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RNA Metabolism During Light-Induced Chloroplast Development in Euglena[1]

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Summary. Methods are described which provide good recoveries of non-degraded chloroplast and non-chloroplast RNAs from *Euglena gracilis* var. bacillaris. These have been characterized by comparing the RNA from W_3BUL (an aplastidic mutant of *Euglena*), with that of wild-type cells which have been resolved into chloroplast and non-chloroplast fractions. Using *E. coli* RNA as a standard, the RNAs from W_3BUL and from the non-chloroplast fraction of green cells exhibit optical density peaks, upon sucrose gradient centrifugation, at 4S, 10S, and 19S. The chloroplast fraction exhibits optical density peaks at 19S and 14S with the 19S component predominating. Application of various techniques for the separation of RNAs to the problem of separating the chloroplast and non-chloroplast RNAs, without prior separation of the organelle, have not proven successful.

$^{32}P_i$ is readily incorporated into RNA by cells undergoing light-induced chloroplast development, and fractionation at the end of development reveals that although chloroplast RNAs have a higher specific activity, the other RNAs of the cells are significantly labeled as well. The succession of labeling patterns of total cellular RNA as light-induced chloroplast development proceeds are displayed and reveal that all RNA species mentioned above eventually become labeled. In contrast, cells kept in darkness during this period incorporate little $^{32}P_i$ into any RNA fraction. In addition, a heavy RNA component, designated as 28S, while representing a negligible fraction of the total RNA, becomes significantly labeled during the first 24 hours of illumination. While there is light stimulated uptake of $^{32}P_i$ into the cells, this uptake is never limiting in the light or dark, for RNA labeling.

On the basis of these findings, we suggest that extensive activation of non-chloroplast RNA labeling during chloroplast development is the result of the activation of the cellular synthetic machinery external to the chloroplast necessary to provide metabolic precursors for plastid development. Thus the plastid is viewed as an auxotrophic resident within the cell during development. Other possibilities for interaction at this and other levels are also discussed.

Euglena gracilis var. bacillaris has been shown to contain 3 different types of DNA. In addition to the nuclear complement, the chloroplasts and mitochondria each contain a unique species (19). While the presence of chloroplast DNA has been shown to be correlated with the ability to construct a chloroplast (6) and appears to be replicated along with the organelle, it is not clear how much of the information necessary to construct the chloroplast resides in the chloroplast DNA. It is also not clear to what extent, if at all, the developing chloroplast is nutritionally dependent on the rest of the cell.

This paper makes a beginning in attacking these questions by providing some observations of RNA metabolism during light-induced chloroplast development. Such observations have provided an opportunity to assess the extent to which the metabolism of chloroplast and non-chloroplast RNAs are activated during chloroplast development.

Materials and Methods

Conditions for Growing of Cultures. *Euglena gracilis* var. bacillaris Pringsheim was grown on Hutner's medium, pH 3.3 (9), as described by Lyman et al. (14) in complete darkness, and resting cells, in the dark, were obtained as described by Stern et al. (25) with the sole exception that the phosphate concentration in the resting medium was reduced to 125 μM. These non-dividing cells were

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used in all experiments unless otherwise noted. Cells were routinely counted in a Coulter Model A Counter in 0.4 % (w/v) NaCl after suitable dilution.

Conditions for Labeling of Euglena. To 700 ml of a resting culture of *Euglena,* prepared as described above, was added 5.6 ml of a sterile, neutral, carrier-free solution of $Na_3{}^{32}PO_4$ (Cambridge Nuclear Corp.) containing about 2×10^9 cpm per ml. After a brief and thorough mixing in the dark, the resting culture was carefully split into equal portions. One was placed on a rotary shaker running at 144 strokes per minute under 150 ft-c provided by cool white and red fluorescent lamps at 26°, and the other was kept on a shaker, running at 144 strokes per minute, in darkness at the same temperature. All stages of chloroplast development are timed from the point at which the cells, rested in darkness, are placed in the light which is taken as time zero. At the end of the desired incubation period, each culture was processed separately in the dark for the initial stages of isolation of RNA as described below. While the modified resting medium used in all experiments contains a lower concentration of phosphate compared to the medium described by Stern et al. (25), *Euglena* chloroplast development is normal in the modified resting medium with respect to: rate of chlorophyll accumulation (25), morphological changes (1), and inception point and rate of photosynthetic carbon dioxide fixation (20, 25).

The uptake of $^{32}P_i$ by *Euglena* was determined by withdrawing 1.0 ml samples from the appropriate resting culture, and centrifuging each sample in the dark at one-half the full speed obtainable on a Clay Adams Table-model Centrifuge for 5 minutes. The pellet was thoroughly resuspended in 5.0 ml of distilled water, centrifuged again, and the cell pellet was then resuspended in 1.0 ml of distilled water. Aliquots were delivered onto glass planchettes, dried, and counted. Such determinations were done in triplicate.

RNA-^{32}P from chloroplasts was obtained by charging 1.4 liters of a dark-grown resting culture with about 20×10^9 cpm of $^{32}PO_4{}^{3-}$ and exposing the culture to light for 72 hours. Then, 3.6 liters of a non-radioactive culture of *Euglena* containing chloroplasts were added as carrier, and chloroplasts were isolated as described by Eisenstadt and Brawerman (7).

RNA-^{14}C from chloroplasts was obtained from cells which were first allowed to complete 20 hours of development at 26° with stirring provided by a sterilized magnetic stirring bar in a rubber-stoppered sealed flask fitted with an entry tube sealed with a serum cap. Then, 4.4 mg of $NaH^{14}CO_3$, representing 1 mc (New England Nuclear Corp.) in 10 mM tris-HCl (pH 7.5) was first passed through a sterile Millipore filter disc held in a Swinney adapter before it was injected into the culture through a sterilized hypodermic needle puncturing the serum cap. After withdrawal of the hypodermic needle, development was allowed to proceed for another 52 hours to complete chloroplast development. Chloroplasts were then isolated as described by Eisenstadt and Brawerman (7), employing non-radioactive cells with fully developed chloroplasts as carrier to provide sufficient material for RNA extraction.

Extraction of RNA from Whole Cells of Euglena. All operations, unless otherwise noted, were carried out at 4°. The initial steps up to and including freezing of cells were carried out in a room equipped with green safelights (14) to avoid induction of further possible chloroplast development.

Two hundred and fifty ml of cells (1×10^6 cells per ml) were harvested by centrifugation at $300 \times g$ for 5 minutes and were washed first with 250 ml of 10 mM tris-HCl containing 5 mM $MgCl_2$ (pH 7.4) (TM) and then with 40 ml of TM by centrifugation at $300 \times g$ for 5 minutes. The cell pellet was thoroughly resuspended in 2.0 ml of TM and the thick cell suspension, held in a glass container, was rapidly frozen in a dry-ice isopropanol bath. When freezing was complete, extraction was continued under ordinary lighting conditions by adding 2.0 ml of a 10 % solution of sodium dodecyl sulfate (SDS), and the frozen cell-soap mixture was rapidly thawed by placing the tube containing the mixture first in a stream of warm tap water followed by mixing with the aid of a Vortex Genie mixer (Scientific Inst. Corp.) at room temperature. This procedure was repeated until the entire contents had thawed. Complete cell lysis was observed within 20 seconds after thawing. Thus, during thawing, intact cells were held in a frozen state, and the cells in the water phase were completely and rapidly lysed. Following complete lysis the volume was then brought to about 20 ml by the addition of 6.0 ml of cold TM followed immediately by the addition of 10 ml of cold water-saturated phenol. The mixture was shaken for 1 hour at 4° on a wrist action shaker running at top speed. The emulsion was resolved into aqueous, interface, and lower phenol phases by centrifugation at $8700 \times g$ for 20 minutes. The aqueous phase, which contains 80 to 98 % of the total RNA, was carefully removed with a large bore pipette and charged with 2 volumes of 95 % ethanol at −20°, and sufficient 1.0 M NaCl to give a final concentration of 0.1 M NaCl. The remaining combined phases contained from 20 % to barely detectable amounts of RNA and were not subjected to re-extraction after it was found that the RNA in the combined phases provided the same profile as was found for RNA recovered from the aqueous phase.

The nucleic acids in the ethanol solution were allowed to precipitate overnight at −20°. The precipitate was harvested by centrifugation at $3000 \times g$ for 5 minutes and was resuspended in 10 ml of TM. The nucleic acids were then re-

precipitated by the addition of ethanol and NaCl as described above. After standing for 3 hours at −20°, the precipitate was recovered by centrifugation, dissolved in 10 ml of TM, and the last traces of phenol removed by repeated ether extractions. Ether was removed by bubbling the solution with nitrogen. The DNA present in the extract was then degraded by a 3 minute incubation at 37° with DNase, 50 μg/ml final concentration. RNase-free pronase was then added to a final concentration of 50 μg/ml and incubation was continued at 37° for 1 hour. At the completion of the pronase step, the extract was chilled and an equal volume of cold water-saturated phenol was added. The mixture was shaken in the cold for 10 minutes and the aqueous phase was recovered as described above. Phenol and ether were removed as indicated above. The nucleic acids, free of detectable DNA and RNase, were then reprecipitated by the addition of ethanol and NaCl for 1 to 2 hours at −20°. The nucleic acids were recovered by centrifugation and dissolved in 2.5 ml of 0.01 M tris-HCl containing 0.1 M NaCl (pH 7.4). The procedure described routinely provided RNA in good yield having a 260 mμ/280 mμ ratio of 1.9 to 2.1.

Chloroplast and S_1 RNA. Chloroplasts from *Euglena* were isolated as described by Eisenstadt and Brawerman (7). The supernatant obtained from the whole cell homogenate (7), free of whole cells, chloroplasts, and paramylum, and called by us "S_1", was made 5 % with respect to SDS and the RNA was extracted as described above. Such RNA is subsequently called S_1 RNA or non-chloroplast RNA.

After chloroplasts were isolated and washed in the buffer described by Eisenstadt and Brawerman (7), they were immediately resuspended in 10 ml of TM containing 5 % SDS, and the RNA extracted as described above.

Separation of RNA Species. RNA profiles and their radioactivity were routinely obtained by carefully layering about 2.0 ml of RNA (about 800 μg) in 0.01 M tris-HCl containing 0.1 M NaCl (pH 7.4) onto 28 ml of a linear sucrose gradient constructed from 15 % and 30 % sucrose containing 0.5 % SDS made up in the same buffer. After centrifugation at 23,000 RPM for 20 hours in an SW 25.1 rotor at a setting of 21.5° in a Beckman Preparative Ultracentrifuge Model L2-50, (avg temp of the gradient was 19.7°), the bottom of the tube was punctured, and the contents were continuously monitored for optical density employing a flow-through cuvette and a Gilford Absorbance Spectrophometer Model 2000. Fraction number 1 in all gradients shown represents the bottom of the tube. The gradient begins at the bottom with 30 % sucrose. Approximately 1.0 ml fractions, each consisting of 30 drops, were collected. For convenience all optical density profiles were adjusted to have an optical density of 1.0 at the peak of the 19S ribosomal RNA. All other parameters were normalized accordingly for graphic display.

Radioactivity in each fraction was determined by first adding 1 ml of carrier yeast RNA (250 μg/ml) followed by the addition of 2.0 ml of cold 30 % trichloroacetic acid. After precipitation at 4° for three-fourths hour, each RNA fraction was collected by suction through Millipore filter discs, which were mounted on planchettes with the aid of Elmers Glue-all (Borden Co.), dried under an infrared spotlight, and counted on a Nuclear Chicago Gas Flow Counter Model D-47 operating in the proportional range.

The total radioactivity of an RNA preparation prior to separation on sucrose gradients was determined by adding carrier yeast RNA (50 μg/ml final concentration) and 2 volumes of cold 20 % trichloroacetic acid to an aliquot of the phenol-extracted RNA solution. After standing at 4° for 10 minutes, the precipitate was collected by suction through a Millipore filter disc, and the disc was dried under an infrared lamp and mounted onto a planchette for counting. Such determinations were carried out in triplicate.

In a typical experiment, 91 % to 95 % of the total acid-insoluble radioactivity was rendered acid-soluble after overnight incubation at 37° with 100 μg/ml DNase-free RNase. From 99.5 % to 100.0 % of the acid-insoluble radioactivity was solubilized after 18 hours of digestion at 37° in 0.3 N KOH or 0.33 N LiOH. Approximately 95 % to 99 % of the radioactivity in such digests was charcoal adsorbable. After treatment with RNase, the phenol-extracted material positioned at the top of the sucrose gradient in the 4S region and above.

Specific activity estimations were made by converting the optical density of a particular fraction read from the optical density profile to μg RNA by multiplying by the factor 40, where an optical density of 1.00 in a 1 cm light path is taken to be equivalent to 40 μg of RNA.

Columns of kieselguhr coated with methylated albumin (MAK columns) were prepared and used according to the methods of Mandell and Hershey (15) and Hayashi, Hayashi, and Spiegelman (11).

Analysis of RNA. Base compositions of RNA were obtained by recovering the RNA after gradient centrifugation by the addition of ethanol. The precipitate was subjected to digestion in 0.33 N LiOH for 18 hours at 37° according to the procedure described by Gebicki and Freed (8). The nucleotides were then separated by paper electrophoresis employing formate buffer (22). The individual nucleotides, identified both by comparison to authentic samples and by spectral analysis, were cut from the paper and eluted in HCl for spectral analysis or were mounted directly on planchettes for counting when the RNA had been previously labeled with $^{32}PO_4^{3-}$.

Determinations of the spectra of the various RNA fractions were made by the methods of Braw-

erman and Eisenstadt (3) employing hot 0.5 N perchloric acid.

Total *Euglena* RNA was determined with orcinol (5) after extraction according to the procedure described by Smillie and Krotkov (21). Ribose was employed to construct standard curves and conversion of ribose to equivalent μg of RNA for samples was made by multiplying the apparent ribose content by the factor 5.88 (5).

Reagents. Phenol solution (88 % w/v) analytical grade was obtained from Mallinkrodt and was saturated with water at 4° before using. Sodium dodecyl sulfate (USP grade) was obtained from Fisher Chemicals and was recrystallized from boiling 95 % ethanol. The ethanol was removed in vacuo and the SDS was dried over $CaCl_2$ in vacuo before the aqueous stock solution (10 %, w/v), made up in distilled water, was prepared.

Pronase, B grade (Calbiochemicals), was made up as a 1 mg/ml stock solution in TM and was self-digested for 2 hours at 37° before being used. DNase, obtained from Worthington Biochemicals, electrophoretically purified and free of RNase, was made up as a 1 mg/ml stock solution in TM. Both pronase and DNase were checked for RNase activity employing *E. coli* RNA-^{14}C as substrate, prepared as described below. Neither enzyme preparation contained detectable RNase after 24 hours incubation with *E. coli* RNA.

E. coli RNA with a specific radioactivity of about 100,000 cpm per μg RNA was obtained by growing *E. coli* (T^-U^-) on a minimal medium supplemented with thymidine and uracil-^{14}C (New England Nuclear) for 4 generations. Then, non-radioactive uracil was added and growth proceeded for 0.9 generation (27). The RNA was then extracted as described by Hayashi and Spiegelman (10). Such RNA consisted of the 2 major RNA components, assigned values of 23S and 16S and a soluble component, 4S. There was excellent coincidence between optical density and radioactivity after centrifugation through the sucrose gradients.

Results and Discussion

Characterization of the RNA of Euglena gracilis *var. bacillaris on Sucrose Gradients.* In order to establish the pattern of non-chloroplast RNA to be expected, the RNA of an aplastidic mutant of *E. gracilis* var. bacillaris (W_3BUL) was first examined. Figure 1 (left) reveals the presence of 3 major peaks in the sucrose gradient and compares their apparent sedimentation values with those of *E. coli* which are well established (12, 24, 26). W_3BUL contains RNA of 19S, 10S and 4S. The RNA prepared from the fraction which sediments as ribosomes from this mutant shows the 19S and 10S components indicating that these are the RNAs

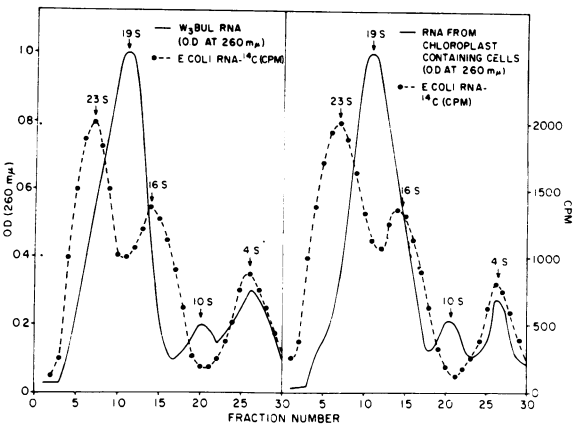

FIG. 1. Comparison of RNA from aplastidic and plastid-containing *Euglena,* as separated on sucrose gradients. Left) Comparison of RNA optical density from an aplastidic mutant of *Euglena* (W_3BUL) with the radioactivity of *E. coli* RNA. *E. coli* RNA-^{14}C (25,000 cpm, representing less than 2 μg) was added to *Euglena* prior to extraction of RNA as described in Materials and Methods. Relative S values are assigned to the *Euglena* RNA peaks by comparison with the reported *E. coli* values (12, 24, 26). Right) Comparison of RNA optical density from chloroplast containing cells of wild-type *Euglena* with radioactivity of *E. coli* RNA. Details as described for left-hand figure.

characteristic of these non-chloroplast particles. The 4S RNA occupies the usual position assigned to s-RNAs (26).

Figure 1 (right) shows the corresponding separation for the RNA of wild-type cells which have completed chloroplast development. This pattern does not differ markedly from that obtained from the aplastidic mutant suggesting that the contribution of RNA by the chloroplast is small compared to the rest of the cell. The same pattern is obtained from dark-grown wild-type cells. These RNA patterns are very similar to those obtained by Brawerman and Eisenstadt (3) for the Z strain of *Euglena gracilis,* although the methods used for extraction differ in detail.

If wild-type cells which have completed chloroplast development are fractionated to obtain a chloroplast fraction and a fraction which contains essentially all the rest of the cellular material (called by us, S_1) the patterns shown in figure 2 are obtained. The left side of figure 2 displays the chloroplast RNA compared with standard *E. coli* RNA. Two major chloroplast peaks at 19S and 14S are present along with a minute amount of 4S material. It is not clear whether this 4S material is adherent cytoplasmic s-RNA or whether it represents the fraction of native chloroplast 4S RNA which remains after leakage during chloroplast preparation. The right side of figure 2 verifies that the RNA of the rest of the cell, exclusive of the chloroplast, is identical with the RNA of W_3BUL (fig 1). It also shows that although at least one of the chloroplast RNA peaks is different

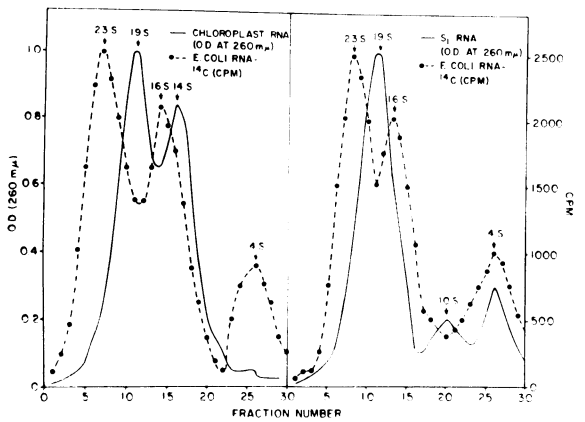

FIG. 2. Comparison of chloroplast and non-chloroplast (S_1) RNA with *E. coli* RNA as separated on sucrose gradients. Left) Comparison of RNA optical density from *Euglena* chloroplasts with the radioactivity of *E. coli* RNA. Conditions as in figure 1, except that 30,000 cpm of *E. coli* RNA-^{14}C (representing less than 2.5 μg) were added to a suspension of *Euglena* chloroplasts prior to processing. Right) Same as left except that S_1, the combined non-chloroplast fractions of the cells, were used in place of chloroplasts.

from any in the rest of the cell (the 14S component) it cannot be detected against the background of the whole cell RNA. It will be seen from figure 2 that in the chloroplast, the 19S component exceeds the 14S. This is contrary to the findings of Brawerman and Eisenstadt (3) for the Z strain chloroplast RNA, where the reverse is reported. We have found that this discrepancy can be traced to the method of preparation and is not strain-dependent. Brawerman and Eisenstadt (3) first prepared chloroplast ribosomes and subsequently extracted the RNA. We have found that the presence of internal nucleases can lead to a change in the ratio of 19S to 14S through the conversion of large to smaller components having lower S values. We believe that our patterns represent the situation most likely to prevail in vivo because the methods we have developed for extraction of chloroplast RNA are rapid and direct (not involving the prior isolation of ribosomes) and are specifically designed to avoid this type of degradation.

Although both the chloroplast and non-chloroplast compartments of the cell contain a 19S RNA, the overall base ratios of RNAs from both sources are different as seen in table I and as reported earlier for the Z strain (3).

Attempts at Separating Chloroplast and Non-Chloroplast RNAs. The fact (table I) that the overall base compositions of the bulk RNA of the chloroplast and non-chloroplast fractions of the cell are different suggested that a separation of some of these components by standard methods might be possible. To this end, ^{14}C labeled chloroplast RNA was prepared and was mixed in tracer amounts with non-radioactive non-chloroplast RNA (i.e. the

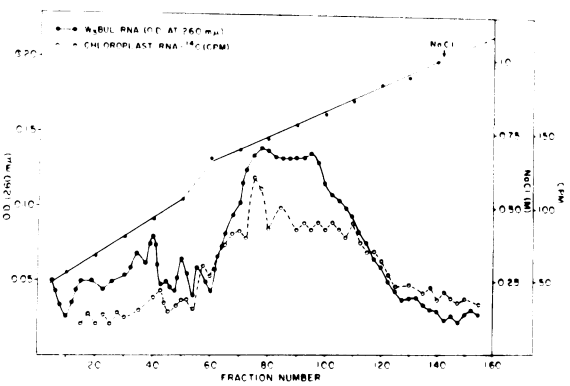

FIG. 3. Attempted separation of RNA from W_3BUL and RNA from *Euglena* chloroplasts on a column of kieseleguhr coated with methylated albumin. A mixture of W_3BUL RNA (800 μg) and *Euglena* chloroplast RNA-^{14}C (10,000 cpm, representing less than 6 μg of RNA) was adsorbed to the column and eluted with NaCl gradients as shown.

Table I. *Base Compositions of Ribosomal RNAs from* Euglena gracilis

	For combined 19S and 10S RNAs of W_3BUL*	For RNA from non-chloroplast ribosomes of strain Z**
	%	%
A	23.0	22.7
U	20.5	20.7
G	30.0	29.5
C	26.5	27.1
	For combined chloroplast 19S and 14S RNAs of bacillaris strain	For RNA from chloroplast ribosomes of strain Z**
	%	%
A	29.0	30.6
U	26.0	25.0
G	30.0	27.0
C	15.0	17.0

* Aplastidic strain derived from bacillaris by UV treatment.
** From Brawerman et al. (3).

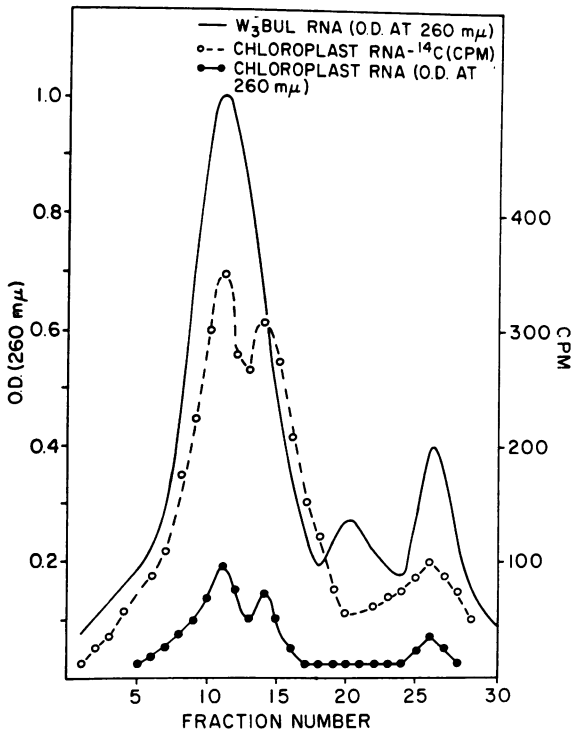

FIG. 4. Comparison of chloroplast RNA with the RNA of an aplastidic mutant of *Euglena* (W_3BUL) as separated on sucrose gradients. The profiles shown represent 2 separate sucrose gradient separations made in the same centrifuge rotor in different centrifuge tubes. One tube received 800 μg of W_3BUL RNA and 8000 cpm of chloroplast RNA-^{14}C. The second tube received an amount of unlabeled chloroplast RNA calculated to represent the amount of chloroplast RNA expected from chloroplast containing cells at the optical density peak at 19S on sucrose gradients. It was assumed that a maximum of 20 % of the total celllular RNA is contributed by chloroplast RNA in cells containing fully developed chloroplasts. The profiles obtained were carefully superimposed for graphic display.

total cellular RNA of W_3BUL). This mixture, when subjected to chromatography on a MAK column revealed that no separation could be achieved under a variety of conditions. A representative attempt is shown in figure 3. A similar profile was found by other workers (23) for the Z strain.

As might be anticipated from the data presented in figure 2, sucrose gradients are no more effective in this type of separation. To emphasize this point, figure 4 compares the actual optical densities expected from the amounts of RNA ordinarily encountered in chloroplasts and S_1. Such comparisons were made on the basis of the observation that the total amount of RNA associated with the isolated chloroplasts comprised 15 % to 20 % of the total RNA extracted from cells containing chloroplasts, while S_1 RNA (non-chloroplast RNA) contributed 80 % to 85 % of the total cellular RNA. As may be seen, the great variance of the S_1 ribosomal peak effectively hides both chloroplast optical density peaks. Figure 4 also demonstrates that the ^{14}C-labeled RNA used to evaluate the MAK separation is indeed pure chloroplast RNA. Thus far, we have found no method which will adequately separate chloroplast and non-chloroplast RNAs including an isopycnic centrifugation in a cesium chloride-cesium sulfate gradient described by Lozeron and Szybalski (13).

The Incorporation of Phosphate into Cellular RNA. Since pyrimidines are not effective precursors for RNA in this strain of *Euglena* in agreement with our earlier findings (17) and those of others that bacillaris prefers purines (2, 18), attempts were made to use purines but the amounts of incorporation were not sufficient to be of practical use. By employing a resting medium low in phosphate, however, sufficient $^{32}PO_4^{3-}$ was taken up by the cells and was incorporated into RNA. Figure 5 shows the sucrose gradient pattern of bacillaris RNA after exposure of the dark-grown resting cells to $^{32}PO_4^{3-}$ and light for 72 hours, the time of complete chloroplast maturation. There is excellent coincidence between RNA as measured by optical density and the curve for radioactivity. The chloroplast RNA, if labeled at all during the course of chloroplast development, is not apparent against the background of non-chloroplast RNA.

Figure 6 reveals, however, that when the chloroplasts are separated from the rest of the cellular material (S_1) not only is the chloroplast RNA labeled, but its specific activity is some 3 times higher than the specific activity of S_1 RNA. In both cases there is excellent coincidence between RNA and radioactivity. It is clear that light-induction of chloroplast development results in the labeling of chloroplast RNA, as might be expected,

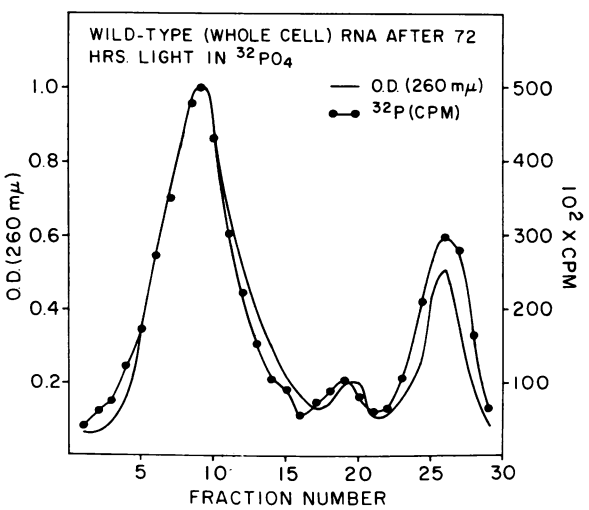

FIG. 5. Radioactivity and optical density profile of RNA from wild-type cells containing chloroplasts, on a sucrose gradient. Dark-grown resting cells were incubated with PO_4^{3-} in the light for 72 hours and processed as described in Materials and Methods.

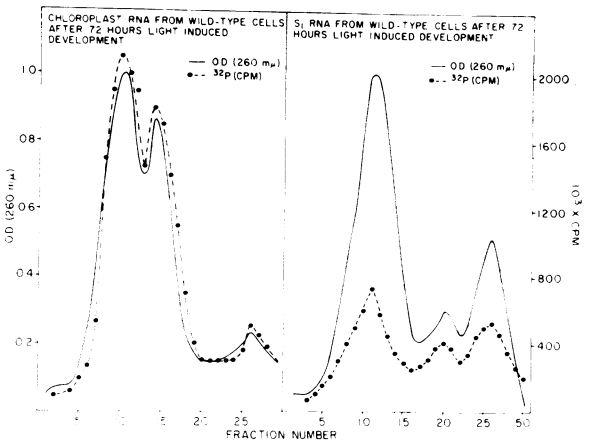

FIG. 6. Comparison of radioactive RNA from chloroplasts with radioactive non-chloroplast RNA as separated on a sucrose gradient. Left) Optical density and radioactivity of chloroplast RNA. Right) Optical density and radioactivity of non-chloroplast RNA (S_1 RNA) prepared from the same homogenate used to isolate chloroplasts containing labeled RNA shown in the left-hand figure.

but also results in a highly significant incorporation of label into non-chloroplast RNAs.

The Patterns of RNA Labeling at Various Times During Chloroplast Development. In the following experiments, zero time represents dark-grown resting cells and all times of development are measured from the time that these cultures are placed in the light. In all experiments, the dark-grown resting cells received an appropriate amount of $^{32}PO_4^{3-}$ and were split into 2 identical aliquots.

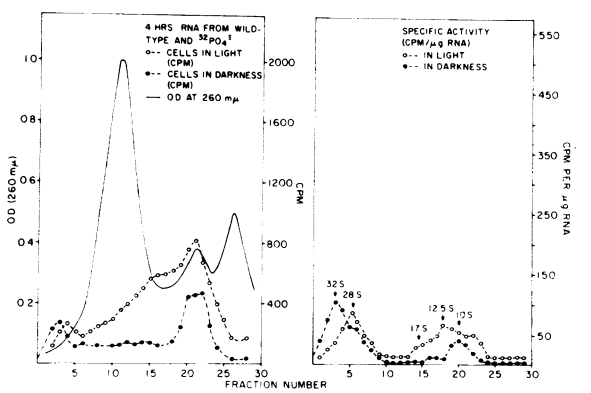

FIG. 7. Radioactivity and specific activity of RNA from wild-type cells incubated with $^{32}PO_4^{3-}$ for 4 hours. Left) Optical density and radioactivity of RNA from wild-type cells incubated in the light and in the dark. The optical density profiles of both type of cells are identical here and throughout development, and, therefore, in each case only 1 optical density profile representing both is shown. Right) Specific activities of RNA fractions from wild-type cell incubated in light and from wild-type cells incubated in the dark. Specific activities were estimated from the radioactivity and optical density of each fraction as described in Materials and Methods.

One aliquot was incubated for the appropriate time in the light, the other for an identical period in the dark.

The first time investigated was 4 hours because previous developmental studies (20, 25) indicated that this was the time of inception of photosynthetic oxygen evolution and the time of completion of the first lamella (1). Figure 7 shows the labeling patterns of these cells in the light and in the dark. Two characteristics seen here and in subsequent patterns are: 1) the optical density profile

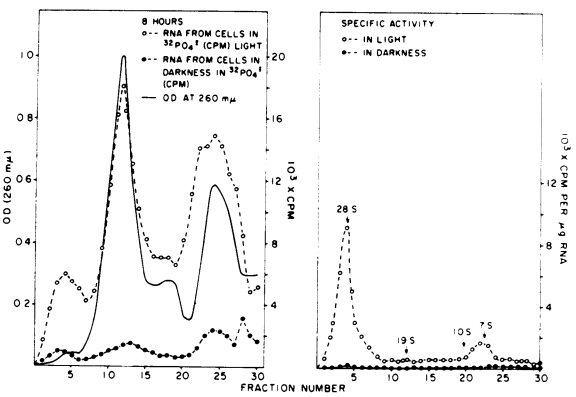

FIG. 8. Radioactivity and specific activity of RNA from wild-type cells incubated with $^{32}PO_4^{3-}$ for 8 hours. Left and right) Details are described in legend of figure 7.

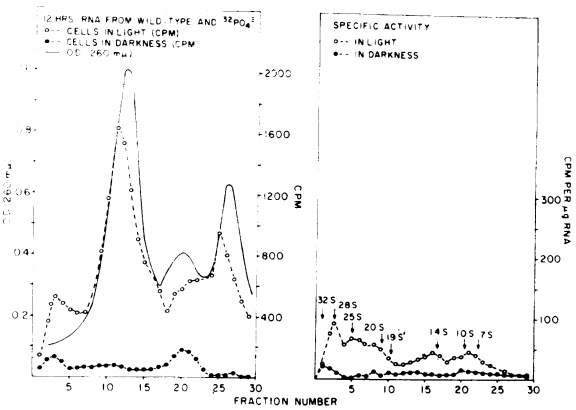

FIG. 9. Radioactivity and specific activity of RNA from wild-type cells incubated with $^{32}PO_4^{3-}$ for 12 hours. Left and right) Details are described in legend of figure 7.

remains identical to that of W_3BUL and the light-grown cells because the chloroplast RNA is completely obscured by the variance of the non-chloroplast RNA optical density, as discussed in detail above (see fig 4), and; 2) the incorporation of ^{32}P into the RNA is always higher in the cells exposed to light than in their dark counterparts, and this difference becomes greatly accentuated with time. The incorporation at 4 hours in the

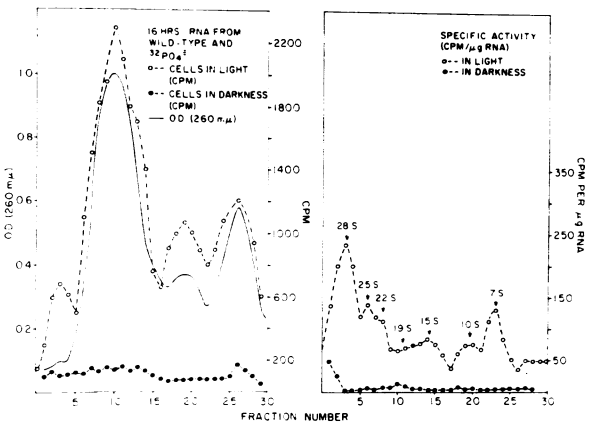

FIG. 10. Radioactivity and specific activity of RNA from wild-type cells incubated with $^{32}PO_4^{3-}$ for 16 hours. Left and right) Details are described in legend of figure 7.

dark is mainly into 32S and 10S RNA and this is reflected in the specific activity peaks for these regions. In addition there is also a hint of incorporation into the 28S region. The higher incorporation into RNA from cells in the light at 4 hours is also found in the 10S and 28S regions with a rather broad distribution through the 19S region, additional specific activity peaks being apparent in the vicinity of 12 and 17S. It should be noted that the 4S, or s-RNA region is extremely low in activity at this time.

During the period of 4 to 24 hours (figs 7–11) the incorporation of the label into RNA from cells in the light continues to preferentially increase the relative specific activity of the 28S component with evidence of increasing incorporation into the 19S region. From 24 hours to the completion of chloroplast development at 72 hours (figs 11–13) incorporation into the heavier 28S RNA ceases while the components which will compose the highly heterogeneous 19S region continue to increase in relative

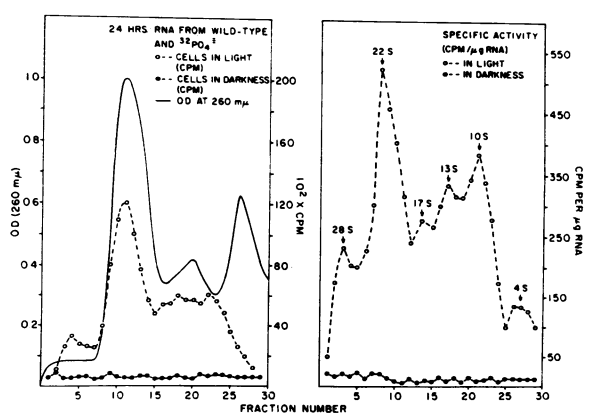

FIG. 11. Radioactivity and specific activity of RNA from wild-type cells incubated with $^{32}PO_4^{3-}$ for 24 hours. Left and right) Details are described in legend of figure 7.

specific activity as does the 10S RNA. Incorporation into the 4S region becomes increasingly apparent and becomes a definite peak in specific activity by 16 hours (fig 10), but from 24 to 48 hours (figs 11 and 12) there is little relative change in this area. By 72 hours (fig 13) it is apparent that the highest specific activities exist in the regions of 4S, 10-14S, and 19S with the 28S remaining at the same relative level it achieved by 24 hours (fig 11).

The overall pattern, then, is that the heaviest RNAs (i.e. the 28S region) reach highest specific activity first and from then on there is an upthrusting of relative specific activity in the highly heterogenous 19S region which eventually greatly exceeds the level achieved earlier by the heavier RNAs. The 10S component increases in specific activity over the entire span of development while the 4-7S components first make their presence known as a definite peak in specific activity at 16 hours, lag for 48 hours, and finally achieve the maximum specific activity (i.e. equal to that of the 19S RNA) by 72 hours.

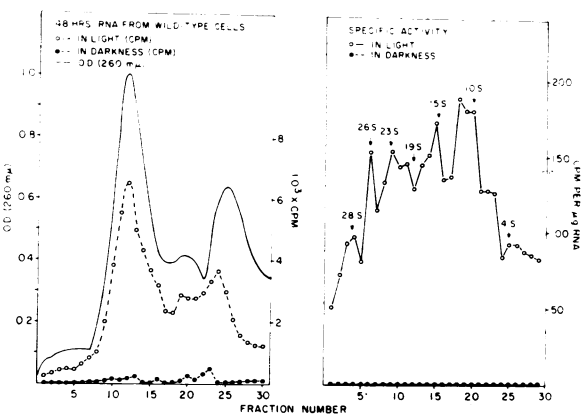

FIG. 12. Radioactivity and specific activity of RNA from wild-type cells incubated with $^{32}PO_4^{3-}$ for 48 hours. Left and right) Details are described in legend of figure 7.

As far as the incorporation of label into RNA of comparable cells in the dark is concerned, aside from the 4 hour pattern already discussed (fig 7) where 32 and 10S predominate, the activities and specific activities are, in general, very low compared with their counterparts in the light. At 72 hours (fig 13) the incorporation in the dark is somewhat higher than these minimal levels and resembles, to some extent, the light-induced pattern.

In order to rule out the possibility that the great differences in the amount of ^{32}P incorporation into RNA between cells in the light and in the dark had its origin in a differential permeability of the cells under the 2 conditions (for example, a light-driven $^{32}PO_4^{3-}$ uptake) measurements were made on the $^{32}PO_4^{3-}$ uptake into the 2 types of cells throughout the developmental period. As may be seen in

Table II. *Comparison of the Uptake of $^{32}PO_4^{3-}$ by Wild-Type* Euglena *Cells with the Total Radioactivity of Their Phenol-Extracted RNA*

Duration of incubation with $^{32}PO^{3-}_4$	1.25 × 10⁸ Cells in darkness		1.25 × 10⁸ Cells in light	
	Total $^{32}PO^{3-}_4$ taken up	Total radioactivity in phenol-extracted RNA	Total $^{32}PO_4^{=}$ taken up	Total radioactivity in phenol-extracted RNA
Hrs.	cpm	cpm	cpm	cpm
4	15 × 10⁶	1.2 × 10⁴	25 × 10⁶	2.6 × 10⁴
8	15 × 10⁶	9.3 × 10⁴	35 × 10⁶	6.3 × 10⁵
24	21 × 10⁶	1.2 × 10⁴	62 × 10⁶	8.4 × 10⁴
50	50 × 10⁶	5.0 × 10⁴	124 × 10⁶	4.0 × 10⁵
72	52 × 10⁶	1.2 × 10⁴	233 × 10⁶	9.5 × 10⁵

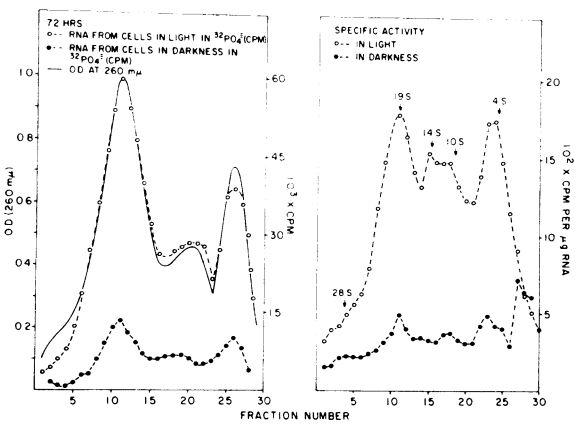

FIG. 13. Radioactivity and specific activity of RNA from wild-type cells incubated with $^{32}PO_4^{3-}$ for 72 hours. Left and Right) Details are described in legend of figure 7.

table II, while light enhances $^{32}PO_4^{3-}$ uptake to some extent, $^{32}PO_4^{3-}$ is never limiting for labeling RNA even in the dark-grown resting cells, when the uptakes are compared with their respective incorporations into RNA.

The data shown indicate that at 4 hours in the dark there is a minimum of $^{32}PO_4^{3-}$ taken up (15 × 10⁶ cpm). Since Smillie and Krotkov have shown that RNA-phosphorous represents 40 % of the total phosphorous of the cell (21), even under these minimal conditions 6 × 10⁶ cpm are theoretically available for RNA labeling. In fact, only 1.2 × 10⁴ cpm are actually incorporated into the RNA of these cells.

To make an even more extreme comparison, this minimum uptake and availability (6 × 10⁶ cpm) is still ample to provide the radioactivity to maximally label the RNA obtained at 72 hours in the light, 9.5 × 10⁵ cpm, the greatest incorporation observed.

Conclusions

Since the *Euglena* cell contains a species of DNA in its chloroplast which is unique and different from the DNAs of the mitochondrion and nucleus (19), one would be tempted, strictly on grounds of parsimony, to suppose that the chloroplast DNA coded largely for the production of chloroplast constituents. Evidence exists that at least a crucial part of the information required for chloroplast development resides in the chloroplast DNA (6). The evidence from higher plants, based on genetic studies (4, 16), indicates a substantial amount of interaction between the genomes of the nucleus and chloroplast.

If the chloroplast DNA of *Euglena* enables the chloroplast to be an autonomous cell within a cell, one would expect that upon illumination of the dark-grown resting cells, the RNA produced should be exclusively that of the chloroplast, coded by its DNA.

It is clear, however, from the data presented in this paper that such a simplistic hypothesis is not tenable. Indeed, illumination of the dark-grown resting cells in the presence of $^{32}PO_4^{3-}$ brings about significant labeling of the 28S and 10S RNA which are known from the studies with the aplastidic mutant W_3BUL, to be associated with structures other than the chloroplast. This has been substantiated by cell fractionation at the end of chloroplast development, and this non-chloroplast assignment of the 10S component is in agreement with work reported on the Z strain of *Euglena* (3).

Fractionation at 72 hours of chloroplast development also indicated that while the distinctive chloroplast RNA was labeled with high specific activity, the bulk RNA of the rest of the cell also incorporated large amounts of $^{32}PO_4^{3-}$.

An explanation which ties together these observations and links them with other events such as the large respiratory stimulation associated with light induction of chloroplast development (20) can be sought by assuming that the chloroplast is not nutritionally autonomous, a possibility already alluded to in previous publications (20, 25). Indeed, since *Euglena* does not become photosynthetic until about 4 hours of development (20, 25), and does not carry out significant rates of photosynthesis until after 10 to 14 hours (25), the developing plastid must rely on the rest of the cell for an

energy supply and for metabolites during this early period of development.

Viewed as a resident auxotroph within the cell, the developing chloroplast must make great demands on the synthetic machinery to complete development. We believe that the increase in respiration and the extensive activation of non-chloroplast RNA metabolism within the cell represents the mobilization of the synthetic capacity of the cell to provide the energy and intermediates for chloroplast development. This leaves moot the question of informational interdependence between the chloroplast and the rest of the cell, but suggests that the chloroplast lacks, at the very least, the information required to produce the simpler metabolites. It is still possible that the chloroplast provides all of the information necessary for the synthesis of its distinctive proteins; some of these are probably enzymes which synthesize chloroplast constituents, and under these circumstances the developing plastid would draw upon the cell only for sources of energy and a supply of the simpler metabolites. But, of course, it is also possible that there is more extensive interaction, extending to informational interdependence, for example, the exchange of informational RNAs between organelles.

At present, one cannot ascertain the degree to which $^{32}PO_4^{3-}$ labeling of the RNA represents net synthesis. Preliminary results, however, indicate that the labeling of RNA is inhibited by actinomycin D. The nucleotides, of course, may be derived from the breakdown of existing RNAs.

These considerations raise, but do not clarify the question of how the cellular machinery outside the proplastids is activated to provide increased RNA metabolism as well as increased rates of respiration.

The first event which can be detected in chloroplast development is the light-dependent conversion of protochlorophyll(ide) to chlorophyll(ide). Unquestionably, this transformation is required to complete chlorophyll biosynthesis, but it is not clear to what extent this photoprocess controls chloroplast morphogenesis. All that can be concluded at present is that the light induction of chloroplast development in dark-grown resting cells is correlated with a light-dependent conversion of protochlorophyll(ide) to chlorophyll(ide). It remains to be shown, however, that the protochlorophyll to chlorophyll conversion step controls derepression of formation of chloroplast proteins. Careful action spectra measuring appropriate developmental parameters, such as chloroplast-specific proteins rather than chlorophyll appear to be indicated. In addition, it remains to be determined whether the stimulus to promote activities outside the chloroplast such as RNA metabolism originates in the chloroplast [perhaps as a result of the protochlorophyll(ide) to chlorophyll(ide) conversion] or whether photoreceptors external to the chloroplasts exist to carry out this function.

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