STUDIES ON CHLOROPLAST DEVELOPMENT
AND REPLICATION IN EUGLEAN

I. Vitamin B₁₂ and Chloroplast Replication

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ABSTRACT
When Euglena gracilis is grown under vitamin B₁₂ deficiency conditions, the amount of protein and of chlorophyll per cell increase with decrease of B₁₂ in the medium and consequently in the cell. The increase in cell protein is proportional to and precedes an increase in the number of chloroplasts per cell. This replication of the chloroplasts under deficiency conditions is not accompanied by nuclear or cell division. It is concluded that chloroplast replication in Euglena gracilis is independent of nuclear and cellular replication, at least under B₁₂ deficiency conditions. We established a graph of the growth of Euglena under different concentrations of vitamin B₁₂ added to the growth medium, which permitted us to calculate that at least 22,000 molecules of vitamin B₁₂ per cell are required to give normal growth.

INTRODUCTION
The formation of chloroplasts from proplastids in Euglena gracilis is separate in time from the replication of the chloroplasts themselves (22). In Euglena deses, for example, Goidjes showed that the cells divided first, apportioning the chloroplast complement to the two daughter cells (22), and that then each chloroplast divided to restore the original plastid complement. This also may be the case in Euglena gracilis. The implication is that chloroplast replication may be geared to cellular and nuclear replication. Dark-grown Euglena contain an average of about 30 proplastids per cell. Upon exposure to light, the proplastids rapidly form chloroplasts by the fusion of three proplastids to form one chloroplast, yielding a final average number of ten chloroplasts per Euglena cell (22).

Vitamin B₁₂ was found to be necessary for the growth of various microorganisms including Euglena gracilis, with the “Z” strain being most sensitive to its deficiency (12, 13, and 21). It was shown (10) that gross B₁₂ depletion in Euglena gracilis resulted in cell gigantism and prolongation of generation time. Cell gigantism in bacteria is characterized by a disproportionate increase in cell size and an irreversibly decreased capacity for cell division. This condition reflects a continued synthesis of protein and RNA without concomitant DNA replication and mitosis (2, 6, and 7).

The coenzyme form(s) of vitamin B₁₂ is implicated to be involved in many biological functions, among which are the reduction of ribonucleotides to deoxyribonucleotides (3 and 4), the C₁-metabolism leading to purine and pyrimidine biosyntheses (16), and the possible methylation of tRNA (24). Whether B₁₂ is involved in the methylation of the different nucleotides and/or nucleic acids is not known yet. It is not known whether or not these processes occur in Euglena. If any one or more of these processes occur, B₁₂ depletion would be expected to lead to impaired DNA synthesis and
defective cell proliferation. Therefore, a study of the effect of vitamin B₁₂ starvation on DNA synthesis and on the replication of both the nucleus and the chloroplasts was initiated. We reasoned that if vitamin B₁₂ is involved in the synthesis of DNA or its precursors, under B₁₂ deficiency nuclear DNA might be synthesized in preference to chloroplast DNA. The chloroplasts might be eliminated under extreme deficiency, since they and their associated DNA are not indispensable to the Euglena cell (11 and 22). Also, if we replete cells with B₁₂, we may be able to follow the origin of the components of the chloroplasts and their interrelationships with the nucleus. Our preliminary results reported here reveal some of the aspects of vitamin B₁₂ deficiency in Euglena.

METHODS AND MATERIALS

Euglena gracilis, strain "Z" was grown in 250-ml Erlenmeyer flasks stoppered with nonabsorbent cotton plugs. Each flask contained 100 ml of Hutner's modified medium (18), with an excess of ethanol as the carbon source. Vitamin B₁₂ (B₁₂) was added at the following concentrations per liter of medium: 10 µg (considered complete medium, and referred to as the control), and 100, 75, 50, 25, and 10 µg. All treatments were done in duplicate or triplicate. Unless specified otherwise, most of the results reported here were obtained with control and 25 µg of B₁₂ concentrations. All flasks, pipettes, etc. were acid washed and rinsed with double-distilled water.

The cells were grown either in the dark or in the light under 100 ft-c of white, cool light on a gyratory shaker at 24 °C ± 1.0 °C. The cells were grown either in the dark or in the light under 100 ft-c of white, cool light on a gyratory shaker at 24 °C ± 1.0 °C.

The culture media were inoculated with 0.1 ml of exponential phase, dark-grown seed cultures containing 50 µg of B₁₂/l. The seed cultures were subcultured three times in 50 µg of B₁₂ medium before such inoculation. Samples for the various determinations were taken directly from the culture flasks during the growth period of up to 9 days or more.

Growth was measured as optical density (OD) by reading the absorbance in a B & L Spectronic 20 (Bausch & Lomb, Rochester, N.Y.) at 540 µm. The samples were always diluted to give an absorbance between 0.10-0.25 OD unit. Cell number was determined from dilutions series in 4% buffered neutral formaldehyde and counted under phase-contrast microscope in a Sedgewick-Rafter cell at 100 magnification and with a Whipple eyepiece reticle. Ten fields were counted in each cell. The dilutions were arranged so that each field contained between 30 and 50 cells. The cell count by this microscopic observation showed less than 5% variation. Chlorophyll was determined by the Arnon method (1), and protein was estimated by the Lowry method (17).

Chloroplast counts were done on only viable cells. Two sets of criteria for viability were established. The first was that individual cells be motile, contracting with their chloroplasts fluorescing bright red color, and with complete integrity of structure (Viab. I). The second was that when samples of the same culture were appropriately diluted and plated on agar plates containing medium with no alcohol added, only the green colonies formed were counted (Viab. II). The viability count by the first criterion was found to vary by 3%, and the viability count by the second criterion varied by 5%. The chloroplast count was done independently by at least two persons, and the variability of the counts ranged between 1 and 4% when the average number of chloroplasts per cell ranged between 10 and about 40, respectively.

In the cases in which the average number of chloroplasts per cell in the whole sample exceeded 40 at extreme deficiency, the count was an estimate rather than an accurate count and is therefore not reported here.

Counting the chloroplasts was performed under 400 or 1000 magnification on a fluorescence microscope (9). The light source consisted of a high-pressure mercury arc lamp (Osram HBO 200 W₁), a quartz field condensor, two heat filters (KG5), a red suppressive filter (BG 38), a blue exciter filter (BG 12), and a yellow (OG 1) Barrier filter (Wild, Farmingdale, N.Y.). A first surface substage mirror, and a cardioid substage condensor oiled to an ordinary glass slide carrying the drop of living sample were used for counts.

Photomicrographs of the fluorescent cells were taken with Polaroid Land Type 413 infrared film (8).

RESULTS

To determine the B₁₂ requirement for the exponential growth of Euglena gracilis, we studied the relationship between the kinetics of growth at different concentrations of B₁₂ in the growth medium. Whereas growth, determined both by optical density of the culture and by cell counts, followed typical sigmoid curves in the semi-log plot (Fig. 1 a and b), the kinetics of the two curves representing any one concentration of B₁₂ were different when the B₁₂ concentration was 100 µg/l and less. This is not surprising, since under B₁₂ deficiency the generation time for cell division increases and the cell size increases (10). In the case of the control (complete) (Fig. 1 b), a reversal is noticed since the cells become smaller in size during the plateau stage. After many such experiments, we attempted to find out if there is a
relationship between cell count and optical density of the culture at different $B_12$ concentrations. A plot of cell number versus optical density of the cultures at different $B_12$ concentrations was established (Fig. 2). A linear curve was noted for the complete (control) culture, but the linearity does not hold true for the case of $B_12$ concentration of 100 µg or less. The cell count deviated from this linearity either early or later during the growth of the cultures, depending on the concentration of $B_12$ in the medium.

To find out if the increase in cell size as detected microscopically was accompanied by an increase in total protein and chlorophyll, we divided the amount of protein/ml and chlorophyll/ml by the number of cells at certain stages and dates of growth. A plot of representative values of protein per cell and of chlorophyll per cell for a control culture and a 25 µg $B_12$/ml culture is shown in Fig. 3. It can be seen that the amount of protein per cell in the control stays practically constant for 9 days, while in the deficient cells it increases very rapidly and then levels off, indicating that enlargement of cells is accompanied by an increase in protein content. The same pattern is found for chlorophyll in these $B_12$ deficient cells. In the control cells, chlorophyll increases, but at a slower rate than in the deficient cells. This is expected because the control cells are continuously dividing and their chloroplasts are replicating, while the deficient cells are dividing either at slower rates or not at all, and therefore their chloroplasts are more developed (20).

We noticed that there is about five times as much protein and chlorophyll in a deficient cell as in a normal cell after 9 days. These data prompted microscopic examination of the cells for finding out whether the increase in protein and chlorophyll which accompanies the increase in cell size also includes an increase in chloroplast number. Microscopic examination of hundreds of cells, at different stages of growth and at different $B_12$ concentrations, revealed no more than one nucleus per cell. However, the number of chloroplasts per cell was found to be higher in all the deficient cells than in the control cells. It was also noticed that the lower the $B_12$ concentration in the medium and the older the culture at any single concentration, the larger the cells became, and the more chloroplasts formed per cell.

Fig. 4 shows the relationships between the average number of chloroplasts per cell and the
protein content per cell for both control and deficient cells over a period of 9 days. It can be seen that in the control the average number of chloroplasts and the amount of protein per cell remain constant. In the deficient cells, both the protein and the average number of chloroplasts increase with time. It can also be noted that in the deficient cells the protein increases sharply first and then slows and levels off. The increase in chloroplast number, however, is gradual but follows the protein. On the 9th day there are five times as much protein and four times as many chloroplasts in the deficient cells as in the control. If the cultures are kept for a longer period, the chloroplast number reaches even higher values than shown here. A representative photograph of a normal viable cell containing nine chloroplasts and a photograph of a viable B_{12} deficient cell containing about 50 large chloroplasts are shown in Fig. 5.

Data characterizing viability by the two criteria described above are given in Table I. It is seen that there is good agreement between microscopic definition of viability and plating definition of viability. Therefore, we concluded that the observations of chloroplast number given above were reliable. A sample of condensed data of an actual chloroplast count is given in Table II.

The finding that the number of chloroplasts in the B_{12} deficient cells increased following an increase in the protein led us to establish the kinetics of the chlorophyll-protein ratio. It can be seen (Fig. 6) that the cells reach a constant ratio of chlorophyll to protein at different concentrations of B_{12} when they stop dividing. At concentrations...
lower than 25 µg/ml of B₁₂ the cells bleach after about 6 days (data not reported here).

For finding out if the proplastids also increase in number under B₁₂ deficiency, it was necessary to grow cells at these concentrations of B₁₂ in the dark for many generations. We found that normal cells had an average of 30 proplastids per cell. The proplastids in the deficient cells were much more numerous, and the entire cells gave off an orange fluorescence. No good estimate count could be made, and therefore data are not listed here.

**DISCUSSION**

The essential results of this paper are the relationship between protein, chlorophyll, and chloroplast number in *Euglena* grown in either complete medium or in media in which B₁₂ has been markedly reduced. The establishment of a relationship between optical density of the culture and cell counts (Fig. 2) reveals some interesting points. First, the linear curve has a slope practically equal to unity (found to be 1.05), corresponding to a direct proportionality between optical density and cell count. The exact functional form of the dependence of optical density on cell count for normal cells is not relevant in this paper. What is significant is the coincidence of the experimental points for the B₁₂-deficient cells with the observed curve for normal cells (see box in Fig. 2 for details) at low cell count and the divergence of the curves for the deficient cells from that of the normal cells at a cell number that depends on the extent of the deficiency. Secondly, taking the value of 1357 daltons as the molecular weight of B₁₂, by simple calculations of the number of cells between any two breakaway points from the linear curve, at two B₁₂ concentrations, we clearly found that the amount of B₁₂ required by a *Euglena* cell to grow normally is around 22,000 molecules per cell. It can also be seen that depletion of B₁₂ in the cell brings about proportional increase in the generation time, accompanied by cell enlargement. Thus, about 22,000 molecules of B₁₂ in whatever active form they may exist in the cell, are required by the cell to grow exponentially, to keep its normal size and normal number of chloroplasts and to maintain chloroplast replication geared to nuclear and cell replication. If we calculate the number of cells at the plateau stage in deficient cells in any one B₁₂ concentration and the amount of B₁₂ added to the medium, we arrive at a value of about 6,000 molecules of B₁₂ per cell. This value, which agrees with those reported by other workers (13, 17, and 19), seems to be the minimum for survival but not for division. Therefore, the value of 22,000 is quantitatively and functionally different since it...
represents a minimum value required by the cell for normal exponential growth. Though the cells are not synchronized, it could be noticed that at all B₁₂ deficiencies the cells seem to be capable of doubling their number only one time after the last division (breakaway points) when they contained the minimum number of B₁₂ required for normal division. We may thus assume that the first daughter cells contain about 11,000 molecules, which we assume to be below the minimum for normal cell division cycle, and thus the cell enlarges. However, the cell seems to contain a certain potential to undergo one more division cycle, and the daughter cell will then contain about 5500 molecules of B₁₂, which would be enough for survival and not division.

The finding of an increase in cell protein, accompanied by an increase in number of chloroplasts per cell (Fig. 4), might suggest that chloroplast replication is dependent upon the amount of (some) protein in the cell. It is not yet known if this increase in cell protein is due to increase in chloroplast protein and/or partially to cytoplasmic protein. Most interesting is that the kinetics of increase of protein formation and the increase in chloroplast number per cell indicate that increase of protein per cell precedes the increase in the number of chloroplasts per cell. We have not yet measured the amount of either nuclear or chloroplast DNA, although work presently is underway to perform such measurements. If, however, we assume either that B₁₂ deficiency in some way inhibited nuclear DNA synthesis or that because of B₁₂ deficiency nuclear DNA cannot function as a

Figure 5  Fluorescence photomicrographs of (a) normal and (b) 25 μg B₁₂/A cells. In a, the cell is small and the chloroplast number per cell can be counted to be 9. In b, the cell is very large, and the chloroplasts are larger in size than normal and their number is increased to about 50. Details are in the text.
**TABLE I**

**Studies of the Viability of Euglena Cells Grown Either in Complete Medium or in 25 µg B_{12}/L Medium, Using Two Sets of Criteria for Viability**

Viability I (Viab I) for direct microscopic observation and viability II (Viab II) for colony formation.

<table>
<thead>
<tr>
<th>Age of culture</th>
<th>Exp. No.</th>
<th>Viab I</th>
<th>Viab II</th>
<th>Ave. No. of Chloroplasts/Cell</th>
<th>Viab I</th>
<th>Viab II</th>
<th>Ave. No. of Chloroplasts/Cell</th>
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<tbody>
<tr>
<td>days</td>
<td>%</td>
<td>%</td>
<td></td>
<td>%</td>
<td>%</td>
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<td></td>
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<td>90</td>
<td>89</td>
<td>9.7 ± 2.0</td>
<td>84</td>
<td>96</td>
<td>11.4 ± 1.8</td>
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<tr>
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<td>89</td>
<td>76</td>
<td>10.5 ± 2.0</td>
<td>90</td>
<td>89</td>
<td>13.8 ± 2.7</td>
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<td>33-2</td>
<td>85</td>
<td>71</td>
<td>12.3 ± 2.0</td>
<td>66</td>
<td>75</td>
<td>15.5 ± 3.9</td>
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<td>90</td>
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<td>92</td>
<td>92</td>
<td>19.1 ± 5.6</td>
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<tr>
<td></td>
<td>35-2</td>
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<td>94</td>
<td>10.8 ± 1.7</td>
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<td>94</td>
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<td>74</td>
<td>95</td>
<td>10.9 ± 2.8</td>
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<td>92</td>
<td>20.7 ± 3.9</td>
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<td>89</td>
<td>23.3 ± 4.5</td>
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<td>83</td>
<td>85</td>
<td>28.4 ± 5.2</td>
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<td>86</td>
<td>10.5 ± 2.4</td>
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<td>79</td>
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<td>91</td>
<td>96</td>
<td>27.1 ± 5.1</td>
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<td>9.2 ± 2.1</td>
<td>98</td>
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<td>91</td>
<td>30.8 ± 5.5</td>
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<td>80</td>
<td>100</td>
<td>10.3 ± 2.9</td>
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</tr>
<tr>
<td></td>
<td>33-1</td>
<td>90</td>
<td>89</td>
<td>9.0 ± 2.8</td>
<td>96</td>
<td>96</td>
<td>40.2 ± 7.1</td>
</tr>
</tbody>
</table>

**TABLE II**

**Condensed Data of an Actual Count of the Number of Chloroplasts per Euglena Cell Grown Either in Complete Medium or in 25 µg B_{12}/L Medium for a 10-Day Duration of the Culture**

Number of cells counted (N), average number of chloroplasts per cell (M), and the standard deviation (using N-1) (σ) are also indicated in the table.

<table>
<thead>
<tr>
<th>Complete medium</th>
<th>25 µg B_{12}/Liter medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td>3 5 7 10</td>
</tr>
<tr>
<td>N</td>
<td>20 20.0 20.0 20.0 20.0 20.0 20.0 20.0 20.0</td>
</tr>
<tr>
<td>M</td>
<td>9.7 9.2 10.0 9.1 11.4 13.8 19.1 23.3 27.1 30.8 33.0 36.05</td>
</tr>
<tr>
<td>σ</td>
<td>2.0 1.7 2.1 2.1 1.8 2.7 5.6 4.5 5.1 5.5 5.4 6.81</td>
</tr>
</tbody>
</table>
template in further replication and consequently that cell and nuclear division was inhibited, then our data of the increase in chloroplast number and presumably chloroplast DNA could only be explained if chloroplast DNA is different, for example, in its base composition, from nuclear DNA. This was shown to be true by the work of Brawerman and Eisenstadt (5) who found the base 5-methyl-cytosine to be present in nuclear DNA and absent in chloroplast DNA of Euglena. B12 coenzymes were found to be involved in methylation processes in many cases (23 and 24), and it would be very important to show that such is also the case for nuclear DNA in Euglena. It is relevant to mention that Lark (14 and 15) found that, when Escherichia coli 15 T- is starved of methionine (a methyl donor of DNA), DNA can be synthesized without concomitant methylation. However, from later work, she proposed that such DNA cannot function as a template in further replication because it is not methylated.

While we have not provided any information regarding the autonomy of chloroplast replication from nuclear division in Euglena, the data do provide evidence of the ability of the cell under B12 deficiency to increase its chloroplast contents far beyond the normal number without observing nuclear replication, and that chloroplast replication seems to be preceded by an increase in cell protein.

Further work is underway to substantiate our findings and their implications.

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REFERENCES