Plant Physiol. (1967) 42, 1179–1186

In Vitro Protein Synthesis by Plastids of Phaseolus vulgaris. I. Localization of Activity in the Chloroplasts of a Chloroplast Containing Fraction from Developing Leaves[1, 2]

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Received March 29, 1967.

Summary. The incorporation of uniformly labeled leucine-^{14}C into protein by a chloroplast containing fraction from developing primary leaves of bean is reported. Chloroplasts, obtained from week old plants grown in darkness, and then illuminated with white light for 12 hours, were shown to be the principal sites of incorporating activity. Incorporation may continue for 2 hours. Rates of up to 50 $\mu\mu$mole leucine incorporated per mg protein per hour are observed when a 1 hour assay period is used. Incorporation is only partially sensitive to ribonuclease.

Chloroplast containing fractions from leaves of higher plants (2, 4, 12, 14, 25, 28, 30, 31), and from algal cells (10, 13) have been reported to incorporate amino acid into protein. Likewise, ribosome preparations from chloroplasts of higher plants (1, 5, 6, 26), and chloroplasts of algae (11) incorporate amino acid into protein. The chloroplast fractions which are used contain contaminating subcellular organelles, and whole cells (bacterial, and leaf, or both). Evaluation of the contribution of these contaminants to amino acid incorporation in vitro has been a continuing problem (see works cited above).

This report deals with amino acid incorporation by a chloroplast preparation from leaves in which chloroplasts are undergoing light dependent development. Its aim was to clearly establish the chloroplasts in this preparation as the sites of the amino acid incorporation that was observed.

Methods

Plant Material. Phaseolus vulgaris L. var. Black Valentine was used throughout. Etiolated plants were grown in the dark for 6 days at 25° (18). The plants were then illuminated for 12 hours with 1000 ft-c of white fluorescent light at a temperature of about 28°.

Preparation of Homogenates and Homogenate Fractions. Leaves were picked and chilled on ice. Thirty g of leaves were soaked for 15 minutes at 0 to 4° in about 300 ml of a saturated solution of calcium hypochlorite, to which about 0.5 ml of a 1:20 (v:v) solution of Dow antifoam B had been added. They were then washed 6 times with 300 ml portions of sterile cold distilled water, thoroughly drained, and blotted dry.

Leaf minces were prepared using Honda medium as described by Spencer and Wildman (30) with the following modifications. The concentration of tris in the medium was increased from 0.025 M to 0.05 M; the pH was adjusted to 7.8 at 20° (8.2 at 0°); 2 ml of medium was used per g of leaves instead of 1 ml per g of leaves. These modifications were necessary to maintain the pH of the mince at 7.8 to 8.0 at 0°. To minimize bacterial contamination, the medium was prepared by dissolving the reagents in sterile distilled water, chilling rapidly, and freezing in small lots. Medium was thawed once, kept on ice, and if not used that day, discarded. The homogenate was squeezed through a pad of glass wool wrapped in 2 layers of bleached cheesecloth, followed by filtration through 4 layers of facial tissue. Filtration removes most whole leaf cells. Disposable plastic gloves were used during chopping, and in expressing liquid from the glass wool pad to minimize contamination with exogenous ribonuclease (15). The homogenate was sequentially centrifuged at 1000 $\times$ g $\times$ 10 minutes, 12,000 $\times g \times$ 30 minutes, and 144,000 $\times$ g $\times$ 120 minutes (30), and pellets were resuspended in fresh medium, the same as used to prepare the mince. The resuspended 1000 $\times$ g pellet is the chloroplast preparation used standardly.

Standard Assay for Determination of Amino Acid Incorporating Activity. Assay was carried out by the procedure of Spencer and Wildman (30). Reagents other than L-leucine-^{14}C, uniformly labeled (New England Nuclear Corporation, Boston, Massachusetts), P-enolpyruvate (Sigma Chemical Corporation, St. Louis, Missouri), and pyruvate kinase (Sigma, type II from muscle; E.C. 2.7.1.40) were

[1] Research was supported in part by funds provided by the United States Atomic Energy Commission Contract No. AT(30-1)2372.

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

[3] The work reported was carried out while Dr. Parenti was holder of a National Research Council Visiting Research Associateship at the Smithsonian Institution.

stored frozen in small batches, thawed once, and the unused portion discarded. The labeled leucine, in 0.01 N HCl, had a specific activity of about 250 mc/mmole, and was used without further purification. Unless otherwise stated, there were 8 μg of chlorophyll, in 0.8 ml of Honda medium in 1 ml of reaction mixture. Incorporation was a linear function of chlorophyll concentration over a range of 2 to 40 μg/ml. Reaction mixture components were combined at 0°, and the incorporation reaction started by adding radioactive amino acid, and warming to 25°. Incubation normally continued for 1 hour. The reaction was stopped by addition of an equal volume of 0.1 M L-leucine in 10 g trichloroacetic acid/100 ml solution to a 0.5 ml sample of reaction mixture. After incubation overnight at 4° to completely coagulate protein, the precipitate was prepared for counting essentially as described by Spencer and Wildman (30), and unless otherwise stated, included extraction of nucleic acids with hot trichloroacetic acid (24). At least 500 counts were observed per sample, using thin window counters of either Geiger or proportional types. Self-absorption was negligible.

Evaluation of Contribution of Bacteria, Leaf Cells, and Nuclei to Incorporation, by Treatment with Triton. At the end of the appropriate incubation times, 0.5 ml samples were taken from assays for incorporation and added to an equal volume of 0.2 ml Triton X-100 (Rohm and Haas, Philadelphia, Pennsylvania) in 100 ml of 0.1 M L-leucine, mixed thoroughly, incubated on ice for 20 minutes, and centrifuged at $6000 \times g \times 20$ minutes at 0 to 4°. This amounted to 2 to 5 mg of detergent/mg of chloroplast protein. The supernatant fluid (Triton supernatant) was carefully removed with a hypodermic syringe. A small white pellet was left behind (Triton pellet) which was resuspended in 0.1 ml of 0.5 mg/ml bovine serum albumin. Trichloroacetic acid was added, and precipitates were then treated and plated for counting as described under Standard Assay. Viable bacteria were determined by plate counts on nutrient agar.

Autoradiography. Procedures were generally those described by Caro (8) and Prescott (21). Slides were cleaned in dichromate cleaning solution and were coated with gelatin. About 0.01 ml of fixed chloroplast suspension, containing 20 μg chlorophyll per ml was smeared on the slides and air dried. In these experiments 45 mμmole L-leucine-4,5-^{3}H (New England Nuclear Corporation) containing 10 μc, and chloroplast suspension containing 40 μg chlorophyll were used per ml final volume of amino acid incorporation reaction mixture. Other reaction mixture components were the same as described under Standard Assay. Immediately after addition of radioactive amino acid, or after 1 hour incubation at 25°, an equal volume of solution containing 0.4 M sucrose, 0.2 M phosphate, and 1 g OsO_4/100 ml at pH 7.8 was added to samples of the reaction mixture, and incubated 1 hour at 0° before samples were taken for smearing. Smeared slides were extracted with 0.05 M L-leucine, in 5 g/100 ml trichloroacetic acid at 0° for 15 minutes, followed by extraction in fresh solution at 80° for 15 minutes, washed overnight in running tap water at 0°, rinsed in distilled water, and air dried. Some slides were extracted with ethanol:ethyl ether (3/1:v/v) at 40° for 1 hour, and air dried. Slides were coated with Kodak NTB-3 emulsion that had been diluted with 1 volume of water, and were dried and stored at 4° over silica gel. Emulsion was developed for 2 minutes with full strength Kodak Dextol at 20°, fixed, washed 1 hour in running tap water, rinsed in distilled water, and air dried. They were then stained 0.25 hour at room temperature with 0.2 g orcein/100 ml 45 % acetic acid, and mounted in Euparol. Slides were examined without the observer knowing which slides were being scored. Random portions of the slides were scored for grains per chloroplast, and grains/unit slide area.

Chemical and Enzymatic Determinations. Protein was routinely determined by a modified Kjeldahl digestion followed by determination of ammonia formed with Nessler reagent (16, 32). Samples were first precipitated in 5 g trichloroacetic acid/100 ml, incubated overnight at 4°, heated at 80° to 90° for 15 minutes to remove nucleic acids (24), the precipitate collected by centrifugation, washed once, and digested. Extraction with hot trichloroacetic acid was necessary since 30 % of the acid precipitable nitrogen in the chloroplast fraction was removed by this procedure. Determination of protein of the chloroplast fraction by the Folin method (17) was inadequate. A chloroplast preparation which contained 0.14 mg/ml of protein as determined by the above procedure, contained 0.91 mg protein by the Folin method. If protein was first precipitated with tungstic acid, to remove reducing substances that interfere with the Folin procedure, only 0.01 mg protein was found. This low value was due to failure of the tungstate precipitate of the chloroplast preparation to dissolve, even in 1 N NaOH.

RNA was determined with orcinol (9) using RNA (Type XI, Sigma Chemical Corporation) as standard. Samples were precipitated, and washed 3 times with cold trichloroacetic acid solution, before extraction of nucleic acids with trichloroacetic acid solution as above. Residues were used for determination of protein nitrogen.

Chlorophyll was determined spectrophotometrically on 80 % acetone extracts (3). Samples in Honda medium no larger than 0.2 ml were diluted to 1.0 ml with water, and 4.0 ml 100 % acetone added, in that order. This procedure was required because of the presence of dextran and ficoll in the sample, which easily trapped, and left chlorophyll unextracted, in the gummy precipitate that was formed if 80 % acetone was added directly.

Cytochrome c oxidase (E.C. 1.9.3.1) activity was determined by following the oxidation of cytochrome c that had been reduced with hydrogen and 5 % palladium asbestos (27). In the range of activities used, oxidation measured was proportional to the amount of homogenate fraction added.

Results

Incorporation by Homogenate Fractions. As has been observed in other chloroplast preparations (30), a portion of the radioactivity incorporated into cold trichloroacetic acid precipitable material by 1000 × *g* × 10 minute pellet can be removed by extraction with hot trichloroacetic acid (fig 1). Incorporation into hot trichloroacetic acid extractable material reaches a maximum, or plateau, in 5 minutes. The exact shape of this curve cannot be clearly defined because of the variability of the determinations in this set of experiments. Incorporation into material insoluble in hot trichloroacetic acid continued for 1 hour, and in some instances was observed to continue for 2 hours.

Heating the chloroplast fraction 3 minutes at 100° destroys incorporating activity. Incorporation by the chloroplast fraction was inhibited 90 % on omission of ATP and the ATP generating system. Approximately 25 % inhibition was observed when either the mixture of unlabeled amino acids, or the mixture of 3 nucleoside triphosphates (other than ATP) was omitted. Of the fractions examined (see Methods), the 0 to 1000 × *g* × 10 minute pellet had both the highest total activity, and highest activity/mg protein of any of the homogenate fractions, as has been observed elsewhere (28, 30).

Approximate calculations of rates of incorporation of leucine were made assuming that the added radioactive leucine was not being diluted by nonradioactive amino acid. Rates of 1000 μμmoles leucine/mg chlorophyll × hour, corresponding to 50 μμmoles leucine/mg protein × hour could be obtained regularly when the chloroplast preparation was prepared quickly and assayed immediately.

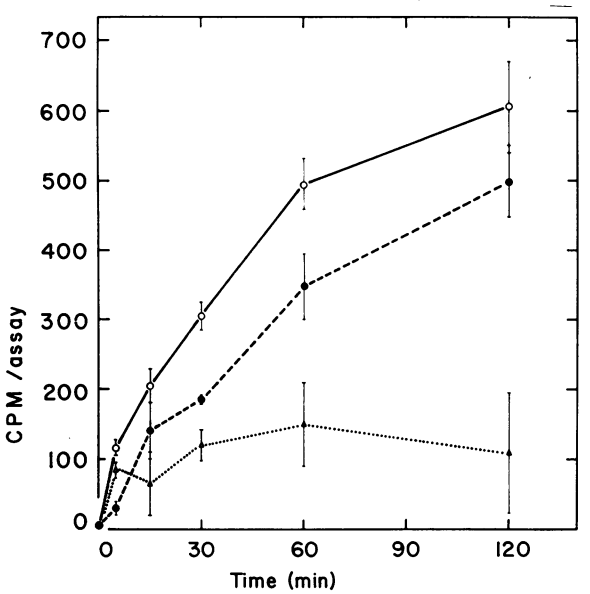

FIG. 1. Time course of incorporation of L-leucine ^{14}C by chloroplast fraction. Incorporation into A) material precipitated with cold trichloroacetic acid (○—○), B) material precipitated with cold, and then extracted with hot trichloroacetic acid (●—●), and C) the difference between A and B (△ . . . △). Dispersions are standard deviations for 3 0.5 ml samples of reaction mixture.

Composition of the Chloroplast Fraction. Microscopic observation of the 0 to 1000 × *g* × 10 minute fraction shows that, in addition to chloroplasts, it frequently contains some whole leaf cells (hair and gland). It always contains numerous starch grains; numerous structures much smaller than chloroplasts, which are probably mitochondria; and on staining with acetocarmine, nuclei can be seen. Further, plate counts show the presence of 10^3 to 5 × 10^4 bacteria per ml of chloroplast suspension containing about 10, and 100 μg chlorophyll, and protein, respectively. The presence of mitochondria is confirmed by finding cytochrome oxidase activity in the chloroplast fraction. Contaminating cytoplasmic ribosomes may also be present in the preparation.

Negligible Contribution of Bacteria, Leaf Cells, and Nuclei to Incorporation by the Chloroplast Fraction. When chloroplasts are prepared from non-sterile buffer solutions, the bacterial contamination ranges from 10^5 to 10^6 bacteria per assay. Such contamination has been reported to contribute significantly to incorporating activity of plastid preparations and other large subcellular particles (2, 33). When leaves are surface sterilized, and sterile or near sterile reagent solutions are used, bacterial contamination can be kept down to 10^3 to 5 × 10^4 cells per assay. Non-ionic detergents have been reported to have no effect on bacterial growth (23). The non-ionic detergent, Triton *X*-100 has been reported to leave nuclei (30) and bacteria (4) unaffected, while fragmenting chloroplasts. The usefulness of Triton X-100 to separate chloroplastic material from bacteria, leaf cells and nuclei of the chloroplast fraction used for these amino acid incorporation studies was examined.

Plate counts showed that Triton had no effect on the number of viable bacteria that were present in chloroplast preparations (table I). Further, few bacteria were found in the 6000 × *g* × 20 minute supernatant after Triton treatment, while most were recovered in the pellet. However, under our conditions, centrifugation at 1000 × *g* × 10 minutes (4) was insufficient to insure removal of more than 50 % of the bacteria from the Triton treated chloroplast preparation. When bacteria were labeled by incubation in the standard amino acid incorporation system, treated with Triton, and Triton pellet and supernatant precipitated with hot trichloroacetic acid, little of the incorporated radioactivity was recovered in the Triton supernatant. The percent of total incorporated radioactivity in the Triton supernatant was the same, both after 60 (table I, expt II), and 30 minute incubation with radioactive amino acid. In this experiment, 10^5 times the number of bacteria usually present in reaction mixture containing chloroplasts was used.

As already mentioned, leaf cells are occasional contaminants of the chloroplast preparations. These cells are recovered in the Triton pellet (table I, expt

Table I. *Effect of Triton X-100 on Bacteria, Whole Cells and Nuclei*

These are representative results from 4 different kinds of experiments. Reaction mixtures, bacterial suspensions, or chloroplast containing 1000 × *g* × 10 minute fraction were treated with Triton (see Methods). In the first (column 3), reaction mixture from a standard assay for incorporation was examined for the effect of Triton on the number of viable bacteria, and on their recovery in the Triton pellet. In the second (column 4), a concentrated suspension of bacteria was incubated with materials used to assay incorporation by chloroplasts, but the chloroplast suspension was omitted. Distribution between Triton supernatant and Triton pellet, of radioactivity incorporated into protein was determined. In the third (column 5), distribution of whole leaf cells in the Triton pellet and Triton supernatant was determined. In this experiment, whole leaf cells were added to the chloroplast fraction, since it normally contains few. In the fourth (column 6), the effect of Triton on nuclei was examined. Here, chloroplast suspension was treated, or not treated with Triton, after 20 minutes, centrifuged at 6000 × *g*, and the pellets suspended, fixed, and stained in small volumes of acetocarmine.

Treatment	Fraction	Expt I Bacteria × 10^{-4}	Expt II Bacteria cpm	Expt III Leaf cells	Expt IV Nuclei
				Relative values	Relative values
None	Total	4.0 ± 1.3	3630 ± 30	51 ± 10	88 ± 40
Triton	Total	3.6 ± 1.2	...	...	...
Triton	6000 × *g* pellet	2.5 ± 0.6	2590 ± 500	39 ± 15	110 ± 27
Triton	6000 × *g* supernatant	0.1 ± 0.07	368 ± 178	1	...

Table II. *Effect of Ribonuclease on Intact Chloroplasts, Broken Chloroplasts, and Cytoplasmic Ribosomes*

In experiment I, 1.2 mg ribosome protein per assay was incubated with radioactive amino acid for 30 minutes. In experiment II, 0.08 mg chloroplast protein per assay, suspended either in isotonic (Honda medium) (30), or hypotonic (0.01 M tris, pH 7.8; 5 mM magnesium acetate; 3 mM mercaptoethanol) medium was incubated with radioactive amino acid for 60 minutes. The numbers in parenthesis are the counts incorporated/assay in the absence of ribonuclease. Incorporation was determined as described in Methods. Ribonuclease was added to the reaction vessels kept on ice, then the chloroplast suspension was added, followed shortly by the remaining reaction mixture components. As usual, the reaction was started by addition of labeled amino acid and warming to 25°.

Ribonuclease conc	Percent inhibition of incorporation Expt I Cytoplasmic ribosomes in isotonic medium	Expt II Chloroplasts in isotonic medium	Expt II Chloroplasts in hypotonic medium
μg/ml			
	0 (341 cpm)	0 (400 cpm)	0 (110 cpm)
0.004	39	44	...
0.04	70	42	95
0.4	91	40	92
4.0	90	50	93
40.0	97	57	87

Table III. *Autoradiography of Chloroplast Suspension Allowed to Incorporate* L-*Leucine-4,5-*^3H

Chloroplasts were either fixed immediately after addition of leucine ^3H, (0 min incubation with leucine ^3H) or after an hour incubation at 25° with leucine ^3H (60 min with leucine ^3H). Grains/10μ^2 represents the grain count in non-plastid areas per area equivalent to that of a chloroplast. Preparation of slides for autoradiography included extraction with ethanol:ether (3/1 :v/v). Dispersion is standard deviation for 3 slides.

Weeks emulsion exposed	Min chloroplasts incubated with leucine ^3H at 25°	Grains/plastid (uncorrected)	Grains/10μ^2 random portions of slides	Grains/plastid (corrected for background)
0	0	0.10 –	0.02	0.08
4	0	0.53 ± 0.09	0.08	0.45
	60	1.17 ± 0.05	0.07	1.10
8	0	0.52 ± 0.12	0.11	0.41
	60	1.62 ± 0.05	0.10	1.52

III), and are probably undamaged by the treatment. Their contents are intact, whole plastids and nuclei being recognizable in them.

Nuclei, which are regular contaminants of the chloroplast preparations, are also unaffected by Triton (table I, expt IV), and are recovered in the Triton pellet. No morphological difference was found between nuclei from standard chloroplast preparations, and preparations treated with Triton. Thus, the Triton pellet can be used to evaluate the contribution of bacteria, leaf cells, and nuclei to the amino acid incorporation observed in crude chloroplast preparations.

A time course of incorporation into Triton pellet, Triton supernatant, and untreated reaction mixture carried out by the chloroplast fraction shows that

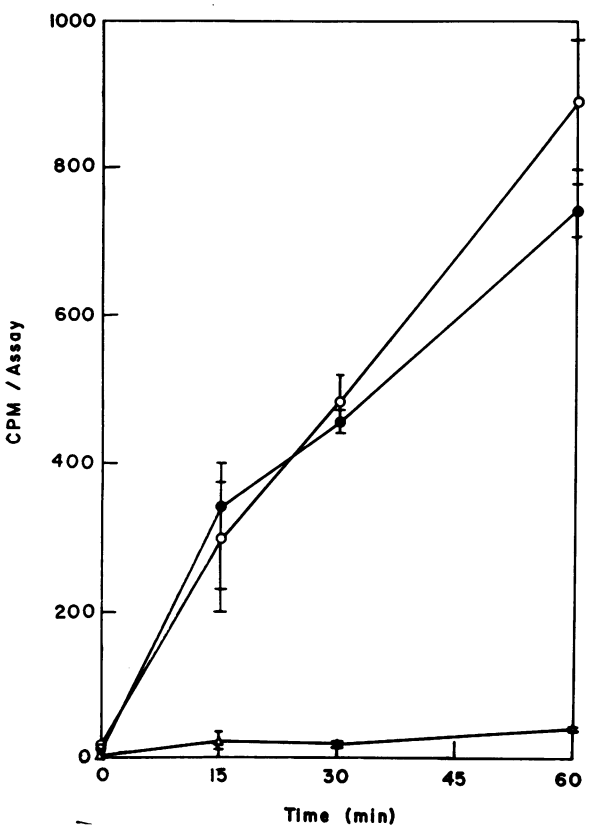

FIG. 2. Evaluation of contribution of bacteria, leaf cells, and nuclei to amino acid incorporation of a chloroplast fraction as determined by fractionation of reaction mixture after treatment with Triton X-100. Chloroplast fraction was incubated with L-leucine ^{14}C, and incorporation into hot trichloroacetic acid precipitated material was determined on: A) samples not treated with Triton (○—○) (chloroplasts, bacteria, leaf cells, and nuclei), and on samples after treatment with Triton and separation into B) a fraction not sedimented at 6000 × *g* × 20 min (●—●) (chloroplasts), and C) a fraction sedimented at 6000 × *g* × 20 min (△—△) (bacteria, leaf cells, and nuclei). Incubation conditions, and determination of incorporation with and without treatment with Triton are described in Methods. Dispersion represents standard deviation for 3 samples.

incorporation by bacteria, leaf cells, and nuclei is small for incubation periods of 0.25 to 1.0 hour (fig 2). At the end of an hour incubation with radioactive amino acid, the contribution of bacteria, leaf cells, and nuclei was 2 to 10 % of the observed total incorporation, as measured by the radioactivity in the Triton pellet.

Contribution of Mitochondria and Cytoplasmic Ribosomes to Incorporation Activity of the Chloroplast Fraction. Mitochondria have been found to make only a small contribution to the amino acid incorporating activity of the chloroplast preparation currently under investigation. The chloroplast rich fraction (0–1000 × *g* × 10 min) of bean leaf homogenates had 0.68 units of cytochrome oxidase/mg protein, while the mitochondria rich fraction (1000–12,000 × *g* × 30 min) had 3.70/units of cytochrome oxidase/mg protein. The chloroplast fraction incorporated amino acid at a rate of 6000 cpm/mg protein × hour while the mitochondria rich fraction incorporated amino acid at a rate of 580 cpm/mg protein × hour. Assuming that the activity of the latter fraction is due only to mitochondria, and that each mitochondrion contributes equally to incorporation, it is calculated that their contribution to incorporation by the chloroplast fraction is less than 2 %. It is worth noting that the ratio of protein to chlorophyll in the chloroplast and mitochondrial fractions is the same. Therefore, the chlorophyll containing particles in the mitochondrial fraction are comparatively inactive in amino acid incorporation.

Twenty percent of the ribosomes in a ribosome preparation from crude chloroplasts may be cytoplasmic ribosome (6, 28). However, cytoplasmic ribosomes are considerably less active in amino acid incorporation than the chloroplast contained ribosomes (28). Similarly, cytoplasmic ribosomes make a small contribution to the incorporation of the chloroplast preparation under study. A ribosome preparation (12,000–144,000 × *g* × 120 min fraction) incorporated amino acid at a rate of 1200 cpm/mg RNA × hour, while a chloroplast preparation (0–1000 × *g* × 10 min fraction) incorporated amino acid at a rate of 17,000 cpm/mg RNA × hour. Thus it is calculated that only 7 % of the observed incorporation by the chloroplast fraction is due to contaminating ribosomes, assuming all the RNA in the chloroplast fraction is in contaminating ribosomes.

Amino acid incorporating activity of chloroplasts is less sensitive to ribonuclease (E.C. 2.7.7.16) than crude cytoplasmic ribosomes (table II). The ribosome preparation is inhibited 90 % by 0.4 mg ribonuclease/ml, while the incorporation by the chloroplast preparation is inhibited only 50 %. Higher concentrations of ribonuclease do not further increase the inhibition of incorporation by the chloroplast preparation. Maximum inhibition of incorporation by the chloroplast fraction can be obtained at concentrations as low as 0.004 μg/ml. It is concluded that the incorporation by the chloroplast preparation is carried out by 2 different systems, one as, or more sensitive

to ribonuclease than the ribosome preparation, and another, insensitive to ribonuclease. Since the activity insensitive to ribonuclease cannot be attributed to incorporation by whole cells, it is hypothesized to be due to intact chloroplasts, impermeable to ribonuclease. Chloroplasts prepared by the method used in the present work are reported to contain a large proportion with chloroplast membranes (29). The remaining activity which is sensitive to ribonuclease may be due to broken chloroplasts, or in part due to free chloroplast ribosomes. If membrane enclosed particles are responsible for the ribonuclease insensitive incorporation, breaking of the membranes should result in sensitivity comparable to that of the cytoplasmic ribosome preparation. Suspending chloroplasts in hypotonic medium renders the resulting incorporating activity sensitive to ribonuclease (table II). However, unlike previous observations (12), suspension in hypotonic medium resulted in a 70 % decrease in the activity of the preparation. Whether this loss occurs at the expense of both the ribonuclease sensitive, and insensitive activities is problematical.

Autoradiography of Plastid Suspensions. Evidence for localization of incorporated amino acid in the chloroplasts of the 1000 × *g* × 10 minute fraction has been obtained by autoradiography. There is an increase in grain count over the chloroplasts as a result of incubation with tritiated leucine for 60 minutes at 25° (table III). The grain count over plastids incubated with tritiated leucine at 25° was 2 times that of control plastids after a 4 week exposure of the emulsion, and 4 times after an 8 week exposure. The grain count over the control plastids increases with exposure time, and cannot be accounted for by the average grain count over the whole slide. The only difference between control plastids, and the experimentals is that the latter were incubated at 25° for 1 hour (see Methods). The high grain count over control plastids is probably due to a nonspecific incorporation of amino acid which occurs as a result of the fixation procedure (20). A large fraction of the plastids incubated with tritiated leucine become radioactive, as is seen by examination of the formation of plastids with 2 or more grains as a function of time of exposure of emulsion (fig 3). After 8 weeks exposure, 45 % of the plastids incubated with tritiated leucine at 25° have become labeled, while only 10 % of those fixed immediately after addition of tritiated leucine have become labeled. In the experiment presented, plastid smears were extracted with both hot trichloroacetic acid, and with ethanol-ether (3/1 :v/v). The smears were stained and plastids identified by staining reaction and appearance. When ethanol-ether extraction is omitted, plastids can also be identified by the color contributed by the phaeophytin they contain. Similar results were obtained when ethanol-ether extraction was omitted, with or without omission of staining. These results indicate that plastids can clearly be identified after ethanol ether-extraction, and that labeling is not due to incorporation into lipid.

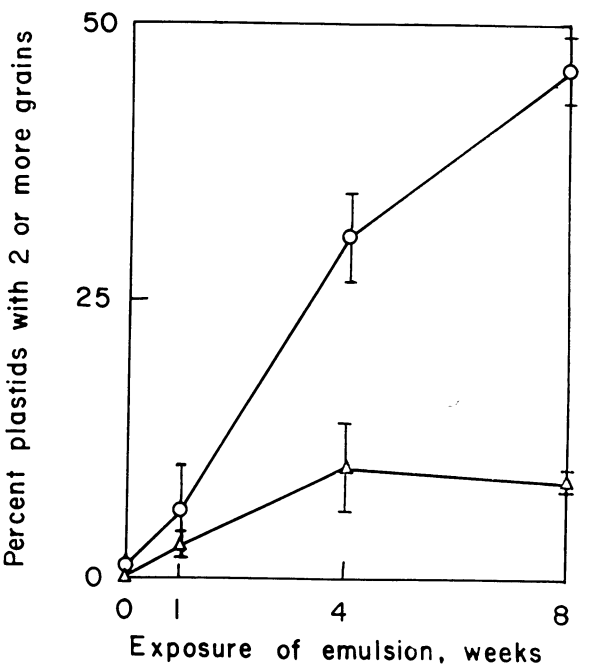

FIG. 3. Autoradiography: grain formation as a function of time of exposure of emulsion. Chloroplasts in complete reaction mixture containing L-leucine-4,5-^{3}H were fixed A) in the cold immediately after addition of radioactive amino acid, (△—△), and B) after incubation for 1 hour at 25° with radioactive amino acid (○—○). Dispersion is standard deviation for 3 slides. Fifty to 100 plastids were scored on each slide.

Discussion

This study of amino acid incorporation by chloroplasts started with the aim of using this in vitro system to learn the factors which limit in vivo protein synthesis of chloroplasts of leaves of higher plants that are grown in the dark. The first step was to obtain a system in which it was clearly shown that the chloroplasts in vitro, prepared from suitable leaf tissue, were responsible for the observed incorporation. A crude plastid fraction was used, rather than a purified plastid preparation, to avoid possible losses of activity during prolonged handling, and because of the decreased yield purification would entail.

We were skeptical of previous reports of amino acid incorporation by crude chloroplast preparations because of the possibility that the observed incorporation was carried out by bacteria, or subcellular particles other than chloroplasts. Bacteria have been found to be responsible for incorporation observed in 1 chloroplast amino acid incorporating system (2) and in preparations of other large subcellular particles (33). Further it was felt that such criteria as ATP dependence, or ribonuclease sensitivity (28, 30) were not sufficient to distinguish between incorporation carried out by bacteria and incorporation carried out by subcellular organelles. In at least 1 instance it has been shown that incorporation of amino acid by

bacteria depends on ATP (33). In addition, some chloroplast amino acid incorporating systems are not sensitive (13), or are only partially sensitive to ribonuclease (14). The present work confirms (4) that treatment of chloroplast preparations with Triton after amino acid incorporation can be used to effectively, and in a routine manner, to distinguish between incorporation carried out by bacteria, and that carried out by chloroplasts. Triton treatment can also be used to distinguish between nuclei and chloroplasts, as previously reported (30), and can also be used to distinguish between leaf cells and chloroplasts. Triton treatment is regarded as the only established procedure for routinely distinguishing between incorporation carried out by bacteria and chloroplasts. Use of an aseptic chloroplast preparation (14) is too difficult for routine use.

Autoradiography further establishes the chloroplasts in the crude preparation as the sites of incorporating activity. As already discussed (see Results), labeling of control chloroplasts is a fixation artifact. The increase in grain count over nonchloroplast areas with exposure of the emulsion is probably not associated with subcellular particles, e.g. mitochondria, since there is no difference in grain count over these nonchloroplast areas between samples fixed before and after incubation at 25°. The increase in grain count in nonchloroplast areas as a function of exposure of emulsion, thus probably is due to exposure from a radiation source external to the incorporation reaction mixture, e.g. the glass slide.

Ribonuclease sensitivity is not a suitable criterion for distinguishing between incorporation carried out by bacteria and chloroplasts. In the present case, 50 % of incorporation activity is resistant to ribonuclease but not more than 10 % of the total incorporation can be ascribed to bacteria. That portion of the activity which is highly sensitive to ribonuclease probably represents plastid messenger RNA and ribosomes accessible to the enzyme, because of the presence of chloroplasts with damaged membranes. Equal or greater insensitivity to ribonuclease has been observed with other chloroplast preparations (13, 14). In both cases chloroplasts were prepared, and incorporation assay carried out in a high osmotic strength medium. However, in some cases, assay in high osmotic strength medium does not result in ribonuclease resistant chloroplast amino acid incorporation (30). Ribonuclease completely inhibits incorporation by ribosomes obtained from chloroplasts (7), or chloroplasts incubated during incorporation, in low osmotic strength medium (4, 5, and table II). Likewise, broken chloroplasts, obtained by suspension in low osmotic strength medium, were completely sensitive to ribonuclease. These broken chloroplasts have decreased amino acid incorporating activity (table II), possibly due to the release of soluble cofactors. In contrast, there have been reports that suspension of chloroplasts in low osmotic strength medium results in increased incorporating activity (12, 25).

Amino acid incorporation has previously been studied with mature chloroplasts. When dark grown bean plants are put in the light, the protein content of the leaf chloroplasts doubles in 48 hours (19). Since protein is actively accumulating in vivo, plastids from leaves undergoing light dependent development should be a suitable starting material with which to obtain good rates of protein synthesis in vitro. This optimism has not been rewarded yet. The rates obtained were better than those obtained with tobacco by Spencer and Wildman (30), equal to those obtained with wheat (4), and considerably less than those obtained with tobacco by Stephenson et al. (31), and *Acetabularia* (13). Rates obtained in the present work were about 50 $\mu\mu$mole leucine per mg protein × hour, which is only one thousandth the rate occurring in vivo. This fraction was calculated assuming: protein synthesis in vivo is linear over 48 hours; there is no pool of nonradioactive leucine in vitro; leucine is 10 % of the weight of the protein synthesized in vitro; the average molecular weight of amino acids is 100. Rates are underestimated since they are calculated for a 1 hour incubation period. During this period the rates in each successive 15 minute period decreases. Both chloroplast systems with the highest rates of amino acid incorporation are insensitive to ribonuclease (13, 31), and one of them is not dependent on an exogenous source of ATP (31). The significance of these differences is not apparent at present. Another difference between other reports and the present, is the continued incorporation of amino acid observed with bean chloroplasts for periods as long as 2 hours. Similar prolonged amino acid incorporation has been observed with mitochondria (22).

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