Plant Physiol. (1968) 43, 504–514

In Vitro Protein Synthesis by Plastids of Phaseolus vulgaris III. Formation of Lamellar and Soluble Chloroplast Protein[1, 2]

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Received November 9, 1967.

Abstract. **Chloroplasts from leaves of plants which had been grown in the dark, and then illuminated for 12 hours were isolated, and allowed to incorporate ^{14}C-leucine into protein, and the products of this incorporation were studied. Lamellar and soluble proteins are the principal products, and are formed in about equal amounts. Only some of the soluble proteins become heavily labeled. Those with highest specific activity have a molecular weight of the order of 140,000, while the higher molecular weight Fraction I protein has a much lower specific activity. The soluble protein as a whole does not serve as a precursor for the lamellar protein, and vice-versa, although a precursor-product relationship between a minor component of the soluble fraction and the lamellar fraction has not been ruled out. The relative protein synthesizing capabilities of chloroplasts and mitochondria are discussed with reference to the data presented.**

It has been reported that chloroplasts have the materials necessary for genetic autonomy. They contain ribosomes which would be necessary to convert the information in their DNA into proteins (8). A previous report from this laboratory showed that chloroplasts, in the crude chloroplast preparation under investigation, were responsible for the amino acid incorporation into protein that was observed *in vitro* (17). Amino acid incorporation into protein by chloroplasts has been reported from a number of laboratories, as reviewed in the first publication in this series (17). The present work reports further studies of the system described previously, and was carried out to find out the nature of the products formed.

Materials and Methods

Plant Material. Chloroplasts of *Phaseolus vulgaris* L. var. Black Valentine were obtained from primary leaves as already described (17). Leaves were obtained from plants grown in the dark for 6 days, and then illuminated with white light for 0.5 day.

Incorporation Assay. Incorporation of uniformly labeled ^{14}C-L-leucine was carried out at a chloroplast concentration which contained 16 to 50 μg chlorophyll/ml of reaction mixture. One μg chlorophyll is equivalent to 10 to 20 μg protein. Within the range used, incorporation is a linear function of chloroplast concentration. Incorporation was carried out in the high osmotic strength Honda medium to which 0.25 volume of other reagent solutions was added (17, 23), except where stated. The contribution of bacteria to incorporation was evaluated only occasionally with the aid of the nonionic detergent Triton X-100 (17). Triton solubilizes chloroplasts without affecting bacteria, making it possible to separate chloroplast contents from bacteria by differential centrifugation. It had been found previously, in experiments conducted over a period of a year, that bacteria in the chloroplast preparation contained less than 10 % of the radioactivity incorporated into protein. Spot checks carried out during the course of the experiments presented here confirmed this observation.

^{14}C-leucine was obtained from New England Nuclear Corporation, Boston, Massachusetts, and had a specific radioactivity of about 250 mc/mmole. Pyruvate kinase, type II from muscle (E.C. 2.7.1.40), and phosphoenolpyruvate, sodium salt, were obtained from Sigma Chemical Corporation, St. Louis, Missouri.

Radioactivity incorporated into protein was determined in a proportional counter after samples had been extracted with hot trichloroacetic acid, and collected on Millipore filters (17). However, in the experiment presented in figure 5, radioactivity in hot trichloroacetic acid insoluble material was determined by scintillation counting (15).

[1] Research was supported in part by funds provided by the United States Atomic Energy Commission Contract No. AT(30-1)2373.

[2] Published with the approval of the Secretary of the Smithsonian Institution.

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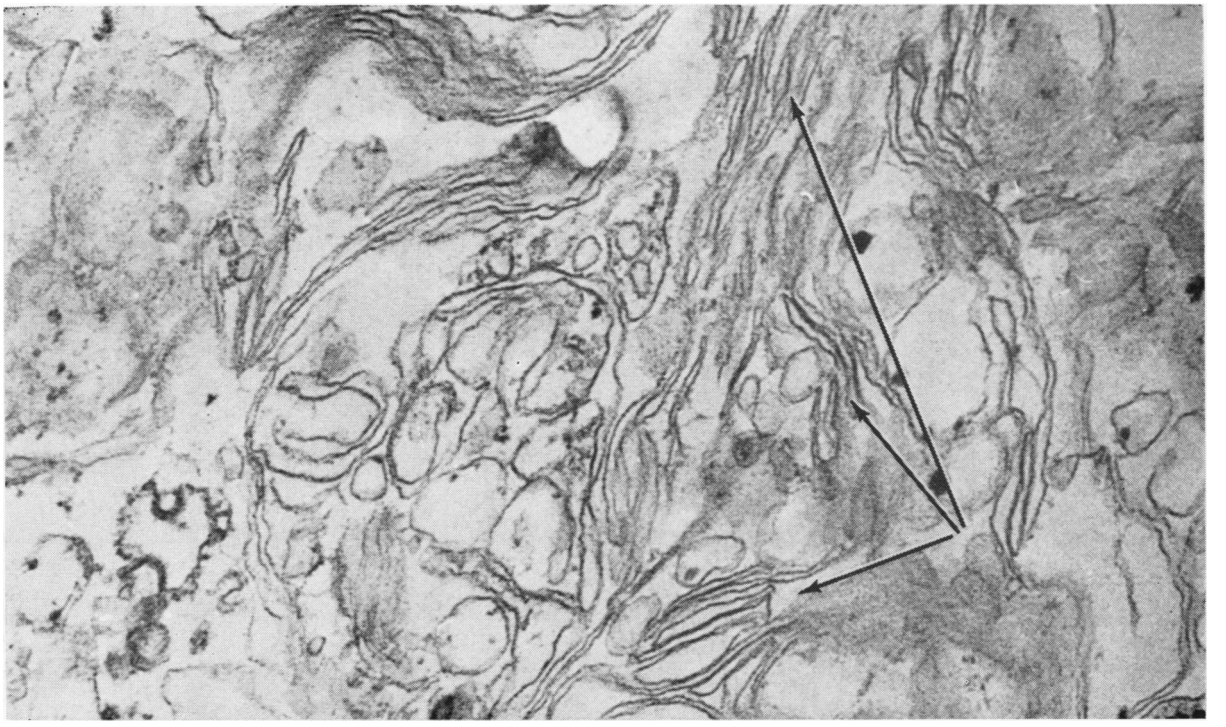

FIG. 1. Electron micrograph of lamellar fraction. × 49.000. See Methods for details. Arrows indicate grana cross sections.

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Chlorophyll was determined spectrophotometrically, and protein of the chloroplast preparations was determined by Kjeldahl digestion and nesslerization (17).

Preparation of "Lamellar" Material Labeled with ^{14}C-Leucine for Analysis in Density Gradients. Chloroplasts were incubated for 60 minutes with ^{14}C-leucine, as described above, at which time unlabeled leucine was added, and the reaction mixture chilled to 0°. The reaction mixture was then centrifuged at 6000 × *g* × 20 minutes. The pellet was resuspended in a small volume of Honda solution, and layered on a density gradient, as described below, or the pellet was washed once or twice in Honda solution, by resuspension in a volume equal to that of the reaction mixture, followed by centrifugation at 6000 × *g* × 20 minutes, and then layered on a density gradient.

A stepwise gradient was prepared by layering solutions of 0.05 M phosphate (pH 7.0) which contained different amounts of sucrose, or in the case of the highest density solution used, sucrose and sorbose. The densities and volumes of the solutions used to prepare the gradient were: ρ = 1.320, 3.0 ml; ρ = 1.305, 3.0 ml; ρ = 1.282, 3.0 ml; ρ = 1.260, 3.0 ml; ρ = 1.230, 3.0 ml; ρ = 1.215, 4.0 ml; ρ = 1.170, 4.0 ml; ρ = 1.155, 4.0 ml. The gradient was allowed to smooth for about 8 hours, and then 3.0 ml of a suspension of labeled chloroplast lamellar material in Honda medium (ρ = 1.05) was layered on top. The gradients were centrifuged at 25,000 RPM for 1 hour in a Spinco SW 25.1 rotor. The tubes were punctured at the level of the interface above the densest layer (which had been marked when the gradients were prepared), and 1 ml samples were collected. These were diluted to 2.0 ml with water, and samples taken for determination of incorporated activity, and chlorophyll.

Preparation of "Soluble" Protein Labeled with ^{14}C-Leucine for Analysis in Density Gradients. Chloroplasts were prepared as described in the preceding section, except that plastids were suspended in Honda medium from which Ficoll and dextran were omitted, and the sucrose concentration was raised to 0.5 M (sucrose medium). These substitutions were made to avoid the need to remove Ficoll and dextran before concentrating the protein containing chloroplast extract. The rates of incorporation of plastids suspended in sucrose medium were less than the rates of plastids suspended in Honda medium. After 60 minutes incubation, unlabeled leucine was added to the reaction mixture, and it was chilled to 0°. The reaction mixture was then centrifuged at 30,000 × *g* × 30 minutes, and the supernatant liquid carefully decanted. The pellet was resuspended in a volume of 1 mM GSH that was one-fifth that of the original incorporation reaction mixture, and incubated at 0° for 30 minutes with occasional stirring. It was then centrifuged at 30,000 × *g* × 30 minutes, and the supernatant liquid was decanted, and combined with the first. This extract was dialyzed for about 12 hours against 100 volumes of 10 % carbowax 6000 (polyethylene glycol, Union Carbide Corporation, New York, New York) in 50 mM potassium maleate, 1 mM GSH (pH 7.0). The dialysis served to concentrate the extract, and to remove enough of the sucrose in it so that the dialyzed extract could be floated on 5 % sucrose.

This soluble protein preparation was analyzed on a linear density gradient consisting of 50 mM potassium maleate, 1 mM GSH (pH 7.0) (24), in which the sucrose concentration was varied from 5 to 20 %. The volume of the gradient was 4.5 ml. Five-tenths ml of soluble protein (0.2–0.4 % protein) was layered on it, and the gradient centrifuged at 40,000 rpm for 6.0 hours in a Spinco SW 39 rotor. Tubes were punctured at the bottom, and their contents were collected in 15 drop fractions. This procedure yielded 27 fractions per gradient. Any residue in the bottom of the tube was resuspended in water. The fractions were analyzed for radioactivity present in protein, and for protein. The latter was determined by a modified Folin procedure (13), using 1 cm path length microcells and a Rodder microcell positioner (Microtech Services Co., Los Altos, California). Fraction I protein from spinach (14) was used as a reference material in replicate density gradient centrifugations.

Preparation of Chloroplast Structural Protein. Chloroplast structural protein was prepared by the procedure of Criddle (5). After chloroplasts had been incubated for 1 hour with labeled leucine, as usual, the reaction mixture was centrifuged at 5000 × *g* × 30 minutes. The pellet was resuspended in an equal volume of water, followed by centrifugation at 30,000 × *g* × 30 minutes. The washed pellet was resuspended in a volume of water half that of the original reaction mixture, and was then sonicated with cooling on ice for 4 15-second periods at 2 to 3 minute intervals. For this procedure, a Branson 75 W machine was operated at 50 % of full power using a 0.5 inch horn. The resulting suspension was centrifuged at 1000 × *g* × 10 minutes, and the pellet discarded. The supernatant was then centrifuged at 140,000 × *g* × 10 minutes, and the pellet was resuspended in one-quarter the original reaction mixture volume of water, and stored frozen. Unlabeled 140,000 × *g* pellet, which was used as carrier in subsequent steps, was prepared as above, except that the chloroplast suspension was incubated without the addition of reagents used to carry out incorporation. One-quarter volume of water was added to the chloroplast suspension instead. After determination of radioactivity in protein, and protein, the labeled and unlabeled preparations were pooled, and structural protein prepared from it using sodium dodecyl sulfate, and urea as solubilizing agents (5). Radioactivity in protein was determined as usual, and the quantity of protein by Kjeldahl digestion and nesslerization (17).

Electron Microscopy. Chloroplasts were incubated in Honda medium plus 0.25 volume of water for 1 hour at 25°. They were then collected by centrifugation at 6000 × *g* × 0.5 hour, and washed once by resuspension in water and recentrifugation at 30,000 × *g* × 0.5 hour. This pellet was resuspended in a small volume of Honda medium which lacked mercaptoethanol, and contained phosphate instead of tris. Then the suspension was fixed in 2% glutaraldehyde, postfixed in 1% OsO_4, embedded, and examined as already described (16).

Results

When the reaction mixture was fractionated by differential centrifugation at the end of 1 hour of incorporation, the results presented in table I were obtained. Forty-two percent of radioactivity incorporated into protein was sedimented at 6000 × *g* × 20 minutes. Only 11% of radioactivity in protein was sedimented at forces above 6000 × *g*. Of this fraction, only 4% was sedimented above 30,000 × *g*. The remaining 50% of radioactivity in protein is found in the supernatant liquid from 140,000 × *g* × 60 minutes centrifugation. A portion of the radioactive protein in the 6000 × *g* pellet was not removed by washing. After 1 washing, the radioactive protein in the 6000 × *g* pellet remains essentially constant (table II). This result was obtained when the washing solution was Honda medium, or a much lower osmotic strength buffer solution.

Once washed 6000 × *g* pellet consists largely of chloroplast membranes (fig 1). Cross sections of stacks of grana in which the thylakoid compartments are swollen are clearly recognizable, and occur frequently. Tangential sections of granum thylakoids are also clearly recognizable. Stroma contents cannot be clearly distinguished. In contrast, freshly prepared chloroplasts, which are used in these studies, are largely intact, containing both the internal membrane system and stroma contents (16). During the incorporation incubation period chloroplasts lose their stroma contents. These chloroplasts appear to differ from washed 6000 × *g* pellet in that starch grains are still contained within the chloroplast membrane system (16). The presence of radioactive protein in a preparation which mainly consists of chloroplast lamellae suggests that leucine was incorporated in the protein of these lamellae.

Additional experiments confirm this conclusion. When 6000 × *g* pellet is washed twice and examined in a density gradient, the chlorophyll sediments forming 1 peak. The principal peak of radioactive protein is almost superimposable on the peak of chlorophyll (fig 2C). With unwashed, or once washed 6000 × *g* pellet, although a peak of radioactive protein is present in the same region as the

Table II. *Partial Removal of Radioactivity in Protein of 6000 × g Pellet Fraction by Repeated Washings*

Chloroplasts were incubated 1 hour, and replicate samples taken. To some trichloroacetic acid was added immediately. Others were chilled to 0°, and centrifuged for 20 minutes at 6000 × *g*. Supernatant fluids were decanted and trichloroacetic acid was added to them, and also to the residues which were first resuspended in water, or the residues were resuspended in washing medium. The latter were centrifuged at 30,000 × *g* × 30 minutes, and the supernatant liquid was pooled with the 6000 × *g* supernatant liquid. After the appropriate number of washings, trichloroacetic acid was added to the pellets which had been resuspended in water, and to the pooled washings. The isotonic medium was Honda solution (23). The hypotonic medium contained 10 mM tris-HCl (pH 7.8); 10 mM $MgCl_2$; and 4 mM mercaptoethanol. There were 458 cpm in the unfractionated reaction mixture in the experiment with isotonic medium and 392 cpm in the experiment with hypotonic medium.

Fraction	Amount of total incorporated radioactivity remaining in pellet after washing with	
	Isotonic medium	Hypotonic medium
	%	%
Total incorporation	100	100
6000 × *g* pellet	64	64
Pellet, 1 × washed	41	42
Pellet, 2 × washed	36	35
Pellet, 3 × washed	...	35

Table I. *Distribution of Radioactivity Incorporated into Chloroplast Protein Among Centrifugal Fractions of Reaction Mixture*

At the end of 1 hour incubation the 12 ml of reaction mixture was centrifuged at 6000 × *g* × 20 minutes, and the supernatant liquid was decanted, and centrifuged at the next higher centrifugal force. Each pellet was resuspended in 12 ml of water, and replicate samples of resuspended pellets, and the final supernatant liquid were taken for determination of radioactivity in protein. Dispersion is standard deviation for 3 replicate samples.

Fraction	CPM/fraction	% Of total
Total	7900 ± 340	100
6000 × *g* × 20 min pellet	3740 ± 224	42
10,000 × *g* × 30 min pellet	530 ± 10	5
30,000 × *g* × 30 min pellet	180 ± 16	2
140,000 × *g* × 60 min pellet	434 ± 18	4
140,000 × *g* × 60 min supernatant	3960 ± 360	50

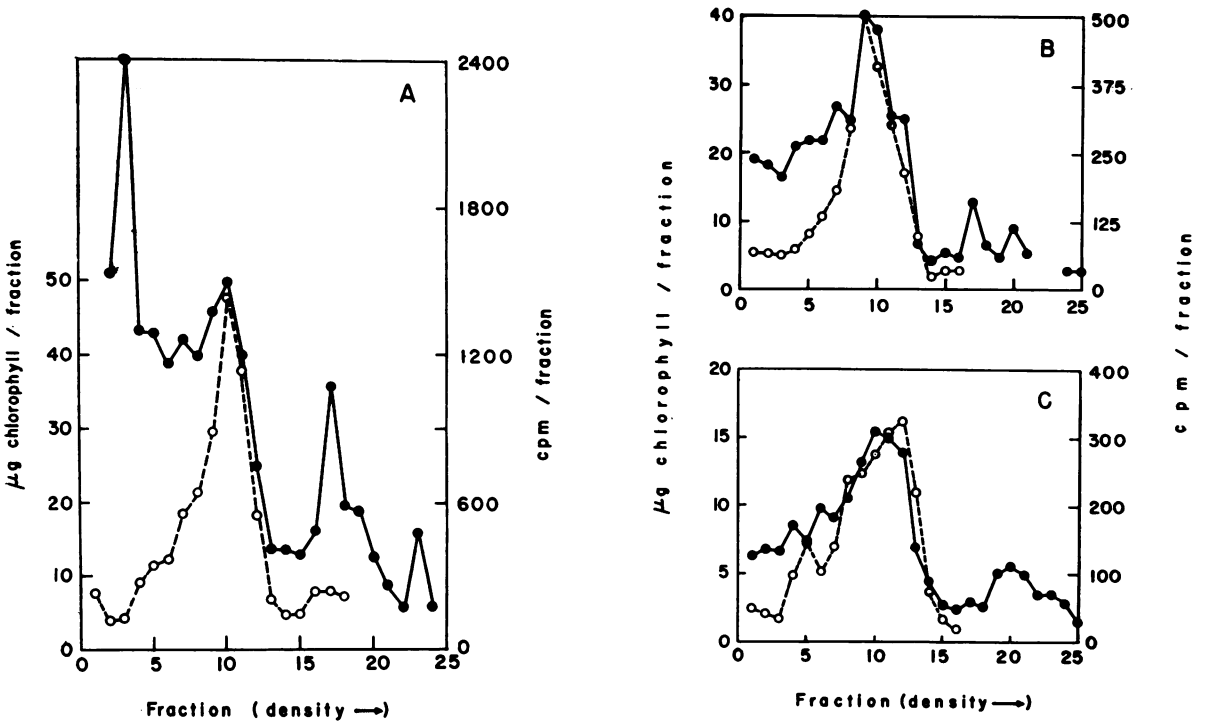

FIG. 2. Density gradient centrifugation of lamellar fraction prepared from labeled chloroplasts. A) Unwashed. B) Washed once with Honda medium. C) Washed twice with Honda medium. Solid circles represent the radioactivity in protein of the fractions. Open circles represent the chlorophyll contents of the fractions.

peak of chlorophyll, the curves are consistently not superimposable in the lighter density regions of the gradient. The discrepancy is greatest with the unwashed material. Here, the ratio of radioactive protein to chlorophyll is much higher than in the region of greatest chlorophyll concentration. Since this does not occur with pellets that have been washed twice, it is probably due to removal of radioactive protein from the chlorophyll containing particles in the suspended pellet, during resuspension, or gradient centrifugation, or both.

Chloroplast structural protein (5) was prepared from 6000 × *g* pellet and was found to be radioactive. The specific radioactivity of the structural protein equaled that of the protein of the chloroplasts from which it was prepared (table III).

The results presented above strongly suggest that the system under study is making protein which is incorporated into the lamellar structure of the chloroplast. However, the possibility exists that incorporation into the 6000 × *g* fraction represents formation of incomplete protein chains bound to ribosomes or other ribonucleoprotein attached to the lamellae, and these serve as precursors of stroma proteins. Evidence presented below indicates that radioactive leucine in the hot trichloroacetic acid

Table III. *Association of Incorporated Radioactivity with Chloroplast Structural Protein*

Details of procedure are given in Methods. Specific radioactivity of the acetone powder, and specific radioactivity of structural protein are corrected for dilution of 140,000 × *g* pellet with unlabeled carrier. A similar correction is applied to values of μg N/ fraction, and cpm in fractions.

Preparation stage	CPM/ fraction	μg N/ fraction	CPM/μg N
Reaction mixture after 1 hr incorporation	72,500	2290	32
6000 × *g* pellet	48 000	1430	34
6000 × *g* pellet, 1 × washed	20,300	765	27
140,000 × *g* × 10 min pellet of sonicated 1 × washed 6000 × *g* pellet	13,200	505	26
Sodium dodecylsulfate-urea extract of acetone powder of 140,000 × *g* pellet	6600	161	41
Structural protein	2890	82	35

Table IV. *Time Independence of Relative Incorporation of Amino Acid into Lamellar and Soluble Protein Fractions*

Incorporation was stopped at the indicated times by chilling samples rapidly to 0°, and unlabeled leucine was added. The samples were centrifuged at 6000 × *g*, and the supernatant fluids decanted. Pellets were resuspended in water, and recentrifuged at 30,000 × *g*. The resultant supernatant fluid was pooled with the first (soluble fraction). The pellet (lamellar fraction) was resuspended in water, and radioactivity in protein of it, and soluble fraction were determined. Percent radioactivity in lamellar fraction = (radioactivity in lamellar fraction)/ (radioactivity in lamellar fraction + radioactivity in soluble fraction). Values followed by (a) are the average of 3 experiments, and those followed by (b) are the average of 5.

Min	Amount of total incorporated radioactivity in protein of lamellar fraction
5	51 ± 1 (a)
15	52 ± 7 (a)
30	51 ± 8 (b)
45	46 ± 13 (b)
60	49 ± 10 (b)

insoluble portion of 6000 × *g* fraction is a stable end product of the incorporation reaction, probably protein. First, no change in the proportion of radioactivity incorporated into protein of the 6000 × *g* fraction as a function of time of incorporation is observed (table IV). That is, the ratio of radioactivity in protein of the 6000 × *g* pellet, to radioactivity in total protein, is constant. If the radioactive protein in the 6000 × *g* pellet fraction represented precursor of soluble protein, it might be expected that the proportion of radioactive protein in this fraction would reach a maximum value which would then decrease with time. Second, the amount of radioactivity in protein of soluble or insoluble fractions remains essentially constant after addition of unlabeled leucine, even though, in the absence of addition of unlabeled leucine, incorporation into each fraction continues (fig 3). For this experiment washed 6000 × *g* pellet is defined as the insoluble fraction, and the 6000 × *g* supernatant pooled with the washing is defined as the soluble fraction.

Analysis of soluble chloroplast protein, prepared as described in Methods, shows that during incorporation *in vitro* the chloroplasts make some soluble proteins in preference to others (fig 4). The curve representing distribution of protein in the gradient shows 2 distinct peaks. The faster sedimenting peak is probably Fraction I protein, since a preparation of Fraction I protein from spinach (14) has the same location in the density gradient when it is run in a separate replicate gradient. Using this protein as reference, and assuming a sedimentation coefficient of 18 S for it (24), it is calculated that the slower sedimenting peak has a sedimentation coefficient of 4 S. The pyruvate kinase added to the reaction mixture contributes only 5% to the protein in the extract which is analyzed by density gradient centrifugation. Distribution of protein in density gradients is the same whether extracts were prepared from chloroplasts suspended in a medium containing pyruvate kinase or lacking it. The curve representing distribution of incorporated radioactivity shows a single peak in the

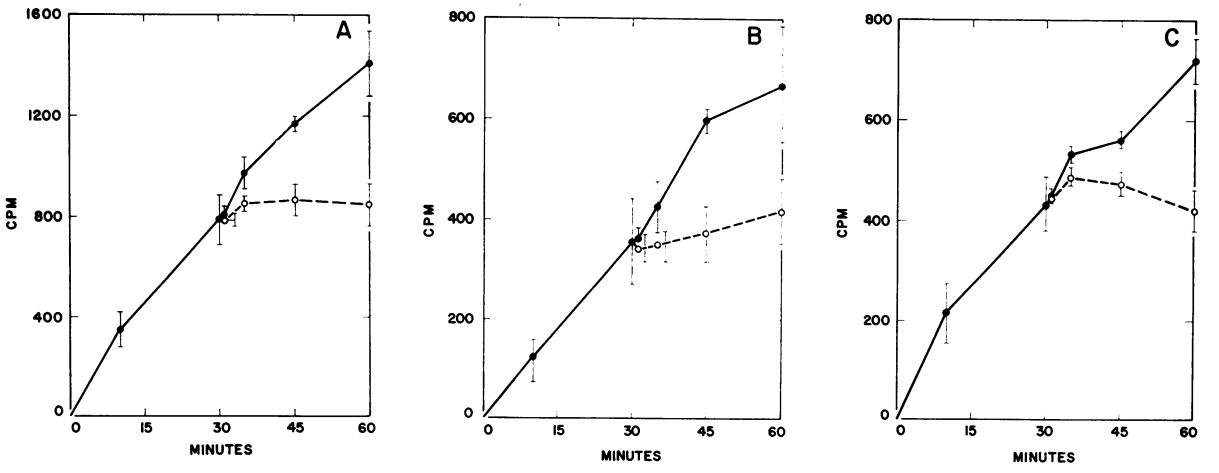

FIG. 3. Effect of addition of unlabeled leucine during amino acid incorporation on the time dependence of incorporation into lamellar and soluble chloroplast fractions. Radioactivity incorporated into protein: A) total; B) soluble fraction; C) lamellar fraction. Reaction mixture was incubated at 25°. After samples were taken at 30 minutes, a portion of the reaction mixture was transferred to a vessel containing 1 μmole unlabeled leucine per ml of reaction mixture. The undiluted reaction mixture contained 5 mμmole leucine/ml. Incubation was continued at 25°, and samples were taken from the vessel which contained undiluted amino acid (●), and from the vessel to which unlabeled amino acid was added (○). Samples were fractionated into lamellar and soluble fractions as described in table IV.

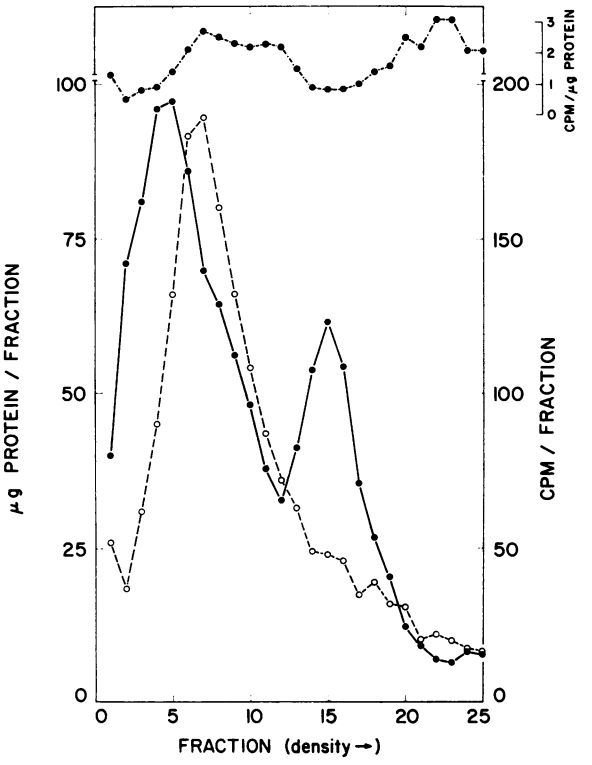

FIG. 4. Density gradient centrifugation of labeled soluble proteins from chloroplasts (30,000 × *g* supernatant. See Methods for details). Protein per fraction, ●. Radioactivity in protein, ○. Each point represents an average value for 3 replicate density gradients.

vicinity of the slower sedimenting protein peak, but somewhat displaced in the direction of the bottom of the density gradient tube. It has a calculated sedimentation coefficient of 7 S. A number of enzymes of the chloroplast photosynthetic carbon reduction cycle have similar sedimentation coefficients (5), corresponding to a molecular weight, in the case of spinach chloroplast aldolase, of 140,000. Protein amounting to 5% of the total applied to the density gradient, and radioactivity amounting to 10%, is found sedimented at the bottom of the tube. This probably represents protein and radioactivity in membrane fragments, and in ribosomes, since the protein preparation examined was obtained by removal of particles sedimenting at forces of 30,000 × *g*.

The assymetry of the peak of radioactivity in the density gradient presented in figure 4 suggests that some labeled Fraction I protein may be formed during incorporation, even though no peak of radioactivity is found in the Fraction I region. An enrichment for Fraction I protein was made by centrifuging labeled soluble protein at 140,000 × *g* × 4 hours. When the pellet obtained was redissolved and analyzed in a density gradient, the major protein peak corresponded to Fraction I protein, and there was a clear indication of a peak of radioactive protein associated with it (fig 5).

Discussion

The protein in the 140,000 × *g* supernatant liquids, obtained from incorporation reaction mixtures, probably consists largely of chloroplast stroma proteins, and may contain some easily solubilizable portions of the internal membrane system, and the spaces this membrane system encloses (18). The calculated sedimentation coefficients of 18 S, and 4 S, based on a comparison with known Fraction I protein, correspond to the sedimentation coefficients of Fractions I and II proteins (18). Since density gradient analyses were run with 30,000 × *g* supernatant, rather than 140,000 × *g* supernatant liquid, intact ribosomes and ribosome fragments would also be present in the soluble protein fraction that was analyzed. However, intact ribosomes would be expected at the bottom of the density gradient tube,

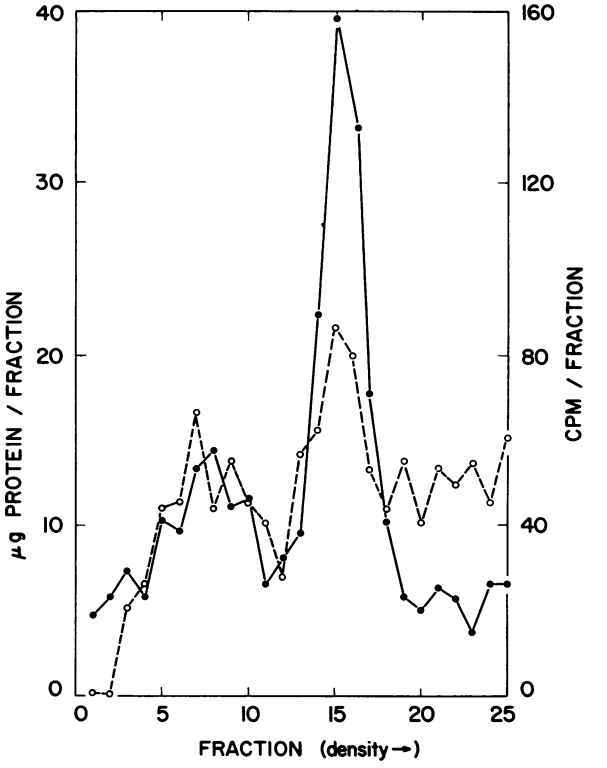

FIG. 5. Density gradient centrifugation of crude Fraction I protein prepared from labeled chloroplast soluble protein. Protein per fraction, ●. Radioactivity in protein, ○. The data represent the analysis of a single density gradient. Crude Fraction I protein was obtained as follows. Thirty thousand × *g* supernatant was centrifuged at 140,000 × *g* × 4 hours. The pellet was dissolved in 50 mM potassium maleate (pH 7.0) and residual insoluble material removed by centrifugation at 1000 × *g*.

since they have a sedimentation coefficient of 70 S (2, 3). Chloroplast ribosome subunits of sedimentation coefficients 35 S and 50 S are observed at low concentrations of magnesium (2, 3). These subunits, if present, would be expected at a position in the density gradient reasonably well removed from the 18 S Fraction I protein peak, probably as part of the ribosomal pellet at the bottom of the tube. For these reasons it is felt that the radioactivity in hot trichloroacetic acid precipitable material, which is found throughout the gradient, is in free protein chains, and does not represent protein bound to ribosomes, or ribosomal subunits.

Analysis by density gradient centrifugation of the soluble protein fraction extracted from chloroplasts shows that the distribution of radioactivity in protein differs from the distribution of protein (fig 4). This suggests that only some chloroplast proteins are being synthesized by this system, or that some are being synthesized more actively than others. This difference in distribution of protein, and radioactivity in protein represents the synthetic capabilities of the chloroplasts *in vivo* at this stage of development, rather than an artifact resulting from chloroplast isolation. When leaves, instead of chloroplasts, are incubated with radioactive leucine, and chloroplasts isolated, extract prepared, and analyzed, it is found that the relative distributions of protein and radioactivity in protein are the same as those presented in figure 4 (unpublished results). Radioactive Fraction I protein, although masked by radioactive 7 S protein (fig 4), is formed *in vitro* (fig 5). Since Fraction I protein is primarily ribulose-1,5-diP carboxylase (24), an enzyme localized in the chloroplasts (11), the results presented are regarded as preliminary evidence that chloroplasts can synthesize this chloroplast protein.

The products formed by the chloroplast amino acid incorporating system being studied differ quantitatively from products formed with other chloroplast systems. Spencer (22) found that incorporation into soluble protein accounted for 25 % of the product formed in his system, compared to the 50 %, or more, found in the present work. Spencer (22) also found indications that "nascent" protein, probably on ribosomes, accounted for 43 % of the amino acid incorporated into protein. In contrast, only 5 % of our radioactive protein product is present in the pellet sedimenting between 30,000 and 140,000 $\times$ g, where one would expect ribosomes. Further, the experiments presented in figure 3 indicate that only a small portion of the radioactive protein in the lamellar fraction is "nascent" protein.

The soluble protein is a large fraction of the radioactive protein product formed by wheat chloroplasts *in vitro* (1). The proportion of soluble protein product of this system more nearly corresponds to that of the bean chloroplast system. With wheat the percent radioactivity in protein in the soluble protein fraction increases with time, and suggests that the insoluble component acts as precursor of the soluble. This result is not obtained with the bean chloroplast system (table IV). This difference may be explained by the differences in conditions used to measure incorporation. With wheat, chloroplasts were suspended in a low ionic strength medium which probably resulted in breakage of chloroplasts, and release of ribosomes into the medium. Thus the wheat chloroplast system can be considered a crude chloroplast ribosome preparation, in contrast to the bean system in which chloroplasts are intact at the start of incorporation. In contrast to the large fraction of soluble protein formed by the wheat and bean systems, the product of amino acid incorporation by ribosomes from *Euglena* chloroplasts was almost entirely bound to the ribosomes (7). Since little radioactivity is incorporated into the ribosome fraction, or into "nascent" protein, the bean chloroplast amino acid incorporating system must be completing protein chains and releasing them from ribosomes.

Spencer (22) presented evidence that some of the protein formed by a spinach chloroplast amino acid incorporating system is incorporated into protein of the insoluble lamellar fraction. Likewise, the bean chloroplast system is incorporating amino acid into lamellar protein. First, chlorophyll and radioactivity in protein of washed lamellar fraction sediment together in a density gradient. These washed lamellar fractions appear to consist primarily of internal chloroplast membranes. No attempt was made to rule out the possibility that another particle fraction, lacking chlorophyll, sediments in the same position as the chlorophyll containing lamellae. However, previous work indicates that radioactivity in mitochondria, the most likely candidate for a contaminant of similar density, contributes only a small portion to the total radioactive protein (17). Second, no more than a small amount of radioactivity in the lamellar fraction is removed from it when chloroplasts are incubated with unlabeled leucine under conditions necessary for incorporation (fig 3). If the radioactivity in the lamellar fraction was an intermediate in the formation of the radioactive protein found in the soluble fraction, removal of label from the lamellar fraction would be expected under these conditions. Third, structural protein which is radioactive can be prepared from chloroplasts that have incorporated amino acid. The interpretation of this experiment depends on what "structural protein" preparations represent. They are prepared from the insoluble fractions of mitochondria (6), and chloroplasts (5). In the case of the former, there is some evidence that the preparation consists of a single protein component (6).

The soluble protein fraction, as a whole, does not seem to be an intermediate in the formation of lamellar protein, and *vice-versa*. This conclusion is drawn from the observation that no appreciable label is transferred from the protein of the soluble fraction to the lamellar fraction, and *vice-versa*.

Interpretation, however, is somewhat complicated due to the fact that the lamellar fraction that was used was 6000 × *g* pellet, and not 140,000 × *g* pellet. However, since only a small portion of the radioactivity in protein sediments between 6000 and 140,000 × *g*, it is felt that the difference between these 2 procedures would not have been significant for the experiment considered. Among the possibilities to be considered for the formation of lamellar protein are the following. A small fraction of the soluble protein might act as precursor for the lamellar protein. Alternatively, chloroplast ribosomes, or other ribonucleoprotein attached to the lamellae might be the sites of lamellar protein formation, in view of the observation that RNA is associated with the lamellar system (10). Still another possibility is that ribosomes in the stroma synthesize lamellar protein, but direct contact between the ribosome bound protein chain and the lamellae is necessary for incorporation into the lamellar system. This possibility is analogous to the hypothesis proposed to explain transfer of protein from cytoplasmic microsomes to mitochondria, which may be involved in the synthesis of the soluble proteins of the mitochondria, and the proteins which are loosely bound to mitochondrial membranes (12).

The protein synthesis which is carried out by intact chloroplasts differs from that carried out by mitochondrial systems. Unlike chloroplasts, mitochondria form only insoluble protein *in vitro* (19, 20, 21, 25). This observation seems to be explained by reports which indicate that the soluble, or readily extractable proteins of the mitochondria are synthesized by cytoplasmic ribosomes and are then transferred to the mitochondria (4, 9, 12). This difference may indicate a higher degree of genetic autonomy for the chloroplast.

Acknowledgment

The authors are indebted to Dr. E. Gantt for the preparation of the electron micrograph which appears as a figure in this article.

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