VITAMIN B_{12} AND THE MACROMOLECULAR COMPOSITION OF EUGLENA

II. Recovery from Unbalanced Growth Induced by Vitamin B_{12} Deficiency

PAMELA LEBAN JOHNSTON and EDGAR F. CARELL

From the Department of Biology, University of Pittsburgh, Pittsburgh, Pennsylvania 15213. Dr. Johnston's present address is the Department of Genetics, University of Washington, Seattle, Washington 98105.

ABSTRACT

When vitamin B_{12} is added to B_{12}-deficient cultures of *Euglena gracilis*, the cells undergo two relatively synchronous cell divisions within a shorter than usual period of time, apparently as a result of a transitory shortening of the cell cycle. The first cell division pulse, occurring 4.5 h after addition of B_{12}, is preceded by the completion of DNA duplication, but appears to involve no net synthesis of RNA or protein. Before the second round of cell division at about 11 h, a significant amount of DNA synthesis is observed. This time it is accompanied by a minor increase in the RNA and protein content of the culture. The cellular contents of RNA and protein were observed to decrease steadily after the resumption of cell division in B_{12}-depleted cultures receiving the vitamin. Ultimately all three macromolecules returned to their nondeficient, plateau stage levels; by this time, cell division had ceased.

INTRODUCTION

Euglena gracilis, when deprived of an adequate supply of exogenous vitamin B_{12}, exhibits classic symptoms of unbalanced growth. Concomitant with vitamin depletion, cellular enlargement occurs, accompanied by an arrest of cell and nuclear division. DNA synthesis appears to cease, in advanced deficiency, when the cells have duplicated approximately 80% of the DNA required for the next round of cell division. In marked contrast, the replication of chloroplasts, and the synthesis of chlorophyll, RNA, and protein continue unhindered until the B_{12}-deficient cells contain 300–400% more of each of these than is normally found in plateau stage, B_{12}-complete cultures (7, 8).

One characteristic feature of B_{12} deficiency in a number of organisms is the reversibility of the condition upon replenishment of the organism with the vitamin, or in some cases, with the product of the B_{12}-dependent biosynthetic reaction. Thus, in *Lactobacillus leichmannii*, correction of the deficiency condition may be brought about via repletion of the culture with either B_{12} itself or deoxyribosides (2), while in *Escherichia coli*, methionine may be substituted for the vitamin (10). Euglena, in contrast, appears to be considerably more demanding in terms of its requirements under cobalamin deficiency, and attempts to replace the vitamin with deoxyribonucleosides (16; Carell, unpublished data), free

purines, pyrimidines, methionine, or folic acid (23) have proved unsuccessful. Nevertheless, preliminary evidence has suggested that B_{12} deficiency in Euglena may be fully reversible upon repletion with B_{12} itself, as is the case in man (1). In addition, since B_{12} deficiency in Euglena appears to bring about the arrest of DNA synthesis and cell division before their completion, there was reason to believe that experimentally-induced B_{12} starvation might prove to be a useful tool for synchronization of Euglena cultures, under conditions in which the macromolecular events leading to cell division potentially could be defined.

The experiments described in this report were undertaken with two goals in mind: first, to define the macromolecular events during reversion of B_{12} deficiency in Euglena and, second, to study the regulatory mechanisms responsible for the regulation of cell division in this organism.

MATERIALS AND METHODS

Growth Conditions

Euglena gracilis Klebs, strain Z (Pringsheim) was grown in 2-liter Erlenmeyer flasks containing 1 liter of modified Hutner's medium as described previously (7). Vitamin B_{12} was added to the culture medium at a final concentration of 25 ng/liter to constitute a B_{12}-deficient medium. Cultures were maintained at 24° ± 1° C on a New Brunswick Model G-10 gyratory shaker (New Brunswick Scientific Co., New Brunswick, N. J.), under constant illumination of 100 ft-c of cool white light. Experimental cultures were inoculated with 0.5 ml portions of a Euglena seed culture containing 1 μg of B_{12} per liter.

Growth of cultures, measured in terms of change in cell number per milliliter culture, was monitored by means of a Coulter Model B electronic particle counter, equipped with a 140 μm aperature and a 500 μl manometer. All cell counts were replicated several times. Viability of cultures was estimated by direct microscope observation of the cells under the fluorescence microscope (7). Chloroplast counts were performed using a fluorescence microscope, and the cell number per milliliter culture and the average chloroplast number per Euglena cell were employed as criteria of the degree of vitamin B_{12} deficiency (7).

Reversion of Vitamin B_{12} Deficiency

When the cells had attained a moderately severe degree of vitamin B_{12} deficiency (average chloroplast number per cell = 32), B_{12} was added to the culture to a final concentration of 10 μg/liter (time = 0 h). Cell samples for determination of cell number per milliliter chloroplast number, DNA, RNA, and protein per cell were withdrawn from the culture at zero time, and at regular intervals during the first 16–26 h after vitamin addition.

Sampling was performed as follows: at each sampling time, two portions of cells were withdrawn from the culture. One was utilized for determination of cell number per milliliter of culture, chloroplast number per cell, and viability. Simultaneously, a second, larger sample of known volume was withdrawn, centrifuged, and resuspended to a known final volume in double-distilled water. Portions for cell counts, protein, RNA, and DNA assays were withdrawn from this concentrated cell suspension. Sample volumes for nucleic acid assays were so calculated that each nucleic acid sample would contain 1.5–3.0 × 10^7 B_{12}-deficient cells. All assays were performed in triplicate, and each experiment was repeated at least four times.

A modified method of Kempner and Miller (18) was used for the extraction of nucleic acids. DNA was determined by the method of Webb and Levy (24), RNA by the method of Dische (11), and protein by the Lowry method (21). Having determined the DNA, RNA, and protein content per milliliter of concentrated cell suspension, it was possible to calculate the cellular concentration of each of the three macromolecules.

RESULTS

The observation that vitamin B_{12} deficiency in Euglena results in an elevation of roughly 180% in the cellular DNA content, as contrasted with the 300–400% increment in both RNA and protein (7, 8), led us to make two predictions regarding the macromolecular requirements for the correction of the B_{12} deficiency condition. First, if the B_{12}-deficient cells are capable of synthesizing all of the species of macromolecules required for cell division other than DNA, then only the completion of DNA duplication should be required before the resumption of cell division in B_{12}-supplemented deficiency cultures. In such a case, we would expect an increase in DNA per cell to precede cell division, while no further synthesis of RNA or protein might be required until the excess of both species of macromolecules had been apportioned to the daughter cells. Alternatively, if B_{12} functions as a cofactor in the synthesis of certain RNA or protein molecules in Euglena, then the synthesis of these macromolecules might be a prerequisite for the correction of the deficiency condition. To test these hypotheses, we elected to study the kinetics of change in the

macromolecular composition of *E. gracilis* after vitamin repletion of B_{12}-deficient cultures.

Fig. 1 shows the kinetics of change in DNA concentration per cell in B_{12}-supplemented deficiency cultures and its relationship to cell division. While no cell division was noted until 4.5 h after B_{12} addition, cellular DNA content began to increase dramatically after 1 h, reaching a maximum concentration of 6.6–6.7 $\times$ 10^{-6} μg by the end of the second hour. This numerical value is roughly twice the minimum DNA concentration observed in plateau stage B_{12}-sufficient cells and is equivalent to the 4C amount of DNA in *E.*

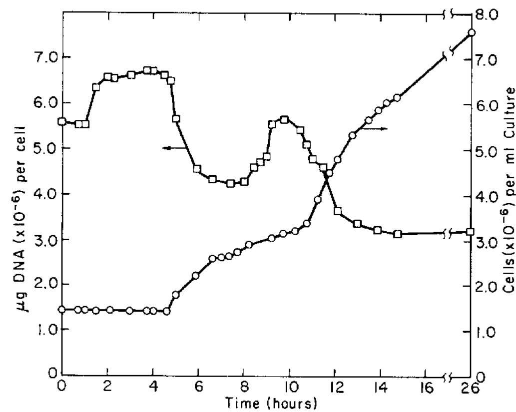

FIGURE 1 Kinetics of change in the DNA concentrations per Euglena cell (□) and the cell number per milliliter culture (○) after addition of vitamin B_{12} to deficient cultures. Details are described in the text.

gracilis, strain *Z* (6, 8). After a burst of cell division which began at 4.5 h and continued until 7.5 h, a second peak of DNA synthesis was noted, followed by an additional round of cell division at 11 h.

Fig. 2 depicts the kinetics of change in cellular RNA and protein concentration during reversion of vitamin B_{12} deficiency in Euglena. No significant change in the cellular content of either macromolecule was observed in the culture before the resumption of cell division at 4.5 h. Concomitant with cell division, both RNA and protein per cell decreased steadily, with brief plateaus between cell division pulses. Within 26 h of vitamin addition, the cellular concentrations of both RNA and protein had attained values characteristic of plateau stage, B_{12}-sufficient Euglena cultures (8). The pattern of decrease in RNA and protein per cell suggests that the correction of vitamin B_{12} deficiency may involve apportionment of the 3–4-fold excess of RNA and protein to daughter cells, at least during initial cell division cycles, rather than *de novo* synthesis of these macromolecules.

Fig. 3 shows the change in average chloroplast number per cell which occurs during correction of B_{12} deficiency in Euglena. Chloroplast number decreased slightly before cell division, then began a steady decline concomitant with cell division, to its normal value of 10 plastids per cell.

The data presented in Figs. 1, 2, and 3 show a decrease in RNA and protein content as well as chloroplast number per cell after the addition

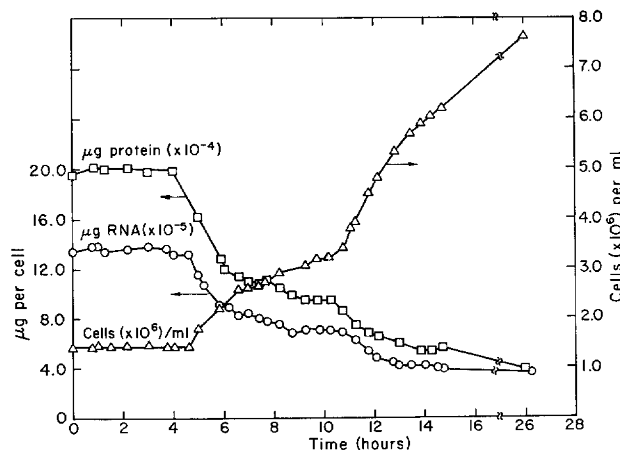

FIGURE 2 Kinetics of change in the RNA concentration (○) and protein concentration (□) per Euglena cell and the cell number per milliliter culture (△) after addition of vitamin B_{12} to deficient cultures. Details are described in the text.

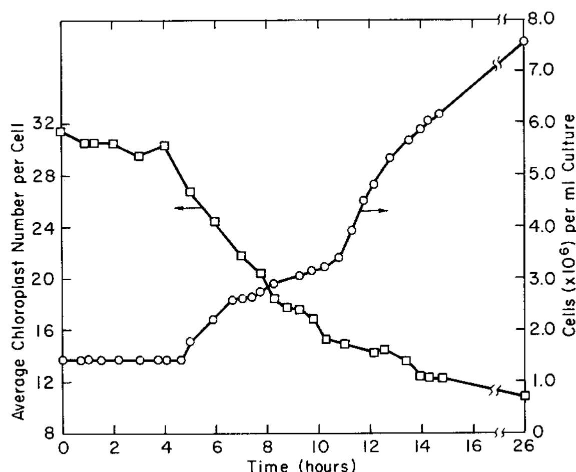

FIGURE 3 Kinetics of change in the average chloroplast number per Euglena cell (□) and the cell number per milliliter culture (○) after addition of vitamin B_{12} to deficient cultures. Details are described in the text.

of B_{12} to deficient Euglena cultures. What these data do not indicate is whether a minor amount of *de novo* synthesis of RNA or protein, or replication of chloroplasts has occurred in the culture. Therefore, it was decided to calculate the concentration of DNA, RNA, and protein, and the chloroplast number per milliliter of Euglena culture after B_{12} supplementation. These data are presented in Fig. 4.

As may be seen from the graph, DNA synthesis, expressed as an increase in the DNA concentration per milliliter of culture, preceded both rounds of cell division which occurred during the first 15 hours after B_{12} addition. On the other hand, no net synthesis of RNA or protein was noted before the onset of the first burst of cell division in B_{12}-supplemented deficiency cultures at 4.5 h. Before the second burst of division, however, RNA per milliliter increased by about 25% in the period between 4.5–10 h after B_{12} addition, while two minor pulses of protein synthesis, at 7 and 12 h, resulted in a 15% increment in protein. During this same time period, chloroplast number per milliliter increased by approximately 90%, plastic replication coinciding with both cell division pulses. It would appear, on the basis of this latter observation, that restoration of chloroplast number to its normal value of 10 per cell is not a passive process of apportionment, but may involve restoration of the control mechanisms normally responsible for synchronization of cell and chloroplast division.

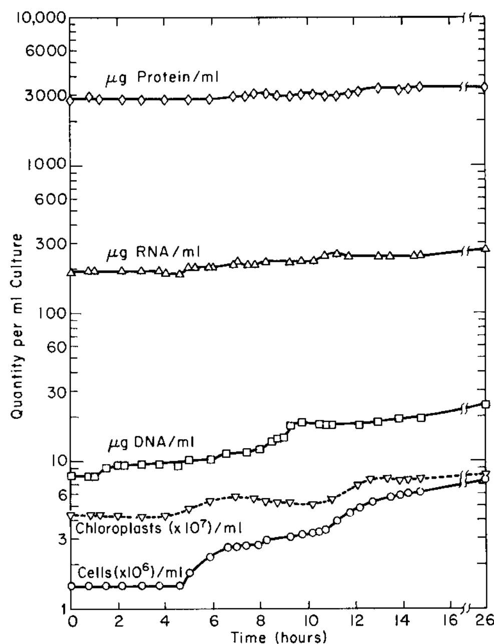

FIGURE 4 Kinetics of change in the DNA concentration (□), RNA concentration (△), protein concentration (◇), average chloroplast number (▽), and cell number (○) per milliliter of Euglena culture after addition of vitamin B_{12} to deficient cultures. Details are described in the text.

DISCUSSION

The results of these experiments have served to substantiate several heretofore suspected, but

undocumented, characteristics of vitamin B_{12} deficiency in *Euglena gracilis*. First, the deficiency condition is fully reversible, in terms of both restoration of cell division capacity and restoration of normal macromolecular composition. Second, resumption of cell division appears to be preceded by the completion of DNA duplication, whereas no significant change in the RNA or protein content of the culture was noted until after the onset of the second burst of cell division. These experiments have not ruled out the possibility that some new RNA or protein synthesis, possibly of minor species of mRNA or protein molecules, might be prerequisite to the correction of B_{12} deficiency. However, it is unlikely that such synthesis could be detected without employing more sensitive assay techniques.

The kinetics of B_{12} deficiency reversal in Euglena are similar in several respects to those observed in *L. leichmannii* and man. In B_{12}-starved Lactobacillus, for example, vitamin supplementation results in the stimulation of arrested DNA synthesis, and is accompanied by the restoration of decreased DNA/RNA and DNA/protein ratios to their normal values (3). In contrast to Euglena, however, only those Lactobacillus cultures which received the vitamin early in deficiency were capable of resuming normal cell division, despite the fact that all cultures receiving B_{12} exhibited extensive DNA synthesis (3). In man, B_{12} deficiency-related pernicious anemia is characterized by megaloblastic erythropoiesis (1), associated with chromosome breakage (17), an elevation of DNA per nucleus to values approaching the 4C DNA complement (25), and a disproportionately greater increase in RNA and protein per cell (9, 19, 25). As in Euglena, vitamin B_{12} addition resulted in stimulation of DNA synthesis and restoration of balanced cell growth and division (25).

The results of these and other studies strongly suggest that vitamin B_{12} functions primarily in the synthesis of nuclear DNA in Euglena. Duplication of nuclear DNA, which appears to be arrested before its completion in B_{12}-deficient cultures (8), is resumed within 1 h of B_{12} addition, and appears to be completed by the end of the second hour. The specific role of the vitamin in vivo is somewhat enigmatic, however.

The Euglenophytes (Euglena and Astasia) have the distinction of being the only eukaryotes in which a cobamide-dependent ribonucleotide reductase has been detected (14, 22). Like that of *L. leichmannii* (4, 5), the Euglena enzyme catalyzes the conversion of ribonucleotide triphosphates to the corresponding deoxyribonucleotide triphosphates in the presence of a 5-deoxyadenosylcobalamin cofactor and a dithiol reducing agent (13). In contrast to the cobamide-dependent reductases of Lactobacillus and certain other prokaryotes, however, the activity of the Euglena reductase is extremely low (13, 14), and the *E. gracilis* strain is most sensitive to B_{12} deprivation; the Z strain (15) exhibits even lower reductase activity in vitro than the less sensitive bacillaris variety (14). These factors, coupled with the observations that B_{12} deficiency in Euglena is not relieved by exogenous deoxyribonucleosides (16; Carell, unpublished data) or methionine (23), suggest that B_{12} may serve some function in Euglena other than as a cofactor for the ribonucleotide reductase reaction.

An additional question of importance remains to be answered regarding the macromolecular requirements for reversion of B_{12} deficiency in Euglena; namely, what biosynthetic processes, if any, occur between the end of DNA synthesis at 2 h and the beginning of cell division at 4.5 h in vitamin-repleted cultures.

It has been reported that, in synchronously dividing, autotrophic Euglena cultures, the cells enter mitosis almost immediately after the completion of DNA synthesis (S phase), with no observable intervening G_2 period (12). The process of mitosis itself and the subsequent cell division then require approximately 4 h for their completion (20). Consequently, in view of the normal timing of DNA synthesis and mitosis in *E. gracilis*, it is not unreasonable to suppose that the entire time period between the completion of DNA synthesis and the onset of cell division in B_{12}-supplemented deficiency cultures might be spent in the process of mitosis.

On the other hand, studies conducted by Edmunds (12) in phototrophic, light-synchronized Euglena demonstrated that while DNA synthesis is prerequisite to cell division, the completion of DNA synthesis alone, in the absence of concomitant or subsequent RNA and protein synthesis, is insufficient to permit cell division. It is difficult to extrapolate these results directly to the control of cell division during correction of B_{12} deficiency, since the B_{12}-deficient Euglena cells accumulate a 3–4-fold excess of both RNA and protein (8).

Consequently, unless certain species of these macromolecules are not synthesized during deficiency, the quantity of both RNA and protein per cell should be adequate to support at least one round of cell division.

It is possible, however, that because of the abundance of both RNA and protein in the B_{12}-deficient cells, a net shortening of the cell division cycle might occur during correction of deficiency. If, as our data suggest, the cell cycle was arrested at a certain point in S phase during B_{12} deprivation, then DNA synthesis should be an absolute prerequisite to the resumption of cell division. Immediately after the completion of S phase, the cells might enter mitosis in synchrony, divide, and then, utilizing their excess of RNA and protein, completely skip the G_1 phase of the subsequent cell cycle and proceed directly through a second S phase, mitosis, and cell division.

This hypothesis is consistent with two major experimental observations made during the correction of B_{12} deficiency in Euglena. First, DNA synthesis does precede both rounds of cell division in B_{12}-repleted cultures (Figs. 1 and 4), while RNA and protein synthesis, which were not observed until before the second round of division, were minimal (Figs. 2 and 4). Second, during reversion, a net shortening of the cell cycle appears to have occurred, since the second burst of cell division was able to commence less than 6 h after the first (Fig. 1), in contrast to the usual 9-h generation time (7).

As noted earlier, we were unable to detect any significant increase in RNA or protein, either per cell or per milliliter culture, before the resumption of cell division in B_{12}-supplemented deficiency cultures (Figs. 2 and 4). However, the results of preliminary experiments, conducted in our laboratory, in which cycloheximide was used to inhibit protein synthesis, suggest that some protein synthesis, occurring as late as 4 h after B_{12} addition, may be an additional prerequisite to cell division in vitamin-supplemented deficiency cultures.

In order to resolve this question of the macromolecular events preceding cell division in B_{12}-repleted Euglena cultures, it will be necessary to employ more sensitive experimental techniques to detect minor synthesis of RNA and protein. Studies are in progress in our laboratory to determine the effects of inhibitors of DNA, RNA, and protein synthesis on the capacity of B_{12}-deficient Euglena to undergo cell division subsequent to vitamin addition.

We wish to thank Miss Elizabeth Janthey and Miss Linda Yurinak for their technical assistance.

This investigation was supported by Grant GB-20903 from the National Science Foundation.

Received for publication 20 November 1972, and in revised form 22 January 1973.

REFERENCES

1. Beck, W. S. 1964. *Medicine (Baltimore)*. **43**:715.
2. Beck, W. S., S. Hook, and B. H. Barnett. 1962. *Biochim. Biophys. Acta*. **55**:455.
3. Beck, W. S., M. Goulian, and S. Hook. 1962. *Biochim. Biophys. Acta*. **55**:470.
4. Blakley, R. L. 1966. *Fed. Proc.* **25**:1633.
5. Blakley, R. L., R. K. Ghambeer, P. F., Nixon, and E. Vitols. 1965. *Biochem. Biophys. Res. Commun.* **20**:439.
6. Brawerman, G., and J. M. Eisenstadt. 1964. *Biochim. Biophys. Acta*. **91**:477.
7. Carell, E. F. 1969. *J. Cell Biol.* **41**:431.
8. Carell, E. F., P. L. Johnston, and A. R. Christopher. 1970. *J. Cell Biol.* **47**:525.
9. Davidson, J. N., I. Leslie, and J. C. White. 1948. *J. Pathol. Bacteriol.* **60**:1.
10. Davis, B. D., and E. S. Mingiolo. 1950. *J. Bacteriol.* **60**:17.
11. Dische, Z. 1955. *In* The Nucleic Acids. E. Chargaff and J. N. Davidson, editors. Academic Press Inc., New York. **1**:285.
12. Edmunds, L. N., Jr. 1964. *Science (Wash. D. C.)*. **145**:266.
13. Gleason, F. K., and H. P. C. Hogenkamp. 1970. *J. Biol. Chem.* **245**:4894.
14. Gleason, F. K., and H. P. C. Hogenkamp. 1972. *Biochim. Biophys. Acta*. **277**:466.
15. Hutner, S. H., M. K. Bach, and G. I. M. Ross. 1956. *J. Protozool.* **3**:101.
16. Hutner, S. H., and L. Provasoli. 1955. *In* Biochemistry and Physiology of Protozoa. S. H. Hutner and A. Lwoff, editors. Academic Press Inc., New York. 39.
17. Keller, R., K. Lindstrand, and A. Norden. 1970. *Scand. J. Haematol.* **7**:478.
18. Kempner, E. S., and J. H. Miller. 1965. *Biochim. Biophys. Acta*. **104**:11.
19. Lajtha, L. G., and R. Oliver. 1960. *In* Ciba Foundation Symposium on Hematopoiesis. G. E. W. Wolstenholme and M. O'Conner, editors. J. & A. Churchill Ltd., London. 289.

20. LEEDALE, G. F. 1968. *In* The Biology of Euglena. D. E. Buetow, editor. Academic Press Inc., New York. **1**:185.
21. LOWRY, O. M., J. N. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **195**:265.
22. MILLARD, S. A. 1972. *J. Biol. Chem.* **247**:2395.
23. ROBBINS, W. J., A. HERVEY, and M. E. STEBBINS. 1950. *Bull. Torrey Botan. Club.* **77**:423.
24. WEBB, J. M., and J. B. LEVY. 1955. *J. Biol. Chem.* **213**:107.
25. YOSHIDA, Y., A. TODO, S. SHIRAKAWA, G. WAKISAKA, and H. UCHINO. 1968. *Blood.* **31**:292.