

# VITAMIN B<sub>12</sub> AND THE MACROMOLECULAR COMPOSITION OF *EUGLENA*

## III. Effect of Cycloheximide on the Recovery from B<sub>12</sub>-Induced Unbalanced Growth

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### ABSTRACT

When cycloheximide is added to (B<sub>12</sub>)-deficient cultures before or after replenishment of the cells with B<sub>12</sub>, reversion of these cells is inhibited. This inhibition is not caused by interference of the inhibitor in the uptake of B<sub>12</sub> as measured by division kinetics. Cycloheximide does not inhibit the initial increase in the rate of DNA synthesis caused by B<sub>12</sub> replenishment, but within 30–45 min the rate decreases and DNA synthesis ceases. Cycloheximide added to replenished deficient cells after completion of DNA duplication inhibits cell division. The total cellular protein and RNA in replenished cells treated with cycloheximide does not change. B<sub>12</sub> added to deficient cells does not stimulate the incorporation of [<sup>14</sup>C]leucine into protein during resumption and completion of DNA duplication. However, there is a large increase in [<sup>14</sup>C]leucine incorporation into the protein of these cells soon after completion of DNA duplication and before resumption of cell division. The addition of cycloheximide to B<sub>12</sub>-replenished or to nonreplenished deficient cells rapidly inhibits the incorporation. We suggest that the addition of B<sub>12</sub> accelerates the rate of DNA synthesis in the deficient cells and that possibly no new protein synthesis is required except for mitosis. However, protein synthesis is needed for continuous DNA synthesis.

### INTRODUCTION

*Euglena gracilis*, when grown with suboptimal amounts of exogenous vitamin B<sub>12</sub> (B<sub>12</sub>), undergoes morphological and biochemical alternations. Concomitant with vitamin depletion, both cell division and DNA synthesis continue at an exponentially decreasing rate, and then both stop at extreme deficiency. At this stage, the amounts of

protein and RNA in these deficient cells are four- to fivefold higher than in normal plateau stage cells, while the DNA is less than doubled (2, 3, 4). This condition is reversed simply by adding B<sub>12</sub> or some of its analogues (12, 13). The B<sub>12</sub>-deficient cells then undergo two relatively synchronous divisions in a shorter than normal period. Before

the first division there is an increase in DNA to the 4C value, while the amounts of protein and RNA are unchanged (13).

The experiments described here using cycloheximide were done for two reasons: first, to find out if there is sufficient protein to support one or more rounds of cell division in post B<sub>12</sub>-repleted cultures; second, to study the effect of cycloheximide on DNA synthesis. Since deficient cells are in an extended S phase, we may deduce that they have qualitatively the proteins required for DNA synthesis. If this is the case, then when B<sub>12</sub> is added to a deficient culture in the presence of cycloheximide, we would expect to find an enhancement of DNA synthesis comparable to that of deficient cultures; whereas if the proteins are not qualitatively the same, we would expect no enhancement of DNA synthesis.

## MATERIALS AND METHODS

### *Growth Conditions and Determinations*

*E. gracilis*, Klebs, strain Z (Pringsheim), and the aplastidic mutant Y<sub>7</sub>ZHL (8) were grown as previously described (2). Growth measurements in terms of cell number per milliliter, production of B<sub>12</sub> deficiency, criteria of deficiency condition, and chloroplast count have been described before (2, 3).

Reversion of B<sub>12</sub> deficiency was performed as before (13). In green cultures, cells containing an average chloroplast number of around 30 were considered moderately deficient and around 40 as extremely deficient. In aplastidic mutants the kinetics of growth and the visual estimation of cell size were used as criteria of deficiency. In the reversion experiments, B<sub>12</sub>, at a concentration of 10 μg/liter, was added to the deficient cells (control). Either B<sub>12</sub> was added to cells in the original culture, or the cells were washed and suspended in a resting medium containing the inorganic salts plus B<sub>12</sub> but no carbon source.

A modified method of Kempner and Miller (14) was used for nucleic acid extraction. Cellular DNA, RNA, and protein were determined by the methods of Webb and Levy (20), Dische (6), and Lowry et al. (15), respectively.

### *Cycloheximide Treatment*

Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was added, in all experiments, at a concentration of 15 μg/ml and at times as indicated in Results.

### *Radioactivity Incorporation Measurements*

In the DNA measurements, cells were grown until they attained moderate deficiency. The culture was then

divided into two portions. One portion was used for the determination of cell counts, DNA, RNA, and protein per cell as described above. B<sub>12</sub>, cycloheximide, and appropriate amounts of nonradioactive formate were added to this portion at times indicated in Results. To the other portion, B<sub>12</sub>, cycloheximide, and [<sup>14</sup>C]formate (Amersham/Searle Corp., Arlington Heights, Ill. sp act 59.9 mCi/mmol diluted 15-fold with nonradioactive formate) at a concentration of 10 μCi/100 ml culture were added. Samples containing about 2 × 10<sup>8</sup> cells were taken for DNA determination.

DNA was isolated by a modified Stutz and Rawson method (19). The purified DNA was hydrolyzed with 5% trichloroacetic acid (TCA). One part of the hydrolyzed DNA was counted in a Beckman LS-250 liquid scintillation counter (Beckman Instruments, Fullerton, Calif.). The amount of DNA in the second part was determined by the method of Webb and Levy (20). From these data, specific activity was calculated as cpm/μg DNA.

In the determination of the incorporation of radioactivity into protein, B<sub>12</sub>, cycloheximide, and 6 μCi of [<sup>14</sup>C]leucine (Amersham/Searle, sp act 325 mCi/mmol) were added, as indicated, to 15 ml of deficient cultures.

Triplicate samples of 0.5 ml were taken at definite intervals. The cells were centrifuged and then washed with ice cold 10<sup>-3</sup> M leucine and recentrifuged. 1 ml of 0.1 N NaOH was added to the cell pellet and the mixture was allowed to stand for 30 min. Then 2 ml of ice-cold 10% TCA was added; the tubes were mixed and left in ice for 20 min. The precipitates were collected on glass fiber filter disks. They were washed with cold 5% TCA, chloroform, and acetone and then the disks were dried. They were counted in a Beckman LS-250 liquid scintillation counter. Cell number was determined by counting appropriate dilutions of the culture in a Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.).

## RESULTS

We studied the possible interference by cycloheximide on the uptake of B<sub>12</sub> by B<sub>12</sub>-deficient cultures. Table I indicates that cycloheximide does not seem to interfere with the uptake of B<sub>12</sub> by these cells. Sufficient B<sub>12</sub> seems to enter the cells rapidly. The data, however, indicate that the longer the inhibitor is left in the cultures before washing, the greater its effect on the length of the period for resumption of cell division and also on the ability of these cells to continue to divide.

The effect of time of addition of cycloheximide to deficient cultures replenished with the vitamin is shown in Fig. 1. In all cases cycloheximide completely inhibits resumption or continuation of cell division whether these cells are not dividing (0-4 h) or have started to divide (6 h). Control cultures divided synchronously within 5 h from the time of

TABLE I  
Percent Increase in Cell Number

Elapsed time	1 min		15 min		30 min		45 min		60 min		Original	
	-	+	-	+	-	+	-	+	-	+	-	+
<i>h</i>												
2	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
6½	41	38	39	35	35	29	13	15	3	0	0	0
7½	76	74	60	55	66	41	41	19	27	0	6	0
9½	89	91	81	84	74	76	56	66	71	38	27	0
30	635	619	627	621	587	570	587	587	554	198	158	0

Effect of incubation of B<sub>12</sub>-deficient cells with cycloheximide on their recovery after B<sub>12</sub> replenishment. Cultures in advanced B<sub>12</sub> deficiency were divided into two portions; one received B<sub>12</sub> (-), and the other received B<sub>12</sub> and cycloheximide (+). Samples of each portion were taken at the times indicated, washed, and resuspended in fresh medium containing no B<sub>12</sub>, and the change in cell number was monitored.

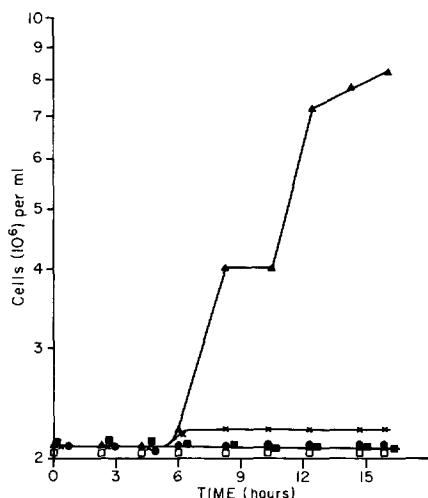


FIGURE 1 The effect of cycloheximide addition at various times on cell division in deficient cultures replenished with B<sub>12</sub>. (▲—▲), Control, only B<sub>12</sub>; (●—●), cycloheximide added at zero time; (□—□), cycloheximide added at 2 h; (■—■), cycloheximide added at 4 h; (×—×), cycloheximide added at 6 h. Detail in text.

B<sub>12</sub> replenishment, doubled within 3 h, entered a plateau stage for 2 h, and practically doubled again within about 5 h.

Studies of the effect of cycloheximide on cellular DNA of B<sub>12</sub>-deficient cells replenished with the vitamin are shown in Fig. 2. There is a slight or no significant increase in DNA per cell when cycloheximide is added simultaneously with B<sub>12</sub>. If cycloheximide is added 2 h after B<sub>12</sub> replenishment, cell DNA increases slightly and then DNA synthesis stops. If cycloheximide is added 4 or 6 h

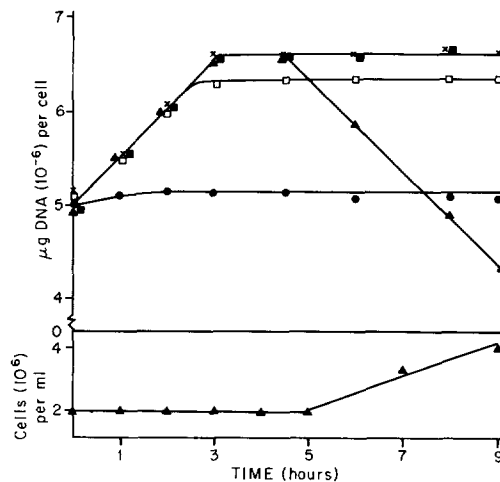


FIGURE 2 Upper part: the effect of cycloheximide addition at various times on DNA content per cell in deficient cultures replenished with B<sub>12</sub>. Details as in Fig. 1 and in text. Lower part: kinetics of cell division in control culture.

after B<sub>12</sub>, at which time cellular DNA duplication is completed, no change in DNA is noticed, and as in all cycloheximide treatments, no cell division occurs. On the other hand, in the control culture the cells show maximum DNA within 3 h, start to divide at 5 h, and therefore the cell DNA decreased but was not halved, indicating resumption of DNA synthesis for the next burst of cell division.

From these data and from our finding (3, 4) that B<sub>12</sub> deficiency causes lengthening of the S phase by decreasing the rate of DNA synthesis, we decided to determine the rates of DNA synthesis by a

method more sensitive than the one we used previously (see Materials and Methods).

Because *Euglena* has been shown to be impermeable to the pyrimidine nucleosides (14, 17), [<sup>14</sup>C]formate was used for these studies. Fig. 3 indicates that B<sub>12</sub>-deficient cells incorporate the radioactivity continuously, but at a very slow rate. Cells receiving cycloheximide 45 min before B<sub>12</sub> replenishment or simultaneously with B<sub>12</sub> are able to synthesize DNA at higher rates than cells receiving neither B<sub>12</sub> nor cycloheximide. However, these high rates drop later and DNA synthesis stops completely. The same result is found in cultures receiving cycloheximide 2.5 h after B<sub>12</sub> replenishment. Control cultures receiving only B<sub>12</sub> continue to incorporate the radioactivity into the DNA at a linear rate for about 4 h. No additional incorporation occurs for about 3–4 h, during which time the cells start to divide, and then [<sup>14</sup>C]formate incorporation resumes.

Fig. 4 a indicates that cultures receiving cycloheximide simultaneously with B<sub>12</sub> or 2 or 4 h after show no alteration in protein content. Cul-

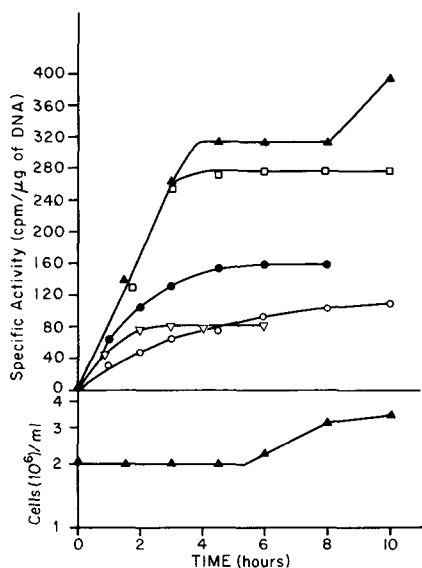


FIGURE 3 Upper part: the effect of cycloheximide addition at various times on the incorporation of [<sup>14</sup>C]formate into DNA in deficient cells replenished with B<sub>12</sub>. (▲-▲) Control, only B<sub>12</sub>; (○-○), no B<sub>12</sub> or cycloheximide; (▽-▽), cycloheximide added 45 min before B<sub>12</sub>; (●-●), cycloheximide added at the same time as B<sub>12</sub>; (□-□), cycloheximide added 3 h after B<sub>12</sub>. Lower part: kinetics of cell division in reverting control culture.

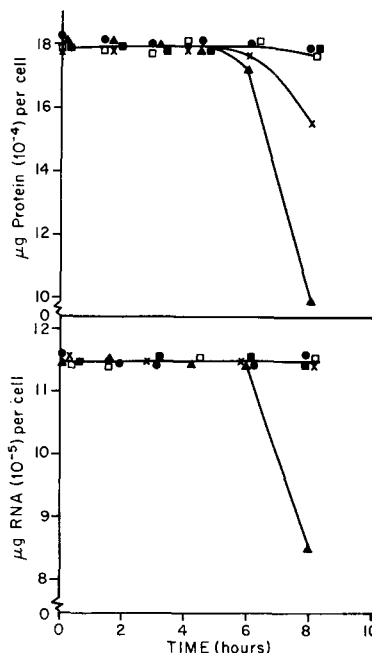


FIGURE 4 The effect of cycloheximide addition at various times on cellular protein content (upper part) and RNA content (lower part) in deficient cultures replenished with B<sub>12</sub>. (▲-▲), Control, only B<sub>12</sub>; (●-●), cycloheximide added at zero time with B<sub>12</sub>; (□-□), cycloheximide added 2 h after B<sub>12</sub>; (■-■), cycloheximide added 4 h after B<sub>12</sub>; (×-×), cycloheximide added 6 h after B<sub>12</sub>.

tures receiving the inhibitor 6 h after B<sub>12</sub> addition, when cells have started division, show a slight decrease in cellular protein as would be expected. In the control cultures, cellular protein is practically halved when cells double in number. The data suggest that at least quantitatively these cells have enough protein for one burst of division. However, it is not clear from these data whether or not cycloheximide specifically inhibits protein synthesis required for resumption of or continuation of DNA synthesis and/or for cell division. Fig. 4 b shows that addition of cycloheximide to B<sub>12</sub>-replenished deficient cultures at different times does not cause any detectable alteration in cellular RNA content. Cellular RNA in control cultures decreases as a result of cell division. It is not, however, halved as would be expected, and the decrease in cellular RNA is noted to be delayed about 1 h after the start of cell division, indicating that new RNA synthesis is taking place during mitosis.

The results of studies of the incorporation of

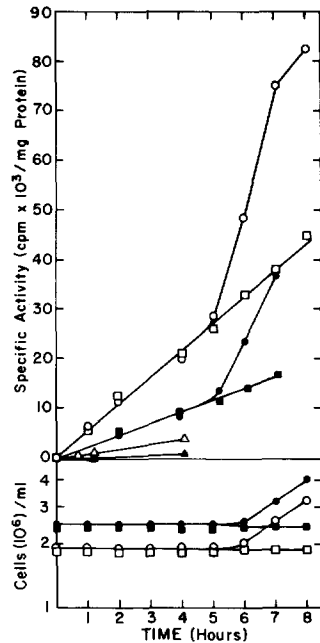


FIGURE 5 Effect of addition of B<sub>12</sub> and B<sub>12</sub> plus cycloheximide on protein synthesis and cell division in B<sub>12</sub>-deficient cultured *Euglena*. Upper part: (○—○), B<sub>12</sub> and [<sup>14</sup>C]leucine in green *Euglena*; (□—□), [<sup>14</sup>C]leucine only added to green *Euglena*; (△—△), B<sub>12</sub>, [<sup>14</sup>C]leucine and cycloheximide added to green *Euglena*; (●—●), B<sub>12</sub> and [<sup>14</sup>C]leucine added to aplastidic mutant; (■—■), [<sup>14</sup>C]leucine only added to aplastidic mutant; (▲—▲), B<sub>12</sub>, [<sup>14</sup>C]leucine, and cycloheximide added to aplastidic mutant. Lower part: (○—○), B<sub>12</sub> and leucine added to green *Euglena*; (□—□), leucine only added to green cells; (●—●), B<sub>12</sub> and leucine added to the aplastidic mutant; (■—■), leucine only added to the aplastidic mutant.

[<sup>14</sup>C]leucine into the protein are shown in Fig. 5. The addition of cycloheximide to deficient cells replenished or not with B<sub>12</sub> (not shown in the figure) almost completely inhibits the incorporation. The slight incorporation seen in the figure may be due to protein synthesis in chloroplasts or mitochondria since protein synthesis in those organelles is not inhibited by cycloheximide (1, 16). It is interesting that both B<sub>12</sub>-replenished and nonreplenished cells incorporate the radioactivity at the same rate for about 5 h, at which time DNA duplication is completed in the replenished cells. However, before and after resumption of cell division, B<sub>12</sub>-replenished cells show an increase in the rate of [<sup>14</sup>C]leucine incorporation by fivefold compared to nonreplenished cells. The bleached mutant Y<sub>7</sub>ZHL shows the same pattern of incor-

poration, but the rate of incorporation is far less than in the Z strain.

## DISCUSSION

We have found that cycloheximide, a specific inhibitor of cytoplasmic protein synthesis in normal exponentially growing *Euglena* (16, 18), inhibits resumption of cell division in B<sub>12</sub>-deficient cells replenished with the vitamin. The inhibition is complete whether cycloheximide is added concomitant with or after the addition of B<sub>12</sub>. Cycloheximide also inhibits continued division in reverting cells which have started to divide. In addition, we found that cells kept in the original medium after B<sub>12</sub> replenishment respond less to B<sub>12</sub> replenishment than those transferred to fresh medium containing no B<sub>12</sub> or to a resting medium (unpublished data). This suggests that the original medium contains inhibitory substances, some of which may possibly be the B<sub>12</sub> binding protein factors secreted by the cells as indicated by Daisley (5). Our data also indicate that cycloheximide does not seem to interfere with the uptake of B<sub>12</sub> in contrast to the finding of Evans (9) that cycloheximide interferes with the uptake of glucose and 2,4-dinitrophenol in *Euglena*.

The inhibition by cycloheximide of cell division, at least when applied to cells up to 2 h after B<sub>12</sub> replenishment, is found to be associated with the failure of cycloheximide-treated cells to continue DNA duplication. This is suggested by the significantly lower DNA content of cells in cultures receiving cycloheximide simultaneously with B<sub>12</sub> or 2 h after (Fig. 2). However, cells that apparently duplicated their DNA before the addition of cycloheximide are unable to divide. It is possible that in the last case the inhibition is not associated with DNA synthesis but with some proteins required for cell division.

Studies of the rate of [<sup>14</sup>C]formate incorporation into the DNA under different treatments in terms of time of application of cycloheximide and/or B<sub>12</sub> revealed the following information. First, B<sub>12</sub>-deficient cells have residual capacity to synthesize DNA at a slow rate which, when calculated from the curve, is found to be about 20–25% of that of the reverting control cultures. This is in agreement with our finding (3, 4) that under B<sub>12</sub> deficiency the S phase of the cell cycle is extended. Second, the addition of B<sub>12</sub> to deficient cells enhances their activity: the cells complete DNA duplication, divide, and then start another

round of DNA synthesis in preparation for the next burst of cell division. Third, B<sub>12</sub> enhances the rate of DNA synthesis even when added 45 min after the addition of cycloheximide; however, the rate decreases and the incorporation stops completely. This indicates that continuous protein synthesis is necessary for normal rates of DNA synthesis in these cells as in many other eucaryotes (10). While, on one hand, there is a lag between the application of cycloheximide and the decrease, followed by cessation, of DNA synthesis, a spontaneous enhancement of DNA synthesis is found upon the addition of B<sub>12</sub> to the deficient cells. Cycloheximide has been shown to inhibit nuclear DNA synthesis in *Euglena* (1, 16) and in other systems (11, 21, 22). Hershey et al. (10) have shown that cycloheximide completely blocks *in vivo* DNA replication within 15 min in HeLa cells. However, in most cases the apparent inhibition of DNA synthesis by cycloheximide seems to be indirect. For example, cycloheximide added to isolated S phase HeLa nuclei shows no effect on DNA replication (10). The inhibition of DNA synthesis by cycloheximide in chick erythroblasts is attributed to its inhibition of chain elongation proteins which are thought to be histones (21), and in the case of HeLa cells the inhibition is due to a protein required for maintenance of replicative activity in nuclei independent of actual DNA synthesis (10).

The kinetic studies of the effect of cycloheximide on protein and RNA synthesis in reverting cultures were done to find out if synthesis of new protein and RNA was a prerequisite for resumption of DNA synthesis at higher rates. We found that no detectable change in the contents of both macromolecules occurred whether cycloheximide was used or not (Fig. 4). Experiments in which the incorporation of radioactivity into the protein was studied showed that the cells in the non-B<sub>12</sub>-replenished culture continued to incorporate the radioactivity at the same rate while the cells in the B<sub>12</sub>-replenished culture increased their rate of incorporation by fivefold immediately before and after resumption of cell division. The data suggested that quantitatively no new protein synthesis was required for DNA replication in these deficient cells, but that some new protein synthesis was needed for mitosis. In the presence of cycloheximide, little or no incorporation into protein took place.

Both the increase in the rate of incorporation of

radioactivity into protein after completion of DNA duplication in the B<sub>12</sub>-replenished culture (Fig. 5) and the inhibition of cell division when cycloheximide is added at 4 h (Fig. 1) after B<sub>12</sub> replenishment, at which time DNA duplication is completed (Fig. 3), suggest that some protein(s) is required for either nuclear division and/or cytokinesis. Edmunds (7) has shown that the completion of DNA synthesis is a necessary condition for cell division in *Euglena*, but that additional macromolecular events are also required before the onset of division. We have noticed that deficient cells undergo two complete bursts of cell division even if they are suspended in a resting medium containing only salts and B<sub>12</sub> (unpublished data). In addition, in most cases of reversion the second burst is always shorter in time than the first, suggesting that these cells contain a large enough pool of proteins, nucleic acids, and their precursors to undergo two divisions and that some repair mechanism may be acting during the first burst of division.

Our results are consistent with our proposal that B<sub>12</sub> plays a direct role in nuclear DNA synthesis, bringing about the lengthening of the S phase and the disproportionate synthesis of the other macromolecules, and resulting in unbalanced growth. The deficient cells contain quantitatively and qualitatively the required protein(s) for at least the initial resumption of DNA synthesis but not for cell division. The inhibition of DNA synthesis by cycloheximide may be due to the breakdown of a highly labile protein or the depletion of the supply of certain proteins which could no longer be synthesized. To determine whether one or more protein factors (species) is involved requires more accurate methods of study. Further studies are in progress in our laboratories.

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