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EVENTS SURROUNDING THE EARLY DEVELOPMENT OF *EUGLENA* CHLOROPLASTS

15. ORIGIN OF PLASTID THYLAKOID POLYPEPTIDES IN WILD-TYPE AND MUTANT CELLS

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Summary

Techniques are described for the isolation of plastid thylakoid membranes from light-grown and dark-grown cells of *Euglena gracilis* var. *bacillaris*, and from mutants affecting plastid development. These membranes, which have minimal contamination with other cell fractions, are localized in sucrose gradients by using the thylakoid membrane sulfolipid as a specific marker. The plastid thylakoid membrane polypeptides isolated from these membranes were separated on SDS polyacrylamide gels and yielded patterns containing 30—40 polypeptides. Light-grown strain Z gave patterns identical with *bacillaris*. Since the plastid thylakoid polypeptide patterns obtained from dark-grown wild-type cells and from a bleached mutant W_3BUL in which plastid DNA is undetectable are identical, it appears that the proplastid thylakoid polypeptides of wild-type cannot be coded in plastid DNA and are probably coded in nuclear DNA. The plastid thylakoid polypeptide patterns obtained from various dark-grown mutants are identical to those obtained from dark-grown wild-type cells. Light-grown mutants, making large but abnormal chloroplasts, show a correlation between the amount of chlorophyll formed and the amount of a plastid thylakoid polypeptide thought to be associated with one of the pigment-protein light-harvesting complexes. Treatment with SAN 9789 (4-chloro-5-(methylamino)-2-(α,α,α,-trifluoro-*m*-tolyl)-3-(2H(pyridazinone) known to block carot-

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enoid synthesis at the level of phytoene, causes a progressive loss of all plastid thylakoid polypeptides during growth in darkness and results in the establishment of a new, lower steady-state level of sulfolipid. At least ten of the plastid thylakoid polypeptides become labeled when isolated chloroplasts are supplied with radioactive amino acids; of these six are undectable in W_3BUL and are, therefore, candidates for coding by plastid DNA.

Introduction

Among the constituents formed in developing chloroplasts are the proteins, and their constituent polypeptides, of the thylakoid membranes. We now know that soluble plastid proteins extractable with buffer solutions are coded in plastid DNA [1,2], nuclear DNA [1,3], or both [4—6] in many organisms including *Euglena* and it is therefore of interest to determine where the insoluble proteins of the thylakoids originate and how their formation is regulated. In this paper we use the thylakoid sulfolipid of *Euglena* to locate the plastid membranes of light-grown and dark-grown wild-type and mutant cells on gradients. The thylakoid membrane polypeptides from these plastid membranes are then separated on sodium dodecyl sulfate (SDS) gels. Comparison of the patterns obtained with those of isolated chloroplasts allows inferences to be made concerning their cellular origins and the regulation of their synthesis. Brief reports of this work have appeared previously [7—10].

Materials and Methods

Organism and culture conditions. Unless noted otherwise, all experiments to be described were performed with the *bacillaris* strain and its mutants.

Euglena gracillis Klebs var. *bacillaris* Cori or strain Z Pringsheim was maintained in light or darkness on Hutner's pH 3.5 medium as previously described [11,12,34]. Some dark-grown cells were treated with 25 μg/ml of SAN 9789 (4-chloro-5-(methylamino)-2-(α,α,α,-trifluoro-*m*-tolyl-3-(2H)pyridazinone) as described previously [13]. Cells were counted using a Coulter counter model Z_B by dilution of the cell suspension with a solution 0.8% in NaCl and 0.1% in thiomersol (merthiolate).

Isolation of cell fractions. For determination of the thylakoid polypeptide composition, three times washed chloroplasts were obtained by differential centrifugation [14]. These plastids were suspended in either buffer IV [14] or buffer IV minus magnesium; the same results as far as plastid membrane polypeptides are concerned are obtained with either buffer. After passage through the French pressure cell and centrifugation [14], the pellet was washed in either 0.15 M EDTA, pH 7.0, or in 50 mM Tris-HCl, pH 7.6, at 4°C and the pellet was used for solubilization of polypeptides. Alternatively, after passage through the French pressure cell, deoxycholate was added to a concentration of 0.1% (w/v), the suspension was shaken for 15 min at 2°C and the pellet was used for solubilization of membrane polypeptides. The same polypeptide patterns were obtained with either procedure. Chloroplasts used for incorporation of radioactive amino acids were obtained by centrifugation of cell homoge-

nates through Ludox gradients [15]. The same thylakoid polypeptide patterns are obtained with any of the above techniques.

All steps in membrane isolation from whole cells were performed at 4°C. 1 l of growin cells ($1—1.5 \cdot 10^6$ cells/ml) was harvested and washed in 50 mM Tris-HCl buffer, pH 7.6. They were then resuspended in 2.5 ml of this buffer and passed through a French pressure cell at 4000 lb/inch^2. In order to break organelles and provide reasonably uniform sizes of membrane fragments, these homogenates were sonicated in a Branson Cell Disruptor for 5 s at the maximum setting for the microtip. This treatment completely disrupted chloroplasts and mitochondria as determined by release of ribulose bisphosphate carboxylase [16] and malate dehydrogenase [17] activity, respectively. 5 ml of sonicate were layered on a step gradient composed of (from top to bottom): 35% (w/v) sucrose (10 ml); 43% (w/v) sucrose (10 ml); 50% (w/v) sucrose (10 ml); and 60% (w/v) sucrose (5 ml) in the above buffer. The gradient was centrifuged for 3 h at 80 000 × *g*. Tubes were punctured at the bottom and 1 ml fractions were collected. To isolate thylakoid membranes the fractions containing the pure plastid membranes were diluted with 0.15 M EDTA, pH 7.0, centrifuged at 100 000 × *g* for 30 min and washed by centrifugation with 50 mM Tris-HCl, pH 7.6, to remove the EDTA. Membrane recovery was judged by the total membrane protein recovered from the appropriate fractions of the sucrose step gradient.

Growth of cells on $^{35}SO_4^{2-}$ *and labeling of sulfolipid.* To label the cells, Hutner's pH 3.5 medium with 0.1 the normal level of sulfate (achieved by replacing $MgSO_4$ with $MgCl_2$ as necessary) was fortified with 1.0 μCi/ml carrier-free $^{35}SO_4^{2-}$. After growth gradient fractions were prepared as above, 1 ml fractions were collected and lipids for determination of lipid-soluble radioactivity were extracted [18]. To each 1.0 ml fraction from the gradient, 2.5 ml of methanol and 1.25 ml chloroform were added. 1.25 ml of water was then added and after shaking, another 1.25 ml chloroform was added. After brief centrifugation in a table-top centrifuge, the upper aqueous phase was removed and 1.0 ml of the lower organic phase was placed in a scintillation vial together with a drop of 1% benzoyl peroxide in chloroform to decolorize the pigments, and the solvents were evaporated. 10 ml of scintillation mixture (5 g/l 2,5-diphenyloxazole (PPO) in toluene) were then added and the samples were counted in a Beckman liquid scintillation counter. When the extracted lipids were separated by two dimensional chromatography on silica plates [19], more than 80% of the radioactivity comigrated with the sulfolipid.

Solubilization and separation of membrane components. The pelleted EDTA and buffer-washed membrane fractions (see above) were solublized by heating at 100°C for 2 min in 0.05 M Tris-HCl, pH 8.3, 8 M with respect to urea, 2% with respect to sodium dodecyl sulfate and 5% with respect to mercaptoethanol. Glycerol to a final concentration of 5% (v/v) was added to the cool solution to facilitate application to the gels. Membrane pellets were of different volumes depending on the strain from which they were isolated. In order to compare the absolute amounts of the polypeptides in cells, the membranes were always isolated from equivalent cell numbers. Equal amounts of sample buffer were added to each membrane sample, usually enough to solubilize all polypeptides of the largest pellet (a protein concentration of 2—3 mg/ml from

membranes of fully green wild-type cells). In order to demonstrate qualitative relationships between the polypeptides of the light and dark-grown wild-type cells and mutants, each pellet was resuspended in enough buffer to give a protein concentration of 2—3 mg/ml.

Gel electrophoresis on polyacrylamide employed the discontinuous buffer system of Laemmli [20] using a slab 0.75 mm thick with a 20 cm linear 7.5—15% acrylamide gradient accompanied by a 5—17.5% sucrose gradient [21]. The samples were subjected to electrophoresis at 50 V through the stacking gel for 1 h and 300 V through the running gel for 6—7 h. The gels were stained overnight with 0.25% Coomassie brilliant blue R in 50% methanol/7% acetic acid and were destained in 10% acetic acid. Molecular weights on the gels were calibrated with the following protein standards: bovine serum albumin, 68 000; ovalbumin, 45 000; chymotrypsin, 25 000; and horse cytochrome *c*, 12 400. Gels were cut longitudinally and scanned at 540 nm in a Guilford Model 2000 recording spectrophotometer equipped with a linear transport scanner.

Protein synthesis in isolated chloroplasts. Protein synthesis in isolated chloroplasts was studied using the method of Vasconcelos [22] using 100 μl of a chloroplast suspension (75—125 μg of chlorophyll) placed in a 10 ml Erlenmeyer flask fitted with a Wratten No. 92 red filter. The dark control flasks were completely wrapped in black tape. 10 μl of a ^{3}H-labeled amino acid mixture (New England Nuclear) equaling 10 μCi was added to each flask along with enough assay buffer (0.33 M sorbitol and 50 mM Tricine/KOH, pH 8.4) to make 0.5 ml. The reaction was carried out at 21°C in an American Instrument Co. shaking water bath illuminated from below with four white flood lights at an intensity of 2200 ft-candles and was initiated by addition of the label. Flasks were illuminated and shaken for 30 min, whereupon synthesis was stopped by one of two methods. For measurement of total incorporation into protein 0.5 ml of cold 10% trichloroacetic acid was added to each sample, which was kept on ice for 30 min, heated to 90°C for 10 min, and the precipitated protein collected on a 0.54 μM Millipore filter. The filter was subsequently washed with 10 ml trichloroacetic acid, 10 ml of a non-radioactive amino acid mixture containing the components of the New England Nuclear mixture, and 10 ml of 80% ethanol at 60°C to remove pigments. Scintillation fluid (see above) was added to the dried filter which was counted in a Beckman liquid scintillation counter. For analysis of incorporation into individual polypeptides, the chloroplast suspension was diluted with 50 mM Tris-HCl, pH 7.6, and sonicated for 10 s at the maximum setting for the microtip of a Branson Cell Disrupter. Membranes were pelleted by a 100 000 $\times g$ centrifugation and were solubilized for electrophoresis.

Radioactivity in polypeptide bands on gels. Radioactivity in the polypeptides was determined by slicing the bands from stained gels. Slices were incubated overnight at 50°C in scintillation vials containing 0.3 ml 30% hydrogen peroxide. The solubilized protein was subsequently counted in a Beckman LS-50 liquid scintillation counter using 3 ml of scintillation fluid consisting of two parts toluene to one part Triton X-100 and containing 5 g 2,5-diphenyloxazole (PPO)/l. Slicing the gels into consecutive 2-mm slices yielded the same data as cutting out the bands, indicating that the radioactivity was localized exclusively in the stained bands.

Experiments with antibiotics. Appropriate antibiotics were added before illumination. The final chloramphenicol concentration was 2 mg/ml added as a filter-sterilized 4 mg/ml stock solution in assay mix. Cycloheximide to 15 μg/ml was added from a filter sterilized 15 mg/ml stock solution.

Enzyme assays. The following enzymes were assayed by diluting about 0.1 ml of each 1.0 ml gradient fraction with the appropriate reaction mixture and buffer given in the original methods; succinate dehydrogenase [23,24], NADH-ferricyanide reductase [24], antimycin A-insensitive NADPH-cytochrome *c* reductase [23], NADH-cytochrome *c* reductase [24], and dicyclohexylcarbodiimide-insensitive Mg^{2+}-dependent ATPase [25]. Inorganic phosphate was determined by the method of Chen et al. [26] and protein by the method of Lowry et al. [27]. Chlorophyll and carotenoid were determined as described previously [12].

Results and Discussion

Sulfolipid as a thylakoid membrane marker in Euglena

The *Euglena* system has been especially useful in determining where plastid constituents are coded and synthesized because dark-grown cells contain proplastids which can be induced by light to form chloroplasts in non-dividing cells where complications due to cell divisions are not present [28—30]. In addition, mutants such as W_3BUL are available which lack detectable plastid DNA, plastid rRNAs and plastid pigments and probably contain only those plastid constituents coded outside of plastid DNA and synthesized on non-plastid ribosomes [28—30]. Reasonably good chloroplasts can be prepared from *Euglena*, especially if only thylakoid membranes are desired, but methods for obtaining large quantities of clean proplastids from dark-grown cells and nuclear-coded plastid membrane remnants from W_3 are not available. (A method for obtaining small amounts of proplastids from dark-grown cells has been reported [31].) The problem, then, in determining where plastid membrane constituents are coded and synthesized is to locate and isolate these membranes when a fully formed intact plastid is not present in the cells. Since we have found (consistent with earlier work of others [48]) that the sulfolipid of *Euglena* is synthesized in light-grown wild-type cells (containing chloroplasts), dark-grown wild-type cells (containing proplastids) and cells of W_3BUL (lacking detectable plastid DNA), this membrane constituent must be synthesized by enzymes coded outside of plastid DNA, probably in nuclear DNA and, therefore, provides a useful marker for plastid thylakoid membranes from all three types of cells. Therefore, the three types of cells were grown on $^{35}SO_4^{2-}$ to label the sulfolipid which they all contain. The total membrane fractions of these cells and of chloroplast preparations were isolated after mild sonication which was found to improve the uniformity of the preparations, probably by reducing fragments to a more or less uniform size distribution.

After centrifugation of these membrane preparations through sucrose step gradients, three peaks of lipid-soluble ^{35}S were observed in membranes prepared from purified chloroplasts, light-grown wild-type cells, dark-grown wild-type cells and cells of W_3BUL (light or dark grown) (Fig. 1). The fact that pure chloroplasts also give three peaks coupled with the observation (Fig. 1) that the

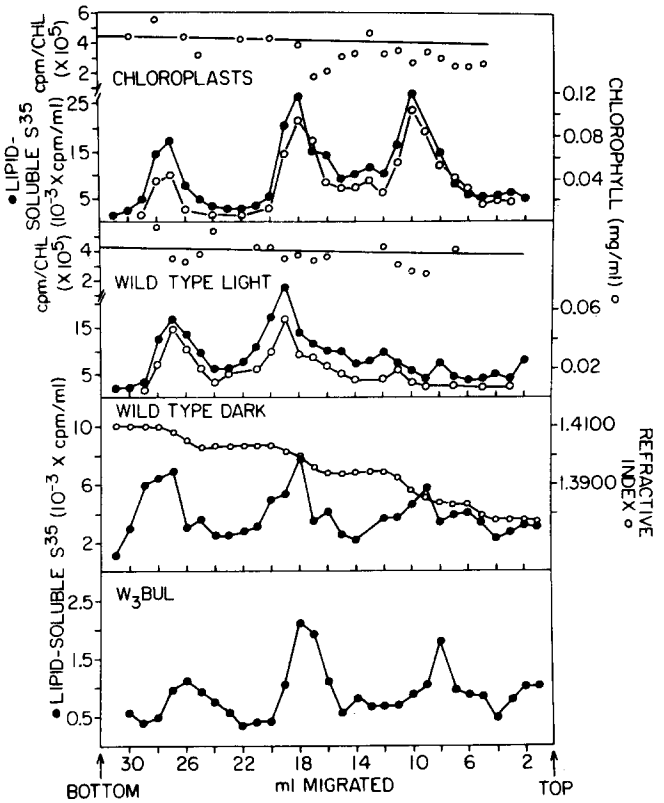

Fig. 1. Membranes from purified chloroplasts and whole cells of light-grown wild type, dark-grown wild type and light-grown W_3BUL separated on sucrose step gradients. Lipid-soluble ^{35}S estimates sulfolipid; chlorophyll content and refractive index are also shown. A lower, middle and upper band is found in each case; the middle band contains fairly clean plastid thylakoids. The plots of cpm/Chl show the constancy of the ratio of lipid-soluble ^{35}S to chlorophyll across the gradients.

ratio of lipid-soluble ^{35}S to chlorophyll is relatively constant across the gradients from chloroplasts and wild-type light-grown cells, suggests that all three fractions contain plastid membranes and that ^{35}S and chlorophyll move together through these gradients establishing the usefulness of lipid-soluble ^{35}S as a plastid thylakoid membrane marker.

Experiments aimed at determining which fractions contained pure plastid membranes (Table I) show that markers [23—25] of mitochondrial membranes such as succinate dehydrogenase, NADH-ferricyanide reductase, and NADH-cytochrome *c* reductase, microsomes (antimycin A-insensitive NADPH-cytochrome *c* reductase and NADH-ferricyanide and cytochrome *c* reductase), and enzymes usually found in the plasma membrane and plastid envelope (Mg^{2+}-dependent ATPase) are high in the upper fraction, appreciable in the lower fraction, but show minimum values in the middle fraction. The lowermost fraction (the interface between 50% and 60% sucrose) probably contains clumped organelles and membranes while the upper fractions contain endoplasmic reticulum, mitochondrial membranes and, perhaps, plastid envelopes and microbody membranes [32]. The mitochondrial membrane fraction bands

TABLE I

ENZYME ACTIVITIES IN UPPER, MIDDLE (PLASTID THYLAKOID) AND LOWER GRADIENT FRACTIONS AND IN MEMBRANES FROM PURIFIED CHLOROPLASTS

Abbreviations used: *Euglena gracilis* var. *bacillaris*: WTL, light-grown wild-type cells; WTD, dark-grown wild-type cells; W_3, light-grown cells of W_3BUL. U, upper fraction; M, middle (thylakoid) fraction; L, lower fraction; n.d., not detectable; cyt, cytochrome.

		μmol cytochrome *c* or ferricyanide reduced · ml^{-1} · min^{-1}					
		Succinate dehydrogenase		NADH-cyt *c* reductase	NADH-ferricyanide reductase	NADPH-cyt *c* reductase	Mg^{2+}-dependent ATPase (μmol P_i · ml^{-1} · h^{-1})
		Cyt *c*	Ferricyanide				
WTL	U	6.6	—	60.0	660	8.4	18
	M	1.3	—	1.1	2	0.84	1.9
	L	5.6	—	12.0	51	2.1	n.d.
WTD	U	8.4	4.1	62.0	295	11.4	6.7
	M	0.47	n.d.	0.62	29	1.6	0.87
	L	8.4	n.d.	4.0	53	2.2	0.35
W_3	U	7.0	4.4	20.0	233	14.2	0.29
	M	0.14	n.d.	1.0	29	0.7	0.16
	L	2.9	n.d.	4.5	46	1.9	0.13
Isolated chloroplasts			n.d.	n.d.	n.d.	n.d.	n.d.

at the interface between 35% and 43% sucrose (density about 1.17 g/cm^3) while the microsomes band above this at a density of about 1.13 g/cm^3. The purest plastid thylakoid membranes are found at the interface of 43% and 50% sucrose (density about 1.21).

In other systems such as spinach, Mg^{2+}-dependent ATPases form at least two groups: those of the envelopes of the cell and plastid which are not inhibited by dicyclohexyl carbodiimide (DCCD) and those of the plastid thylakoids which are inhibited by DCCD [25]. Table II shows that in gradient fractions prepared from total cell membranes of light-grown wild-type cells, all fractions contain DCCD-inhibitable ATPase as might be expected from the distribution of chlorophyll and sulfolipid. Only the upper fraction, however, contains appreciable activity resistant to DCCD indicating that cell and plastid evelopes are present in this fraction and do not contaminate the middle fraction used as a source of reasonably pure thylakoid polypeptides in the experiments to be described.

For purposes of quantitation it is important to know whether the middle fraction of the gradients is a reliable and constant sample of the total thylakoid membranes, since these membranes appear in all three gradient fractions. Table III shows that in all four types of cells, a constant fraction of the sulfolipid, chlorophyll (when present) and membrane protein appears in the middle fraction. This means that the techniques employed always yield the same fraction of the thylakoid membranes present regardless of the cells from which they are taken, and that the middle fraction can be used as a constant sample of the

TABLE II

Mg^{2+}-DEPENDENT ATPase ACTIVITY IN SUCROSE STEP GRADIENTS FROM WILD-TYPE CELLS GROWN IN THE LIGHT

Thirty 1.0 ml fractions were collected and the activity of the Mg^{2+}-ATPase was determined on an 0.1 ml aliquot of each fraction. The three fractions above represent the three interfaces where the cell membranes accumulate. Dicyclohexyl carbodiimide (DCCD).

Fraction	Chlorophyll (μg/ml)	Mg^{2+}-ATPase (μmol P_i released · ml^{-1} · h^{-1})	
		+DCCD	—DCCD
Upper	20	18	44
Middle	77	1.9	87
Lower	55	0	62

total thylakoid material for purposes of quantitation. Sulfolipid and chlorophyll in the middle fraction represent a higher fraction (about 0.45 of the total) than protein (about 0.2) probably because the two lipid materials are specific to the plastid thylakoids while membrane protein can arise from any of the membranes of the cell. Why the plastid membranes break up during isolation and sonication to give three separate fractions in constant ratios is not clear.

Polypeptides from purified thylakoid membranes of wild type and W_3BUL

Figs. 2 and 3 show the polypeptide patterns obtained on detergent treatment of the thylakoid membranes isolated from the middle gradient fractions. In Fig. 2 and the left side of Fig. 3, equal amounts of protein from the various types of cells was placed on the gradients to permit a comparison of the types of bands present in cells of dark-grown and light-grown wild type and W_3BUL. The right-hand side of Fig. 3 displays the amounts of plastid thylakoid polypeptides from equal numbers of cells permitting a quantitative comparison of membrane recoveries from the various types of cells.

Fig. 2 shows that the pattern of bands recovered from purified chloroplasts

TABLE III

RECOVERY OF SULFOLIPID MEMBRANE PROTEIN AND CHLOROPHYLL IN THE MIDDLE (THYLAKOID MEMBRANE) FRACTION OF SUCROSE GRADIENTS

In each case, the sulfolipid in the middle fraction was divided by the sum of the sulfolipid in all three fractions; recoveries of protein and chlorophyll were computed similarly. n.d., not detectable.

Membrane origin	Chlorophyll	[^{35}S]sulfolipid fraction recovered	Protein
Isolated chloroplasts	0.42	0.42	—
WTL	0.44	0.47	0.26
WTD	n.d.	0.40	0.19
W_3L	n.d.	0.48	0.20
W_3D	n.d.	0.46	0.26

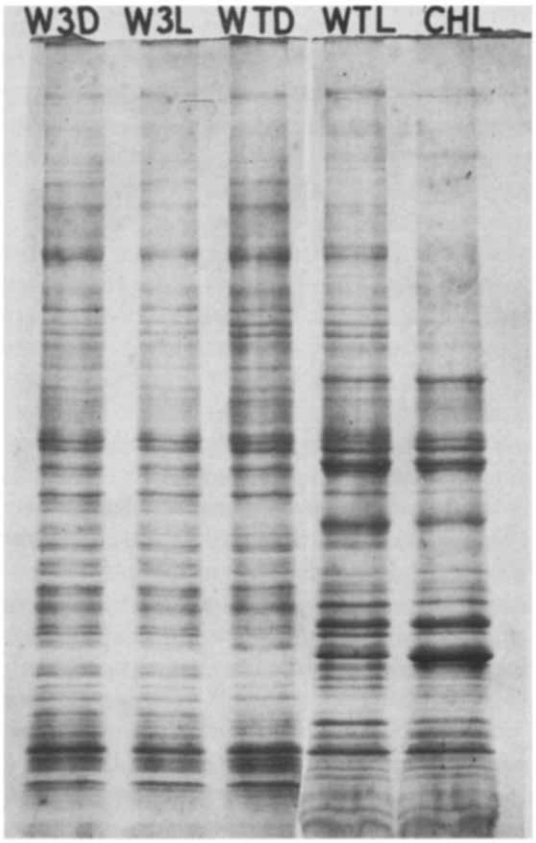

Fig 2. Polypeptide patterns from plastid thylakoid (middle) fractions of dark-grown W_3BUL (W_3D), light-grown W_3BUL (W_3L), dark-grown wild-type (WTD) and light-grown wild-type (WTL) cells and from thylakoid membranes of purified chloroplasts (CHL) separated on SDS-polyacrylamide gels. The samples placed on the gels contained equal amounts of protein.

is identical to the pattern from thylakoids isolated from cell homogenates of light-grown wild-type cells in the middle fraction of the gradients, below a molecular weight of about 60 000. This indicates that the membranes isolated directly from the cells are indeed plastid thylakoids as inferred from chlorophyll and sulfolipid determinations. Identical patterns were obtained from thylakoids isolated from cell homogenates of light-grown wild-type cells of the Z strain. Above a molecular weight of 60 000, bands are present in the cell preparations which are absent from the chloroplasts. Either these represent contaminations from other cellular fractions, which seems unlikely in view of the close correspondence of the bands below a molecular weight of 60 000 or these higher molecular weight polypeptides were removed from the plastid thylakoids during isolation and washing of the chloroplasts.

Fig. 3 (left) shows that the thylakoid polypeptide patterns from dark-grown wild-type cells and cells of W_3BUL (either light grown or dark grown) are nearly identical. Fig. 3 (right) shows that quantitatively, the patterns are also very nearly the same from equal numbers of cells. Since plastid DNA is

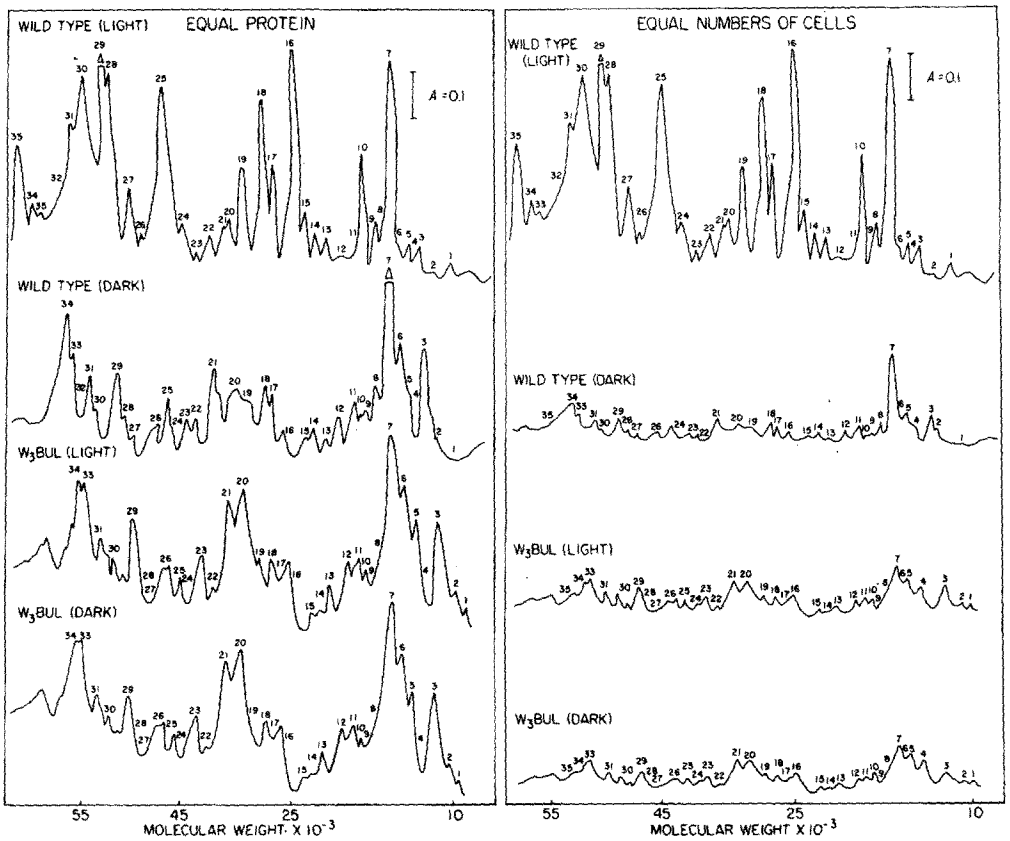

Fig. 3. Densitometer tracings of polypeptide patterns from plastid thylakoid (middle) fractions of dark-grown W_3BUL, light-grown W_3BUL, dark-grown wild-type and light-grown wild-type cells and from thylakoid membranes of purified chloroplasts separated on SDS-polyacrylamide gels and stained with Coomassie blue. Left. Equal amounts of protein were placed on the gel for each sample. Right. Protein from equal numbers of cells was placed on the gel.

undetectable in W_3BUL [33] the polypeptides of this mutant cannot be coded in plastid DNA but rather in nuclear (probably) or mitochondrial (much less likely) DNA. Thus it appears very likely that all of the proplastid thylakoid polypeptides in dark-grown cells are nuclear coded. The finding that W_3BUL contains all of these bands is consisitent with recent findings that this mutant contains atypical proplastid-like bodies [30,35,36,50] which are undoubtedly proplastic remnants composed of the nuclear-coded constituents of normal proplastids. The fact that the middle fraction has the same density when isolated from dark-grown wild-type cells and light and dark-grown cells of W_3BUL and that it yields the same thylakoid polypeptide patterns qualitatively and quantitatively, suggests that the three types of membranes have very similar compositions and that there is no extensive synthesis of new components on placing dark-grown cells of W_3BUL in the light. Thus the changes in structure from a homogenous body to a rudimentary prolamellar body seen in electron micro-

graphs [30,50] of dark-grown and light-grown cells of W_3BUL must be due to reorganization of existing structures or incorporation of material which does not change the density or polypeptide composition of the membranes.

Polypeptides from purified thylakoids of plastid mutants

We have investigated several mutants of *Euglena* which make chloroplasts with abnormal thylakoid contents or arrangements. These mutants [47] may be conveniently grouped into classes: (1) mutants which make fairly normal-sized plastids in the light but have altered amounts of chlorophyll and membranes and abnormal plastid morphology; these include P_1BXL, O_1BS, O_2BX, and G_1BU. (2) Mutants which develop little beyond the proplastid stage in light including Y_1BXD and Y_3BUD. (3) Mutants which have abnormal proplastids in light or dark, and in which plastid DNA is undetectable [33]: W_3BUL, W_8BHL, and W_{10}BSmL [37]. In the following discussion, one mutant from each group will serve as an example but it may be assumed unless stated otherwise that all other mutants in that group show the same characteristics.

The dark-grown mutants all share the same characteristics (Figs. 2—4); the thylakoid polypeptide patterns are qualitatively and, within the variability of the preparations, quantitatively the same as those of dark-grown wild-type cells. The membrane protein recovered from each of the mutants is also the same within experimental error (Table IV), even though carotenoid content varies, particularly in W_3BUL, W_8BHL and W_{10}BSmL. Although proplastid abnormalities exist in W_3BUL, W_8BHL, and W_{10}BSmL and in Y_3BUD grown in darkness, this is not reflected in the thylakoid membrane polypeptide patterns. Mutant G_1BU is known to be constitutive for plastid tRNAs in darkness, i.e. dark-grown cells of wild type contain low levels of plastid tRNAs but dark-grown cells of G_1BU have levels of tRNAs comparable to light-grown wild-type cells [38]. Fig. 4 shows that the plastid membrane polypeptides are not depressed in G_1BU since the levels are the same as in dark-grown wild-type cells.

For cells of wild type and mutants grown in the light (Table IV), the recovery of thylakoid membrane protein in the middle fraction is proportional to the chlorophyll content of the cells for all mutants which develop normal-size chloroplasts (group 1). The ratio of chlorophyll/cell to the amount of band 16 recovered from these cells is also fairly constant as might be expected since this band is thought to be associated with major chlorophyll binding proteins of the thylakoids [39,40]. For mutants which do not develop beyond the proplastid stage in light (group 2) little chlorophyll is formed and membrane recoveries from light-grown cells are comparable to those from dark-grown cells. The same is true for light-grown cells of group 3 which lack detectable plastid DNA and have only abnormal proplastid remnants.

Fig. 5 shows the thylakoid membrane polypeptide bands from these light-grown mutants. As already mentioned, band 16 is reduced in the mutant cells on a protein basis (Fig. 5, left; Figs. 2 and 3, left). Other bands in this region of the gel are seen to vary as well. Otherwise, all of the polypeptides in the mutants are very similar qualitatively. Compared on a quantitative basis (amount of each band from equal numbers of cells; Figs. 2 and 3 right, Fig. 5 right) the amounts of most bands is related to the extent of chloroplast

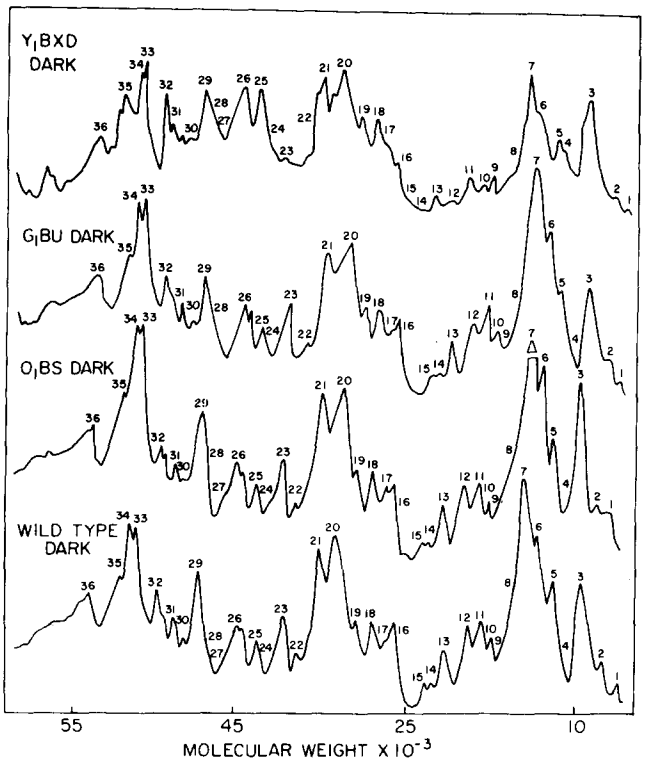

Fig. 4. Densitometer tracings of polypeptide patterns from plastid thylakoid (middle) fractions of various dark-grown mutants and dark-grown wild-type cells separated on SDS-acrylamide gels and stained with Coomassie blue. The same patterns are obtained from equal amounts of protein or from equal numbers of cells since the thylakoid membrane protein recovered from equal numbers of cells was very nearly equal for all types of cells.

development in the particular mutants. The greater the extent of plastid development in each type of mutant the greater the amounts of each band recovered. Mutants of groups 2 and 3 (the Y and W mutants) show the same patterns qualitatively and quantitatively in light and dark, indicating that all of these bands, which are presumed to be nuclear coded are not induced to form or to increase by light in these strains. This agrees with the lack of significant morphological development beyond the proplastid in these strains. The mutants of group 1 show extensive unstacked thylakoid regions [37]. Perhaps this is related to the changes in band 16 since it has been shown that bands in this region are associated with light-harvesting pigment-protein complexes [41,42] thought to comprise the larger membrane particles which are quantitatively correlated with the degree of stacking [43].

Thylakoid polypeptides in wild type during inhibition of carotenoid synthesis

Inhibition of carotenoid biosynthesis with SAN 9789 which blocks the pathway at the level of phytoene has been shown to interfere with thylakoid membrane formation in *Euglena* [13]. Fig. 6 shows that SAN 9789 does not inhibit

TABLE IV

CHARACTERISTICS OF LIGHT-GROWN AND DARK-GROWN WILD-TYPE AND MUTANT PLASTID THYLAKOID MEMBRANES

The data in the left and right halves of the table were obtained, respectively, from two separate experiments. n.d., not detectable.

Strain	Carotenoid (pg/cell)		Chlorophyll (pg/cell; light)	Band 16 (% total membrane protein) *	Chlorophyll in light/% membrane protein in in Band 16	Chlorophyll (pg/cell; light)	Thylakoid membranes (middle fraction; mg protein/ml)		Chlorophyll in light/ membranes in light (pg^{-1} cell · mg^{-1} protein)
	Light	Dark					Light	Dark	
Wild type	3.52	0.43	6.4	12.0	0.53	6.7	4.7	1.4	1.4
O_2BX	1.72	0.43	4.8	5.0	0.56	5.8	3.9	1.2	1.5
O_1BS	1.60	0.41	2.3	4.2	0.54	2.6	1.9	1.0	1.4
P_1BXL	1.58	0.42	2.0	3.9	0.51	2.0	1.8	1.1	1.1
G_1BU	0.69	0.40	1.3	n.d.	—	1.3	1.5	1.0	0.87
Y_1BXD	0.43	0.38	0.3	n.d.	—	0.3	1.2	1.0	—
Y_3BUD	0.58	0.40	0.2	n.d.	—	0.2	1.2	1.0	—
W_3BUL	0.22	0.08	n.d.	n.d.	—	n.d.	1.1	0.8	—
W_8BHL	0.11	0.04	n.d.	n.d.	—	n.d.	1.0	0.9	—
W_{10}BSmL	n.d.	n.d.	n.d.	n.d.	—	n.d.	0.9	0.8	—

* Protein in band 16 divided by total membrane protein on gel (sum of all bands).

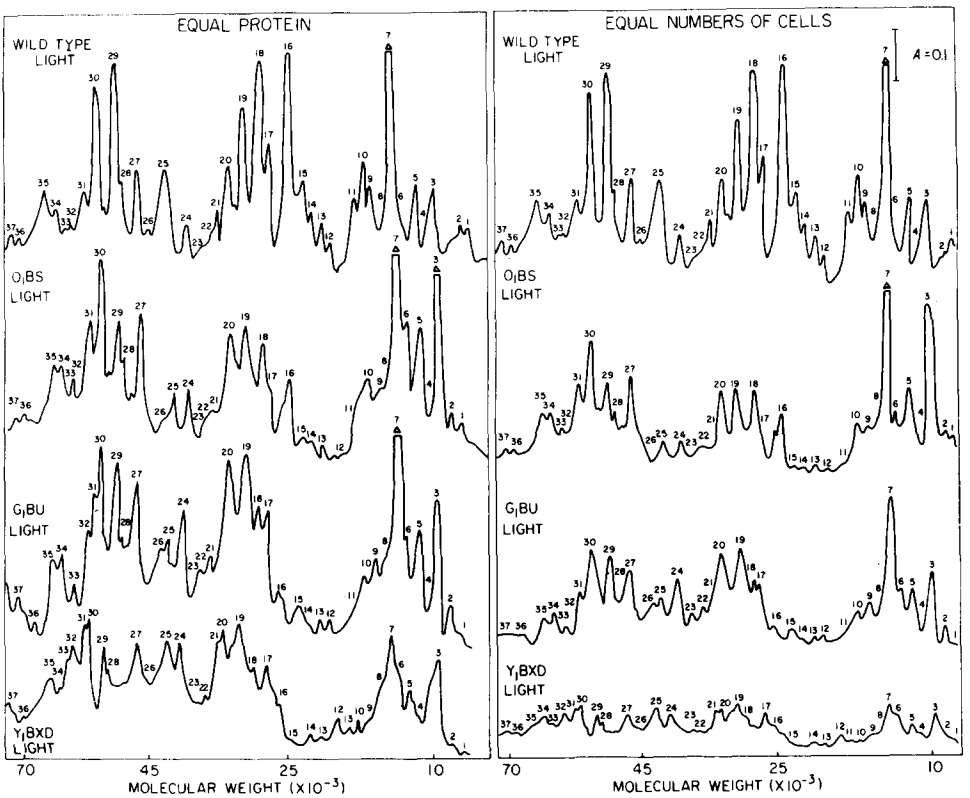

Fig. 5. Densitometer tracings of polypeptide patterns from plastid thylakoid (middle) fractions of various light-grown mutants and light-grown wild-type cells separated on SDS-acrylamide gels and stained with Coomassie blue. Left. Equal amounts of protein were placed on the gel for each sample. Right. Protein from equal numbers of cells was placed on the gel.

the uptake of sulfate into dark-grown dividing cells. The consequences of SAN inhibition however, are different for carotenoid, sulfolipid and membrane thylakoid polypeptide synthesis (Fig. 6). Treatment of dividing cells with SAN 9789 causes an immediate drop in the carotenoid/cell as a result of the inhibition of carotenoid synthesis and the dilution of remaining carotenoid among the progeny cells. Inhibition is not complete since the rate of loss is far less than the 0.5/generation expected for 100% inhibition. The rate of loss of thylakoid membrane polypeptides is less than that of the carotenoids leading to a loss of about 65% by the end of 24 generations. The sulfolipid content of the cells showns a somewhat different pattern (Fig. 6). After achieving constant specific activity, the SAN-treated cells maintain a constant, but lower level of sulfolipid than the controls. Since SAN 9789 is known to block the biosynthesis of carotenoids in *Euglena* at the level of phytoene, the consequences of this inhibition seem to be different for the thylakoid polypeptides and the sulfolipid. In the case of the polypeptides, the rate of formation of these molecules is reduced below the level necessary to maintain constant amounts/cell during cell division. The effect on the synthesis of the sulfolipid, on the

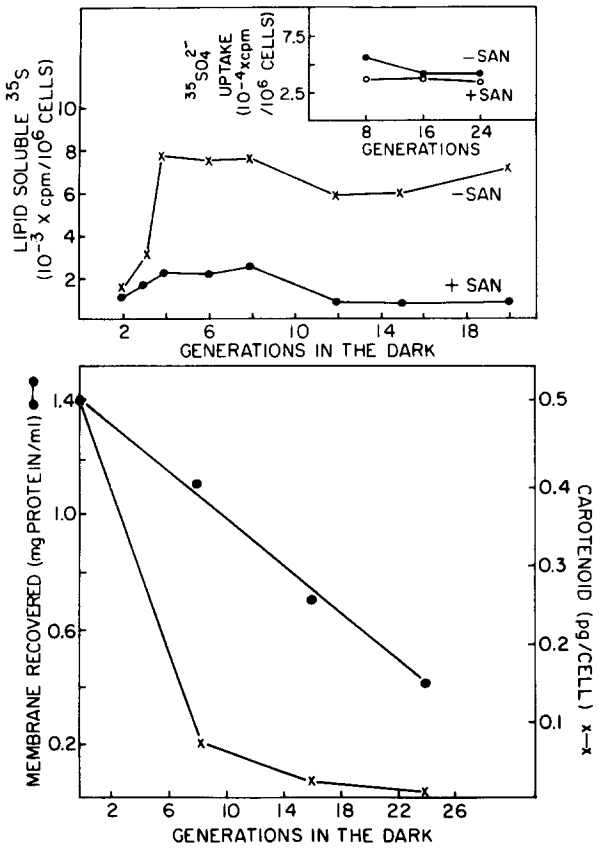

Fig. 6. Labeling of sulfolipid, amounts of membrane recovered in the middle fractions of the gradients, and amount of carotenoid in dark-grown cells growing in the dark in the presence or absence of SAN 9789. The inset shows uptake of $^{35}SO_4^{2-}$ with and without SAN 9789.

other hand, is to affect the regulation of the level of this material in such a manner that a lower level is achieved rapidly and is then maintained for many generations of division. In any case, inhibition of carotenoid biosynthesis has pronounced regulatory effects on the formation of plastid thylakoid membrane polypeptides and sulfolipid in agreement with previous findings by electron microscopy [13].

In agreement, Fig. 7 shows that all of the individual thylakoid membrane polypeptides are reduced to the same extent by growth of the cells on SAN 9789, the final pattern being qualitatively the same as that of untreated cells. Thus the regulation of thylakoid membrane polypeptide synthesis by carotenoids is quite general and must be exerted at an early control point common to the biosynthesis of all polypeptides. This would be expected since all of these polypeptides of the dark-grown cells are thought to be nuclear coded. The same type of experiments cannot be done in normal light-grown cells since the lower carotenoid concentrations brought about by SAN treatment do not protect the cells against photokilling.

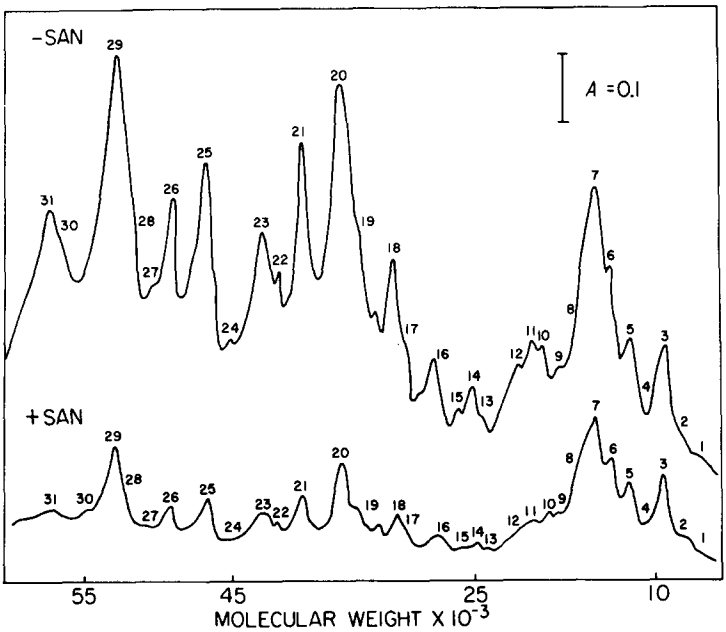

Fig. 7. Densitometer tracings of polypeptide patterns from plastid thylakoid (middle) fractions of dark-grown cells growing in the presence and absence of SAN 9789 for 24 generations in darkness, separated on SDS gels and stained with Coomassie blue. Protein from equal numbers of cells was placed on the gel.

Labeling of thylakoid polypeptides in isolated Euglena chloroplasts

Since most of the plastid thylakoid polypeptides in *Euglena* appear to be nuclear coded, it was of some interest to determine whether any of the polypeptides are synthesized in the chloroplast, since these might be coded in plastid DNA. On feeding radioactive amino acids to isolated chloroplasts of *Euglena* in red light, incorporation into plastid thylakoid polypeptides was observed (Fig. 8). If the same experiment was conducted in white light, in darkness, or in the presence of chloramphenicol almost complete inhibition of incorporation was observed. Cycloheximide, however, produced no inhibition. This demonstrates (as has been found by others [22,44]) that the light-dependent labeling occurs through translation on 70 S plastid ribosomes, not on any contaminating 87 S cytoplasmic ribosomes.

At least ten polypeptides become labeled in the isolated chloroplasts (Fig. 8) as found by others [22]. Fig. 9 summarizes all of the data in this paper in a manner that permits a comparison among the various polypeptide patterns found. The total number of plastid thylakoid polypeptides found is about 34 in general agreement with data from other systems employing optimal conditions [2,21,45,46]. Of these, the majority are present in W_3BUL and light and dark-grown cells of wild type. Since they are present in W_3BUL they cannot be coded in plastid DNA and, therefore, are probably coded in nuclear DNA. Our experience thus far with the *Euglena* system, and the experience of others with various organisms indicates that nuclear-coded plastid proteins are synthesized on cytoplasmic ribosomes while those coded in plastid DNA are translated on

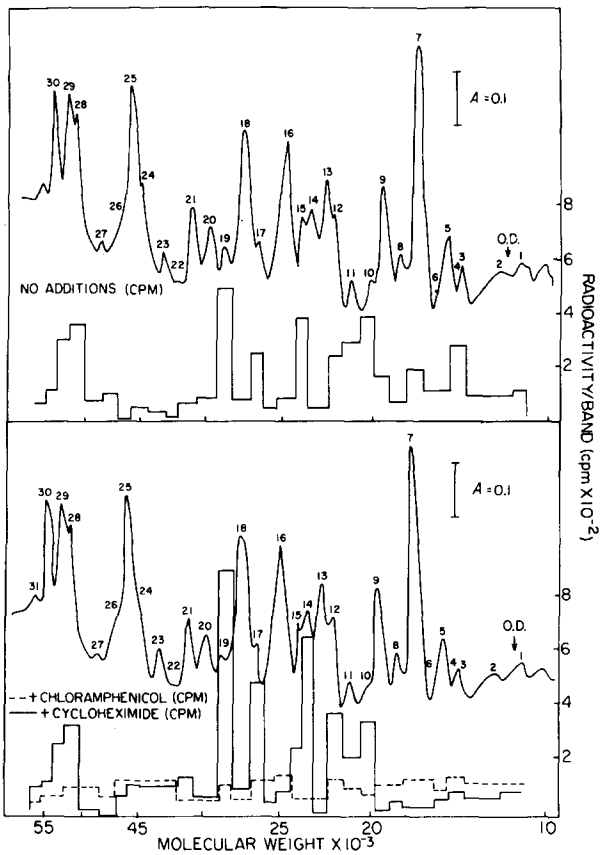

Fig. 8. Incorporation of label from ^{3}H-labeled amino acids into thylakoid membrane polypeptides in illuminated chloroplasts of *Euglena* in the presence and absence of inhibitors of protein synthesis. Densitometer tracings after staining with Coomassie blue and radioactivity across the SDS gels are shown.

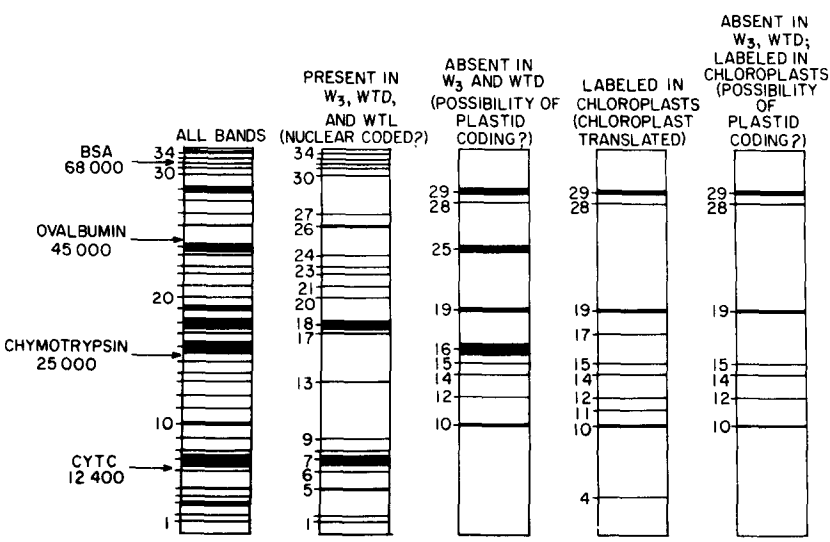

Fig. 9. Comparison of thylakoid polypeptide patterns obtained from W$_3$BUL and wild-type cells under various conditions. Shown are comparisons of W$_3$BUL (W$_3$), wild-type dark-grown cells (WTD), wild-type light-grown cells (WTL), and polypeptides shown to be labeled in isolated chloroplasts. See text for details.

plastid ribosomes [28—30]. Those bands which are absent from W_3BUL, and from dark-grown wild-type cells (within our limits of detection), are candidates for being coded in plastid DNA. To verify this requires further work since the structural gene could be in a DNA other than the plastid DNA but the absence of plastid DNA could prevent the formation of plastid ribosomes and of any regulatory molecules which are gene products of plastid DNA and might control the transcription of nuclear (or mitochondrial) genes. Still better candidates for coding in plastid DNA are the polypeptides which are absent from W_3BUL and are found to be labeled in isolated chloroplasts. These number some six polypeptide bands and further explorations seeking plastid-coded thylakoid membrane polypeptides should concentrate on these as possibilities.

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