Plant Physiol. (1966) 41, 992–1003

Effect of Chloramphenicol on Formation of Chloroplast Structure and Protein During Greening of Etiolated Leaves of Phaseolus Vulgaris[1, 2]

Maurice M. Margulies

Received February 3, 1966.

Summary. Greening of leaves of *Phaseolus vulgaris* in the presence of chloramphenicol inhibits formation of A) total chloroplast protein, B) an easily extractable fraction removed during isolation of chloroplasts in isotonic media by differential centrifugation, and C) the insoluble lamellar fraction which remains after extracting osmotically shocked freeze-dried plastids. The inhibition of insoluble chloroplast protein formation is correlated with decreased formation of lamellae and increased formation of vesicular structures. In contrast, chloramphenicol increases the formation of a fraction not removed during differential centrifugation, but removed by water extraction after osmotic shock and freeze-drying of chloroplasts. Analysis of this fraction by electrophoresis and column chromatography, indicates that the increased accumulation of this protein fraction is largely due to accumulation of a protein which is normally present in this fraction in small quantities. It was suggested that this protein may be a precursor which is normally incorporated into the lamellae. The protein extracted from freeze-dried chloroplasts of chloramphenicol treated chloroplasts contains a smaller proportion of one or more proteins than a similar extract of untreated plastids. However, per plastid, no such difference exists.

Chloramphenicol inhibits the formation of photosynthetic activities of chloroplasts when present during greening of etiolated leaves (14, 16, 17) and inhibits greening and accompanying protein synthesis of etiolated *Euglena* (22). Since chloramphenicol is a specific inhibitor of protein synthesis in bacteria, it was suggested that chloramphenicol inhibited development of Hill and photosynthetic phosphorylation reaction activities of chloroplasts by inhibiting the formation of particular proteins required for these reactions (16). In support of this it was shown that chloramphenicol inhibits light dependent synthesis of total chloroplast protein, and light dependent increase in the activity of the soluble chloroplast contained enzyme, ribulose-1,5-diP carboxylase (16). The results presented in this publication stem from an attempt to determine whether proteins necessary for photosynthetic phosphorylation and Hill reactions are absent in chloroplasts from chloramphenicol treated plants. Evidence for absence of protein necessary for Hill reactions and photosynthetic phosphorylation was not obtained. Instead it was found that chloramphenicol treated plants accumulated a water soluble protein fraction accompanied by a decrease in lamellae formation. A preliminary account of some aspects of this work has already been presented (15).

Materials and Methods

Plant Materials. Seedlings of *Phaseolus vulgaris* var. Black Valentine were grown and treated with chloramphenicol as described previously (14). The designation, "treated," refers to p'ants sprayed with 4 mg/ml chloramphenicol after 6 days growth in darkness, followed by exposure to white fluorescent light at an intensity of 1000 ft-c for 48 hours. The designation, "untreated," refers to plants to which chloramphenicol treatment only was omitted.

Electron Microscopy. Leaf tissue was fixed by cutting pieces 1 mm^2 or less into buffered permanganate at 0° (20). Pieces of tissue were taken from a point midway between the midrib and margin, and halfway between the leaf tip and base. After 20-hours fixation at 4° the tissue was washed with ice water. It was then dehydrated with alcohol and embedded in epoxy resin (2). Sections were mounted on Formvar coated grids and were examined with an RCA-EMU-3 (F) microscope usually at an original magnification of 2600×. However, for measurement of thylakoid thickness, examination was at an original magnification of 55,000 ×.

Preparation of Chloroplasts and Determination of Nitrogen Content per Plastid. Once washed chloroplasts were prepared by differential centrifu-

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

[2] Research was supported in part by funds provided by the United States Atomic Energy Commission Contract AT(30-1)2373, 655 M.

gation as described for preparation of plastids for measurements of photosynthetic activities (16), and are referred to as crude chloroplasts. Purified chloroplasts were prepared by centrifuging a suspension of crude chloroplasts in a density gradient (21). Plastid pellets (crude or purified) were suspended in water, freeze-dried, and stored over silica gel at 4°. Freeze-dried crude plastids were used for preparation of chloroplast extracts while either crude or purified chloroplasts were used in the preparation of antisera. Before freeze-drying, the crude chloroplast preparation is contaminated with large numbers of starch particles and with large numbers of fairly uniform particles about 1 μ in diameter. The latter are probably mitochondria. Although present in numbers equal to or greater than the plastids, calculation of relative total volume of chloroplasts and mitochondria from their diameters indicate that the latter would not be more than about 5 % of the protein of the chloroplasts plus mitochondria. Density gradient centrifugation removes all the starch and greatly increases the chloroplast to mitochondrion ratio. Extracts of freeze-dried chloroplasts, prepared as described below, contained RNA and DNA equivalent to 5 % and 0.3 % of the freeze-dried preparation after correction was made for sucrose and starch in the preparations. If the aqueous extraction procedure extracts a large fraction of the DNA present in the chloroplast preparation, it would appear that the nuclear contamination of the chloroplast preparation is small, since nucleii consist of about 10 % by weight DNA (24).

To determine the nitrogen content per plastid, crude plastids were: fixed in 4 % formaldehyde containing 0.4 M sucrose and 0.05 M Na phosphate, pH 7; purified by density gradient centrifugation; and nitrogen content and number of plastids determined (16).

Preparation and Analyses of Plastid Extracts. Two-tenth g portions of freeze-dried crude chloroplasts were ground in a Ten Broeck homogenizer with about 10 ml of water at 0°. The homogenates were centrifuged at 100,000 × *g* (avg) × 60 minutes. The supernatant fraction was carefully decanted so that all the green pellet was left behind. The supernatant fractions, termed extracts, of treated chloroplasts contained 200 to 500 μg/ml of trichloroacetic acid precipitable nitrogen per ml, and untreated 100 to 200 μg/ml. Extracts of treated were diluted to the same protein nitrogen concentration as untreated and both untreated and treated were concentrated by partially drying them in dialysis sacs in a stream of air at 4° for 2 to 3 days. Concentration of extracts by freeze-drying was unsatisfactory since a good part of the resulting dry protein could not be redissolved in water or dilute buffers.

For determination of soluble and insoluble plastid protein, replicate portions of freeze-dried plastids homogenized in water were taken. Protein of some portions was precipitated with 10 % (w/v) trichloroacetic acid, while other portions were first separated into soluble and insoluble fractions by centrifugation at 100,000 × *g*, before addition of trichloroacetic acid. Trichloroacetic acid precipitates were collected by centrifugation after standing overnight at 4° to insure quantitative collection. The precipitates were washed with 10 % trichloroacetic acid and digested by a modified Kjeldahl procedure (9). The ammonia formed was determined with Nessler reagent (29). Protein was calculated by multiplying ammonia nitrogen by 6.25. The protein of extracts used for chromatography was also determined with the Folin phenol reagent (12). Bovine serum albumin (Armour) was used as standard. Agreement within 10 % between Folin and Nessler methods was obtained with either extract from treated or untreated.

Carbohydrate content of freeze-dried plastids was determined with anthrone (13). The protein containing chloroplast extracts were assayed for DNA and RNA with diphenylamine (4) and orcinol (29), respectively.

Preparation of Sera. Rabbits were injected intramuscularly with freeze-dried chloroplast preparation suspended in Freund's complete adjuvant and saline, followed by a second injection of antigen in incomplete adjuvant after a 2-week interval. Fifty mg of untreated or 25 mg of treated plastid preparation was used per injection. Bleedings were carried out for 4 weeks at weekly intervals starting 4 weeks after the first injection. Serum was cleared of fat and particulates by centrifuging at 100,000 × *g* × 60 minutes. Antiserum from 4 bleedings of each of 2 rabbits injected with the same antigen was pooled. Control sera had been obtained previously from the same animals before initiation of the injection schedule.

Antisera were prepared to: 1) untreated crude chloroplasts; 2) an acetone powder of untreated crude chloroplasts; 3) untreated purified chloroplasts; and 4) treated crude chloroplasts. All antisera agglutinated suspensions of freeze-dried chloroplasts from untreated or treated leaves. However, residues of freeze-dried chloroplasts that had been extracted with water 3 times did not give a clear cut agglutination reaction. The antisera gave a precipitin reaction with extracts from freeze-dried chloroplasts. With a 1:8 dilution of serum about 1.5 μg of extract protein from either treated or untreated was needed to give a precipitate with sera to crude plastids, but about 10 μg was required with serum to purified plastids. Similarly, the titers of sera to crude plastids were about the same, a serial dilution of about 1:128 being the limit which gave a precipitate with 10 μg of extract protein. However, serum to purified plastids yielded a precipitate only when dilutions no greater than 1:8 were used. Antigen-antibody precipitates were not markedly soluble in excess antigen. The shape of the titration curve for serum (1) was typical for all antisera (fig 6). In the region of excess antigen sera (1) and (4) yielded about 4 times as much antigen-antibody precipitate as sera (2) and (3).

These results contrast with a report in which acetone extraction of chloroplasts was required to obtain antibody (11).

Antigenic Analyses. Precipitin reactions were carried out in solution or agar gel. In solution a mixture of varying amounts of chloroplast extract was incubated with replicate half ml portions of serum for an hour at 36° followed by 72 hours at 4°. Precipitates were collected by centrifugation and were washed 3 times with 1 % NaCl. These were dissolved in 1 N NaOH and protein determined with the Folin phenol reagent. The supernatant fractions from the precipitin reactions were tested for excess antibody and antigen by double diffusion in agar using chloroplast extract containing 150 μg protein/ml or undiluted serum as test reagents. Double diffusion precipitin reactions in agar were carried out by standard procedures (3, 8).

Chromatography of Chloroplast Extracts. Extract protein (25 mg) in 25 ml was dialyzed with stirring against 1.1 0.005 M phosphate pH 8.0 for 2 hours and the dialysis repeated with fresh buffer. The contents of the dialysis tube were quantitatively transferred to a 1 × 16 cm column of DEAE-cellulose (Whatman DE 50), and the column washed with 10 ml of buffer. This was followed by a convex gradient of 0.0 to 0.3 M NaCl in 0.005 M phosphate pH 8.0 until an additional 465 ml had been collected. The column was then washed with 50 ml of 4 M NaCl in 0.005 M phosphate pH 8.0. Effluent was collected in 5.0 ml fractions. Elution was carried out at 4° at a flow rate of about 25 ml/hour. The resin was removed and extracted 3 times with 0.1 N NaOH and extracts pooled. Protein content of eluate fractions was determined with Folin phenol reagent. Resin was prepared for chromatography by washing with 0.1 N NaOH till filtrates were colorless; with water till neutral, and with 0.005 M phosphate pH 8.0 till the eluate was pH 8.0 to 8.5.

Electrophoresis of Chloroplast Extracts. Either 0.2 or 0.5 mg of extract protein in 0.1 ml of water was placed in a well, 1 × 0.2 × 0.5 cm, in a sheet of 5 % acrylamide in 0.02 Tris-maleate pH 8.0. Equal amounts of protein from treated and untreated were compared. Separations were carried out at 1 to 2 ma/cm with a voltage gradient of 10 to 20 v/cm at 20°. Protein was fixed and stained with 0.5 % light green in 5 % acetic acid, 25 % ethyl alcohol and destained with 2 % acetic acid.

Results

Once washed freeze-dried chloroplast preparations contained about 50 % by weight carbohydrate which consisted of starch and sucrose. After subtracting this carbohydrate contamination, the remainder contained 60 to 70 % protein by weight, whether chloroplasts were isolated from treated or untreated leaves. Correction was made for nitrogen contained in chlorophyll, but not for nucleic acid.

The 100,000 × *g* pellets of freeze-dried crude plastids extracted with water contained all the green pigment of the freeze-dried preparation. The supernatant fraction from untreated was a pale brown, while that from the chloroplasts of treated leaves was paler and more yellowish even though the latter had twice as much protein as the former. When a chloroplast sample was extracted successively 3 times, the first extract contained 85 to 90 % of the trichloroacetic acid precipitable nitrogen removed by the 3 extractions. No difference in percent of trichloroacetic acid precipitable nitrogen removed by the first extraction was observed between chloroplasts from untreated or treated leaves. However, the first 100,000 × *g* supernatant fraction from treated contained 62 % of the trichloroacetic acid precipitable nitrogen present in the freeze-dried chloroplast preparation, while the supernatant fraction from untreated contained only 23 % (table I). Acetone extraction in the cold of freeze-dried chloroplast preparations of untreated leaves did not increase the amount of protein that could be extracted subsequently by homogenization with water.

Once washed plastids were fixed with formaldehyde and were purified further by centrifugation in a density gradient. These chloroplasts contained the same amount of trichloroacetic acid precipitable nitrogen per plastid whether the preparations were made from treated or untreated leaves (table I).

Table I. *Protein Content of Chloroplasts*

Source of chloroplasts	Chloroplasts fixed during homogenization of leaves.* μg N/plastid × 10^7	Chloroplasts fixed after isolation by differential centrifugation μg N/plastid × 10^7	Unfixed chloroplasts, freeze-dried after differential centrifugation			
			Extractable N		Unextractable N	
			% of total	μg/plastid** × 10^7	% of total	μg/plastid*** × 10^7
Dark	4.0 ± 0.1	...	...	...	...	...
Untreated	7.8 0.4	5.0 ± 0.2	23 ± 16†	1.1	73 ± 16	3.6
Treated	5.9 0.1	5.4 0.9	62 6	3.3	38 7	2.0

* Data from published experiments (16).
** Calculated from data in columns 3 and 4.
*** Calculated from data in columns 3 and 6.
† Standard deviation for determinations of 4 separate chloroplast preparations.

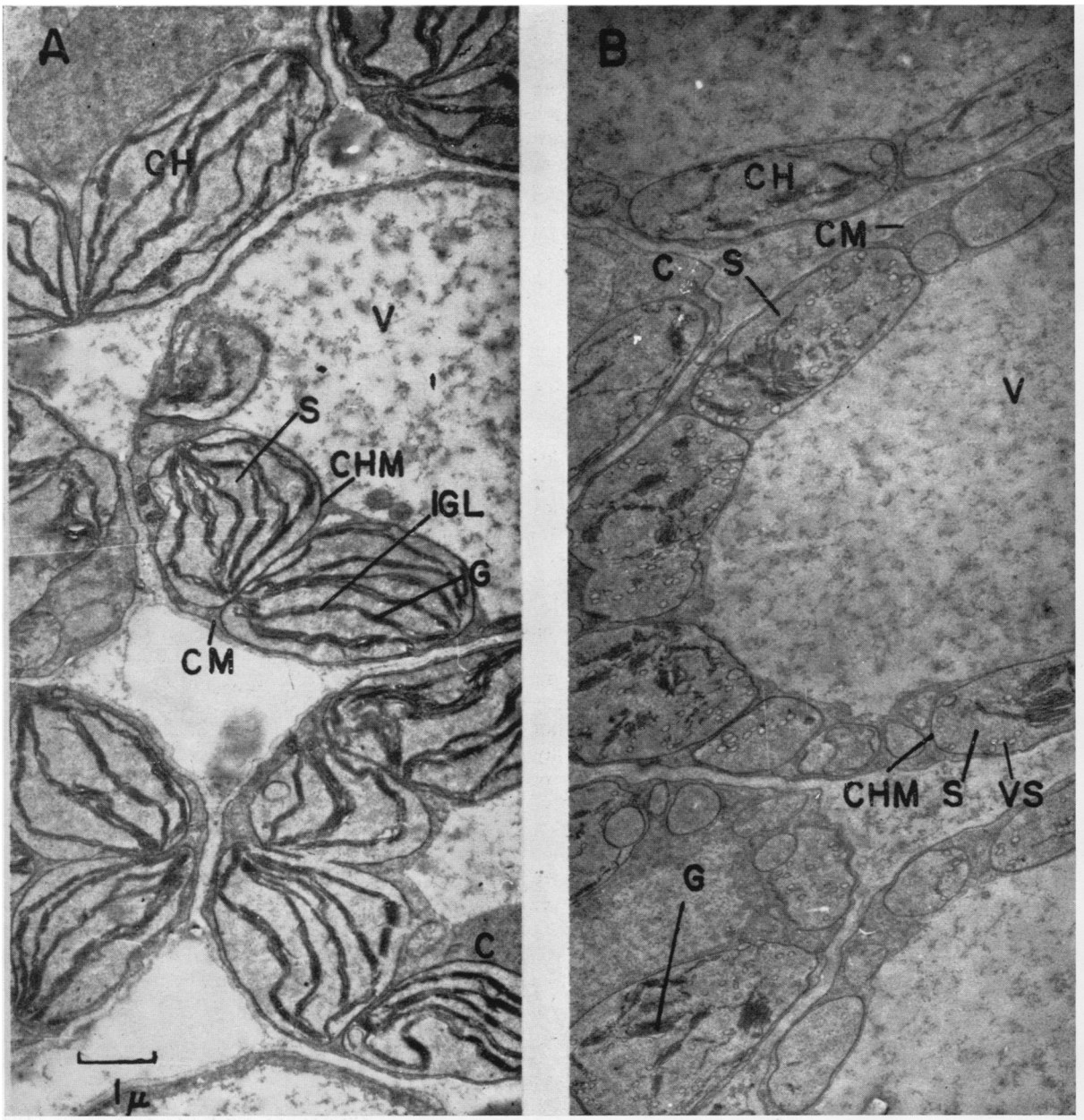

FIG. 1. Chloroplasts from (a) untreated and (b) treated leaves. Low magnification. C, cytoplasm; CM, cell membrane; CH, chloroplast; CHM, chloroplast membrane; G, granum; IGL, intergrana lamellae or frets; S, stroma, V, vacuole, VS, vesicle.

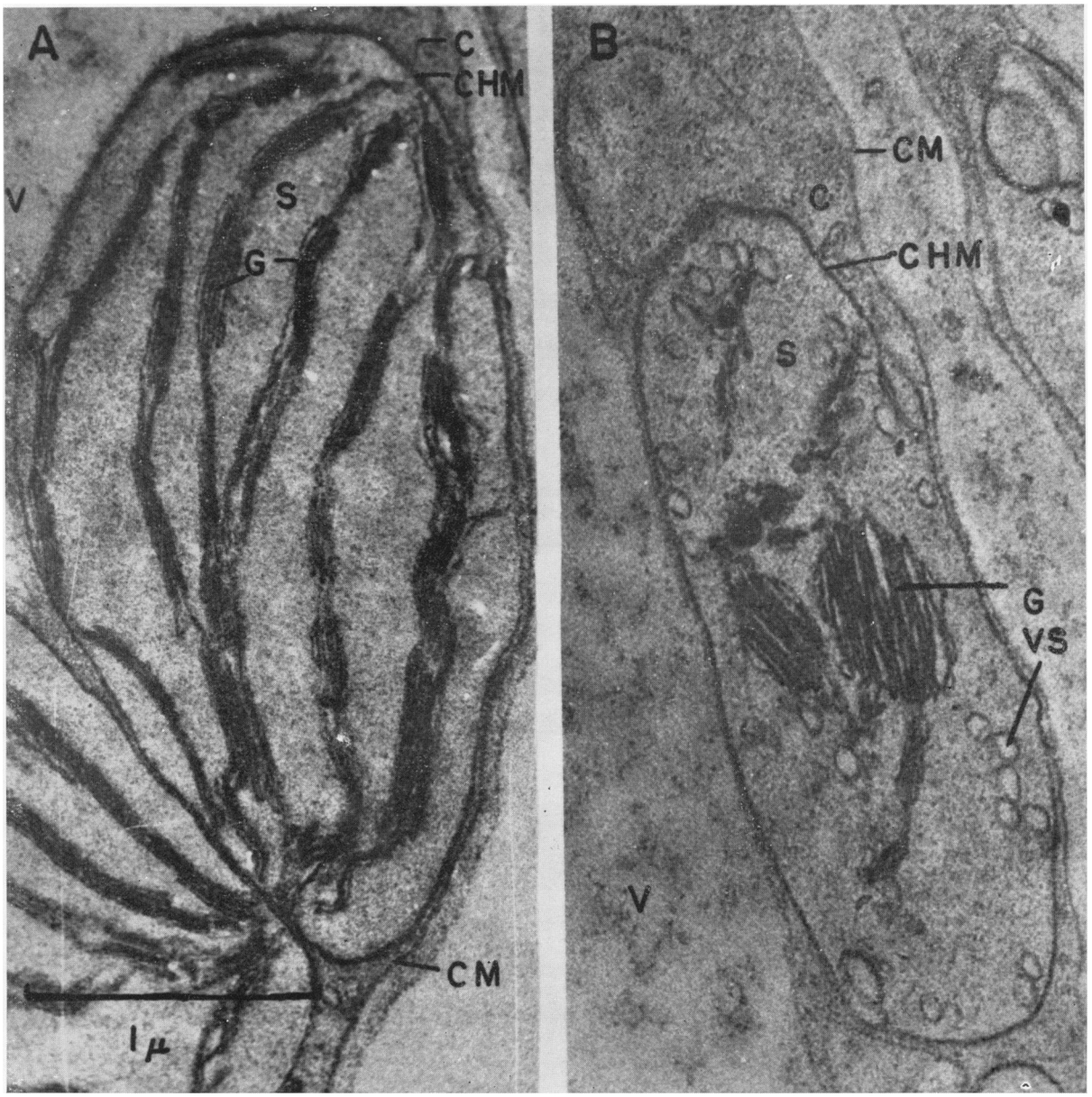

FIG. 2. Chloroplasts from (a) untreated and (b) treated leaves. High magnification. For abbreviations see legend to figure 1.

This was surprising in view of previous determinations of plastids fixed during grinding of leaves which showed treated plastids had less protein than untreated (table I; 16). It was calculated from the nitrogen per plastid of fixed plastids, and percentage soluble protein of freeze-dried plastids, that once washed plastids from treated leaves have less insoluble protein nitrogen per plastid which is replaced by an increase in the soluble protein fraction. The absolute quantities of protein nitrogen per plastid for crude plastids, isolated without formaldehyde fixation, and those fixed with formaldehyde before isolation, agree well with published values for plastids of the primary leaves of Black Valentine bean plants (16, 18).

The extractability of more protein per plastid from once washed freeze-dried plastids of treated than untreated leaves is correlated with decreased lamellar structure in electron micrographs of permanganate fixed leaf pieces (fig 1, 2). The prolamellar body, present in plastids of etiolated leaves (18), is absent in both treated and untreated. Numerous grana are observed in each chloroplast section from untreated. The grana are regularly arranged and connected by intergrana lamellae or frets. In treated there are chloroplasts identifiable by the presence of grana. These grana are fewer in number, tend to be larger and more irregular in shape, and often contain thylakoids with enlarged loculi. However, where thylakoids are closely packed their thickness in untreated and treated is about 160 A. This agrees well with previously published figures for similarly fixed bean leaf material (32). In treated, in addition to the grana, there are numerous vesicle like structures (fig 1, 2). These may be sections of spherical or tubular structures. They appear to replace the frets present in the untreated plastids. In leaves of treated, some sections of organelles which are probably plastids, have only vesicles. Some organelles present in leaves of treated may be chloroplasts, but are not definitely identifiable as such since they lack both grana and vesicles.

Extracts of chloroplasts from treated and untreated leaves were chromatographed on Whatman DEAE-cellulose powder (fig 3). About 80 % of the protein applied to the column could be eluted. A small amount of protein was not held by the resin and was washed through with the equilibrating buffer. A large amount of protein was eluted with the 0.0 to 0.3 M NaCl gradient. In the particular experiment presented in figure 3, 54 % of the extract protein from untreated and 64 % of the extract protein of the treated was eluted by equilibrating buffer and the salt gradient. The salt gradient was followed by 100 ml of 4 M NaCl which eluted an additional 4 and 3 % of protein from untreated and treated, respectively. Extraction of the resin with 0.1 N NaOH removed an additional 30 % of untreated extract protein applied and 14 % of treated. Twelve percent of the untreated protein applied and

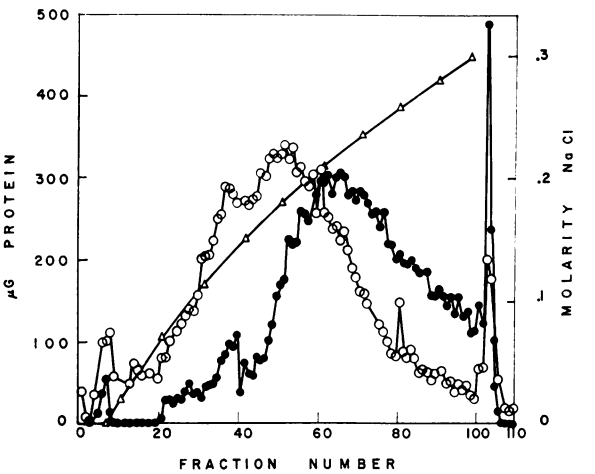

FIG. 3. Column chromatography of soluble extracts. Folin protein per fraction of chromatograms of extracts from untreated (filled circles), and chloramphenicol treated (unfilled circles) chloroplasts; calculated NaCl concentration of solution added to column (triangles).

19 % of the treated was not accounted for. These results are typical. No consistent differences were noted between treated and untreated with regard to the percent protein applied removed by one eluant. However, a consistent difference was noted in the pattern of elution by the salt gradient (fig 3). In both treated and untreated, a small peak was observed before the start of the salt gradient. The salt gradient produced one large peak with both extracts, with indications that the large peak is composed of a number of separate peaks. For example, a shoulder or sub-peak was consistently observed in fractions 30 to 40 of untreated. A consistent difference in the location of the principal peak was noted depending on whether protein from untreated or treated chloroplasts was being eluted. The peak from untreated occurred at a higher salt concentration than the peak from treated. More protein from treated than untreated was eluted in fractions 10 to 50, about equal amounts in fractions 50 to 70, and more from untreated than treated in 70 to 100. One can explain these results by the following: both extracts are composed of a number of different proteins eluted by 0 to 0.3 M NaCl; the 2 extracts differ quantitatively in respect to the relative amounts of these different proteins. It is suggested that both extracts have proteins in common, but treated contains more of some components and less of others than untreated.

Support of this viewpoint was obtained from examination of the extracts by electrophoresis. The protein of extract from untreated moves largely as one band (β) at 0.30 to 0.45 (relative to serum albumin at 1.0), (fig 4). An additional but faint band is found at 0.50 to 0.70 (α). Protein, usually without distinct band formation, occurs from the origin than the B band of treated. This indiband at about 0.13. The extract from treated also

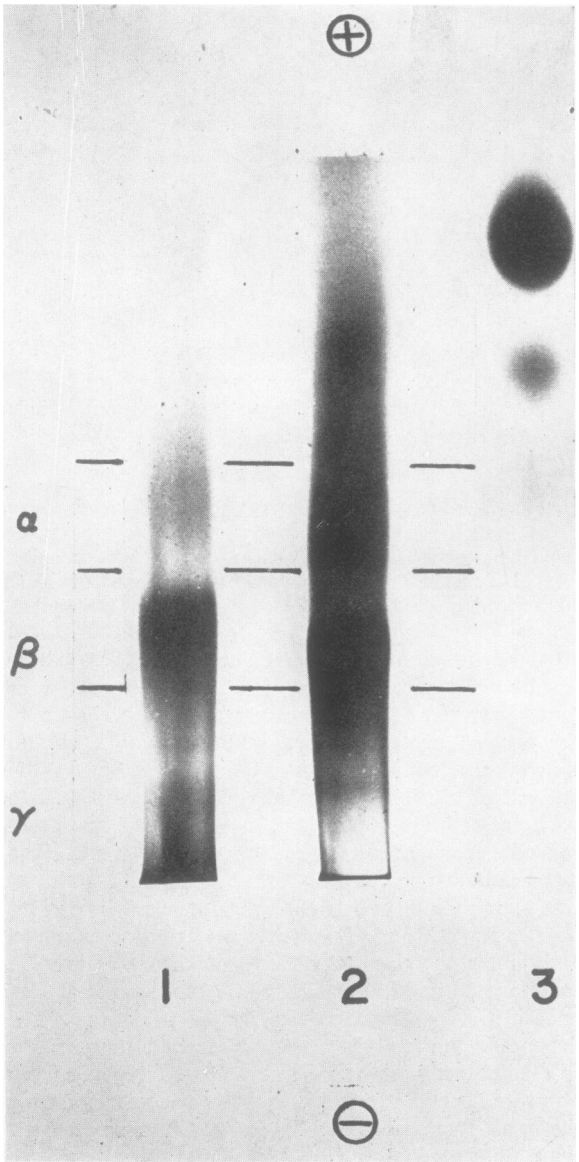

FIG. 4. Electrophoretogram of extracts of (1) untreated and (2) treated chloroplasts and of (3) bovine serum albumin. Five-tenth mg protein was placed in wells 1 and 2 and 0.2 mg in well 3.

forms bands in regions α and β. More protein is present in the α band of treated than untreated, while there are about equal amounts of protein in the β bands of treated and untreated. The front of the β band of untreated is slightly further from the origin than the β band of treated. This indicates a lack of identity between the β bands of treated and untreated, which could be due to A) the presence of multiple components in the β band of untreated which are unresolved, fewer components being present in the β band of treated, or B) the lack of identity could be due to the presence of 2 different β band components, one present in untreated, and the other present in treated. Co-electrophoresis of protein from untreated and treated results in a blurring of the separation between the α and β bands, indicating a real difference in the mobility of the β bands. Extract of untreated regularly contains more protein (stained material) in region γ than does extract of treated, even though this is not shown clearly in the photograph (fig 4). Thus, when equal amounts of protein of extract from treated and untreated are compared, it is found that extract of treated contains more of one component, and probably less of another than extract of untreated, while both contain about equal amounts of a third component.

Additional evidence to support the conclusion that treated contains less of at least one component present in untreated was obtained by use of antiserum to untreated plastids. When antiserum to crude untreated plastids was reacted with extract of untreated by double diffusion, 2 precipitin bands were formed. One of these fused with the single band forms as a result of reaction of antiserum with extract of treated (fig 5). With higher concentra-

PRECIPITIN BANDS FORMED BY DIFFUSION IN AGAR

Serum	Plastids used to prepare serum	mg protein/ml in diffusion wells	Precipitin band pattern
1	Crude, normal	160	Antigen Antigen
		1300	
3	Purified, normal	160	
		1300	
4	Crude, chloramphenicol treated	160	
		1300	

FIG. 5. Diagramatic representation of precipitin bands formed by double diffusion in agar of chloroplast extracts and antisera. The circles indicate wells in agar containing (a) extract from untreated chloroplasts, (b) extract from treated chloroplasts, and (c) antiserum. The lines between wells indicate the formation of sharp precipitin bands and stippling the formation of diffuse regions of precipitation. The distance between the centers of the well containing antiserum and those containing extract was 2 cm. Wells were 8 mm in diameter.

tions of extract this difference remained. Although additional bands were observed at higher extract concentrations, no additional regularly occurring differences were found. Thus there appears to be a component in extract of untreated which is absent in extract of treated. With antiserum to purified plastids from untreated leaves, a slightly different picture is obtained. With this reagent, extract of treated forms a single heavy precipitin band, while with extract of untreated, no band, or one that was barely detectable was formed. Here one can only conclude that one component in the extract of treated is present in a lower concentration than in extract of untreated.

An attempt was made to prepare an antiserum to the component lacking in extract of treated by absorbing antiserum to crude untreated plastids with extract of treated (fig 6). Possible involvement of this component in Hill and photosynthetic phosphorylation reactions could be tested by looking for an inhibitory effect of antiserum to it on these plastid reactions. Additions of 60 to 400 μg of extract protein from chloroplasts of untreated leaves precipitated more antibody-extract protein than equal amounts of extract protein from chloroplasts of chloramphenicol treated leaves. With larger quantities, as much extract-antibody protein could be precipitated by extract of treated. Sixty μg or less of extract from untreated was required to remove antibody and 250 μg for excess antigen to be detectable. However, 250 μg or more of treated was required to remove antibody and 440 μg for excess antigen to be detectable. These results can be explained by assuming that each extract consists of a mixture of antigens, of which, some represent a smaller proportion of the total extract protein of treated than untreated.

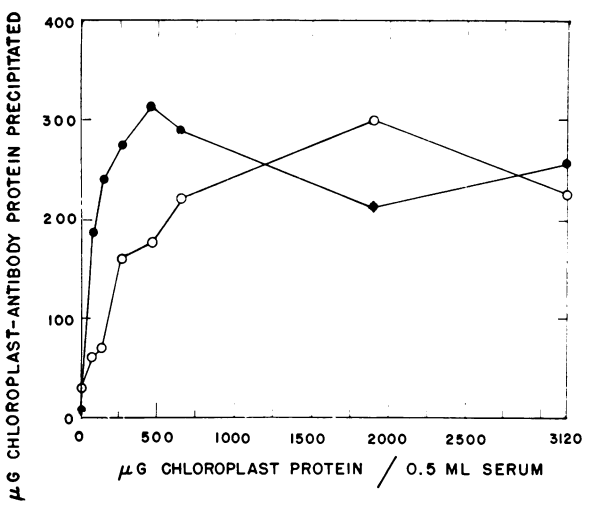

FIG. 6. Titration of antiserum (1) to crude untreated chloroplasts with chloroplast extract from untreated (filled circles), and chloramphenicol treated chloroplasts (unfilled circles). Values are averages of 3 replicate experiments carried out with the same batch of serum but with different extract preparations. Standard deviations from 60 to 625 μg chloroplast protein added are about 25.

Chromatography and electrophoresis show that there are components that represent a larger proportion of the total extract protein in treated than untreated. Consistent evidence to support these observations using antiserum to crude treated plastids was not obtained. Although faint precipitin bands were formed with low concentrations of extract of treated which were not formed with extract of untreated, these differences disappeared at higher extract concentrations (fig 5). Furthermore, the amounts of extract-antibody protein precipitated by equal amounts of extract protein from untreated or treated chloroplasts were the same over the range 30 to 3000 μg extract protein per half ml of antiserum.

Discussion

The effect of chloramphenicol on the development of chloroplast structure has been studied previously by Dobel (5). In that study chloramphenicol was applied to the shoot apex and developing leaves at one-fourth the concentration used in the present study. Thus, the effects observed are the effects brought about by application of chloramphenicol at different times during plastid development. In the present study chloramphenicol was applied at the stage of leaf development when all plastids had presumably reached a similar stage of growth in which they contain a well-developed prolamellar body (18). Then plants were placed in the light to permit chloroplasts to undergo light dependent development in what would be expected to be a synchronous manner. In spite of this, the variability in plastid structure of treated leaves seems to be as great as that observed by Dobel (5). Although some unusually large grana were formed, the thylakoid loculi were not as swollen as reported by Dobel. There was no readily identifiable fretwork structure, but vesicles or tubuli were present as previously reported. In some instances 2 or more vesicles appear close together and probably represent swollen thylakoids. Many of the vesicles appear singly and might indicate aborted thylakoid or fret development. The vesicles vary in size from about 500 A to 2500 A, and are comparable in size to those observed by Dobel (5). The chains of vesicles that sometimes appear to extend from the grana indicate that, in part, the vesicles represent a reduced fretwork structure. Although thylakoid lamellae in the plastids from chloramphenicol leaves are not noticeably different in structure from those of chloroplasts of normal leaves, there must be some difference in structure to explain the inactivity of the plastids in Hill and photosynthetic phosphorylation reactions (16).

Structural deformations showing similarity to those observed as a result of chloramphenicol treatment also can be produced by some mineral deficien-

cies (23, 26, 30), darkness (1), treatment with chemicals that interfere with metabolism (1, 5, 25), or mutation (7, 10). The greatest similarity is found with the plastid mutation grandigrana (7) in which large grana are formed without enlargement of locules, as well as vesicles which occur singly and in pairs.

The protein of the chloroplasts of untreated leaves can be divided into 3 categories: A) that fraction which is lost during isolation by differential centrifugation in isotonic media, but is not lost when chloroplasts are fixed during homogenization of leaves; B) a fraction which is extractable with water after osmotic shock and freeze-drying of isolated chloroplasts; C) the insoluble chlorophyll containing residue left after the above-mentioned extractions. Fraction A protein probably is the same as that lost in aqueous media, but not in nonaqueous, which consists of fraction I and II proteins (6). The source of the easily lost protein probably is the plastid stroma since, in aqueous preparations, the plastid envelope seems to be severely damaged with much loss of stroma (6, 19, 31, 32). Another interpretation of the similarity in protein content of treated chloroplasts fixed before and after isolation is that chloramphenicol modifies the properties of the plastid membrane so that proteins are no longer easily lost. Fraction B probably corresponds to the extract of Heber and Tyzkiewicz (6) that was prepared by sonication of plastids prepared in an aqueous medium. It too probably largely consists of stroma protein. However, some protein might be extracted from the lamellae, possibly more from the lamellae of treated. It is not possible, however, to assign definitely fractions A and B to a particular plastid structure. Fraction C undoubtedly consists of the lamellar portion of the chloroplasts, or part of it, since it contains the photosynthetic pigments.

Analysis of equal amounts of fraction B from treated and untreated by electrophoresis shows differences in composition which indicate accumulation in treated of component α (fig 4). Since treated plastids contain 3 times as much fraction B it is obvious that in each plastid chloramphenicol results in a very great increase in the α component of fraction B.

Examination of chromatograms can also be interpreted to indicate the massive accumulation, in fraction B of chloramphenicol treated plastids, of a component normally present in much smaller quantities (fig 3). Here, too, the experiments represent examination of equal quantities of proteins from untreated and treated plastids. Correcting to a per plastid basis by multiplying the curve for treated by 3 shows that very much more of the components present in fractions 10 to 50 occurs in a treated than untreated plastid. Whether the chromatogram component which has a peak at fractions 30 to 40 is the same as electrophoretogram component A has not been determined, although their identity is a distinct possibility. It is suggested that a portion of the extractable protein accumulating during chloramphenicol treatment might be a precursor of protein normally occurring, for the most part, in the lamellae. This seems possible since fraction I protein can be found both in the insoluble lamellae and soluble chloroplast fraction (27, 28).

No evidence was obtained to show accumulation of an unusual component in fraction B of treated plastids by titration of antiserum to treated plastids. This could be due to the failure of some plastid proteins to generate antibody. It is estimated that antibody is formed to only 20 to 35 % of the fraction B protein, assuming molecular weights of the proteins of 200,000, and from this an antibody-antigen combining ratio of 5:1 (8).

Although double diffusion using antibody to untreated plastids indicated the lack of a component in fraction B of treated, comparison by titration indicates that this difference is only quantitative. These results differ from those obtained with *Euglena* in which it was shown that antiserum to chloroplasts of cells grown in light could be absorbed with extract of cells grown in darkness or cells of certain mutants and still retain antibody to extracts of cells grown in light (19). When the titrations of antiserum to untreated plastids (fig 6) are replotted to compare additions of extract from equal numbers of untreated and treated plastids, it is found that equal amounts of antigen-antibody protein are precipitated by equal numbers of untreated or treated plastids. Thus, although the ratio of one component to the total protein may be lower in extract of treated, the total amount of this component in a plastid of treated is about the same as in a plastid of untreated.

Acknowledgments

I express my sincere thanks to the following people for their assistance: to Mr. C. B. Brubaker for capable technical assistance in the laboratory and for preparation of illustrations; to Mr. Warfield and Mr. King of the NIH and Walter Reed Animal production facilities, respectively, for a supply of rabbits; to Drs. Fahey of NIH and Spies of the Food and Drug Administration for advice on immunological techniques; to Dr. H. Ursprung of Johns Hopkins University for use of the electron microscope; and to his technical assistant, Mrs. P. Siger, for assistance in sectioning and examination of sections.

Literature Cited

1. Ashton, F. M., E. M. Gifford, Jr., and T. Bisalputra. 1963. Structural changes in *Phaseolus vulgaris* induced by atrazine. II. Effects on fine structure of chloroplasts. Botan. Gaz. 124: 336–43.
2. Cargille, R. P., Laboratories, Inc., Little Falls, New Jersey. Procedure for embedding with NYSEM epoxy resin A.

3. CROWLE, A. J. 1961. Immunodiffusion. Academic Press, New York.
4. DISCHE, Z. 1955. Color reactions of nucleic acid components. In: The Nucleic Acids, Vol. I. E. Chargaff and J. N. Davidson, ed. Academic Press, New York. p 285–305.
5. DOBEL, P. 1963. Untersuchung der Wirkung von Streptomycin-chloramphenicol, und 2-thiouracil Behandlung auf die Plastidenentwicklung. Biologisches Zentralblatt 82: 275–95.
6. HEBER, U. AND E. TYSZKIEWICZ. 1962. The rate of photosynthesis in isolated chloroplasts. J. Exptl Botany 13: 185–200.
7. HESLOP-HARRISON, J. 1963. The ultrastructure of some chemically-induced somatic chloroplast mutants in *Cannabis sativa* L. Port. Acta Biol. Ser. A. 8: 13–40.
8. KABAT, E. A. AND M. M. MAYER. 1961. Experimental Immunochemistry, 2nd edition. Charles C. Thomas, Springfield, Illinois.
9. KOCH, F. C. AND M. E. HANKE. 1953. Practical Methods in Biochemistry. Williams and Wilkins Company, Baltimore, Maryland. p 184–87.
10. LE FORT, M. 1959. Etudes de quelques mutants chlorophylliens induits chez *Lycopersicum esculentum* Mill. Rev. Cytol. Biol. Vegetales 20: 1–160.
11. LEWIS, S. C., J. A. SCHIFF, AND H. T. EPSTEIN. 1965. Studies of chloroplast development in *Euglena*. 9. Chloroplast antigens and their appearance during chloroplast development. J. Protozool. 12: 281–90.
12. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–75.
13. MCREADY, R. M., J. GUGGOLZ, V. SILVIERA, AND H. S. OWENS. 1950. Determination of starch and amylose in vegetables. Anal. Chem. 22: 1156–58.
14. MARGULIES, M. 1962. Effect of chloramphenicol on light-dependent development of seedlings of *Phaseolus vulgaris* var. Black Valentine, with particular reference to development of photosynthetic activity. Plant Physiol. 37: 473–80.
15. MARGULIES, M. 1963. Chloroplasts from chloramphenicol treated leaves. Plant Physiol. 38: iii.
16. MARGULIES, M. 1964. Effect of chloramphenicol on light-dependent synthesis of proteins and enzymes of leaves and chloroplasts of *Phaseolus vulgaris*. Plant Physiol. 39: 579–85.
17. MARGULIES. M. 1965. Relationship between red light mediated glyceraldehyde-3-phosphate dehydrogenase formation and light dependent development of photosynthesis. Plant Physiol. 40: 57–61.
18. MEGO. J. L. AND A. T. JAGENDORF. 1961. Effect of light on growth of Black Valentine bean plastids. Biochim. Biophys. Acta 53: 237–54.
19. PARK, R. B. AND N. G. PON. 1961. Correlation of structure with function in *Spinacia oleracea* chloroplasts. J. Mol. Biol. 3: 1–10.
20. PEASE, D. C. 1960. Histological Techniques for Electron Microscopy. Academic Press, New York. p 43–46.
21. PIERPONT, W. S. 1962. Mitochondrial preparations from leaves of tobacco (*Nicotiana tabacum*). IV. Separation of some components by density-gradient centrifuging. Biochem. J. 82: 143–48.
22. POGO, B. G. T. AND A. O. POGO. 1965. Inhibition by chloramphenicol of chlorophyll and protein synthesis and growth in *Euglena gracilis*. J. Protozool. 12: 96–100.
23. POSSINGHAM, J. V., M. VESK, AND F. V. MERCER. 1964. The fine structure of leaf cells of manganese-deficient spinach. J. Ultrastruct. Res. 11: 68–83.
24. SERRA, J. A. 1955. Chemistry of the Nucleus. In: Encyclopedia of Plant Physiology, Vol. I. W. Ruhland, ed. Springer, Berlin. p 413–44.
25. SIGNOL, M. 1961. Comparison de l'action de la dihydrostreptomycine a celle de l'acide 3-(α-iminoethyl)-5-methyltetronique sur l'infrastructure des chloroplastes de *Zea mays* L. Compt. Rend. Acad. Sci. Paris 252: 1993–95.
26. THOMSON, W. W. AND T. E. WEIER. 1962. The fine structure of chloroplasts from mineral deficient leaves of *Phaseolus vulgaris*. Am. J. Botany 49: 1047–55.
27. THORNBER, J. P., J. L. BAILEY, AND S. M. RIDLEY. 1964. The relation of fraction I protein to the proteins of the lamellae of spinach beet chloroplasts. Biochem. J. 92: 18 p.
28. TROWN, P. W. 1965. An improved method for the isolation of carboxydismutase. Probable identity with fraction I protein and the protein moiety of protochlorophyll holochrome. Biochemistry 4: 908–18.
29. UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1949. Manometric Techniques. Burgess, Minneapolis.
30. WEIER, T. E. AND W. W. THOMSON. 1962. Membranes of mesophyll cells of *Nicotiana rustica* and *Phaseolus vulgaris* with particular reference to the chloroplast. Am. J. Botany 49: 807–20.
31. WEIER, T. E., C. R. STOCKING, C. E. BRACKER, AND E. B. RISLEY. 1965. The structural relationships of the internal membrane systems of in situ and isolated chloroplasts of *Hordeum vulgare*. Am. J. Botany 52: 339–52.
32. WEIER, T. E., A. H. P. ENGELBRECHT, A. HARRISON, AND E. B. RISLEY. 1965. Subunits in the membranes of chloroplasts of *Phaseolus vulgaris*, *Pisum sativum*, and *Aspidistra* sp. J. Ultrastruct. Res. 13: 92–111.