STUDIES ON CHLOROPLAST DEVELOPMENT AND REPLICATION IN *EUGLENA*

I. Vitamin B$_{12}$ and Chloroplast Replication

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ABSTRACT

When *Euglena gracilis* is grown under vitamin B$_{12}$ deficiency conditions, the amount of protein and of chlorophyll per cell increase with decrease of B$_{12}$ 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 B$_{12}$ deficiency conditions. We established a graph of the growth of *Euglena* under different concentrations of vitamin B$_{12}$ added to the growth medium, which permitted us to calculate that at least 22,000 molecules of vitamin B$_{12}$ 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, Gojdics 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 B$_{12}$ 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 B$_{12}$ 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 B$_{12}$ is implicated to be involved in many biological functions, among which are the reduction of ribonucleotides to deoxyribonucleotides (3 and 4), the C$_1$-metabolism leading to purine and pyrimidine biosyntheses (16), and the possible methylation of tRNA (24). Whether B$_{12}$ 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, B$_{12}$ depletion would be expected to lead to impaired DNA synthesis and

defective cell proliferation. Therefore, a study of the effect of vitamin B_{12} starvation on DNA synthesis and on the replication of both the nucleus and the chloroplasts was initiated. We reasoned that if vitamin B_{12} is involved in the synthesis of DNA or its precursors, under B_{12} 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 B_{12}, 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 B_{12} 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 B_{12} (B_{12}) 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 mμg. All treatments were done in duplicate or triplicate. Unless specified otherwise, most of the results reported here were obtained with control and 25 mμg of B_{12} 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° $\pm$ 1.0°C.

The culture media were inoculated with 0.1 ml of exponential phase, dark-grown seed cultures containing 50 mμg of B_{12}/l. The seed cultures were subcultured three times in 50 mμg of B_{12} 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 mμ. 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 W4), 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 B_{12} requirement for the exponential growth of *Euglena gracilis*, we studied the relationship between the kinetics of growth at different concentrations of B_{12} 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 B_{12} were different when the B_{12} concentration was 100 mμg/l and less. This is not surprising, since under B_{12} 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
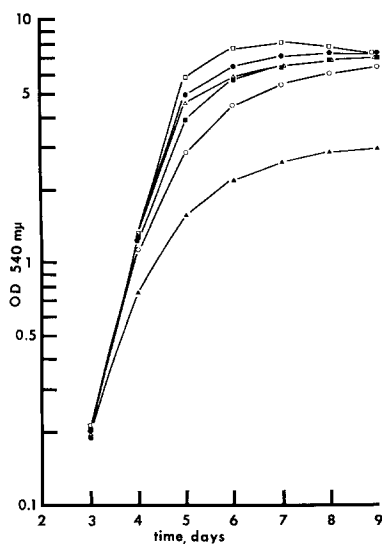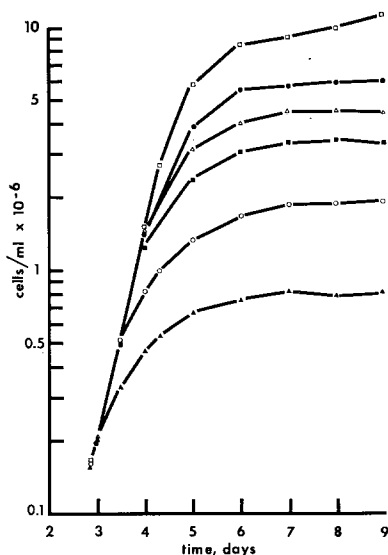
FIGURE 1 a

FIGURE 1 b

FIGURE 1   Time-course of growth of *Euglena gracilis* in the light at different concentrations of added B_{12} to the medium. B_{12} added per liter of medium as follows: 10 μg (complete), ($\square$); 100 mμg, ($\bullet$); 75 mμg, ($\triangle$); 50 mμg, ($\blacksquare$); 25 mμg, ($\bigcirc$); and 10 mμg, ($\blacktriangle$). *a*, Growth measured as optical density units at 540 mμ in a B & L Spectronic 20 with a standard ½-in. tube. *b*, Growth measured as cell count per 1 ml of the growth culture.

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 mμg 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 mμg B_{12}/l 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.

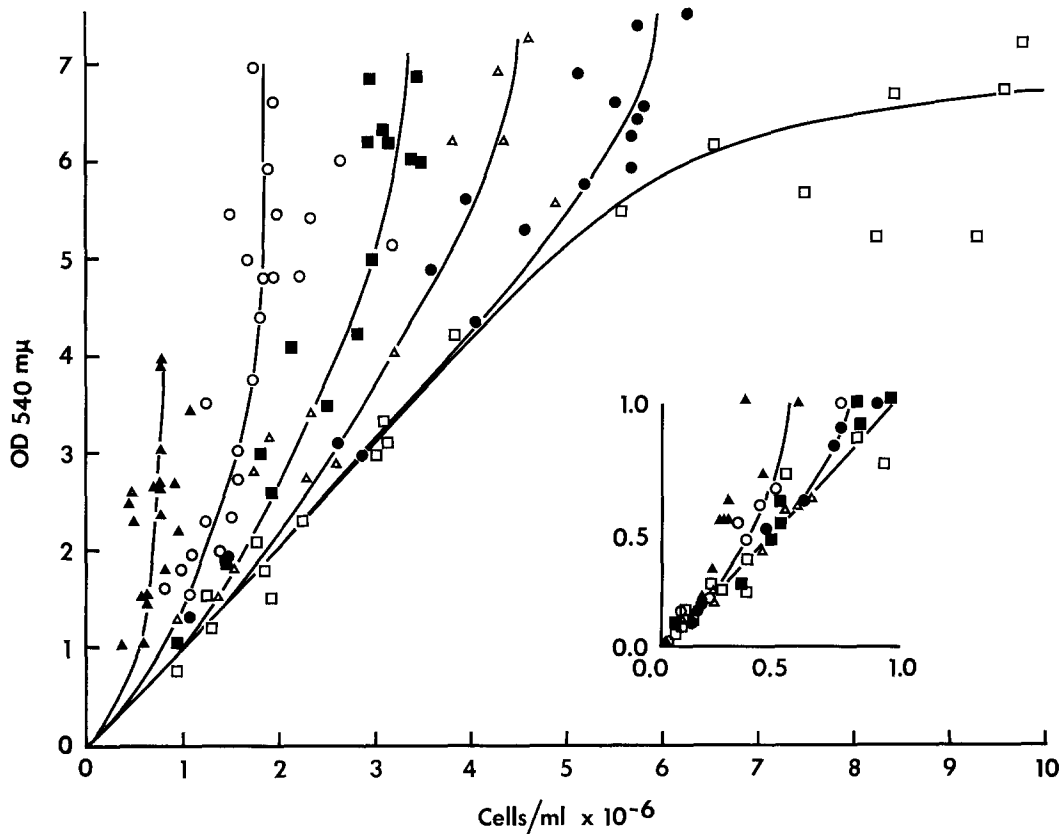Fig. 4 shows the relationships between the average number of chloroplasts per cell and the

FIGURE 2   Behavior of growth of *Euglena* in response to B$_{12}$ added to the growth medium. Coordinates are growth measured as optical density units at 540 mμ of the cultures versus growth measured as cell count per ml of the culture. The straight line represents normal growth at optimal and excess B$_{12}$ in the cells. The branching curves represent growth when cells are deficient in B$_{12}$. Growth conditions and B$_{12}$ concentrations as in Fig. 1.

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
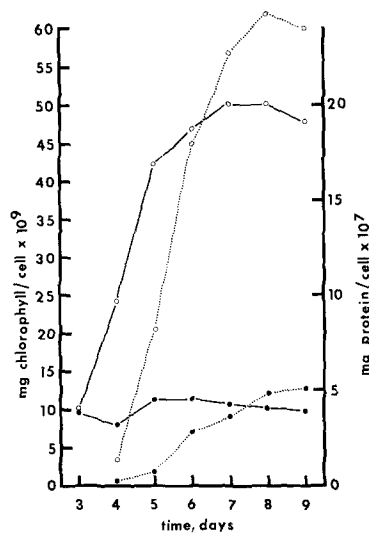
FIGURE 3 Time-course of protein synthesis (solid line) and of chlorophyll synthesis (dotted line) per *Euglena* cell grown in complete medium (●) and in 25 mμg B$_{12}$/l medium (○). Details are in the text.
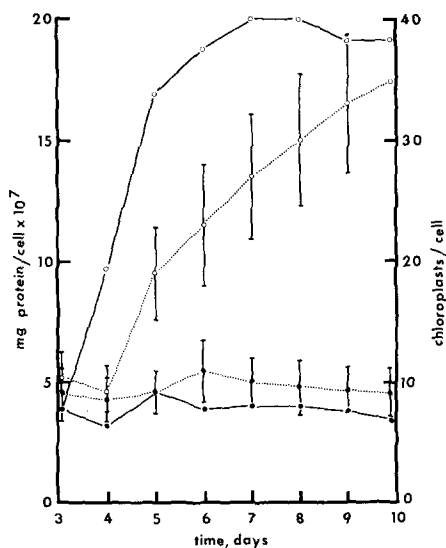

FIGURE 4 Time-course of the amount of protein (solid line) and the average number of chloroplasts (dotted line) per *Euglena* cell grown in complete medium (●) and in 25 mμg B$_{12}$/l medium (○). Details are in the text.

lower than 25 mμg/ml of B$_{12}$ the cells bleach after about 6 days (data not reported here).

For finding out if the proplastids also increase in number under B$_{12}$ deficiency, it was necessary to

grow cells at these concentrations of B$_{12}$ 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 B$_{12}$ 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 B$_{12}$-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 B$_{12}$, by simple calculations of the number of cells between any two breakaway points from the linear curve, at two B$_{12}$ concentrations, we clearly found that the amount of B$_{12}$ required by a *Euglena* cell to grow normally is around 22,000 molecules per cell. It can also be seen that depletion of B$_{12}$ in the cell brings about proportional increase in the generation time, accompanied by cell enlargement. Thus, about 22,000 molecules of B$_{12}$, 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 B$_{12}$ concentration and the amount of B$_{12}$ added to the medium, we arrive at a value of about 6,000 molecules of B$_{12}$ 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 B_{12} 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 B_{12} 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 B_{12}, which would be enough for survival and not division.

The finding of an increase in cell protein, ac-companied 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 B_{12} deficiency in some way inhibited nuclear DNA synthesis or that because of B_{12} deficiency nuclear DNA cannot function as a
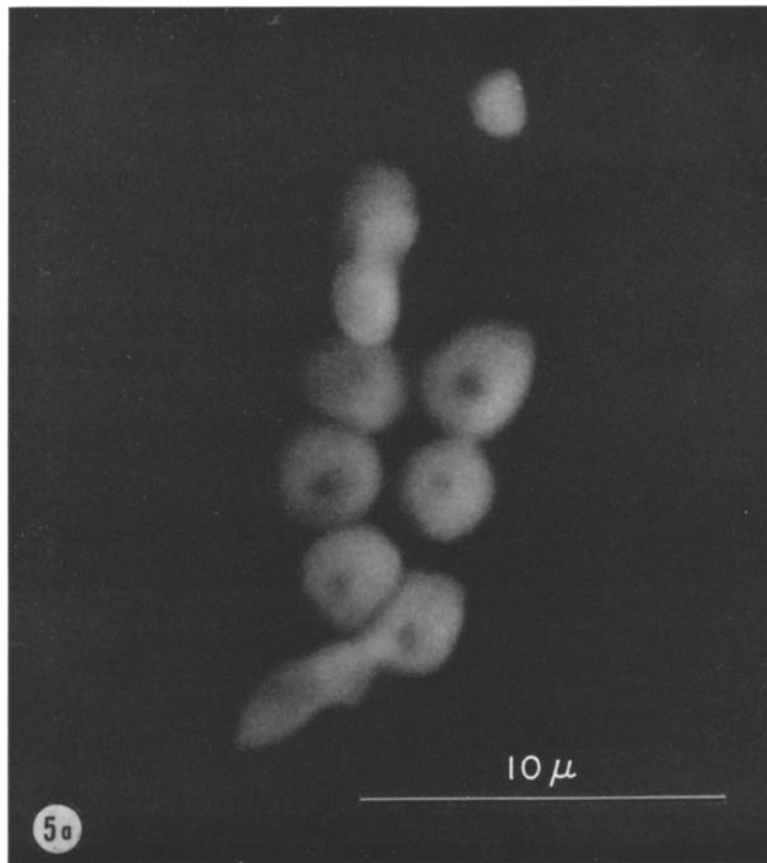

FIGURE 5 Fluorescence photomicrographs of (a) normal and (b) 25 mμg B_{12}/l cells. In a, the cell is small and the chloroplast number per cell can be counted to be 9. In b, the cell is very large, and the chloroplasts are larger in size than normal and their number is increased to about 50. Details are in the text.
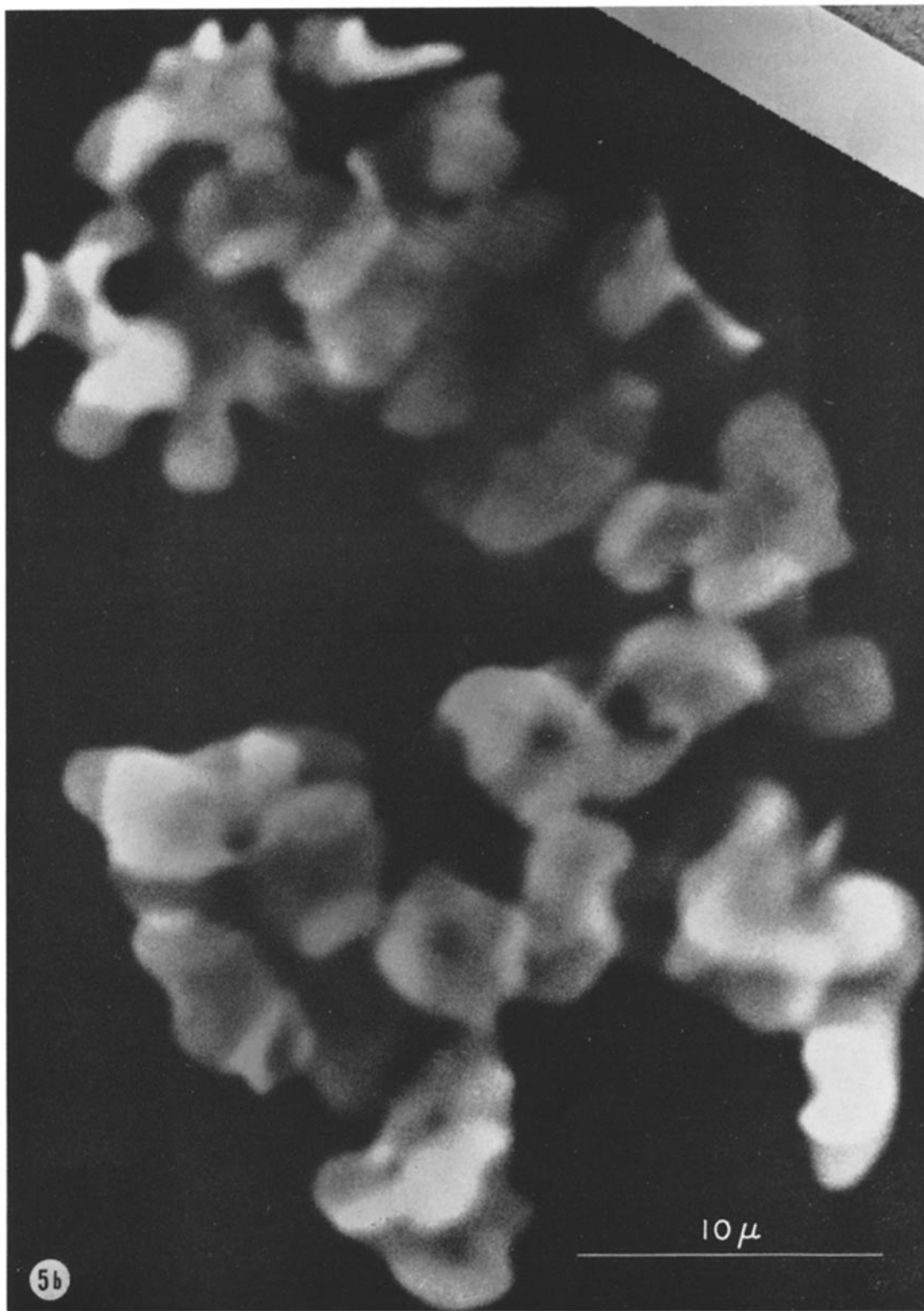
10 μ

5b

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TABLE I

Studies of the Viability of Euglena Cells Grown Either in Complete Medium or in 25 mμg
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.

Age of culture	Exp. No.	Complete medium			25 mμg B_{12}/Liter medium		
		Viab I	Viab II	Ave. No. of Chloroplasts/Cell	Viab I	Viab II	Ave. No. of Chloroplasts/Cell
days		%	%		%	%	
3	35-1	90	89	9.7 ± 2.0	84	96	11.4 ± 1.8
4	35-1	89	76	10.5 ± 2.0	90	89	13.8 ± 2.7
	33-2	85	71	12.3 ± 2.0	66	75	15.5 ± 3.9
5	35-1	85	90	9.2 ± 1.7	92	92	19.1 ± 5.6
	35-2	83	94	10.8 ± 1.7	97	94	21.3 ± 5.7
	33-1	74	95	10.9 ± 2.8	85	92	20.7 ± 3.9
6	35-1	85	73	10.3 ± 1.7	90	89	23.3 ± 4.5
	35-2	50	100	8.6 ± 1.8	83	85	28.4 ± 5.2
	33-1	84	86	10.5 ± 2.4	76	79	26.4 ± 6.1
7	35-1	—	—	—	91	96	27.1 ± 5.1
	35-2	90	92	9.2 ± 2.1	98	100	34.1 ± 5.8
	35-2	98	86	10.9 ± 2.2	96	100	33.0 ± 5.4
8	35-1	—	—	—	78	91	30.8 ± 5.5
	33-1	80	100	10.3 ± 2.9	70	95	36.3 ± 4.9
9	35-1	79	83	9.9 ± 1.6	71	89	33.0 ± 5.4
	33-1	87	92	8.3 ± 2.0	73	91	39.0 ± 7.2
10	35-1	93	100	9.0 ± 2.0	90	95	35.8 ± 6.6
	33-1	90	89	9.0 ± 2.8	96	96	40.2 ± 7.1

TABLE II

Condensed Data of an Actual Count of the Number of Chloroplasts per Euglena Cell Grown Either in Complete
Medium or in 25 mμg 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 devia-
tion (using N-1) (σ) are also indicated in the table.

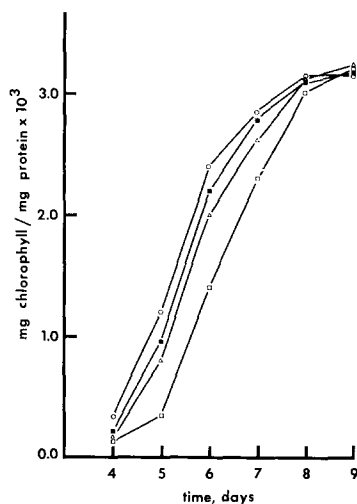| Age of culture | Complete medium | | | | 25 mμg B_{12}/Liter medium | | | | | | | |
	Days				Days							
	3	5	7	10	3	4	5	6	7	8	9	10
N	20	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20
M	9.7	9.2	10.0	9.1	11.4	13.8	19.1	23.3	27.1	30.8	33.0	36.05
σ	2.0	1.7	2.1	2.1	1.8	2.7	5.6	4.5	5.1	5.5	5.4	6.81

FIGURE 6 Time-course of amount of chlorophyll per amount of protein per *Euglena* cell as a function of the concentration of vitamin B_{12} added to the growth medium. Curves represent complete medium ($\square$); 75 mμg B_{12}/l medium, ($\triangle$); 50 mμg B_{12}/l medium, (∎); and 25 mμg B_{12}/l medium (○).

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*. B_{12} 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 B_{12} 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.

REFERENCES

1. ARNON, D. 1949. *Plant Physiol.* **24**:1.
2. BECK, W. S., M. GOULIAN, and S. HOOK. 1962. *Biochim. Biophys. Acta.* **55**:470.
3. BECK, W. S., and J. HARDY. 1965. *Proc. Nat. Acad. Sci. U.S.A.* **54**:286.
4. BLAKLEY, R. L., and M. A. BARKER. 1964. *Biochem. Biophys. Res. Commun.* **16**:391.
5. BRAWERMAN, G., and J. M. EISENSTADT. 1964. *Biochim. Biophys. Acta.* **91**:477.
6. COHEN, S. S., and H. D. BARNER. 1954. *Proc. Nat. Acad. Sci. U.S.A.* **40**:885.
7. COHEN, S. S., and H. D. BARNER. 1956. *J. Bacteriol.* **71**:588.
8. EPSTEIN, H. T., and E. ALLAWAY. 1967. *Biochim. Biophys. Acta.* **142**:195.
9. EPSTEIN, H. T., E. BOY DE LA TOUR, and J. A. SCHIFF. 1960. *Nature.* **185**:825.

10. EPSTEIN, S. S., J. B. WEISS, D. CAUSELEY, and P. BUSH. 1962. *J. Protozool.* **9**:336.
11. GRANICK, S.., and A. GIBOR. 1967. Progress in Nucleic Acid Research. Academic Press Inc., New York. **6**:143.
12. HUTNER, S. M., A. SCHUTZ, and C. P. HASKINS. 1950. *Proc. Amer. Phil. Soc.* **94**:42.
13. HUTNER, S., H. M. K. BACH, and G. I. M. ROSS. 1956. *J. Protozool.* **3**:101.
14. LARK, C. 1968 a. *J. Mol. Biol.* **31**:389.
15. LARK, C. 1968 b. *J. Mol. Biol.* **31**:401.
16. LINDSTRAND, K. 1967. *Scand. J. Clin. Lab. Invest.* **19**(Suppl. 95): 3.
17. LOWRY, O. M., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**: 265.

18. PRICE, C. A., and B. L. VALLEE. 1962. *Plant Physiol.* **37**:428.

19. ROBBINS, W. J., A. HERVEY, and M. E. STEBBINS. 1950. *Bull. Torrey Bot. Club.* **77**:423.

20. ROSEN, W. G., and S. R. GAWLIK. 1961. *J. Protozool.* **8**:90.

21. ROSS, G. I. M. 1952. *J. Clin. Pathol. (London).* **5**:250.

22. SCHIFF, J. A., and H. T. EPSTEIN. 1965. The con-tinuity of the chloroplast in *Euglena. In* Reproduction: Molecular, Subcellular and Cellular. Michael Locke, editor. Academic Press Inc., New York.

23. STÄHLBERG, KARL-GUSTAV. 1967. Studies on methyl-B_{12} in man. *Scand. J. Haematol. Suppl.* 1.

24. WALERYCH, W. S., S. VENKATARAMAN, and B. CONNER JOHNSON. 1966. *Biochem. Biophys. Res. Commun.* **23**:368.