$ may be related to the finding that under in vitro conditions, the coding acuity of bacterial preparations can be seriously impaired by suboptimal $Mg^{2+}$ concentrations (*18*). The stability of ribosomes at low concentrations of $Mg^{2+}$ in vitro may reflect a stability in vivo toward fluctuating metabolic conditions of functional value to the organism and therefore preserved in the evolution of higher forms.

Why, then, did bacteria and *Chlamydomonas* fail to evolve ribosomes with the presumably advantageous stability to low concentrations of $Mg^{2+}$ achieved by higher forms? An answer to this question requires more knowledge about the detailed structure of ribosomes, especially the proteins and the functionally permissible mutationally possible alterations they can undergo.

The presence of chloroplast ribosomes with properties different from those in the cytoplasm is not surprising in view of the evidence that chloroplasts contain DNA and exhibit partial genetic autonomy. The existence of special classes of organelle genes and protein synthesizing machinery suggests some degree of independence in their evolutionary origin. Regardless of origin, however, a dual or multiple genetic system would not survive evolutionary pressures unless it possessed continuing selective value. The intriguing biological problem posed by the genetic autonomy of organelles is the nature of the selective advantage they confer.

*Note added in proof.* The most probable explanation for the effect of sodium deoxycholate on the chloroplast ribosomes is its contribution to the ionic strength of the solution rather than a detergent action. In separate experiments the effect was reproduced by the addition of KCl to the lysate in an equivalent final concentration. Moreover, the ratio of $K^+$ to $Mg^{2+}$ was the critical factor. The 70*S* ribosomes were stable at ratios of 6 to 1 and 8 to 1, but not 16 to 1.

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19. This work was supported by PHS Grants GM-13970 (R.S.), CY-3190, and CA-08748, and by AEC contract AT(30-1)-910 (Sloan-Kettering Institute for Cancer Research).

11 May 1967