Plant Physiol. (1972) 50, 19–23

Protein Synthesis by Isolated Etioplasts and Chloroplasts from Pea and Wheat and the Effects of Chloramphenicol and Cycloheximide[1]

Received for publication October 19, 1970

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ABSTRACT

Etioplasts capable of incorporating ^{14}C-leucine into protein have been isolated from dark-grown pea and wheat plants. The requirements for leucine incorporation for etioplasts were similar to those for chloroplasts. An ATP-generating system, Mg^{2+}, and GTP were required. The amino-acid-incorporation activity of etioplasts from wheat was comparable to that of chloroplasts on an RNA basis, whereas the activity of pea etioplasts was about 50% of the activity of pea chloroplasts. The incorporation of leucine into protein by etioplasts and chloroplasts from pea and wheat was inhibited by chloramphenicol, and to a slight extent by cycloheximide.

There have been several studies made of protein synthesis in chloroplasts isolated from higher plants, most notably from bean (9, 23), pea (29), spinach (35), tobacco (4, 5, 11, 14), tomato (20), and wheat (2). Little is known, however, about the development of the capacity for protein synthesis during chloroplast biogenesis, and with one exception (9) studies comparable to those cited above have not been carried out using isolated etioplasts or immature chloroplasts. Indeed, the development of an extraction medium for isolating structurally intact etioplasts (from maize) has only recently been reported (21).

Characterization of the ribosomes and ribosomal RNA of etiolated tissues suggests that the ribosomes which are evident in electron micrographs of etioplasts (18) are of the same 70S type as those found in chloroplasts. Boardman (3) has shown that 70S ribosomes are present in etiolated leaves of bean, and Scott *et al.* (28) have demonstrated the existence of rRNA characteristic of 70S ribosomes in etiolated leaves of pea and wheat plants. With respect to the role of light in regulating chloroplast biogenesis, it would be valuable to know whether etioplast ribosomes are part of a functional system for protein synthesis, and if so how the activity of this system compares to that of chloroplast systems.

Protein synthesis on the 70S ribosomes of chloroplasts can be distinguished from protein synthesis on the 80S ribosomes of the cytoplasm by its much greater sensitivity to inhibition by chloramphenicol and related antibiotics (10, 11). Therefore it has been presumed that the chloroplast 70S system is insensitive to cycloheximide, an inhibitor of protein synthesis in eucaryotes but not in procaryotes (12). Both compounds have been used extensively in experiments designed to determine whether chloroplast proteins are synthesized on the 70S ribosomes of chloroplasts or the 80S ribosomes of the cytoplasm (17, 30, 31, 33, 34), but published data on the effects of these two chemicals on protein synthesis by isolated chloroplasts remains incomplete. Chloramphenicol has been shown to inhibit amino acid incorporation by chloroplasts isolated from a number of higher plants (2, 4, 11, 24, 29, 35) and algae (10, 15), but only one or, in a few cases, two concentrations of chloramphenicol were used. There are two reports that cycloheximide does not inhibit amino acid incorporation by isolated chloroplasts, one from Spencer (35), who used spinach chloroplasts, and the other from Ellis (11), who used chloroplasts from tobacco.

In this paper we show that etioplasts isolated from pea and wheat leaves can incorporate amino acids into protein. The activities of etioplasts and the corresponding chloroplasts are compared and the effects of several concentrations of chloramphenicol and cycloheximide on the incorporating activities of etioplasts and chloroplasts are reported.

MATERIALS AND METHODS

Growth of Wheat and Pea Seedlings. Wheat seeds (*Triticum aestivum* L. "Monon") were surface-sterilized by soaking in a 2 to 5% sodium hypochlorite solution for 15 min. Seeds were then washed with flowing tap water for 24 hr in the dark and planted in sterile vermiculite in trays. In order to compare etiolated and green tissues, seedlings were grown in darkness for an additional 4 days. Then a portion of them was trans-

[1] Research was jointly sponsored by the National Institute of General Medical Sciences, National Institutes of Health, Grant GM 15595, and by the United States Atomic Energy Commission under contract with the Union Carbide Corporation.

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Table I. *Composition of Extraction Medium and Reaction Mixture*

Component	Concentration
Plastid Extraction Medium:	
Sucrose	0.33 mM
HEPES (N-2-Hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) (pH 8.0)	10 mM
Dithiothreitol	1 mM
$MgCl_2 \cdot 6H_2O$	15 mM
KCl	100 mM
Bovine serum albumin	0.2%
Reaction Mixture (0.5 ml) for Amino Acid Incorporation:	
Plastid extraction medium	As above
ATP	0.4 μM
P-enolpyruvate	0.5 μM
Pyruvate kinase	20 μg
GTP	0.02 μM
^{12}C-Amino acids (minus ^{12}C-leucine)	0.025 μM
L-Leucine-U-^{14}C (263 mc/mmole)	3.8 nmoles
Plastids	0.5 mg RNA

ferred to another room and exposed to light (cool white fluorescent lamps, 150 ft-c) for 24 hr. The growth temperature was 21 C. Etiolated leaves were harvested under green safelight. The time period between the initial washing and the harvest was 6 days for both etiolated and green seedlings.

Bush pea seeds (*Pisum sativum* L. "Thomas Laxton") were handled in a similar manner, except that they were washed with tap water for 48 hr prior to planting, and the period of exposure of the seedlings to light was 48 hr. The total growth period was 10 days.

Isolation of Plastids. The composition of the medium used for the extraction of etioplasts and chloroplasts is given in Table I. Strict attention was given to working rapidly and maintaining very cold conditions (0 to 2 C) throughout plastid extractions. Harvested leaves were chilled on ice, extraction medium was added, the leaves were hand chopped with a razor blade to 1- to 2-mm sections and then crushed using a mortar and pestle. The slurry was passed through Miracloth and centrifuged twice at 30*g* for 30 sec. Each time all but approximately 1 ml was decanted from the centrifuge tubes and the supernatant retained. The supernatant was then centrifuged at 1000*g* for 4 min, and the pellet was resuspended and washed twice in extraction medium by suspension and centrifugation at 1000*g* for 4 min. The final washed pellet was resuspended in extraction medium. Aliquots of this suspension were used for determinations of amino acid-incorporating activity and RNA content. RNA was determined by extraction with hot perchloric acid (32).

Amino Acid Incorporation Assay. The reaction mixture for the assay is given in Table I. The temperature of incubation was 25 C. At various time intervals 100-μl aliquots were removed and placed on discs. The filter-paper disc method of Bollum (6) as described by Mans and Novelli (22) was used to determine amino acid incorporation into peptides. Bray's scintillation liquid, 10 ml (7), was used for counting each disc in a Nuclear Chicago scintillation counter.

RESULTS

Preparation of Etioplasts and Chloroplasts and Assessment of Amino Acid Incorporating Activity. The extraction and assay media shown in Table I were used for both etioplasts and chloroplasts. Since comparisons between etiolated and green tissues were required, a method suitable for isolation of plastids from either type of tissue was developed. The extraction medium was based on one developed by Jacobson (21). High concentrations of $MgCl_2$ and KCl were included in both the extraction and assay media since the 70S ribosomes of chloroplasts usually require high concentrations of these salts to stabilize them (4, 25). A single medium was suitable for obtaining active preparations of either etioplasts or chloroplasts. If $MgCl_2$, KCl, and dithiothreitol were omitted from the extraction medium, but included in the assay medium, activities were much lower.

Sissakian *et al.* (29) observed that the supernatant fraction from pea leaves inhibited the amino acid incorporating activity of isolated pea chloroplasts. We likewise found that the supernatant fraction from either etiolated or green pea leaves inhibited the activities of etioplasts and chloroplasts from pea and wheat. The supernatant fraction from etiolated or green wheat leaves was also inhibitory. To minimize the harmful effects of the supernatant fluid, the latter was separated from the plastids by centrifugation as soon as possible after tissue homogenization, and precautions were taken to prevent the temperature from rising above 2 C during these procedures. Table II illustrates the importance of washing the plastid preparations until they are free of residual supernatant fraction. The inhibiting agent in the supernatant fraction might have been ribonuclease, but the effect of the supernatant was not overcome by adding an excess of soluble RNA to the extraction medium.

Table III shows the requirements for protein synthesis by the various preparations. Although there were some variations among the different types of plastids, the same general pattern emerged. The provision of a system for generating ATP was essential, and activity was increased by the inclusion of GTP

Table II. *Effect of Washing Wheat Etioplast on Amino Acid-Incorporating Activity*

Etioplasts were isolated, washed, and assayed as described in "Materials and Methods."

No. of Washes	Specific Activity
	cpm mg RNA·min
0	770
1	1160
2	1365

Table III. *Effect of Various Components on ^{14}C-Leucine Incorporation by Plastids*

System	Activity of Control			
	Wheat etioplasts	Wheat chloroplasts	Pea etioplasts	Pea chloroplasts
	%			
Control[1]	100	100	100	100
Plus ^{12}C-amino acids (minus ^{12}C-leucine)		120	108	176
Minus ATP, P-enolpyruvate, and pyruvate kinase	14	0	42	8
Minus GTP	30	18	84	57
Plus EDTA (20 mM)	10	6	34	52
Plus ribonuclease (0.2 μg/ml)	21	23		26

[1] As in Table I except for ^{12}C-amino acids.

and a complete mixture of amino acids. Ribonuclease and in most cases EDTA inhibited the amino acid incorporating activity.

Possible Interference by Contaminating Bacteria, Nuclei, and Mitochondria. Experiences reported by other investigators point to the importance of avoiding bacterial contamination of preparations of isolated organelles used in studies of protein synthesis (2). Accordingly, portions of the reaction mixtures were plated out routinely so that the extent of bacterial contamination could be estimated. When the precautions mentioned in "Materials and Methods" were adhered to, the bacterial counts were very low. Young etiolated wheat plants proved to be the chief exception. These plants were harvested just prior to the emergence of the first leaf through the surrounding coleoptile. In the initial experiments the leaves were not dissected from the coleoptile. However, if the coleoptile was removed, most of the contaminating bacteria were eliminated and activity was greater. In the experiments reported here the coleoptile was removed to reduce potential sources of interference.

In addition to contamination by bacteria, plastid preparations are subject to contamination by other organelles which might also incorporate amino acids. Table IV shows experiments in which counts were made of the numbers of bacteria, chloroplasts and other particles in preparations of chloroplasts from wheat. The bacterial count was insignificant, and the fact that the ^{14}C incorporated was soluble in Triton X-100 confirms this and also shows that nuclei, which should not have been solubilized by this treatment (36), were not a problem either. In a similar experiment, however, it was noted that particles smaller than chloroplasts (mitochondria, chloroplast fragments) were almost as numerous as whole chloroplasts (Table IV). The contribution of contaminating mitochondria to amino acid incorporation by chloroplast preparations does not appear to have been considered in other studies, although it is known that plant mitochondria can incorporate amino acids (8). For estimation of the possible contribution by mitochondria, two particulate fractions, one obtained at 1000*g* (the normal chloroplast fraction) and one at 15,000*g* were prepared. The amino acid-incorporating activity of each fraction was estimated, as well as NAD-isocitrate dehydrogenase (to estimate the relative proportions of mitochondria in the two fractions) and NADH-malate dehydrogenase (to estimate the relative proportions of mitochondria plus peroxisomes). The results are shown in Table V, and it is apparent that the smaller particles did not contribute significantly to the amino acid incorporating activity of the chloroplast fraction. Although the 1000*g* fraction was nearly six times as active in amino acid incorporation as the 15,000*g* fraction, on the basis of the enzyme assays it contained only 0.4% of the mitochondria and 3.8% of the mitochondria plus peroxisomes of the 15,000*g* fraction.

Table IV. *Numbers of Bacteria, Plastids, and Other Particles in 1000g Fraction from Wheat Leaves*

Particle	Etiolated Wheat	Green Wheat	Ratio of Plastids to Bacteria	Ratio of Chloroplasts to Small Particles
	Number per ml			
Bacteria	800	1180		
Etioplasts	17×10^8		2×10^6	
Chloroplasts		9.8×10^8	0.8×10^6	
Mitochondria and other particles smaller than chloroplasts		7.4×10^8		1.3

Table V. *Specific Activities of Particulate Fractions from Green Wheat Leaves*

Assay	Fraction	
	1,000*g*	15,000*g*
	cpm/mg RNA·min	
^{14}C-Leucine incorporation	806	142
	μmoles/min·ml	
NAD-isocitrate dehydrogenase	0.0003	0.073
NADH-malate dehydrogenate	0.72	18.7

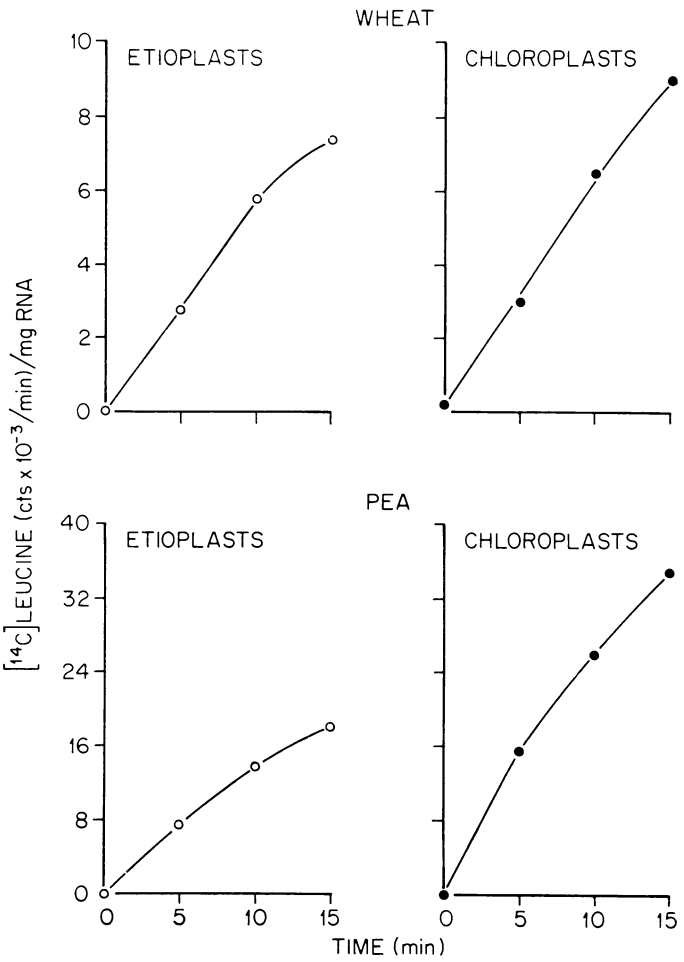

FIG. 1. Incorporation of ^{14}C-leucine into peptides by isolated etioplasts and chloroplasts from wheat and pea. Plastids were isolated as described in Materials and Methods.

Amino Acid Incorporating Activities of Etioplasts and Chloroplasts. Figure 1 shows the results of experiments in which the activities of etioplasts and chloroplasts from pea and wheat leaves were compared. The activity (on an RNA basis) was approximately twice as great in pea chloroplasts as in etioplasts in plants of the same chronological age, whereas the activities of etioplasts and chloroplasts from wheat were essentially equal.

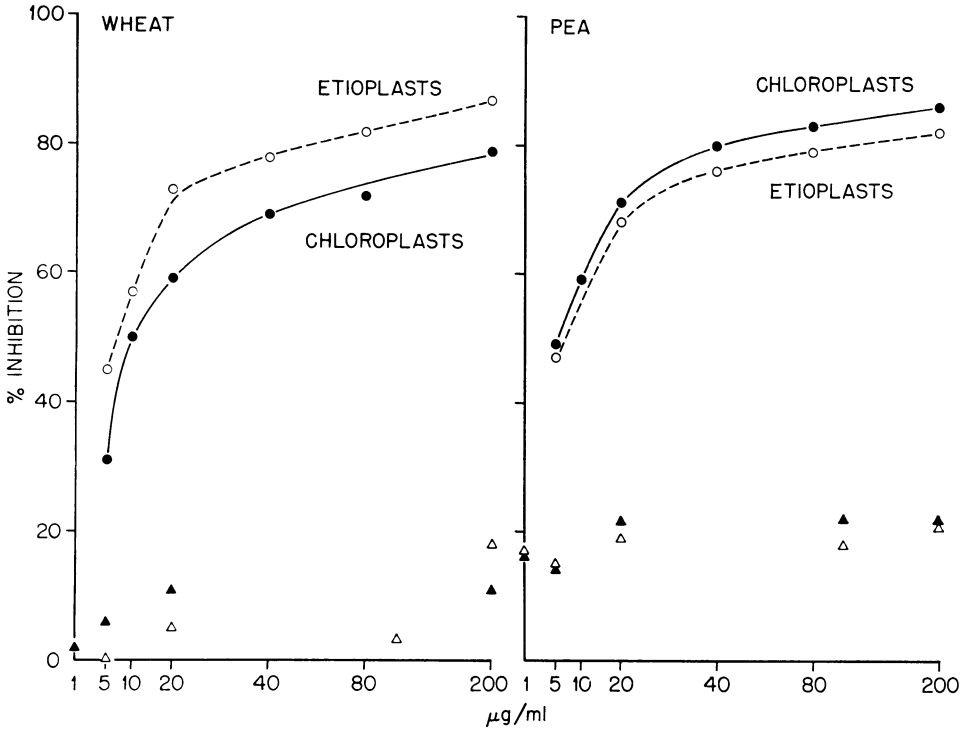

FIG. 2. Inhibition of ^{14}C-leucine incorporation into peptides by chloramphenicol and cycloheximide treatment of etioplasts and chloroplast from wheat and pea. Plastids were isolated as described in "Materials and Methods." Chloramphenciol: ○: etioplasts; ●: chloroplasts. Cyclo heximide: △: etioplasts; ▲: chloroplasts.

The Effect of Chloramphenicol and Cycloheximide. Figure 2 shows the effects of different concentrations of chloramphenicol and cycloheximide on the amino acid incorporating activities of etioplasts and chloroplasts from wheat and pea. Similar results were obtained with all four types of plastids; chloramphenicol showed a concentration-dependent inhibition, whereas cycloheximide showed little or no inhibition. With chloramphenicol 50% inhibition was obtained at a concentration between 5 and 10 μg/ml. The highest concentration of cycloheximide used (200 μg/ml) was 200-fold higher than the concentration which is usually sufficient for inhibition of the synthesis of cytoplasmic protein in plants.

Evans *et al.* (13) have observed that inhibition of chlorophyll synthesis in *Euglena* cells by chloramphenicol is considerably reduced if low concentrations of cycloheximide (1 to 2 μg/ml) are also present. Table VI shows that cycloheximide did not decrease inhibition of amino acid incorporation by chloramphenicol in isolated plastids.

DISCUSSION

The data in this report show that etioplasts which have been isolated from wheat and pea leaves have the necessary enzymic machinery for incorporation of amino acids into peptide chains. Similarly, Drumm and Margulies (9) have shown that etioplasts obtained from bean leaves can carry out protein synthesis. Since etioplasts are known to contain DNA (19, 21), RNA (37), and ribosomes (18), this is not surprising. In the case of wheat etioplasts, however, it is interesting that their activity on an RNA basis is comparable to that of chloroplasts. Since the RNA content of the etiolated and green wheat leaves is also comparable (28), wheat etioplasts appear to contain a fully developed system for the synthesis of plastid proteins. Thus the development of a protein-synthesizing system in wheat leaf plastids is independent of light, except perhaps in a nutritional sense.

Table VI. *Effect of Chloramphenicol and Chloramphenicol plus Cycloheximide on Amino Acid Incorporation by Wheat Etioplasts and Chloroplasts*

Plastids	Control	10 μg/ml Chloramphenicol	10 μg/ml Chloramphenicol + 1 μg/ml Cycloheximide
	cpm/mg RNA·min		
Etioplasts	569	269	269
Chloroplasts	632	297	307

In the dicotyledonous pea (Fig. 1) and bean (9) plants, the protein synthetic capacity of etioplasts is not as great as that of chloroplasts. In etiolated pea seedlings, activation of the phytochrome system by brief irradiation with light at about 660 nm results in marked increases in plastid rRNA (18) and plastid proteins (16). The amount of rRNA in the etioplasts of the irradiated plants does in fact reach the same levels found in chloroplasts, and the protein synthetic capacity of the etioplasts might show a similar increase. If they did, it would be unnecessary to postulate a direct effect of light on the development of protein synthetic capacity in plastids, since the phytochrome-induced increases in plastid protein and rRNA can be explained by the action of the phytochrome system on leaf growth, rather than by a direct effect on plastid development (33).

A different situation is found in *Euglena gracilis*. In this organism, light is required for the full development of protein synthetic capacity in the chloroplasts (27), as well as for the synthesis of chloroplast rRNA (28), and certain chloroplast tRNAs and aminoacyl-tRNA synthetases (26). Thus both the extent of development of a functional protein synthetic capacity for protein synthesis in plastids of plants maintained in the dark and the influence of light on the further development

of this capacity can vary greatly in different organisms. In particular, in higher plants the role of light in this phase of plastid development does not appear to be as important as has often been presumed.

Etioplasts and chloroplasts from wheat and pea show comparable responses to chloramphenicol and cycloheximide, *i.e.*, inhibition by chloramphenicol and little or no inhibition by cycloheximide. The sensitivity towards chloramphenicol is not as great as in the case of bacterial ribosomes, and this correlates with the lower binding capacity for chloramphenicol in chloroplast ribosomes (1). The small inhibition shown by cycloheximide is unexplained, and the possibility that some fraction of the protein synthetic capacity of plastids is sensitive to cycloheximide cannot be discounted.

Acknowledgment—We are indebted to Mr. Nicholas Rigopoulos for excellent technical assistance.

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