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Variation in Plastid Number

EFFECT ON CHLOROPLAST AND NUCLEAR DEOXYRIBONUCLEIC ACID COMPLEMENT IN THE UNICELLULAR ALGA *OLISTHODISCUS LUTEUS*[1]

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ABSTRACT

Changes in the physiological state of the multiplastidic alga *Olisthodiscus luteus* result in a shift in chloroplast complement from 33 to 21 plastids. The effect of this induced change in organelle complement on nuclear and chloroplast DNA levels has been analyzed. Data suggest that the absolute amount of chloroplast and nuclear DNA found within a cell remains constant but that the amount of chloroplast DNA per plastid is inversely proportional to the number of chloroplasts to which that DNA must be distributed.

Eukaryotic cell function is dependent upon a highly refined metabolic coordination between the nucleus and other compartmentalized cellular structures. Of the many organelles present within the cell, chloroplasts probably rank highest in biochemical and structural complexity (6). Light availability (25, 30), light quality (23), temperature (25), the presence of plant hormones and specific carbon compounds (4) or the entrance of the organism into a specific differentiation sequence (7) may induce a cell to vary its chloroplast complement significantly. It has been known for some time (1) that nuclear gene products are necessary for organelle maintenance. Recent data (13, 26) obtained by investigators attempting to describe the specific coding function of chloroplast DNA indicate that the per cent chloroplast genome transcribed may change during different phases of cell growth and development. It has been reported (12, 27) that the total amount of chloroplast DNA/cell may shift in response to conditions of cellular maintenance of differentiation. Although a positive correlation has been postulated (8) to exist in higher plant systems between an increase in chloroplast number and a maintenance of nuclear DNA synthesis, little is known of the relationship between plastid number and organelle DNA levels. Feedback loops must exist between the nuclear and organelle genome and the macromolecular communication effected by these loops is, no doubt, of primary importance both in the regulation of organelle development and in the determination of cellular replication.

Measurement of the relationship between plastid number, chloroplast DNA complement, and nuclear DNA content should provide background data of critical importance to studies concerned with the elucidation of the macromolecular interaction of genome and plastome in organelle biogenesis.

The unicellular alga *Olisthodiscus luteus* (9) was chosen as the test system for these studies. This organism contains many small plastids which replicate at a defined period of the synchronous cell cycle (9) and plastid number may be manipulated by small changes in the growth regime of the cell. In addition, the chloroplast complement of these wall-less cells may be easily and stringently monitored.

MATERIALS AND METHODS

Reagents. Crystallized phenol obtained from Baker Chemical Co. was redistilled before use and stored at −20 C. Sigma Chemical Co. supplied the sodium-*n*-lauryl sarcosinate, T_1 ribonuclease, pancreatic ribonuclease, and *Micrococcus luteus* DNA. Optical grade CsCl was purchased from Harshaw Chemical Co. (Solon, Ohio). All other chemicals were of reagent grade.

Culture Maintenance. Cells were grown axenically in wide mouth 2.8-liter Fernback flasks which contained 800 ml of 0–3 medium (21). The cultures were maintained at 20 C on a 12-hr light:12-hr dark cycle. Unless otherwise indicated, illumination (Westinghouse cool-white bulbs) was 612 ft-c at the culture surface. All cell counts, chloroplast counts, and DNA extractions were made with cells harvested between hours 6 to 8 of the light portion of the synchronous growth cycle when no cell division, chloroplast replication, or nuclear DNA synthesis was taking place (9).

Cell Counts. Approximately 50 μl of 5% (v/v) formalin were added/ml of cells contained in growth medium. Fixed cells were counted using a Levy-Hausser hemocytometer. Alternatively, unfixed cells appropriately diluted 0–3 medium were counted using a model ZB_1 Coulter Counter equipped with a 100-μm aperture.

Chloroplast Counts. *Olisthodiscus* cells were centrifuged at 20 C in a 12-ml conical test tube for 3 min at 3,000 rpm. The pellet was resuspended in 0–3 medium to give a final concentration of approximately 10^6 cells/ml. Five μl of this suspension were placed on a washed glass slide, and the droplet was covered with a coverslip (22 × 22 mm) of 1.5 thickness. The droplet begins to desiccate almost immediately, and the cells flatten into a single plane of focus, allowing the chloroplasts to be counted easily (Fig. 1).

Cell and Chloroplast Volume Measurements. Cell volumes were determined using a model ZB_1 Coulter Counter equipped with a 100-μm aperture, whereas chloroplast volume determinations were made using a 70-μm aperture. Details of the isolation of osmotically stable chloroplasts from *Olisthodiscus* will be presented elsewhere (K. J. Aldrich and R. A. Cattolico, manuscript in preparation).

DNA Extraction. Cells were harvested in a Sorvall RC-5 centrifuge by centrifugation at 5 C and 3,000 rpm for 5 min using a GSA rotor or by centrifugation at 5 C and 5,000 rpm using an SS-34 rotor with a Szent-Gyorgi-Blum continuous flow attachment. The pelleted cells were resuspended at 2.3×10^6 cells/ml in SSC buffer (0.15 M NaCl, 0.15 M $Na_3C_6H_5O_7 \cdot 2H_2O$) to which 2.5% sodium *n*-lauryl sarcosinate had been added. The cells were allowed to lyse at 5 C in this buffer-detergent mixture for 5 min after which 1.5 volumes of phenol, previously extracted with 1 ×

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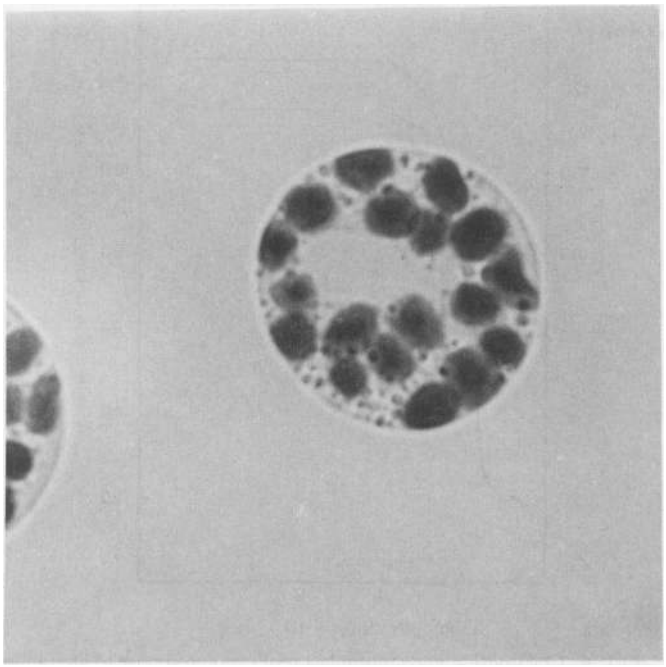

FIG. 1. Example of partially desiccated *Olisthodiscus* cell used to obtain chloroplast counts.

SSC buffer were added. This mixture was kept at 5 C and gently mixed for 20 min. The mixture was centrifuged at 5 C and 7,500 rpm for 15 min using an HB-4 rotor. The supernatant was extracted twice again with 1 volume of phenol. The final supernatant was extracted five times with ice-cold ether, and the DNA was quickly spooled out after the addition of 2 volumes of 95% ethanol (−20 C). Spooling was critical to the extraction of clean *Olisthodiscus* DNA for this step eliminated contamination by co-precipitating polysaccharides. The spooled DNA was dissolved at a concentration of 20 A_{260} units/ml in a pH 8.5 buffer which contained 0.01 M Tris-HCl and 0.02 M NaCl. This solution was treated with 10 units/ml of T_1 ribonuclease and 30 μg/ml of pancreatic ribonuclease for 1 hr at 37 C. Stock nuclease solutions were made in a pH 8.5 buffer containing 0.01 M Tris-HCl and 0.02 M NaCl and were heated at 60 C for 30 min before use. These stock solutions were stored at −20 C. After enzymic treatment, the solution was cooled at 5 C for 5 min. In some experiments one further extraction with phenol was done to remove RNase; in others, DNA was used directly with no further purification. DNA was precipitated by the addition of 95% ethanol and resuspended at a final concentration of 1 mg/ml in a pH 8.5 buffer which contained 0.02 M Tris-HCl. The DNA product obtained by this method had an A_{260}:A_{280} ratio of 1.83 and an A_{230}:A_{260} ratio of 0.51.

Analytical CsCl Density Gradient Centrifugation. A Beckman model E ultracentrifuge equipped with UV optics was used to determine buoyant density. Purified DNA was dissolved in a 0.02 M Tris-HCl (pH 8.5) to which optical grade CsCl was added until a refractive index of 1.4020 was obtained. The final concentration of DNA was 1 μg/ml for *Olisthodiscus* and 0.5 μg/ml for the *M. luteus* marker (ρ = 1.731 gm/cm^3). The DNA was centrifuged for 20 to 24 hr at 20 C and 44,700 rpm. The buoyant density of *Olisthodiscus* DNA was determined by its position relative to the reference density marker.

DNA Quantitation. Total cellular DNA was determined using the microfluorometric technique of Cattolico and Gibbs (10). The amount of chloroplast DNA/cell was determined as follows. *Olisthodiscus* DNA species were separated by analytical CsCl centrifugation. The distribution of these species was monitored photographically. A 2-min exposure time was used which is well within the linear range of the photographic film. Negatives were scanned using a Gilford spectrophotometer equipped with a 2410 linear scanning device. By integrating the areas under each peak, the relative amounts of DNA in main band nuclear and chloroplast species were determined.

Data Evaluation. Kolmogorov-Smirnov and Mann-Whitney tests found in the NPAR subprogram of SPSS were used in the statistical analysis of chloroplast data. Computations were performed on a CDC 6400 computer at the University of Washington.

RESULTS

Variation in Chloroplast Population: Induction and Analysis. A critical aspect of this study was to establish cell populations which had reproducible but varied mean chloroplast numbers. Advantage was taken of the observation that *Olisthodiscus* cells do not maintain a constant number of chloroplasts through all phases of synchronous cell growth. This shift in chloroplast number is demonstrated in the representative experiment of Figure 2. Cells in the linear growth phase which contained 26 plastids were inoculated into fresh growth medium. After a short lag period, a population of cells containing a mean plastid number of 33 was attained. Chloroplast number/cell remained at this high value as long as the culture displayed a logarithmic growth response. At the onset of the linear growth phase, cell division and chloroplast replication rate were no longer equivalent. This resulted in the production of a cell population which has a mean plastid complement of 21. A Mann-Whitney analysis demonstrated conclusively (v = 199579.5, Z = −30.6587, n_1 = 1418, n_2 = 1025) that a population of cells having a mean chloroplast number of 33 and a population having a mean chloroplast number of 21 are statistically different ($P << 0.001$) even though a significant variation (Fig. 3) in the number of plastids/cell is observed for each population studied. The variation in the number of

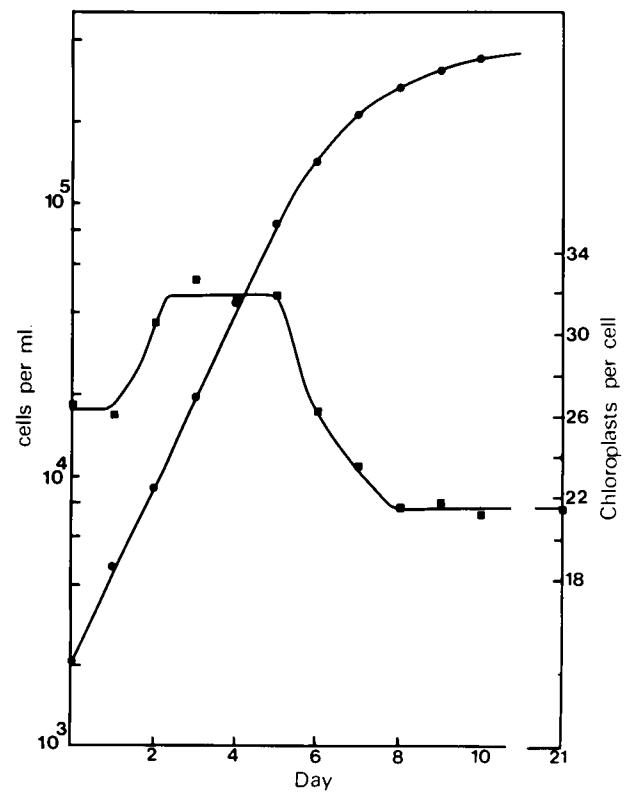

FIG. 2. Change in chloroplast number/cell during different phases of culture growth. Cultures were maintained on a 12:12 light:dark cycle. Both cell and chloroplast counts were made between L4 and L8 when neither cell division nor chloroplast replication occurred (9).

chloroplasts/cell within both the logarithmic and linear *Olisthodiscus* populations is not normally distributed. A positive asymmetry (skewness) and leptokurtosis (flat peakedness) are evident. The deviation of each population from binomial and Poisson distributions with respect to these two variables is presented in Table I.

Given the obligate light requirement of *Olisthodiscus,* it was probable that the observed drop in chloroplast number/cell during culture maturation was induced by self-shading resulting from high culture densities. To test this hypothesis, cultures were inoculated at L4 with cells which contained a mean chloroplast number of 24. These cultures were maintained for the next 24 hr on a normal 12:12 light:dark cycle except that each culture was subject to a light regime of either 85, 140, 335, or 612 ft-c. At the end of this time period, both cell and chloroplast counts were made. Cell division response shows a moderate decrease as light intensity is reduced (Fig. 4). This slow transition in division capability does not hold for plastid replication. Light levels seem critical to this event, for a sharp transition point occurs in the light intensity required to effect chloroplast division.

It was of interest to determine whether chloroplast size would change as a cell altered its chloroplast complement. As seen in Table II, the mean chloroplast volume of 18.9 ± 3.0 μm^3 for cells containing 33 plastids was almost identical to the 17.2 ± 3.7 μm^3 value observed for cells containing 21 chloroplasts. Total chloroplast volume (mean chloroplast number times mean chloroplast volume) represents 44 and 69% of the total volume of *Olisthodiscus* cells sampled in the linear or logarithmic growth phases, respectively.

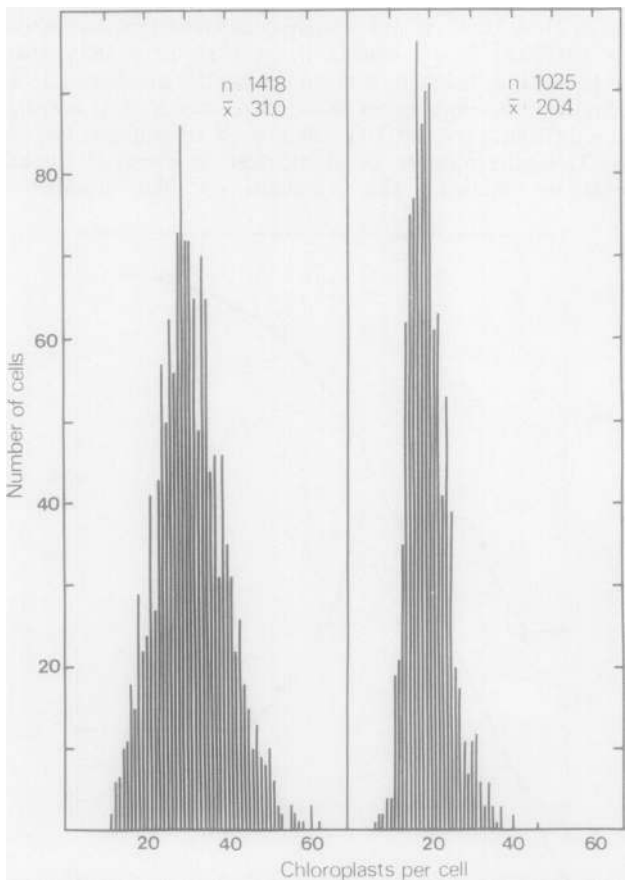

FIG. 3. Distribution of chloroplasts within an *Olisthodiscus* population. Details of cell sampling and chloroplast counting are given in Figure 1.

Table I
Analysis of chloroplast distribution per cell

Chloroplast Mean Number	Coefficient of Skewness	Kurtosis	Kolmogorov-Smirnov (Goodness of fit) Test Normal	P	Poisson	P
31.0 ± 8.42	0.319	0.159	1.9626	< .001	4.5943	<< .001
20.4 ± 5.07	0.855	1.564	3.2539	<<.001	2.9221	<< .001

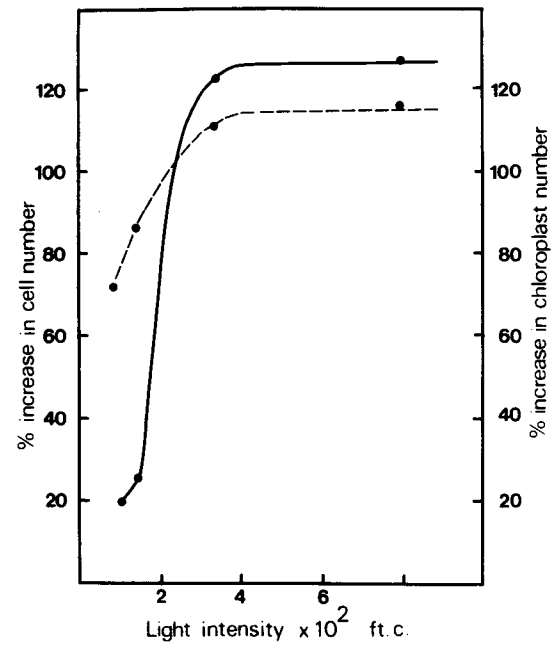

FIG. 4. Cell division and chloroplast replication response to alterations in light intensity.

Table II
Mean chloroplast volume measurements

Mean Chloroplast Number	Mean Chloroplast Volume (μm^3)	Mean Chloroplast Number	Mean chloroplast Volume (μm^3)
21.4	14.1	32.0	21.4
18.9	24.0	33.0	20.0
19.8	18.5	33.3	21.6
20.8	15.3	34.0	16.2
21.0	14.8	33.4	15.1
21.4	16.2		
Mean ± S.D. 20.6	17.2 ± 3.7	33.1	18.9 ± 3.0
Total Volume of Chloroplast Material per cell	354.		626.

Total DNA Complement of *Olisthodiscus* Cells. It has been shown in a number of algal systems that total DNA levels may shift in response to environmental perturbation (15, 22) or an alteration (1) in the growth phase of an organism. For this reason, *Olisthodiscus* cultures at all phases of maturity were sampled and DNA complement/cell determined. A mean DNA level of 2.17 × 10^{-12} g/cell was observed for all samples analyzed independent of source culture density (Fig. 5).

To determine the buoyant density distribution of *Olisthodiscus* DNA species, whole cell DNA was extracted by the sarkosyl-phenol method described under "Materials and Methods." Analysis by neutral CsCl equilibrium density gradient centrifugation using a Beckman model E ultracentrifuge (Fig. 6) indicates that main band nuclear DNA has a buoyant density of 1.702 ± 0.001 whereas a major satellite species was shown to have a buoyant density of 1.691 ± 0.001. Plastids which had been exhaustively washed and passed through a density gradient to remove mitochondria were subject to DNase treatment, phenol extraction, and ethidium bromide CsCl centrifugation. The DNA obtained in this manner had a buoyant density of 1.691 and contained covalently closed supertwisted circular molecules as evidenced by electron microscopic analysis (K. J. Aldrich and R. A. Cattolico, manuscript in preparation). These observations give good experimental support to the chloroplast origin of the 1.691 satellite species. It

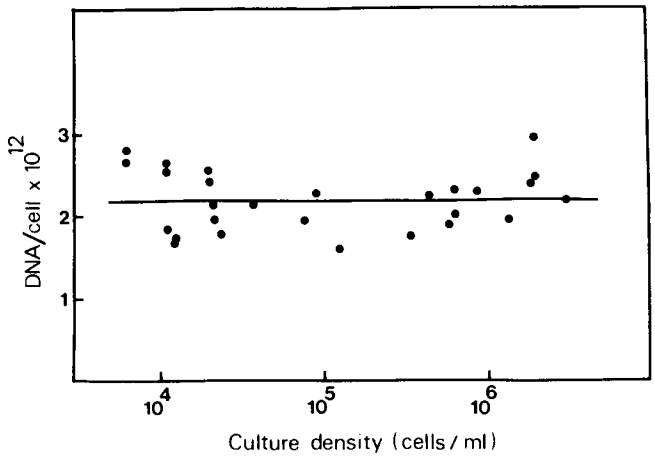

FIG. 5. Relationship between total DNA complement/cell and culture density used as sample source.

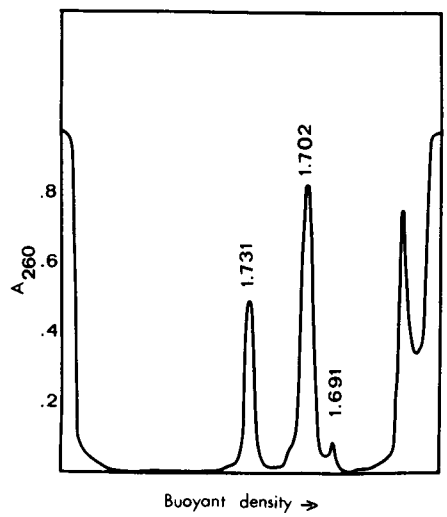

FIG. 6. Buoyant density distribution of *Olisthodiscus* DNA species. *M. luteus* DNA has a buoyant density of 1.731 whereas nuclear and chloroplast DNA species of *Olisthodiscus* have buoyant densities of 1.702 and 1.691, respectively.

should be noted that all observed DNA bands were sensitive to DNase digestion thus eliminating the possibility that the light satellite band was a polysaccharide material which has been shown (14) to contaminate bacterial, plant, insect, and mammalian DNA preparations.

DNA Complement/Chloroplast. The amount of chloroplast DNA/organelle was calculated using the following formula:

$$\frac{\left(\begin{array}{l}\text{Cellular DNA from}\\ \text{fluorometric analysis}\end{array}\right)\left(\begin{array}{l}\text{\% chloroplast DNA}\\ \text{from model E scans}\end{array}\right)}{\left(\begin{array}{l}\text{Mean no. of chloroplasts/}\\ \text{cell from direct counts}\end{array}\right)} = \text{DNA content/chloroplast}$$

The data which were used in this computation are presented in Table III. The information in this table indicates that total cellular DNA content ($2.14 \pm 0.32 \times 10^{-12}$ g) and the proportion of total cellular DNA represented by the chloroplast DNA species ($4.85 \pm 0.7\%$) remain constant. As a result, the total amount of chloroplast DNA/cell ($.103 \pm .018 \times 10^{-12}$ g) is a stable quantity. The amount of DNA found in each chloroplast is therefore determined by the number of plastids to which the DNA must be distributed. The regression curve for chloroplast DNA amount *versus* chloroplast number/cell is presented in Figure 7. A reciprocal relationship between chloroplast number and chloroplast DNA content is evident. As chloroplast number/cell increases, the amount of chloroplast DNA present/organelle decreases.

Table III
Effect of chloroplast number on cellular DNA values

Exp.	Chloroplast number	Total DNA/Cell x10^{+12}	Percent Chloroplast* DNA	Total Chloroplast DNA/Cellx10^{+12}	DNA/Chloroplast x10^{+15}
1	14.0+	2.38	3.32	0.079	5.68
2	16.3+	1.92	5.22	0.100	6.15
3	17.8+	2.74	4.47	0.124	6.88
4	18.6	2.17	5.45	0.118	6.36
5	20.2	1.93	4.88	0.094	4.66
6	23.5	2.04	4.50	0.092	3.91
7	24.2	2.00	5.46	0.109	4.51
8	26.9	2.34	4.49	0.105	3.91
9	33.9	2.29	5.81	0.133	3.92
10	38.3	1.58	4.88	0.077	2.01
Mean ± S.D.		2.14 ± 0.32	4.85 ± 0.70	0.103 ± .018	
Coefficient of Determination (r^2)		0.19 (P>0.2)^	0.17 (P>0.2)°	0.10 (P>0.2)□	0.77 (P<0.001)#

+0-2 medium (9) was used in the growth of these cells. Chloroplast number in stationary cultures was 14 whereas chloroplast number in logarithmic cultures was 21.
*Transformed to arcsine values for statistical computations
^Chloroplast number vs total DNA/cell x 10^{12}
°Chloroplast number vs total chloroplast DNA/cell x 10^{12}
#Chloroplast number vs DNA/chloroplast x 10^{15}

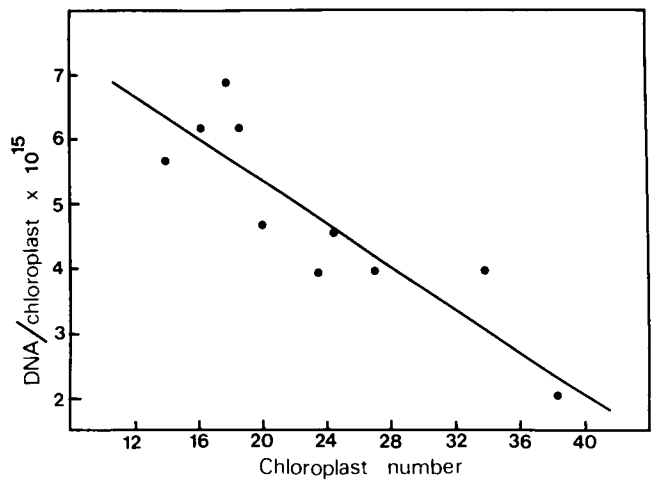

FIG. 7. Change in chloroplast DNA complement effected by an alteration in plastid number/cell.

DISCUSSION

For this study it was necessary to establish a system wherein chloroplast number could be accurately determined. Tissue complexity (4, 8), a variation in the timing of cell and organelle division events (7, 25), combined with the need for sophisticated fixation and/or microscopic techniques (19, 35), often make plastid complement analysis extremely difficult in many plant systems. *O. luteus* provides an excellent experimental organism wherein a uniform population of cells may be analyzed at a time in synchronous growth where neither cell division nor chloroplast replication takes place. No fixation or complicated tissue preparation is required and chloroplast counts can be made quickly using simple light microscopy.

The chloroplast complement of a cell both in higher (4, 23) plants and in algal species (15, 30) seems to have an inducible plasticity which is dependent upon environmental and developmental cues. *Olisthodiscus* is no exception to this observation. Although a given population of these cells can maintain a specific mean chloroplast level, chloroplast complement will shift to a new stable mean value (*e.g.* 21 *versus* 33 plastids) in sensitive response to small changes in a particular growth regime.

Recent evidence obtained using *in vitro* cultured chloroplasts (28), chloroplasts exposed to different light qualities (23), or metabolic inhibitors (34) and observations of chloroplasts in anucleate cells (30) suggests that chloroplast division and chloroplast growth are separable events. For this reason, a difference in

chloroplast number alone is not sufficient to define total plastid material within a cell. Under the growth conditions in which we have maintained *Olisthodiscus,* chloroplast volume does not change, remaining at 17 to 19 nm^3, independent of chloroplast complement. However, preliminary studies indicate that a chloroplast shape change may occur.

A second major consideration of this study was to determine whether shifts in total cellular DNA amount take place in *Olisthodiscus* concomitant to changes in plastid complement. Although an increase in cellular DNA content has been indicated to occur (24, 29) in higher plant cells where plastid numbers are increasing, precise DNA measurements are often difficult to obtain in these systems due to the structural complexity of the tissue under analysis. In many multiplastidic, unicellular algal systems, however, data indicate that dramatic shifts in total cellular DNA values might occur. For example, *Astasia* (22) cells with a generation time of 124 hr have one-fifth as much DNA as cells with a generation time of 10 hr, and a number of dinoflagellate species (1) contain a 2-fold difference in DNA when logarithmic and stationary cells are compared. The evidence presented by Butterfass (8) that plastid number in higher plants might be influenced (controlled?) by nuclear DNA amount is quite convincing. Indeed, *Euglena* cells maintained (15) in phosphate-depleted medium contain half the nuclear DNA amount and half the plastid complement of cells maintained under a normal growth regime. The fact that *Olisthodiscus* cells maintain a constant DNA amount regardless of chloroplast complement indicates that perhaps this "control" method is not universal or at least not primary in some plant systems. More studies of other multiplastidic algal species are needed before accurate comparisons may be made.

To determine whether the proportion of *Olisthodiscus* chloroplast DNA changes as chloroplast number increases, total cellular DNA was separated into its component species by analytical CsCl ultracentrifugation. The buoyant density obtained for *Olisthodiscus* mainband (1.702) and chloroplast (1.691) are quite similar to that observed for the chrysophyte *Ochromonas* (11). It has recently been suggested (20) that *Olisthodiscus* be transferred from the Chrysophyseae to the Chloromonadaceae. As far as we can determine, no DNA literature exists for this algal group.

The chloroplast DNA species of *Olisthodiscus* represents approximately 4.9% of the total DNA of the cell. Plastid DNA amounts of 8.6, 16, and 4.9 to 16% have been observed in *Chlamydomonas* (33), *Polytoma* (31), and *Euglena* (12) respectively. The constancy in the proportion of chloroplast to nuclear DNA observed in *Olisthodiscus* is not unique. Vegetatively reproducing diploid strains of *Chlamydomonas* have been shown (34) to maintain the same ratio of nuclear to plastid DNA when compared to the normal haploid algal strain. However, given the fact that plastid to nuclear DNA ratios of *Euglena* may remain the same (15) or change (12, 17) dependent upon the nutritional or developmental state of the cell, it seems premature to invoke a stringent genome-plastome relationship in *Olisthodiscus* until studies with cells maintained under varying growth regimes have been completed.

The DNA complement of a chloroplast is present as many small circular molecules (18) which segregate randomly (16, 29) during plastid replication. In this study it has been shown that the amount of DNA/plastid is inversly related to the number of chloroplasts present/cell. This work plus data from *Chlamydomonas* (2), guinea grass (32), and pea (3) which also suggest that chloroplast DNA amount/organelle may vary, indicate that the plastid may contain "excess" DNA. Studies using FUdR-treated tobacco (5) and irradiated spinach leaf tissue (24) show that chloroplast division may occur in the absence of chloroplast DNA synthesis. Exactly what minimal DNA level may occur without affecting the survival (maintenance) of the organelle is presently unknown.

One of the most interesting of the many regulatory programs expressed during cell growth is the production and maintenance of a specific organelle complement. A highly refined coordination between plastid biogenesis and cell growth, differentiation, and development must be present to insure the functional efficiency and survival of the cell. This study provides an analysis of chloroplast and nuclear DNA species from the alga *O. luteus* and provides information on the effect of physiological determinants on the genome and plastome complement of this multiplastidic cell.

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