He pointed out that in higher plants, the absorption and accumulation of phosphate from dilute solution requires active respiration, and that phosphate accumulation is markedly affected if metabolic activity is inhibited by reduction of oxygen tension in the medium, by low temperature, or by poisons of the cytochrome system.

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Functional Chloroplast Polyribosomes from Tobacco Leaves

Abstract. *Incubation of isolated tobacco chloroplasts with ingredients required for protein synthesis resulted in liberation of 70S ribosomes and polyribosomes that no longer sedimented with the chloroplasts. With increasing time of incubation, polyribosomes broke down to 70S monosomes. Similarly, microgram quantities of ribonuclease caused chloroplast polyribosomes to break down into monosomes. Both polyribosomes and 70S ribosomes that were isolated on sucrose density gradients and tested separately in cell-free systems were capable of protein synthesis; however, polyribosomes formed more protein per unit of RNA than monosomes did.*

Whether protein synthesis by isolated chloroplasts occurs on polyribosomes is a question that has not been answered satisfactorily. Using analytical centrifugation, Clark *et al.* (*1*) demonstrated polyribosomal aggregates, by Schlieren optics, when Chinese cabbage leaves were homogenized in the presence of polyvinyl sulfate, a nuclease inhibitor. Boardman *et al.* (*2*) confirmed this observation for extracts of tobacco leaves but showed that polyvinyl sulfate strongly inhibited protein synthesis by the isolated chloroplasts. In another study (*3*), these investigators concluded that monosomes were mainly responsible for the protein-synthesizing activity in a chloroplast supernatant. The procedure they utilized was first to obtain a chloroplast supernatant by resuspending intact chloroplasts in a buffer of low molarity and then to centrifuge it at 17,000*g*. In their experiments, they measured the protein-synthesizing activity in the supernatant.

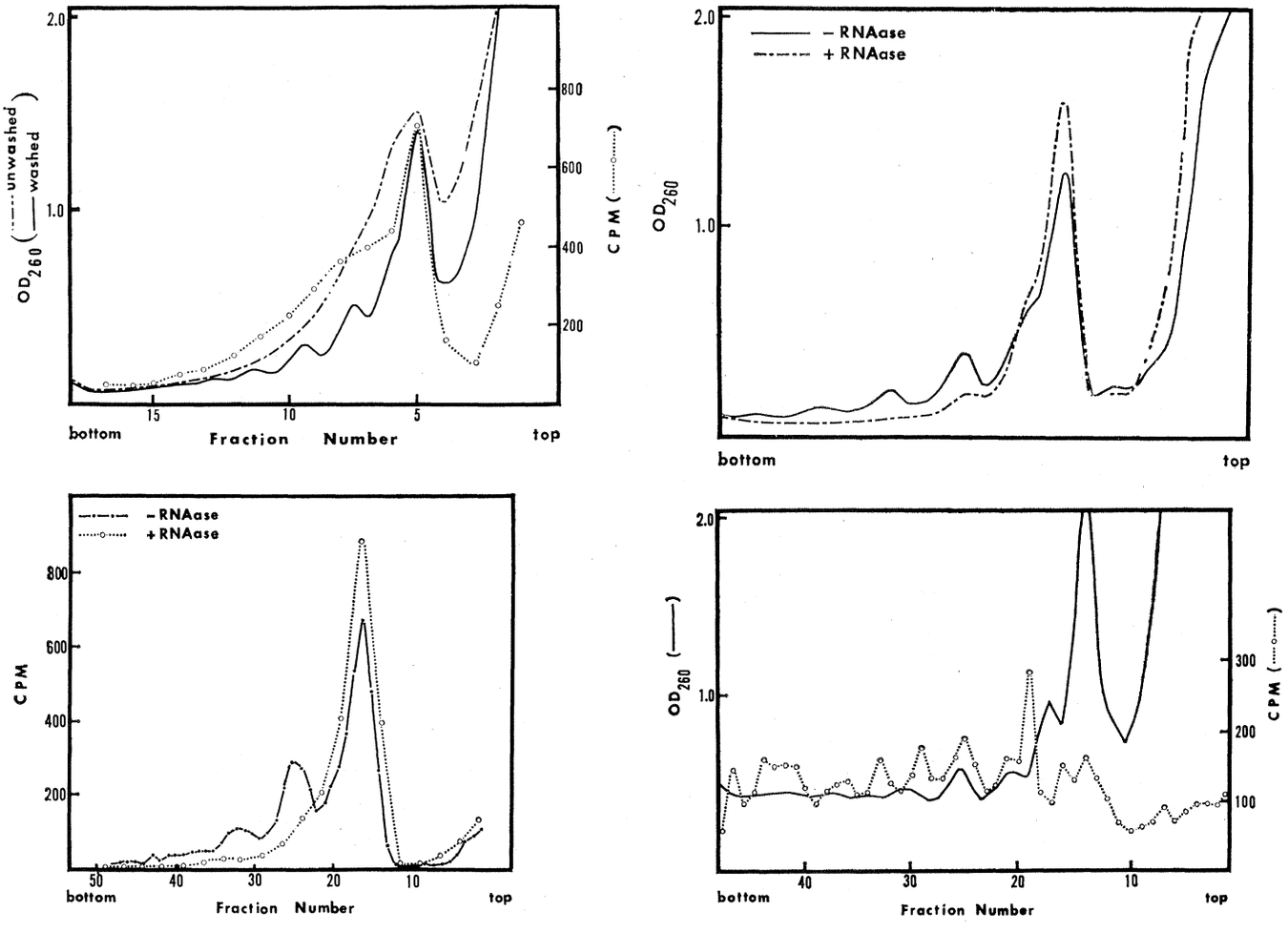
In contrast to their procedure, we first incubated intact chloroplasts and then fractionated each incubated mixture into a 17,000*g* pellet and supernatant. By subjecting these supernatants to sucrose density gradient centrifugation, it was possible to fractionate the materials that had synthesized protein and, simultaneously, to locate their positions in the gradient by optical-density measurements. Further investigation showed that functional chloroplast polyribosomes could be identified in rapidly growing leaves of young tobacco plants.

Chloroplasts were prepared from leaves of young tobacco plants by methods already described (*4*). The chloroplast pellet from 5 g of leaves was resuspended in 0.35 ml of a medium containing 0.025*M* tris-HCl (*p*H 7.8), 0.015*M* magnesium acetate, and 0.004*M* 2-mercaptoethanol. To each suspension of chloroplasts was added 0.20 ml of a combined reagent mixture that contained tris, (*p*H 7.8), 4.6 μmole; magnesium acetate, 4.6 μmole; KCl, 27.6 μmole; mercaptoethanol, 2.6 μmole; adenosine triphosphate, 0.4 μmole; phosphoenolpyruvate, 2.5 μmole; pyruvate kinase, 50 μg; and a mixture of 0.02 μmole each of uridine, guanosine, and cytidine triphosphates. Next, 2 μc (20 μl) of an amino acid mixture (uniformly labeled with C^{14} and having a specific activity of 0.1 mc/0.067 mg) was added and incubation was carried out for 1 minute (or longer, where stated) at 28°C. The incubated mixture was immediately chilled in an ice bath and then centrifuged at 17,000*g* for 15 minutes. All operations before and after incubation were conducted at a temperature close to 0°C. Portions (0.3 to 0.5 ml) of the 17,000*g* supernatants were layered directly on linear 5 to 20 percent sucrose density gradients that contained 0.015*M* magnesium acetate and 0.025*M* tris-HCl, *p*H 7.8. Gradients were centrifuged for 2 hours in the Spinco model L-2 centrifuge at 24,000 rev/min with the rotor coasting to a stop without braking. Contents of the gradients were pushed up by a 30 percent sucrose solution that was pumped into the bottom of the tube at a constant speed with a motor-driven syringe; they were then passed through a Vanguard automatic ultraviolet spectrophotometer and the optical density, at 260 mμ, was monitored continuously with a graphic recorder. The flow rate was 0.7 ml/min. Radioactivity incorporated into protein in each fraction was precipitated by hot 5 percent trichloroacetic acid (TCA) (for 20 minutes at 80°C) and collected on glass fiber filters. Precipitates were first washed four times with 5-ml portions of 5 percent TCA; this was followed by three 5-ml washes with 80 percent acetone. They were then dried under an infrared lamp before being placed in a vial with 5 ml of scintillation fluid; radioactivity was counted with a liquid scintillation counter.

When intact chloroplasts were incubated with a mixture of uniformly labeled amino acids in the presence of the mixture of combined reagents, as radioactivity was incorporated into protein, about 50 percent of this radioactivity would no longer sediment at 17,000*g*, whereas the other 50 percent did sediment with the chloroplasts. This ratio of incorporated radioactivity which did not sediment at 17,000*g* to that which did sediment remained about the same whether incubation was for 5 or 30 minutes. The nonsedimentable incorporated radioactivity present in 17,000*g* supernatants that resulted from incubation periods of 1, 5, and 15 minutes was fractionated on sucrose density gradients. Measurements of optical density (OD) revealed a broad peak of ribosomes that increased in amount without significant change in the OD profile as the time of incubation was increased. However, there was a marked change of the incorporated radioactivity from the heavy ribosome regions to the monosome region as the incubation period lengthened.

The possibility was suggested to us that a polyribosome OD profile was being concealed and that it might be revealed by washing the chloroplasts before incubation for protein synthesis. Isolated chloroplasts were therefore

washed once by resuspending them in a small volume of buffered medium, containing 0.025*M* tris-HCl (*p*H 7.8) and 0.015*M* magnesium acetate, and again centrifuging the chloroplasts into a pellet at 12,000*g* for 10 minutes. Similarly, the washed pellet of chloroplasts was resuspended in buffered medium and incubated, in the usual manner, with ingredients necessary for protein synthesis. As before, incubation with these ingredients resulted in incorporated radioactivity remaining in the supernatant after the washed chloroplasts had been centrifuged at 17,000*g*. When the supernatants from washed chloroplasts were fractionated on sucrose density gradients, there was a marked change in OD profiles. Figure 1 shows the profiles of ribosomes obtained from both unwashed and washed chloroplasts. Incubation of washed chloroplasts with ingredients required for protein synthesis for 1 minute resulted in release of clearly defined components, heavier than 70*S* monomers, into the 17,000*g* supernatant. Components larger than monosomes constitute about 50 percent of the total extracted ribosomes, as determined by measuring the relative areas. In this sucrose density gradient profile, aggregates as large as five ribosomes were detected; in other profiles, aggregates of up to seven ribosomes have been observed. Figure 1 also shows that much of the radioactivity from washed chloroplasts was associated with polyribosomes but appreciable amounts were also found in the monosome regions. The specific radioactivity (counts per minute per unit of optical density at 260 mμ) of the polyribosomes was approximately twice that of monosomes and accounted



Figs. 1–4. Fig. 1 (top left). Effect of washing chloroplasts prior to incubation for protein synthesis and subsequent fractionation of the 17,000*g* supernatant by sucrose density gradient centrifugation. For radioactivity measurements, the gradient was fractionated into 17 1.5-ml portions. CPM, radioactivity in counts per minute; OD_{260}, optical density at 260 mμ. Fig. 2 (top right). Effect of ribonuclease on chloroplast polyribosomes; absorbancy measurements. Washed chloroplasts were incubated for 1 minute for protein synthesis and were then centrifuged at 17,000*g*. The supernatant was divided into two equal portions and incubated at 0°C for 10 minutes, one without ribonuclease and the other with 2 μg of ribonuclease per milliliter. Both portions were then fractionated by the sucrose density gradient techfiique. RNAase, ribonuclease. Fig. 3 (bottom left). Effect of ribonuclease on chloroplast polyribosomes. Same experiment as for Fig. 2 except that this gives the radioactivity measurements. Fig. 4 (bottom right). In vitro protein synthesis by chloroplast polyribosomes. Washed chloroplasts were obtained from 10 g of leaves and resuspended in 1.0 ml of 0.025*M* tris-HCl buffer, *p*H 7.8, that contained 0.015*M* magnesium acetate and 0.004*M* 2-mercaptoethanol. A portion (0.2 ml) of the combined-reagents mixture (see text) was added; the suspension was maintained at 0°C and then centrifuged at 17,000*g* for 15 minutes. The entire supernatant was applied to a linear, 5 to 20 percent sucrose density gradient (volume 25 ml) that contained 0.025*M* tris-HCl (*p*H 7.8), 0.015*M* magnesium acetate, and 0.004*M* 2-mercaptoethanol. After centrifugation at 24,000 rev/min for 2 hours, 20-drop fractions (approximately 0.5 ml) were collected. To each fraction was added 0.2 ml of a supernatant obtained by high-speed centrifugation (*8*), 0.1 ml of a combined-reagents mixture, and 0.5 μc of an amino acid mixture, uniformly labeled with C^{14}; the final preparation was incubated for 45 minutes at 28°C. Radioactivity incorporated into protein was determined as described in the text.

for about 60 percent of the label incorporated into the ribosomes. Although washing the isolated chloroplasts resulted in about a 40-percent loss of activity, the washing procedure was routinely carried out in subsequent experiments, as it revealed more distinctly the position occupied by heavy ribosomes in sucrose density gradients.

The OD profile of the polyribosome region was shifted to the monosome region by pancreatic ribonuclease (Fig. 2). This treatment also shifted much of the incorporated radioactivity from the polyribosome to the monosome region (Fig. 3), although there appears to be a small amount of heavier material that is less sensitive to ribonuclease.

With the evidence that polyribosomes could be detected by use of washed chloroplasts, the question arose whether the polyribosomes could be released from washed chloroplasts and fractionated on sucrose density gradients before testing the fractions for their capacity to incorporate radioactivity into protein. It was not possible to release sufficient ribosomes for OD analysis by suspending washed chloroplasts at 0°C in a buffered medium of low molarity, according to the method that Boardman *et al.* (*3*) used with unwashed chloroplasts. However, addition of the mixture of combined reagents to the washed chloroplasts at 0°C caused release of ribosomes, generally to about one-half the extent of the ribosomes released during a 1- to 5-minute incubation at 28°C for protein synthesis. By use of combined reagents at 0°C, sufficient ribosomes were obtained in a 17,000*g* supernatant to permit their fractionation by sucrose density gradient centrifugation prior to the incubation of each fraction with reagents necessary for protein synthesis. As shown in Fig. 4, the shape of the absorbancy profile of the ribosomes obtained in this manner is similar to that obtained in previous experiments in which labeled ribosomes were released from washed chloroplasts into the 17,000*g* supernatant as protein synthesis occurred. Figure 4 also shows that more of the protein-synthesizing activity was associated with the polyribosome region than with the monosome region.

This result may be compared with results obtained when washed chloroplasts were first made to incorporate labeled amino acids, and the resulting labeled supernatant was fractionated by sucrose density sedimentation. The specific radioactivity of the polyribosomes was only twice that of monosomes (Fig. 1). Apparently, most of the monosomes carry nascent, or completed, polypeptide chains, and we assume that these monosomes arise by breakdown of the polyribosomes during protein synthesis. Longer incubation periods, as well as mild treatment with ribonuclease, results in the conversion of both radioactivity and OD, originally associated with polyribosomes, to monosome material.

The polyribosome profile of tobacco chloroplasts consists of a much smaller proportion of larger ribosome aggregates, in comparison with monosomes, than those encountered in other plant tissues (*5*) and other organisms (*6*). It can be questioned, therefore, whether the low yields of larger aggregates from the chloroplasts arose from nuclease action on the polyribosomes during the extraction procedure. Boardman *et al.* (*3*) examined mixtures of reticulocyte polyribosomes and tobacco chloroplast supernatants in the analytical centrifuge and concluded that chloroplast supernatants contain little active nucleases. Our chloroplast supernatants were also incubated with reticulocyte polyribosomes (*7*) at 0°C for 15 minutes before resolving the mixture by sucrose density gradient centrifugation. The several humps of the reticulocyte polyribosomes appeared undegraded. Thus, it seems unlikely that the chloroplast polyribosome profile had been altered by nucleases.

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Antibodies to Rabbit Cytochrome c Arising in Rabbits

Abstract. *Antibodies reactive with rabbit cytochrome* c *have been observed in rabbits immunized with several heterologous cytochromes. Such antibodies have also been observed in rabbits immunized with rabbit cytochrome* c *conjugated to bovine gamma globulin. The serum of a rabbit immunized with human cytochrome* c *reacted with the cytochrome* c *of the same rabbit.*

The elicitation of autoantibodies by crude tissue preparations, either by autoimmunization or by heteroimmunization, has been amply described (*1*). Autoantibodies have also been observed after autoimmunization or heteroimmunization with purified extracellular or cytoplasmic proteins such as thyroglobulin (*2*), γ-globulin (*3*), and adenylate kinase (*4*). We describe here the regular appearance of antibody reactive with a mitochondrial protein of the immunized species. Rabbits immunized with a variety of cytochromes *c* from other species produced antibodies to rabbit cytochrome *c* prepared from a pool of several hundred rabbit hearts. Moreover, serum of a rabbit immunized with human cytochrome *c* reacted with cytochrome *c* subsequently isolated from the same rabbit. We have also observed the formation of antibody to cytochrome *c* in rabbits immunized with rabbit cytochrome *c* coupled covalently to acetylated bovine γ-globulin.

White New Zealand rabbits were immunized by either of two methods (*5*). The tuna, turkey, and rabbit cytochromes *c* were injected as conjugates to acetylated bovine γ-globulin while the human and horse proteins were injected as the free native proteins (*5*). Methods used for conjugation and for quantitative estimation of specific antibody have been described (*5*); antibody was estimated by a modification of the Farr technique with the use of I^{125}-labeled cytochrome *c*, by precipitin analysis and by complement fixation.

Cross reactions of antibodies elicited by a particular cytochrome *c*, with the cytochromes *c* of other species, were also investigated by measuring the competition between the homologous I^{125}-labeled protein and the heterologous unlabeled proteins for binding to antibody. On the basis of initial experiments