is presented to show that ribosome monomers are mainly responsible for the incorporating activity of *Chloroplasts*. Unpublished experiments have also shown that two classes of ribosomes, 70 S and 80 S, exist in leaves, in confirmation of previous results by Lyttleton (1962). The two classes of ribosomes are present in about equal amounts. When *Chloroplasts* prepared in *Honda* medium are removed from a leaf homogenate as a 1000 *g* pellet, ultracentrifugal examination of the remaining "cytoplasmic" supernatant shows that over 80% of the cytoplasmic ribosomes are of the 80 S class, in marked contrast to a *Chloroplast supernatant* where over 80% of the ribosomes are of the 70 S class. Thus removal of *Chloroplasts* with mobile phase intact also preferentially removes the 70 S class of ribosomes. Preferential removal of the 70 S ribosomes is most likely the result of the 70 S ribosomes' being contained within the boundaries of the mobile phase during isolation of *Chloroplasts*.

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Protein Synthesis by Cell-free Extracts from Tobacco Leaves. II. Association of Activity with Chloroplast Ribosomes*

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ABSTRACT: The amino acid incorporating activity of isolated tobacco leaf chloroplasts was released from the mobile phase of the chloroplasts and fractionated by centrifugation on density gradients of sucrose. It was found that most of the activity was associated with ribosome monomers.

Analytical ultracentrifugation showed the presence of two classes of ribosomes in the chloroplast extracts with sedimentation coefficients of about 70 S and 80 S. The 70 S ribosomes predominated and accounted for over 80% of the ribosomes of the chloroplast fraction. There was no evidence from these analyses for the presence of substantial amounts of polyribosomes in any of the preparations.

Evidence was presented in the preceding paper (Francki *et al.*, 1965) that most of the activity responsible for amino acid incorporation into protein in isolated tobacco leaf chloroplasts is located in the mobile phase of the chloroplasts. The activity could be separated from the chlorophyll-containing stationary component without drastic alteration in the structure of the stationary component. Protein synthesis was not dependent upon the structural integrity of the chloroplasts since there was no decrease in the incorporating activity after the chloroplasts were solubilized by the nonionic detergent, Triton X-100. Lyttleton (1962) has reported the isolation from spinach chloroplasts of ribosome monomers which were distinguishable from other plant ribosomes by their low sedimentation coefficient of 66 S. We have confirmed this result for tobacco leaf chloroplasts. The question arose, therefore, as to whether the 66 S ribosomes, or some form of polyribosomes such as those identified in *Escherichia coli* (Marks *et al.*, 1962), reticulocytes (Warner *et al.*, 1963), mammalian cells (Gierer, 1963), or plant tissues (Clark *et al.*, 1963) were responsible for amino acid incorporation by chloroplasts. From the results to be presented, it is evident that ribosome monomers released from the mobile phase of chloroplasts account

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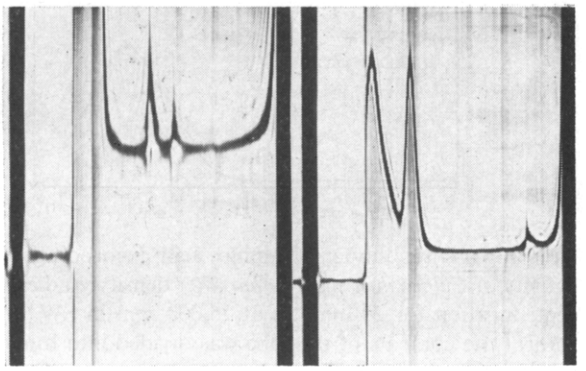

FIGURE 1: Ultracentrifuge analysis of *Chloroplast supernatant*. (A, left) Pattern obtained at a bar angle of 25° after 13 minutes at 42,040 rpm. (B, right) Pattern obtained at a bar angle of 60° after 29 minutes at 42,040 rpm.

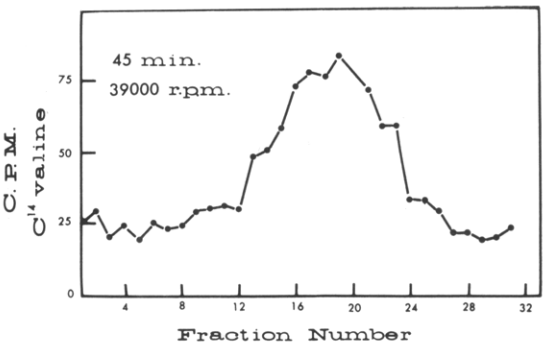

FIGURE 2: Distribution of amino acid incorporating activity of *Chloroplast supernatant* after density gradient centrifugation for 45 minutes at 39,000 rpm. Abscissa shows increasing fraction number from the bottom of the tube.

for virtually all the activity of isolated chloroplasts.

Materials and Methods

Isolation of Chloroplasts. Using the terminology adopted in the previous paper (Francki *et al.*, 1965), *Chloroplasts* were isolated from tobacco leaves in Honda medium and were resuspended in Tris-Mg-SH medium. Separation of the amino acid incorporating activity into *Chloroplast supernatant* and *Chloroplast pellet* was accomplished by centrifugation at 17,000 *g* for 15 minutes. In the present experiments, *Chloroplasts* extracted from 10 g fresh weight of leaves were resuspended in 1.0 ml of Tris-Mg-SH medium, in contrast to previous experiments where the ratio was 10 g to 7.0 ml of medium. The ratio of amino acid incorporating activity in the *Chloroplast supernatant* to that in the *Chloroplast pellet* did not change significantly when the smaller volume of suspending medium was used. The smaller volume permitted the contents of the *Chloroplast supernatant* to be analyzed directly by ultracentrifugation. In some experiments the incorporating activity still remaining with the *Chloroplast pellet* was released by 1% Triton X-100.

Analytical Ultracentrifugation. Analyses were carried out in a Spinco Model E centrifuge at 42,040 rpm and temperatures from 2 to 10°, using a standard 12-mm cell.

Density Gradient Centrifugation. Linear density gradients were made in 5-ml Spinco SW 39 tubes (Beckman Instruments) using 5% and 20% sucrose solutions. Where incorporating activity was to be measured, the sucrose was dissolved in Tris-Mg-SH medium. In some experiments dealing with ultraviolet absorbancy measurements, mercaptoethanol was omitted and the sucrose was dissolved in 0.01 M Tris, *p*H 7.8, and 5 mM magnesium acetate.

Assay of Amino Acid Incorporating Activity. A chloroplast (0.2 ml) high-speed supernatant, prepared as described by Francki *et al.* (1965), was added to each fraction from the density gradient column, and the fractions were assayed for their amino acid incorporating activity by the methods used by Francki *et al.* (1965).

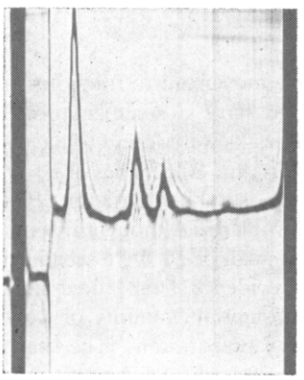

FIGURE 3: Ultracentrifuge analysis of resuspended pellets obtained by centrifuging *Chloroplast supernatant* for 2 hours at 40,000 rpm in Spinco 40 rotor. Pattern obtained at bar angle of 40° after 13 minutes at 42,040 rpm.

Results

Analysis of Chloroplast Supernatant in the Ultracentrifuge. When *Chloroplast supernatant* was examined in the analytical ultracentrifuge, two small peaks (Figure 1A) were seen to move away from the starting boundary shortly after the rotor reached its operating speed of 42,040 rpm. Within 25 minutes these two peaks had almost reached the bottom of the cell but were replaced by two additional peaks which were much greater in area (Figure 1B). The latter two peaks are the protein boundaries usually observed in extracts of green leaves, have sedimentation coefficients of 16–18 S and 3–4 S, and have previously been designated as fraction I and fraction II proteins, respectively (Eggman *et al.*, 1953; Dorner *et al.*, 1957). When calculated

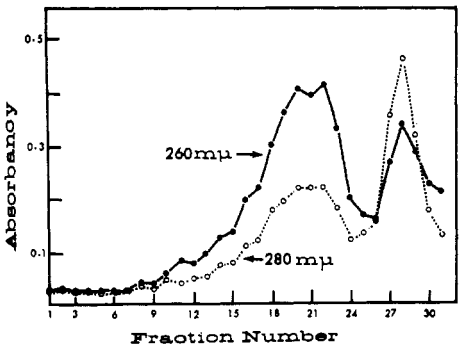

FIGURE 4: Density gradient centrifugation of resuspended pellets obtained by centrifuging *Chloroplast supernatant* for 2 hours at 40,000 rpm in Spinco 40 rotor. Centrifugation was for 45 minutes at 39,000 rpm in the SW 39 rotor. -·-·-·, absorbancy at 260 mμ; O- -O- -O, absorbancy at 280 mμ.

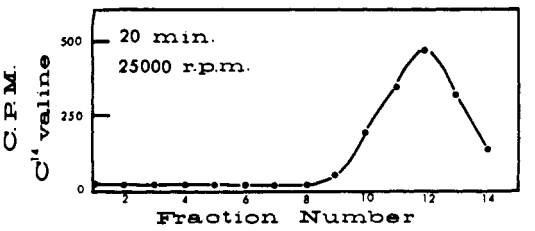

FIGURE 5: Distribution of amino acid incorporating activity of *Chloroplast supernatant* after density gradient centrifugation for 20 minutes at 25,000 rpm in SW 39 rotor. (The contents of the tube was divided into fourteen fractions.)

by their relative movement to fraction I protein, which was assumed to have a sedimentation coefficient of 18 S, the small peaks were found to have sedimentation coefficients of 70 and 80 S. Thus the small peaks correspond to those seen by Lyttleton (1962) in extracts of spinach chloroplasts. Although in some preparations additional very small peaks were seen in the centrifuge, there was no evidence from these analyses for the presence of substantial amounts of polyribosomes in any of the many preparations. The sharp spikes associated with the 70 and 80 S boundaries may indicate that the sedimentation of these components is highly dependent upon their concentration.

In order to determine what components in *Chloroplast supernatant* were responsible for the amino acid incorporating activity, the activity was fractionated by density gradient centrifugation.

Fractionation of the Incorporating Activity of Chloroplast Supernatant by Density Gradient Centrifugation. Chloroplast supernatant (0.3 ml) was layered on a sucrose gradient column and centrifuged for 45 minutes at 39,000 rpm before the tube was punctured and the column was divided into thirty-one equal fractions. The amino acid incorporating activity was distributed as shown in Figure 2. Unfortunately, absorbancy measurements could not be used to locate the position of the ribosomes in the gradient column. The absorbancy, both at 260 and 280 mμ, showed a continuous rise in passing from the bottom to the top of the gradient column, apparently owing to the presence of nucleic acid throughout the gradient. Therefore, in order to locate the positions of the 70 and 80 S ribosome components as well as the fraction I protein, resort was made to the use of sedimented ribosomes.

Pellets obtained by centrifuging *Chloroplast supernatant* for 2 hours at 40,000 rpm in a Spinco 40 rotor were resuspended by being allowed to stand overnight in 0.01 M Tris buffer, *p*H 7.8, containing 5 mM magnesium acetate. Material which did not disperse completely was removed by centrifugation at 17,000 *g* for 15 minutes. The resuspended pellets produced the ultracentrifuge pattern shown in Figure 3, where it was evident that the 3–4 S components had been eliminated and the amount of the 18 S component had been reduced in relation to the 70 and 80 S ribosome components. When such a preparation was subjected to density gradient centrifugation (Figure 4) it was found that the ribosome components and the 18 S protein could now be located by absorbancy measurements at 260 and 280 mμ. There is a good separation of the fraction I protein from the ribosomes, but the 70 S ribosomes are not resolved from the 80 S ribosomes. On the assumption that the location of ribosomes found in this experiment would also apply to their location after density gradient centrifugation of *Chloroplast supernatant*, it can be concluded that the components in the *Chloroplast supernatant* responsible for incorporating activity are the ribosome monomers (Figure 2). Since the recovery of activity from the density gradient column was only 70%, the possibility remained that some of the activity in the *Chloroplast supernatant* was associated with large polyribosomes or ribosomal aggregates which sedimented to the bottom of the tube during density gradient centrifugation. Therefore a sample of *Chloroplast supernatant* was subjected to density gradient centrifugation for 20 minutes at 25,000 rpm in the SW 39 rotor. The result (Figure 5) shows that all the activity added to the gradient was recovered from the upper part of the tube. There was no activity in regions of the tube which would correspond to positions occupied by large ribosome aggregates.

A criterion used for presumptive identification of polyribosomes has been the change in the ultracentrifuge pattern resulting from incubation of the preparation with RNAase[1] (Clark *et al.*, 1963). Incubation of *Chloroplast supernatant* with pancreatic RNAase (0.2 μg/ml) for 30 minutes at 25° did not produce a significant change in the centrifuge pattern when compared with a sample incubated in the absence of RNAase, or with a nonincubated sample.

[1] Abbreviation used in this work: RNAase, ribonuclease.

Fractionation of Activity Released from the Chloroplast Pellet. Incorporating activity which remained associated with the *Chloroplast pellet* was released by the neutral detergent, Triton X-100. Analytical ultracentrifugation showed that the relative proportion of the 70 and 80 S ribosomes was similar to that found for the *Chloroplast supernatant.* Sucrose gradient analyses (Figure 6) showed that the activity was confined again to the regions of the column occupied by the ribosome monomers. There was no significant difference in the distribution of activity throughout the gradients from that observed with the *Chloroplast supernatant.*

Discussion

The amino acid incorporating activity of isolated tobacco leaf chloroplasts may be accounted for in terms of ribosome monomers. While two classes of ribosomes are seen in the ultracentrifuge patterns of the dispersed mobile phase of chloroplasts, recent unpublished experiments show that the 70 S ribosomes, which constitute over 80% of the ribosomes of the *Chloroplast supernatant*, are of chloroplast origin whereas the 80 S ribosomes appear to be of cytoplasmic origin. Lyttleton (1962) also found two types of ribosomes in extracts of spinach leaves and concluded that one kind was of chloroplast origin. Because of the poor resolution of the two classes of ribosomes on density gradient centrifugation, it was not possible from the work presented in this communication to estimate their relative amino acid incorporating activities. However, in a subsequent communication evidence will be presented to show that most of the activity of the *Chloroplast supernatant* is associated with the 70 S ribosomes.

In some preparations of *Chloroplast supernatant* additional peaks were observed in the analytical ultracentrifuge, but were small even in comparison with the 80 S peak. The minor peaks had sedimentation coefficients of about 105 and 125 S, suggesting that they may be due either to dimers of the 70 and 80 S particles, respectively, or to dimers and trimers of the 70 S particle. They did not apparently contribute significantly to the incorporating activity of the *Chloroplast supernatant.* The sedimentation coefficients reported in this paper are only approximate; more accurate measurements will be reported in a future publication which will deal with the properties of purified ribosomes from leaves.

The lack of polyribosomes in our preparations indicates either that they are not present in the mobile phase of chloroplasts, or that they are too rapidly degraded during the time necessary to isolate the mobile phase of chloroplasts to be seen in the analytical centrifuge. It seems unlikely that complete degradation of polyribosomes by nucleases could be accomplished without simultaneously destroying the amino acid incorporating activity of the system. The extreme sensitivity of the amino acid incorporating activity to inhibition by pancreatic RNAase (Spencer and Wildman, 1964), irrespective of whether the activity is

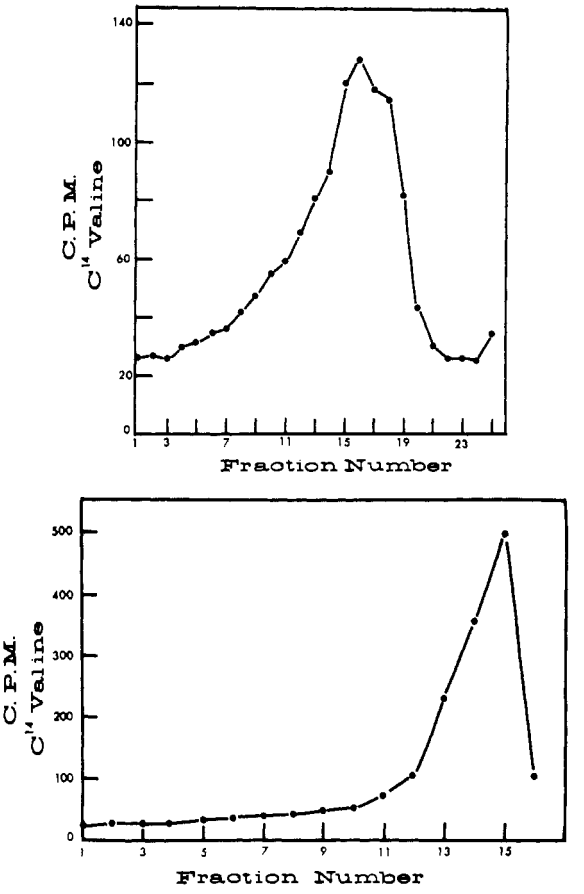

FIGURE 6: Distribution of amino acid incorporating activity after its release from *Chloroplast pellet* by Triton X-100 followed by density gradient centrifugation. (A, upper) Centrifugation for 45 minutes at 39,000 rpm in SW 39 rotor. (B, lower) Centrifugation for 20 minutes at 25,000 rpm in SW 39 rotor.

located in *Chloroplasts* or *Chloroplast supernatant*, is difficult to interpret except on the basis that ribosome monomers are the agents responsible for the activity. Additional support for this view is found in an experiment where *Chloroplast supernatant* was incubated for 1 hour at 0° with a preparation of reticulocyte polyribosomes kindly supplied by Dr. Alex Miller of this institution. When the mixture was subjected to analytical ultracentrifugation, the several peaks of the reticulocyte polyribosomes were still readily resolved from the chloroplast ribosome monomers, although some reduction in the amount of polyribosomes was apparent. Thus it appears that nucleases in *Chloroplast supernatant* capable of degrading animal polyribosomes are nominal in activity and probably of little consequence as agents capable of rapidly altering the ribosome composition of *Chloroplast supernant.*

Analysis of the composition of the mobile phase of *Chloroplasts* suggests a need for caution in the identification of ribosomes in sectioned chloroplasts examined by electron microscopy. Since about 50% of the protein released from the mobile phase is fraction I protein

having a particle diameter of about 110 A, and the concentration of fraction I protein is 10-fold higher than the 70 and 80 S ribosomes having particle diameters of about 200 A, it may be difficult to recognize the latter by size alone. Disappearance of particles after RNAase treatment of the sections would not be conclusive evidence for ribosomes because fraction I protein particles might also be held in the mobile phase by binding to RNA and disappear when this material is hydrolyzed.

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Protein-Carbohydrate Interaction. II. Inhibition Studies on the Interaction of Concanavalin A with Polysaccharides*

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ABSTRACT: Concanavalin A, a lectin isolated from jack bean meal, has been reported to form a precipitate with certain types of ramified α-glucans such as glycogens, amylopectins, and dextrans, and α-mannans such as yeast mannan. This interaction is now shown to be amenable to investigation by the Landsteiner inhibition technique.

A large number of mono- and oligosaccharides and modified sugars have been studied for their capacity to inhibit the precipitation reaction between concanavalin A and a dextran. In this manner it has been possible to determine some of the stereochemical requirements of the combining sites of the concanavalin A molecule. Inhibition data suggest that unmodified hydroxyl groups at the C-3, C-4, and C-6 positions of the D-glucopyranose (or D-mannopyranose) ring are essential for binding to the active sites of the protein. A hydroxyl group at C-2 is not essential but the D-*manno* configuration is bound more firmly than the D-*gluco* configuration. The α-glycopyranosides of these sugars are much more active than the free sugars, whereas the corresponding β-glucosides are poor inhibitors. In like manner, the α-linked glucobioses are very tightly bound to the protein whereas the β-linked glucobioses that were tested are inactive. On a molar basis, the almost identical patterns of inhibition for maltose and maltotriose and for isomaltose and isomaltotriose further suggest that the interaction of concanavalin A with simple and complex saccharides is directed primarily toward the terminal, nonreducing portion of these molecules. These results suggest that the interaction of concanavalin A with polysaccharides involves the chain ends rather than intact inner branches, as has been suggested previously.

The specific interaction of protein with polysaccharide is well known in the field of immunology (Heidelberger, 1960). Thus, well documented studies have been reported on the immunochemical reactions of dextrans (Kabat, 1961; Sugg and Hehre, 1942), specific capsular polysaccharides of pneumococci (Heidelberger, 1956), and the *O*-antigens of *Salmonella* (Staub and Tinelli, 1957).

In addition to antibody proteins of higher animals, proteins are found in the seeds of certain plants which are capable of a similar type of interaction. These

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