AUTORADIOGRAPHIC EVIDENCE FOR MANY SEGREGATING DNA MOLECULES IN THE CHLOROPLAST OF *OCHROMONAS DANICA*

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ABSTRACT

Light-grown cells of *Ochromonas danica*, which contain a single chloroplast per cell, were labeled with [*methyl*-^{3}H]thymidine for 3 h (0.36 generations) and the distribution of labeled DNA among the progeny chloroplasts was followed during exponential growth in unlabeled medium for a further 3.3 generations using light microscope autoradiography of serial sections of entire chloroplasts. Thymidine was specifically incorporated into DNA in both nuclei and chloroplasts. Essentially all the chloroplasts incorporated label in the 3-h labeling period, indicating that chloroplast DNA is synthesized throughout the cell cycle. Nuclear DNA has a more limited S period. Both chloroplast DNA and nuclear DNA are conserved during 3.3 generations. After 3.3 generations in unlabeled medium, grains per chloroplast followed a Poisson distribution indicating essentially equal labeling of all progeny chloroplasts. It is concluded that the average chloroplast in cells of *Ochromonas* growing exponentially in the light contains at least 10 segregating DNA molecules.

INTRODUCTION

Studies of the rate of renaturation of chloroplast DNA from a variety of plants have shown that chloroplast DNA has a kinetic complexity, or genomic size, of approximately 1–2 $\times$ 10^8 daltons (2, 12, 22, 24, 26, 27). Recently, it has been shown by electron microscopy that the chloroplasts of *Euglena* (14, 17), spinach (16), corn (16), and peas (12) contain circular DNA molecules 39–44 μm in contour length. The molecular weight of a 44-μm molecule (0.9 $\times$ 10^8 daltons) is sufficiently close to the values observed for genetic complexity to indicate that a single molecule of chloroplast DNA carries the total genetic information of the chloroplast. However, since it is well known that a single chloroplast contains considerably more than 44 μm of DNA (values between 300 and 3,000 μm of DNA per chloroplast are commonly reported), it is assumed that chloroplasts contain numerous identical DNA molecules which are segregated among the progeny chloroplasts. However, to date, it has not been directly demonstrated that chloroplasts contain a number of DNA molecules which segregate at chloroplast division. To show this, we have employed the unicellular alga, *Ochromonas danica*, which contains a single chloroplast which divides once each cell generation. Light-grown cells were labeled with [^{3}H]-thymidine and chased in medium containing excess unlabeled thymidine for a number of generations. The distribution of labeled DNA among the progeny chloroplasts was determined from autoradiographs of serial sections of entire chloroplasts. The results demonstrate that the average chloroplast in cells of *Ochromonas danica*

growing exponentially in the light contains at least 10 DNA molecules which segregate to the progeny chloroplasts.

MATERIALS AND METHODS

Culture Conditions

Stocks of *Ochromonas danica* Pringsheim were obtained from the Culture Collection of Algae at Indiana University, Bloomington, Ind. (culture no. 1298). All experimental cultures were grown at 29°C in Aaronson and Baker's (1) complete medium under a bank of fluorescent and incandescent lamps adjusted to give a light intensity of 450 foot candles at the culture surface. The cells were grown in 500- or 1,000-ml Erlenmeyer flasks which were shaken gently on a New Brunswick rotary shaker (New Brunswick Scientific Co., Inc., New Brunswick, N. J.). Cell counts were made with a hemocytometer.

Experimental Procedure

5 mCi of [*methyl*-^{3}H]thymidine (New England Nuclear, Boston, Mass.; sp act 18.4 Ci/mmol) were added sterilely to a 300-ml culture of exponentially growing cells of *Ochromonas* to give a final concentration of [^{3}H]thymidine in the culture of 16.4 μCi/ml or 0.9×10^{-6} M. The cells were returned to the light and allowed to grow logarithmically in the presence of isotope for 3 h. The labeled culture was then divided into four appropriately sized aliquots; each aliquot was collected by centrifugation at 1,000 *g* for 5 min and rinsed twice in growth medium containing 10^{-3} M unlabeled thymidine (Schwarz Bio Research Inc., Orangeburg, N. Y.). After rinsing, one aliquot of cells was fixed immediately in glutaraldehyde whereas the other three aliquots were each resuspended in 200 ml of growth medium containing 10^{-3} M unlabeled thymidine and allowed to grow in the light for 1.0, 2.2, and 3.3 generations. The cells resumed exponential growth immediately. The size of each aliquot had been adjusted so that the cells in each culture would remain in the logarithmic phase of growth throughout the experiment. Light-grown cells of *Ochromonas* enter the linear phase of growth between 10 and 20×10^6 cells/ml. All cultures when fixed contained between 5.7×10^6 and 8.9×10^6 cells/ml. The presence of 10^{-3} M thymidine had no inhibitory effect on the growth rate of the cells. In all exponentially growing cultures, the cells divided once every 8.25 h. Sterile techniques were employed during the centrifuging, washing, and resuspending procedures, and the cultures were carefully monitored for contamination both by light microscopy of living cells and by electron microscopy of the fixed cultures. No contamination was observed.

All cultures were fixed in 2.5% glutaraldehyde in 0.05 M potassium phosphate buffer, pH 7.3, for 2 h at 4°C. After fixation, the cells were washed in five changes of cold 0.1 M phosphate buffer for a total of 2 h and then postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.3, for 2 h at 4°C. After rinsing twice in buffer, the cells were embedded in small agar blocks, dehydrated in a graded ethanol series followed by propylene oxide, and embedded in Araldite (Ciba Products Co., Summit, N. J.).

Preparation of Serial Sections

For each culture 10–15 slides were prepared each containing a series of 10–12 consecutive 1-μm thick Araldite sections. Sections were cut with glass knives on a Porter-Blum MT-2 Ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) set at 1 μm. The sections were usually kept in order by arranging them along the unused part of the knife edge. They were then picked up individually with a stiff hair and transferred to a drop of water on an ethanol-cleaned slide. This slide had been marked by a vertical black line on its under surface and placed on the stage of a dissecting microscope with its left-hand edge propped up by another slide. A large drop of water had been placed on the slide slightly downhill from the black line. After each section had been transferred to the drop of water, the edge of the water drop was momentarily pulled to and slightly across the black line with a hair loop and at the same time the section was maneuvered into place with the stiff hair so that its left-hand edge was aligned with the black line. After all 10–12 sections had been arranged in order by this method, the remainder of the water drop was slowly eased off the slide with a camel hair brush. The slide was then heated for 15 min on a slide warmer at 70°C to thoroughly attach the sections to the slide.

Autoradiography

The slides were dipped into undiluted Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N. Y.), maintained at 40°C, and allowed to dry very slowly in a darkroom held at 28°C and 80% relative humidity. The slides were stored at 4°C in light-tight boxes containing Drierite (W. A. Hammond Drierite Co., Xenia, Ohio). After exposure times of 25 or 51 days, the slides were developed in Kodak D-19 for 1 min at 20°C, rinsed briefly in dilute acetic acid, and fixed in 24% sodium thiosulfate for 5 min at 20°C. After washing and drying, the slides were stained through the emulsion with an aged solution of 1% methylene blue in 1% borax for 2 h at 40°C. The excess methylene blue was removed by dedifferentiation in a dilute acetic acid solution. Slides were mounted in Permount (Fisher Scientific Co., Pittsburgh, Pa.) and examined with a Zeiss microscope

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with a 100 × planapochromat oil immersion lens. To facilitate the analysis of specific cells in serial sections, a cluster of cells would be diagrammed in every section on a slide, each cell in the cluster being identified by number. Only those cells in which both the entire chloroplast and the entire nucleus were sectioned were analyzed. For each section of a cell, the grains lying over the chloroplast, nucleus, cytoplasm, and leucosin vacuole were recorded and later summed.

DNase Digestion Experiment

A preliminary experiment was performed in order to determine if [methyl-^{3}H]thymidine is specifically incorporated into nuclear and chloroplast DNA in *Ochromonas*. Light-grown cells in the logarithmic phase of growth were labeled with 50 μCi/ml [methyl-^{3}H]thymidine (sp act 16.1 Ci/mmol) for 18 h. After a 5-min rinse in unlabeled medium, the cells were fixed for 30 min in 10% formaldehyde in 0.1 M potassium phosphate buffer, pH 7.5, at 4°C. The cells were then washed in four changes of cold 0.1 M phosphate buffer, pH 7.2, for a total of 1 h. One aliquot of cells was immediately postfixed in osmium tetroxide. A second aliquot was suspended in an 0.1% solution of DNase I (Worthington Biochemical Corp., Freehold, N. J.) and incubated for 2 h at 37°C. The DNase was dissolved in 0.003 M $MgSO_4$ and adjusted to pH 6.5 with NaOH. A third aliquot of cells was incubated in 0.1 M phosphate buffer, pH 6.5, for 2 h at 37°C. All cells were postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer, pH 7.3, for 2 h at 4°C and embedded in Araldite as described above. Sections 1 μm thick were mounted on glass slides and coated with Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) diluted 1:2 with distilled water. These slides were dried at 20°C and 50% relative humidity and then stored in light-tight boxes containing Drierite. After 6–36 days exposure at 4°C, the slides were developed for 8 min in Kodak D-170, fixed in 24% sodium thiosulfate, washed, dried, and mounted in Permount. The slides were examined by phase microscopy.

Determination of Chloroplast Volume

The percent of the total cell volume occupied by the chloroplast in the light-grown, log-phase cells employed in this experiment was determined by electron microscopy. Thin sections of each of the four cultures used in this study were stained with 4% uranyl acetate in 40% ethanol followed by lead citrate (18) and viewed in a Philips EM 200 electron microscope. For each culture approximately 100 cell sections, each from a different cell, were photographed at low magnification and printed at a final magnification of × 20,800. The relative volume occupied by the chloroplast was determined by the tracing and weighing method described previously (10).

RESULTS

Specificity of [Methyl-^{3}H]Thymidine Labeling

In the control experiment, digestion of formaldehyde-fixed whole cells with DNase for 2 h at 37°C reduced the number of grains/cell section by 96% whereas digestion in warm buffer alone for 2 h reduced the number of grains/cell section by 12%. Although preservation of cytological detail in the formaldehyde-fixed, DNase-digested cells was distinctly inferior to that obtained after glutaraldehyde fixation (Fig. 1), in a few slides, grain counts were made separately for the nucleus, chloroplast, and cytoplasm. No differential effects of DNase digestion on the different cell compartments were observed. Thus, it is concluded that thymidine is an effective precursor of both chloroplast and nuclear DNA in *Ochromonas* and that virtually all the observed radioactivity in the cells is in labeled DNA.

Use of Serial Sections of Entire Chloroplasts for Quantitative Autoradiography

Log-phase, light-grown cells of *O. danica* are 5–12 μm wide and 8–15 μm long and contain a single large chloroplast which lies in the anterior half of the cell and partially encircles the cell's nucleus. The chloroplast in its simplest form consists of two flat platelike lateral lobes which are joined by a narrow bridge dorsal to the nucleus (9, 20). The chloroplast DNA is not scattered at random throughout the chloroplast, but instead lies just inside the rim of the chloroplast, interior to the girdle bands of thylakoids which loop around the rim of the chloroplast (10, 11). In three dimensions, the chloroplast nucleoid has the shape of a long narrow cord, approximately 0.25 μm in diameter, which encircles the periphery of each chloroplast lobe and is continuous across the top and bottom of the bridge.

In this experiment, it was necessary both to be able to determine whether or not an individual chloroplast contained labeled DNA and also to have a quantitative measure of the relative amount of radioactivity in each population of chloroplasts. Clearly, since data was needed for an entire chloroplast, random thin sections could not be used. Moreover, autoradiographs of squash prep-

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arations of whole cells or of thick 10-μm sections which would include an entire chloroplast would not give quantitative results, due to the very short path length of tritium β-particles. Also, the chloroplast would not be clearly resolved in squashes or in sections thick enough to include a whole chloroplast. Thus it was necessary to make serial sections of entire chloroplasts and prepare these for autoradiography.

It was found in preliminary experiments that sections 1-μm thick were most suitable. Series of 10–12 such sections were mounted on glass slides as described above and prepared for autoradiography. As a rule, all cells which were sectioned at the level of the nucleolus in the middle section of a series would include an entire chloroplast and thus could be used for grain counts. The number of sections it took to traverse an entire chloroplast depended both on the size of the chloroplast and on the angle at which the cell was sectioned, but was usually 5–8 sections (average, 6.9 sections; range, 4–11 sections). Fig. 1 illustrates a representative series of sections of a complete chloroplast from a cell which had been chased for 2.2 generations in unlabeled thymidine. With the exception of Fig. 1 *g*, each section has been focused at the level of the cell, so the reader can see how clearly the chloroplast is resolved in these methylene blue-stained, 1-μm sections. In Fig. 1 *a* and *b*, just one lobe of the chloroplast has been cut; in Fig. 1 *c*, both lobes of the chloroplast are cut and the nucleus can be seen as a light homogeneous area lying between the two chloroplast lobes. In Fig. 1 *d*, the nucleolus is sectioned; two out-of-focus grains are present over the posterior end of the right chloroplast lobe. In Fig. 1 *e* and *f* the two

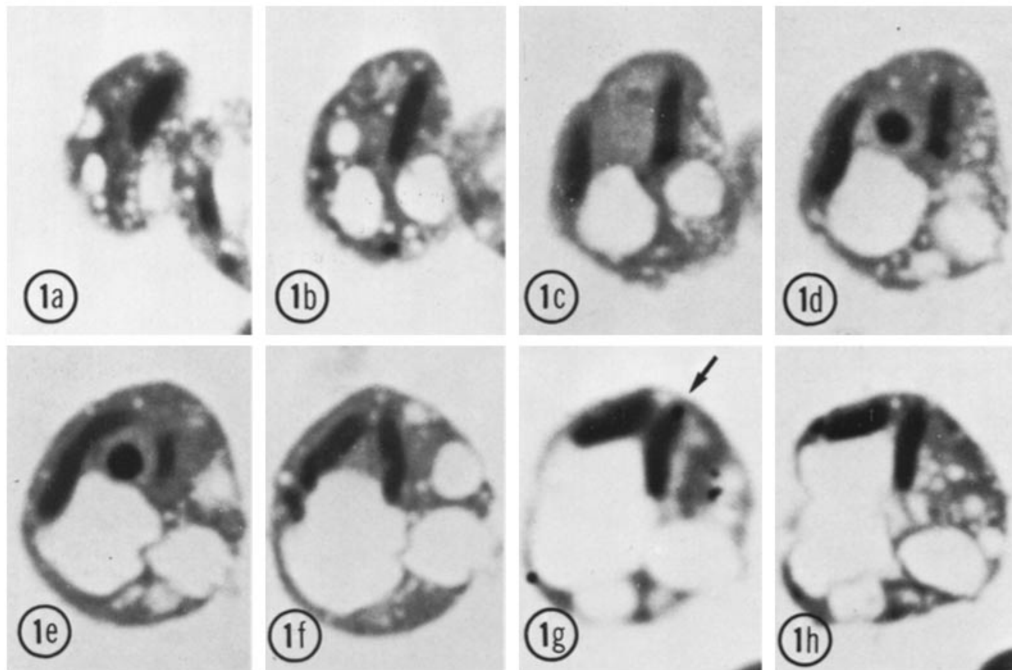

FIGURE 1 Serial 1-μm sections through an entire chloroplast of a light-grown cell of *O. danica*. This cell was labeled with [^{3}H]thymidine for 3 h and chased for 2.2 generations in unlabeled medium. The slide was exposed for 51 days and stained through the emulsion with methylene blue. Since the purpose of this figure is to demonstrate the clarity with which an entire chloroplast can be distinguished in a series of sections, a cell with comparatively few grains was chosen and each section, except for Fig. 1 *g*, was focused at the level of the cell. Fig. 1 *g*, which is a section through the bridge region of the chloroplast, was focused at the level of the silver grains. A single silver grain (*arrow*) lies over the anterior extremity of the right-hand chloroplast lobe. Two out-of-focus grains are located over the posterior extremity of the right-hand chloroplast lobe in Fig. 1 *d*. No nuclear grains are present. The section immediately preceding Fig. 1 *a* and that following Fig. 1 *h* have not been included as the chloroplast was not present in those sections. × 3,000.

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chloroplast lobes come closer to each other and in Fig. 1 *g* can be seen to be joined by a narrow bridge behind the nucleus. In the last section, Fig. 1 *h*, the two chloroplast lobes jut out behind the bridge like two shoulder blades. Fig. 1 *g* has been focused at the level of the silver grains, and a single grain can be seen at the upper end of the right chloroplast lobe (arrow). The grain lies over the region of the chloroplast known to be occupied by the DNA-containing chloroplast nucleoid. In this study, as in a previous electron microscope study (10), virtually all chloroplast grains were associated with the extremities of the chloroplast where the chloroplast nucleoid is located.

The autoradiographs of serial sections of chloroplasts employed in this study meet the requirements for measuring relative radioactivity (see discussion by Rogers [19]) with one exception. There will be some variation in the efficiency of measuring the radioactive DNA in the different individual chloroplasts of a population depending on exactly how the chloroplast nucleoid is located in the series of sections. It will be realized that since the chloroplast nucleoid is a ring-shaped structure encircling the periphery of the chloroplast, almost every chloroplast section will include it. In most sections, the nucleoid will lie perpendicular to the section, and, assuming that tritium is distributed uniformly in the 1-μm section of nucleoid, the grain counts observed will reflect the concentration of tritium. However, in those sections where the nucleoid is sectioned tangentially, the observed grain count will vary depending on the depth of the nucleoid in the section due to the self-absorption of the β-particles of tritium. This small variation in the efficiency with which tritium is measured in different series of sections of entire chloroplasts is no doubt a cause of some of the variation of the observed grain counts from perfect Poisson distributions. However, when comparing one population of chloroplasts (60–80 series) with another, this consideration no longer applies for the geometry of the chloroplast nucleoid within the two sets of serial sections will be essentially the same.

Background Grain Counts

Counts of background grains were made on the cell-free areas of the Araldite sections using a Whipple disk. The average background grain counts observed were: for the unchased cells, 25 days exposure, 0.19 grains/100 μm^2; for the first generation cells, 25 days exposure, 0.15 grains/100 μm^2; for the second generation cells, 25 days exposure, 0.08 grains/100 μm^2; for the second generation cells, 51 days exposure, 0.13 grains/100 μm^2; and for the third generation cells, 51 days exposure, 0.10 grains/100 μm^2. The percentage of the cell occupied by the chloroplast was determined for each culture and shown to be approximately the same in each case, averaging 13.6% of the cell volume. Log-phase, light-grown cells of *Ochromonas* average approximately 560 μm^3 in volume (21); thus, the average absolute volume of the chloroplast is 76 μm^3. Knowing the three-dimensional shape of the chloroplast, it is possible to calculate the area occupied by an average chloroplast when cut into serial 1-μm sections. This area varies slightly depending on the angle at which the chloroplast is cut and in how many sections the chloroplast falls, but in almost all cases, the total area occupied by an entire chloroplast is less than 100 μm^2. Clearly, therefore, the number of background grains which would be found over an entire chloroplast is not significant and it was not necessary to make any corrections to the observed chloroplast grain counts. Since the average volume of the nucleus is only 18 μm^3, it also was not necessary to correct the observed nuclear grain counts for the background.

Chloroplast Division

In an electron microscope study of mitosis in *O. danica*, Slankis and Gibbs (20) have shown that the single chloroplast of this species divides in two shortly before mitosis by a progressive narrowing of the bridge region. Since in 1-μm sections it is usually not possible to distinguish a recently divided chloroplast from an undivided chloroplast, all the chloroplast grains in a single cell were tabulated as belonging to a single chloroplast. Because of this method of analysis, chloroplast division can for the purposes of this experiment be considered as occurring at the moment of cell division. This equation of one chloroplast generation with one cell generation considerably simplifies the analysis of the experiment. It should be realized though that strictly speaking our calculations refer to the number of chloroplast DNA molecules per cell rather than per chloroplast. However, since only a small percentage of the cells of a population contain a divided chloroplast (about 5%), the errors introduced by this method

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of analysis do not significantly alter our conclusions.

Initial Pattern of Labeling: Synthesis of Chloroplast DNA throughout the Cell Cycle

In order to determine whether the chloroplast of *Ochromonas* contains one, several, or many segregating molecules of DNA, the chloroplast DNA was labeled with [^{3}H]thymidine and the distribution of label among the progeny chloroplasts was observed during subsequent growth in unlabeled medium. Because of the relatively long generation time of *Ochromonas* (8.25 h), the number of generations the cells could be chased in unlabeled medium was a limiting factor in the experiment. Thus it was deemed desirable to start the chase period with chloroplast DNA labeled in one strand only. Consequently, exponentially growing cells of *Ochromonas* were labeled for 3 h (0.36 of a generation) with [^{3}H]thymidine and then prepared for autoradiography after 0, 1.0, 2.2, and 3.3 generations in fresh medium containing excess unlabeled thymidine.

Fig. 2 shows the distribution of grains per chloroplast in cells fixed at the end of the labeling period. It can be seen that essentially all of the chloroplasts are labeled during the 3-h labeling period. If every cell at the time of fixation had exactly the same amount of tritium in its chloroplast DNA, then due to the random decay of tritium, the number of grains per chloroplast observed in the autoradiographs would follow a Poisson distribution, such that

$$P(n) = \frac{e^{-m}m^n}{n!}$$

where m is the mean number of grains per chloroplast and $P(n)$ is the proportion of chloroplasts with n grains (25). The black circles in Fig. 2 give the expected Poisson distribution of grains per chloroplast which would have been observed if the chloroplast of every cell had contained equal amounts of radioactivity. Clearly the results do not follow a Poisson distribution. A probable explanation why a number of chloroplasts had less grains than would be expected from a Poisson distribution is that some cells would have divided near the end of the labeling period and thus would have fewer grains per chloroplast. Indeed, many of the cells which had seven or less grains per chloroplast could be seen to be small cells with small chloroplasts. The chloroplasts of these cells, for example, occupied on average 5.9 sections, whereas those containing 8 grains or more occupied on average 7.2 sections. It will be shown below that after one or more generations in unlabeled medium

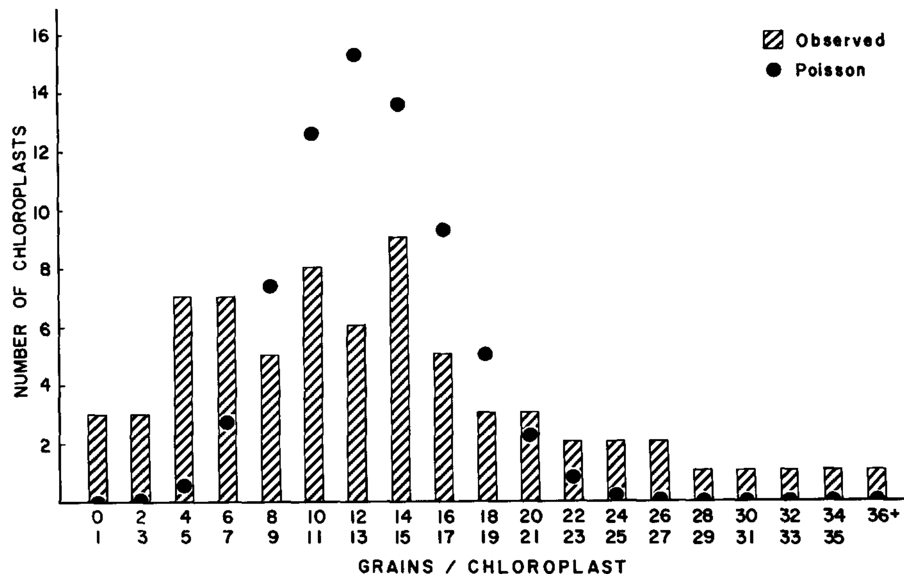

FIGURE 2 Frequency distribution of grains per chloroplast in autoradiographs of cells fixed immediately after labeling with [^{3}H]thymidine for 3 h (0.36 generations). 25 days exposure. Mean number of grains per chloroplast = 13.2. Only one of the 70 chloroplasts had no grains. The observed distribution differs significantly from a Poisson distribution having the same mean (chi-square test: $P < 0.01$).

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the distribution of grains per chloroplast approaches much more closely to a Poisson distribution. This suggests that differences in division time between cells have tended to equalize the amount of label in different chloroplasts. This supports the view that initial inequalities of label were correlated with cell size and should not be attributed to lack of chloroplast DNA synthesis during part of the cell cycle.

These two observations that essentially all the chloroplasts of an asynchronous culture are labeled in 0.36 of a generation and that the progeny chloroplasts receive equal amounts of label indicate that chloroplast DNA is synthesized throughout the cell cycle.

Conservation of DNA during the Chase Period

In Fig. 3 the average number of grains per chloroplast is plotted against the number of generations the cells were grown in unlabeled medium. The solid line represents values which would be observed if the amount of labeled chloroplast DNA per cell cut exactly in half at each cell division. The slope of the regression line calculated from the five experimental points is not significantly different ($P > 0.2$) from the theoretical line shown in Fig. 3. Thus chloroplast DNA is conserved during 3.3 generations in chase medium. This observation that chloroplast DNA in *O. danica* is stable for at least 3.3 generations is at variance with the recent report of Manning and Richards (15) that one-third of the total chloroplast DNA of *Euglena gracilis* is degraded every cell generation.

In Fig. 3, the solid circles are the actual averages obtained from autoradiographs exposed for 25 days. The open circles are adjusted values which were obtained from autoradiographs exposed for twice as long (51 days). The fact that the corrected average grain count per chloroplast for the second generation cells after an exposure time of 51 days is actually somewhat higher than that observed after an exposure time of 25 days indicates that there is not significantly more fading of the latent image at the longer exposure time.

Fig. 4 is included to show that, as expected, nuclear DNA is also conserved during 3.3 generations in unlabeled medium. The fit of the observed values to the theoretical values is not as good as for chloroplast DNA, no doubt because the sample of labeled nuclei is considerably smaller than that

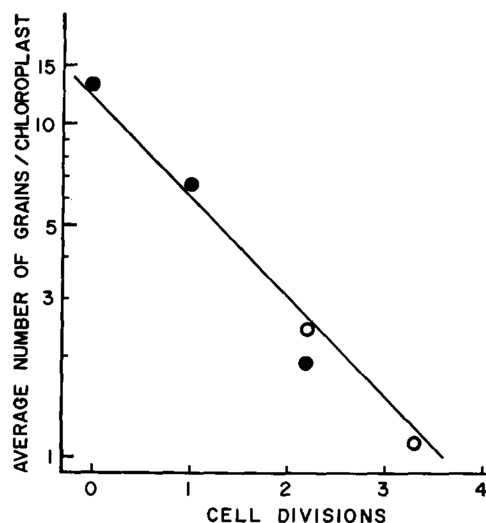

FIGURE 3 Semilogarithmic plot of the average number of grains per chloroplast versus the number of cell divisions in unlabeled medium. The solid line represents the slope expected if the number of grains per chloroplast is halved at each cell division. The solid circles are the observed grain counts for cells exposed for 25 days; the open circles are 0.49 times the observed grain counts for cells exposed 51 days.

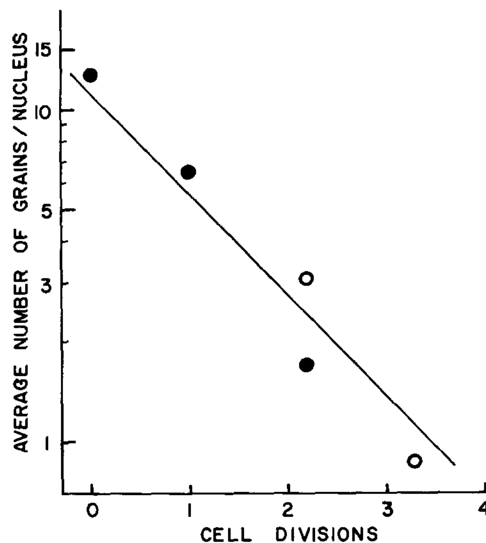

FIGURE 4 Semilogarithmic plot of the average number of grains per nucleus versus the number of cell divisions in unlabeled medium. The solid line represents the slope expected if the number of grains per nucleus is halved at each cell division. The solid circles are the observed grain counts for cells exposed for 25 days; the open circles are 0.49 times the observed grain counts for cells exposed 51 days.

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of chloroplasts since all cells did not synthesize nuclear DNA in the 3-h labeling period.

Distribution of Labeled DNA among the Progeny Chloroplasts

Fig. 5 shows the distribution of labeled DNA among the progeny chloroplasts after 1.0 generation in chase medium. It can be seen that, as before, essentially all the chloroplasts are labeled and that all the chloroplasts now contain approximately equal amounts of label since the observed distribution of grains per chloroplast does not differ significantly from a Poisson distribution (chi-square test, $P > 0.05$).

The observation that all the chloroplasts are still labeled after one generation in unlabeled medium would be expected whether the chloroplast contains one or many molecules of DNA. However, since chloroplast DNA replicates in a semiconservative manner (6, 15), one would expect in the case of a single DNA molecule per chloroplast that half of the progeny chloroplasts would be unlabeled by the second generation. With more than one DNA molecule per chloroplast, the predicted results will depend on whether or not the chloroplast DNA molecules segregate randomly at chloroplast division. If the labeled molecules are distributed equally (i.e., in a nonrandom manner) among the progeny chloroplasts, then the number of grains per chloroplast would continue to follow a Poisson distribution. However, as soon as the number of progeny chloroplasts exceeds the number of DNA strands initially labeled, a larger zero class than that predicted by a Poisson distribution would appear. If the chloroplast divides in such a way that the labeled molecules are distributed randomly among the progeny, a large zero class will appear more quickly.

The distribution of label among the progeny chloroplasts after 2.2 divisions in chase medium is shown in Figs. 6 and 7. The data in Fig. 6 is from autoradiographs exposed for 25 days; that in Fig. 7 from autoradiographs exposed for 51 days. In each case it is clear that essentially all of the chloroplasts still contain labeled DNA, for the zero class is not markedly different from that predicted by a Poisson distribution. Also every chloroplast contains approximately the same amount of label since the observed distribution of grains

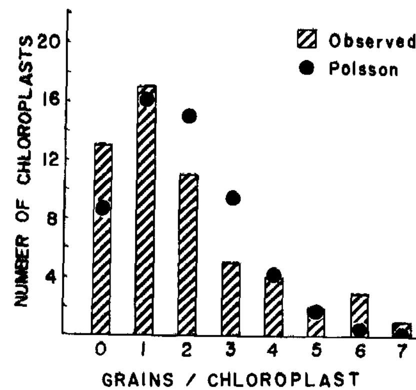

FIGURE 6 Frequency distribution of grains per chloroplast in autoradiographs of [^{3}H]thymidine-labeled cells grown in unlabeled medium for 2.2 generations. 25 days exposure. Mean number of grains per chloroplast = 1.9. The observed distribution does not differ significantly from a Poisson distribution having the same mean (chi-square test: $P > 0.05$).

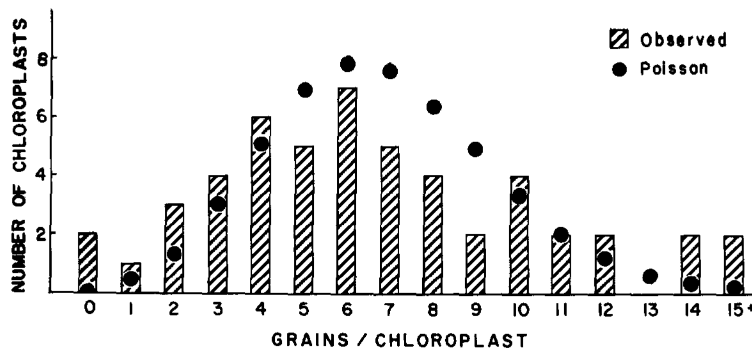

FIGURE 5 Frequency distribution of grains per chloroplast in autoradiographs of [^{3}H]thymidine-labeled cells grown in unlabeled medium for 1.0 generation. 25 days exposure. Mean number of grains per chloroplast = 6.8. The observed distribution does not differ significantly from a Poisson distribution having the same mean (chi-square test: $P > 0.05$).

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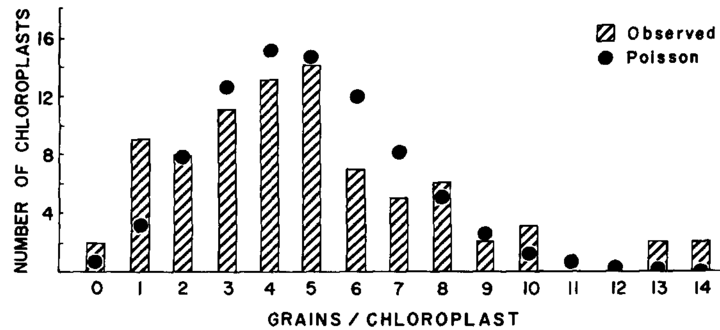

FIGURE 7 Frequency distribution of grains per chloroplast in autoradiographs of [^{3}H]thymidine-labeled cells grown in unlabeled medium for 2.2 generations. 51 days exposure. Mean number of grains per chloroplast = 4.8. The observed distribution does not differ significantly from a Poisson distribution having the same mean (chi-square test: $P > 0.05$).

per chloroplast does not differ significantly from a Poisson distribution.

Fig. 8 shows the distribution of labeled DNA among the progeny chloroplasts after 3.3 generations in chase medium. Since the distribution of grains per chloroplast fits a Poisson distribution, it can be concluded that essentially all of the chloroplasts still contain labeled DNA and furthermore that the chloroplasts have received approximately equal amounts of label.

Clearly, these observations follow the pattern predicted for a chloroplast which contains a number of segregating DNA molecules. A minimum estimate of this number is calculated below.

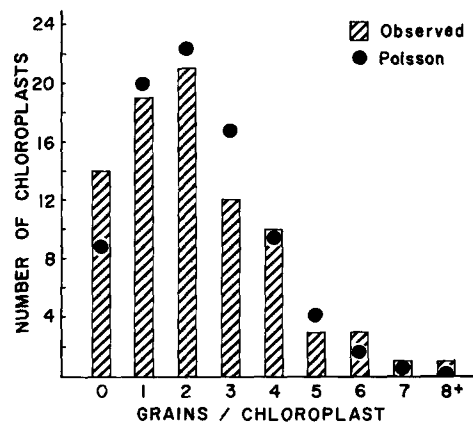

FIGURE 8 Frequency distribution of grains per chloroplast in autoradiographs of [^{3}H]thymidine-labeled cells grown in unlabeled medium for 3.3 generations. 51 days exposure. Mean number of grains per chloroplast = 2.2. The observed distribution does not differ significantly from a Poisson distribution having the same mean (chi-square test: $P > 0.3$).

DISCUSSION

Minimum Number of DNA Molecules per Chloroplast

In the present experiment, ^{3}H-labeled chloroplast DNA was conserved during 3.3 generations (Fig. 3) and at the end of this period the distribution of grains per chloroplast followed a Poisson distribution (Fig. 8). It may be concluded that at 3.3 generations virtually every chloroplast contained at least one molecule of labeled DNA. Since the total number of chloroplasts in the culture increased by a factor of $2^{3.3}$ (i.e., 9.8-fold) during the chase period, it follows that there must have been an average of at least 9.8, or approximately 10, labeled strands of DNA per chloroplast at the end of the labeling period. We have assumed that there has been no significant amount of recombination. In a similar study on *Escherichia coli*, van Tubergen and Setlow (25) found very low levels of recombination. Since the cells were labeled for only 0.36 of a generation, the DNA was labeled in one strand only. An average of 10 labeled strands thus corresponds to an average of 10 labeled molecules of DNA per chloroplast at the end of the labeling period. Without making any further assumptions, this may be taken as a minimal estimate of the number of DNA molecules in the average chloroplast.[1]

[1] It may be noted that if the DNA content of the chloroplast increases linearly during the cell cycle, the average chloroplast will contain 1.44 times the number of DNA molecules present in a newly divided chloroplast (7).

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There are, however, two considerations which suggest that we have underestimated the probable number of molecules of chloroplast DNA. First, the period of exposure to [^{3}H]thymidine was only 0.36 generations. If the time taken to replicate one molecule of chloroplast DNA is short compared with the generation time (8.25 h), only about half of the chloroplast DNA molecules would have been undergoing replication during the labeling period.[2] Therefore, in addition to the minimum of 10 labeled DNA molecules per chloroplast shown to be present at the end of the labeling period, it is likely that there were an additional 10 unlabeled DNA molecules. This would clearly double our minimal estimate of the number of DNA molecules in the average chloroplast. A reasonably rapid rate of replication of individual chloroplast DNA molecules is indicated by the observation of Chiang and Sueoka (6) that the entire complement of chloroplast DNA in *Chlamydomonas reinhardtii* underwent a round of replication within a 2-h period.

Secondly, the actual number of DNA molecules in the chloroplast of *Ochromonas* can be as low as our calculated minimum only if the labeled DNA is distributed as equally as possible, i.e., in a nonrandom manner, among the progeny chloroplasts. If, in fact, the labeled DNA molecules are distributed randomly, it would require approximately twice the initial number of labeled molecules to account for the observed low number of unlabeled chloroplasts at 3.3 generations. However, the shape of the chloroplast nucleoid and the possibility (3, 4, 13, 23) that chloroplast DNA molecules are attached to the thylakoid membranes make it unlikely that the segregation of chloroplast DNA molecules is completely random.

In conclusion, our data indicate that there are at least 10 molecules of DNA per chloroplast. If it is assumed that a single chloroplast DNA molecule is replicated in 20 min or less, it can be calculated that an average chloroplast contains at least 20 DNA molecules. Both these minimal estimates would have to be raised if chloroplast DNA molecules segregate randomly among the progeny chloroplasts.

[2] If the replication time for individual molecules of chloroplast DNA is taken as 20 min, a given strand will incorporate label if it is completed within the labeling period (0.36 generations) or up to 20 min (0.04 generations) later. In the culture as a whole, the total number of completed strands increases during 0.40 generations by a factor of $2^{0.40}$, or 1.32-fold. Thus 0.32/1.32, or 24% of the strands, i.e., 48% of the molecules, will be labeled.

Other Estimates of the Number of DNA Molecules per Chloroplast

Estimates of the number of DNA molecules per chloroplast have been made for a variety of other plant species by comparing the amount of DNA calculated to be present in a single chloroplast with the observed size of the chloroplast DNA molecules or with the kinetic complexity of the chloroplast DNA. In *E. gracilis*, Manning et al. (17), on the basis of their observation that chloroplast DNA molecules are 40 μm long, have calculated that a single chloroplast in a light-grown cell contains between 9 and 72 molecules of DNA. Their lower estimate is based on the report of Edelman et al. (8) that the total amount of DNA per chloroplast in *Euglena* is 1.2×10^{-15} g, whereas the higher figure is based on Brawerman and Eisenstadt's (5) estimate of 1×10^{-14} g of DNA per chloroplast. In *C. reinhardtii* which has only a single chloroplast per cell the amount of DNA per chloroplast is more accurately known (6), and Bastia et al. (2) and Wells and Sager (27) have postulated on the basis of the observed kinetic complexity of chloroplast DNA that there are 24–26 copies of DNA in the chloroplast of gametes and 52 copies in the chloroplast of vegetative cells. In peas, Kolodner and Tewari (12), using both the observed size of the chloroplast DNA molecules (39 μm) and the kinetic complexity of the DNA, have estimated that there are approximately 30 molecules of DNA per chloroplast. Similarly, in tobacco, Tewari and Wildman (24) on the basis of the kinetic complexity of its chloroplast DNA have estimated that there are 20 DNA molecules per chloroplast. Our estimate of 10 or 20 DNA molecules per chloroplast in light-grown cells of *O. danica*, although obtained by an entirely different method, agrees well with these studies.

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