VITAMIN B₁₂ AND THE MACROMOLECULAR COMPOSITION OF EUGLENA

III. Effect of Cycloheximide on the Recovery from B₁₂-Induced Unbalanced Growth

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ABSTRACT

When cycloheximide is added to (B₁₂)-deficient cultures before or after replenishment of the cells with B₁₂, reversion of these cells is inhibited. This inhibition is not caused by interference of the inhibitor in the uptake of B₁₂ as measured by division kinetics. Cycloheximide does not inhibit the initial increase in the rate of DNA synthesis caused by B₁₂ 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. B₁₂ added to deficient cells does not stimulate the incorporation of [¹⁴C]leucine into protein during resumption and completion of DNA duplication. However, there is a large increase in [¹⁴C]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 B₁₂-replenished or to nonreplenished deficient cells rapidly inhibits the incorporation. We suggest that the addition of B₁₂ 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 B₁₂ (B₁₂), 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 B₁₂ or some of its analogues (12, 13). The B₁₂-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_12-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_12 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 YZH (8) were grown as previously described (2). Growth measurements in terms of cell number per milliliter, production of B_12 deficiency, criteria of deficiency condition, and chloroplast count have been described before (2, 3).

Reversion of B_12 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_12, at a concentration of 10 #g/liter, was added to the deficient cells (control).

Either B_12 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_12 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 #g/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_12, cycloheximide, and appropriate amounts of nonradioactive formate were added to this portion at times indicated in Results. To the other portion, B_12, cycloheximide, and [1^4C]formate (Amersham/Searle Corp., Arlington Heights, Ill. sp act 59.9 mCi/mmoll diluted 15-fold with nonradioactive formate) at a concentration of 10 mCi/100 ml culture were added. Samples containing about 2 x 10^6 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 is the second part was determined by the method of Webb and Levy (20).

RESULTS

We studied the possible interference by cycloheximide on the uptake of B_12 by B_12-deficient cultures. Table I indicates that cycloheximide does not seem to interfere with the uptake of B_12 by these cells. Sufficient B_12 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 1
Percent Increase in Cell Number

<table>
<thead>
<tr>
<th>Elapsed time (h)</th>
<th>1 min</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
<th>Original</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6½</td>
<td>41</td>
<td>38</td>
<td>39</td>
<td>35</td>
<td>35</td>
<td>29</td>
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<tr>
<td>7½</td>
<td>76</td>
<td>74</td>
<td>60</td>
<td>55</td>
<td>66</td>
<td>41</td>
</tr>
<tr>
<td>9½</td>
<td>89</td>
<td>91</td>
<td>81</td>
<td>84</td>
<td>74</td>
<td>76</td>
</tr>
<tr>
<td>30</td>
<td>635</td>
<td>619</td>
<td>627</td>
<td>621</td>
<td>587</td>
<td>570</td>
</tr>
</tbody>
</table>

Effect of incubation of B12-deficient cells with cycloheximide on their recovery after B12 replenishment.

Cultures in advanced B12 deficiency were divided into two portions; one received B12 (-), and the other received B12 and cycloheximide (+). Samples of each portion were taken at the times indicated, washed, and resuspended in fresh medium containing no B12, and the change in cell number was monitored.

B12 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 B12-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 B12. If cycloheximide is added 2 h after B12 replenishment, cell DNA increases slightly and then DNA synthesis stops. If cycloheximide is added 4 or 6 h after B12, 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 B12 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), \(^{14}\text{C}\)formate was used for these studies. Fig. 3 indicates that B\(_{12}\)-deficient cells incorporate the radioactivity continuously, but at a very slow rate. Cells receiving cycloheximide 45 min before B\(_{12}\) replenishment or simultaneously with B\(_{12}\) are able to synthesize DNA at higher rates than cells receiving neither B\(_{12}\) 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\(_{12}\) replenishment. Control cultures receiving only B\(_{12}\) 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 \(^{14}\text{C}\)formate incorporation resumes.

Fig. 4 a indicates that cultures receiving cycloheximide simultaneously with B\(_{12}\) or 2 or 4 h after show no alteration in protein content. Cultures receiving the inhibitor 6 h after B\(_{12}\) 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\(_{12}\)-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 $B_{12}$ and $B_{14}$ plus cycloheximide on protein synthesis and cell division in $B_{12}$-deficient cultured Euglena. Upper part: (O--O), $B_{12}$ and $[^{14}C]$leucine in green Euglena; ($\Delta$--$\Delta$), $[^{14}C]$leucine only added to green Euglena; ($\Delta$--$\Delta$), $B_{12}$, $[^{14}C]$leucine and cycloheximide added to green Euglena; ($\bullet$--$\bullet$), $B_{12}$ and $[^{14}C]$leucine added to aplastic mutant; ($\bigcirc$--$\bigcirc$), $[^{14}C]$leucine only added to aplastic mutant; ($\triangle$--$\triangle$), $B_{12}$, $[^{14}C]$leucine, and cycloheximide added to aplastic mutant. Lower part: (O--O), $B_{12}$ and leucine added to green Euglena; ($\square$--$\square$), leucine only added to green cells; ($\bigtriangleup$--$\bigtriangleup$), $B_{12}$ and leucine added to the aplastic mutant; ($\blacktriangle$--$\blacktriangle$), leucine only added to the aplastic mutant.

$[^{14}C]$leucine into the protein are shown in Fig. 5. The addition of cycloheximide to deficient cells replenished or not with $B_{12}$ (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 $B_{12}$-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, $B_{12}$-replenished cells show an increase in the rate of $[^{14}C]$leucine incorporation by fivefold compared to nonreplenished cells. The bleached mutant $Y_{7}Z_{HL}$ shows the same pattern of incorporation, 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 $B_{12}$-deficient cells replenished with the vitamin. The inhibition is complete whether cycloheximide is added concomitantly with or after the addition of $B_{12}$. 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 $B_{12}$ replenishment respond less to $B_{12}$ replenishment than those transferred to fresh medium containing no $B_{12}$ or to a resting medium (unpublished data). This suggests that the original medium contains inhibitory substances, some of which may possibly be the $B_{12}$ 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 $B_{12}$ 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 $B_{12}$ 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 $B_{12}$ 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 $[^{14}C]$formate incorporation into the DNA under different treatments in terms of time of application of cycloheximide and/or $B_{12}$ revealed the following information. First, $B_{12}$-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 $B_{12}$ deficiency the S phase of the cell cycle is extended. Second, the addition of $B_{12}$ 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, B₁₂ 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 B₁₂ 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 protein was studied showed that the cells in the non-B₁₂-replenished culture continued to incorporate the radioactivity at the same rate while the cells in the B₁₂-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 resumption of DNA replication in the B₁₂-replenished culture (Fig. 5) and the inhibition of cell division when cycloheximide is added at 4 h (Fig. 1) after B₁₂ 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 B₁₂ (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 B₁₂ 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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