VITAMIN B_{12} AND THE MACROMOLECULAR COMPOSITION OF *EUGLENA*

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INTRODUCTION

Carell (1) observed two abnormal characteristics of vitamin B_{12}-deficient *Euglena* cells. First, their chloroplast number increased by 4–5-fold as the cells entered advanced deficiency, and second, chlorophyll and protein levels per cell concomitantly increased by the same factor.

In many organisms including *Euglena* and *Lactobacillus leichmanii*, vitamin B_{12} deficiency has been shown to be accompanied by cell enlargement and an increase in generation time (2, 3). However, earlier studies of the effects of B_{12} deficiency on cell composition have considered only ratios of DNA:RNA and DNA:protein, not the absolute amounts of these macromolecules per cell (4).

We therefore explored the effects of vitamin B_{12} deficiency on the DNA and RNA content of *Euglena* cells. In the present paper, we provide evidence that B_{12}-deficient *Euglena* increase their DNA, RNA, and protein contents.

MATERIALS AND METHODS

Euglena gracilis, strain Z, was grown as described previously (1). Vitamin B_{12} was added to the culture medium at concentrations of 10 μg/liter (complete B_{12} medium) or 25 mμg/liter (B_{12} deficient medium).

Samples for kinetic studies were withdrawn from identical cultures of complete or deficient cells. Initial samples were withdrawn at a culture OD of 0.1 (chloroplast number of 10 per cell), and at regular time intervals thereafter. Cells were harvested by centrifugation and resuspended to a known volume in double-distilled water. Aliquots for cell counts, protein, DNA, and RNA determinations were taken from this concentrated suspension. Sample volumes for nucleic acid assays were so calculated such that each nucleic acid sample would contain $1.5–3.0 \times 10^7$ cells for the deficient cultures, or $1.5–10 \times 10^7$ of the complete cultures. All samples were run in triplicate, and each experiment was repeated four times. A modified method of Kempner and Miller (5) was used for the extraction of nucleic acids. Chloroplast and cell counts were performed as described previously (1). DNA was determined by the method of Webb and Levy (6), RNA by the method of Dische (7), and protein by

the Lowry method (8). Concentrations of DNA, RNA, and protein were calculated on a cell basis.

For the determination of DNA per nucleus, aliquots of cells were fixed in 3:1 methanol:acetic acid, washed in methanol until the cells were colorless, smeared on albuminized coverslips, and air dried. The cells were then stained and bleached (9), dehydrated in ethanol, passed to xylene, and mounted in Synthetic Neutral Mountant, refractive index 1.5240 (Jaymar Scientific Co., Kenilworth, N.J.). The refractive index of this mounting medium approximates that of the cytoplasm and paramylon granules of *Euglena* as determined by the extinction transfer method of Richards (10). The samples were air dried for 2 days.

The cells were assayed for DNA content on a "per nucleus" basis by the two-wavelength method of Patau (11), using approximately 590 and 570.5 nm as wavelengths one and two, respectively. This method yielded values of DNA expressed as arbitrary units (AU). The microscope used was essentially identical to the one described by Pollister (12).

RESULTS

To determine the effects of vitamin B_{12} deficiency on the macromolecular composition of *Euglena gracilis*, we studied the kinetics of the change in the concentrations of DNA, RNA, and protein per cell under both normal and deficiency conditions. We expected that during the very early stages of growth in B_{12}-deficient medium, the cells would behave as normal, B_{12}-complete cells. As they continue to divide and deplete the vitamin, they will exhibit a change in their macromolecular composition corresponding to the decrease in B_{12} available to the cell (1). The two parameters consistently used to determine the extent of B_{12} deficiency were chloroplast number per cell, and the graph of cell number per milliliter of culture vs. optical density (1).

Fig. 1 shows the change in protein in cells grown in complete or deficient medium. The protein increased slightly during exponential growth in complete cultures, then decreased to a lower level during the stationary phase. The decrease represents a change of about 30% between the minimum and maximum protein values. However, the protein per cell in B_{12}-deficient cultures began to increase before the cells entered deficiency and ultimately reached three times its normal concentration.

Fig. 2 shows the change in RNA in normal and deficient cells. The RNA content of the normal cells was constant during exponential growth, then dropped by about 30% as the cells entered stationary stage, in a manner similar to the protein. The RNA of the B_{12}-deficient cells increased at the beginning of deficiency and climbed steadily to three times the level found in normal cells.

Fig. 3 shows the changes in DNA and chloroplast number per cell in both the normal and deficient cultures. As is evident, while the chloro-

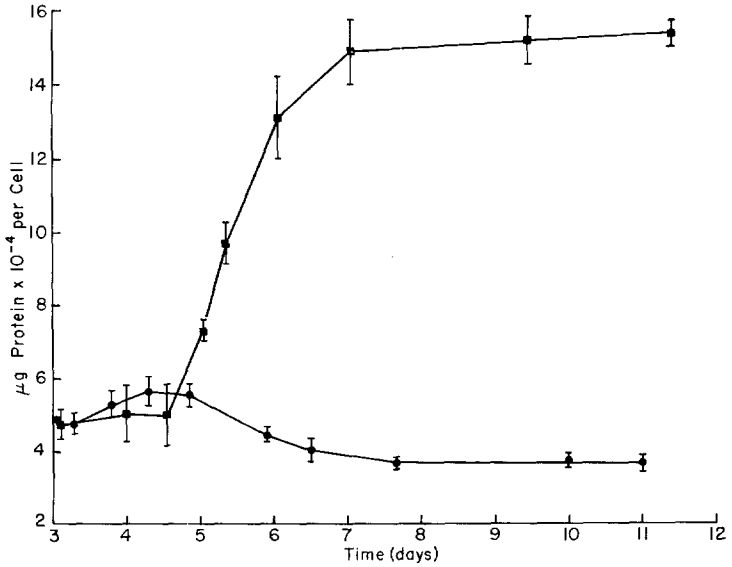

FIGURE 1 Time course of protein synthesis per *Euglena* cell grown in complete medium (●) and in B_{12}-deficient medium (■). Details are described in the text.

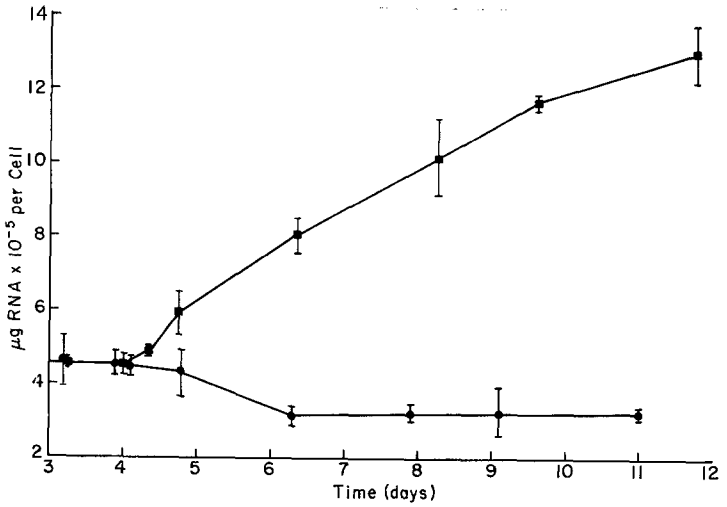

FIGURE 2 Time course of RNA synthesis per *Euglena* cell grown in complete medium (●) and in B_{12}-deficient medium (■). Details are described in the text.

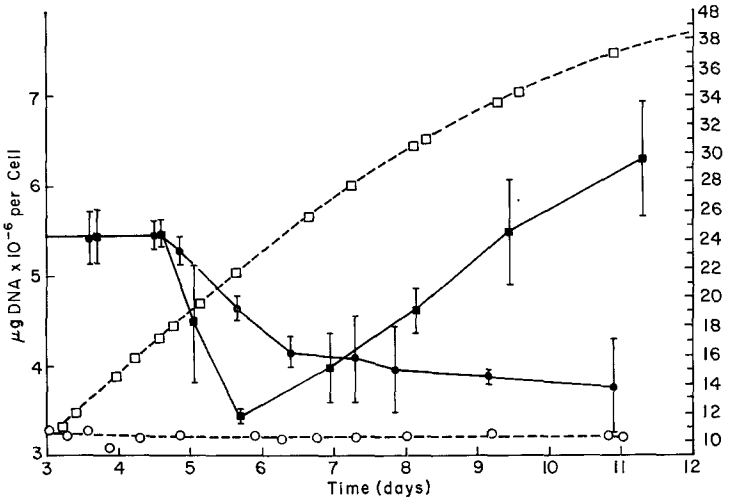

FIGURE 3 Time course of the amount of DNA per *Euglena* cell grown in complete medium (●——●) or in B_{12}-deficient medium (■——■); and of the average number of chloroplasts per *Euglena* cell grown in complete medium (○-----○) or in B_{12}-deficient medium (□-----□).

plast number in normal cells did not change, the DNA concentration dropped when the cells entered stationary phase. Although we expected that the DNA of deficient cells would behave like the RNA and protein by increasing steadily after the cells entered stationary phase, this was not the case. The DNA decreased to a minimum at a chloroplast number of approximately 22, then began a continuous increase as the cells became more B_{12} depleted and further increased their chloroplast complement. Deficient cell DNA finally attained a value slightly less than twice its minimum, while the chloroplast number increased 3–4-fold.

To verify the DNA kinetics reported in Fig. 3 and to determine whether or not this increase was due entirely to an increase in chloroplast DNA, a microspectrophometric assay of DNA per nucleus was performed. The data in Fig. 4 show the same pattern of DNA change as in Fig. 3.

Table I presents the ratios of DNA:RNA, DNA:protein, and RNA:protein at four points

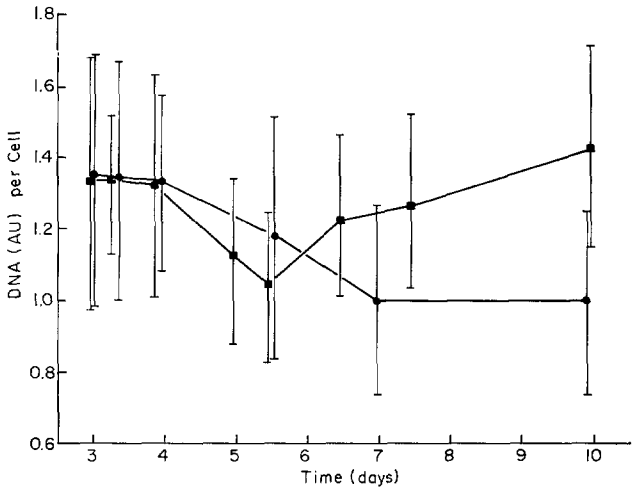

FIGURE 4 Time course of the amount of DNA per nucleus, measured microspectrophotometrically, of *Euglena* cells grown in complete medium (●) or in B_{12}-deficient medium (■). Details are described in the text.

TABLE I

Changes in the DNA:RNA, DNA:Protein, and RNA:Protein Ratios per Euglena Cell Grown in Complete Medium and in B_{12}-Deficient Medium, at Different Stages of Growth

Culture	Day	DNA:RNA range ($\times 10^{-1}$)	DNA:Protein range ($\times 10^{-2}$)	RNA:Protein range ($\times 10^{-1}$)
Complete B_{12}	3.5	1.05–1.36	0.99–1.24	0.81–1.06
	5.7	1.16–1.42	0.93–1.08	0.69–0.88
	7	1.05–1.54	0.87–1.29	0.71–0.97
	11	0.95–1.39	0.84–1.24	0.79–0.98
25 mμg B_{12}	3.5	1.10–1.30	0.96–1.30	0.83–1.05
	5.7	0.43–0.53	0.27–0.34	0.53–0.76
	7	0.39–0.50	0.23–0.30	0.52–0.66
	11	0.40–0.56	0.34–0.44	0.75–0.89

Ratios were calculated from the curves appearing in Figs. 1, 2, and 3. Details are described in the text.

during the growth of normal and B_{12}-deficient *Euglena*. Day 3.5 represents the exponential phase of growth of both complete and deficient cultures, and it is from this day that the deficient cultures decrease in growth rate. Days 5.7 and 7 represent points at which the growth of normal cultures enters the plateau stage, and the deficient cultures stop dividing completely. Day 11 is a point in growth at which normal cells are in late plateau phase and the deficient cells are greatly enlarged. In the case of the complete cultures, all three ratios remained essentially constant throughout the various stages of growth. In the deficient cultures, however, it is noticed that during early growth, the three ratios are similar to those obtained in the log phase complete cultures, then decrease progressively with B_{12} depletion. The decrease in the DNA:RNA and DNA:protein ratios is especially pronounced.

DISCUSSION

The effect of vitamin B_{12} on the growth and macromolecular composition of cells has been investigated by many workers. Our findings (1) that vitamin B_{12} deficiency in *Euglena* brought about an increase in protein, chlorophyll, and chloroplast number per cell raised the question of the effects of the vitamin deficiency on the cellular content

of DNA and RNA. Our data indicate that in the normal cells, the protein, RNA, and DNA decrease as the cells enter the plateau stage, with 140% variation between the exponential and plateau stages in these three macromolecules. These data agree with those of Pogo et al. (13) for RNA and protein, but not for DNA. In contrast, the amount of DNA per cell in *Escherichia coli* B decreases by 40% in the plateau stage (14). However, the kinetics of change exhibited by the macromolecules in deficient cultures are not the same. The protein increases first with the appearance of the earliest deficiency symptoms, continues to rise, then levels off at a chloroplast number of 28–30. The RNA increment follows that in protein, and the DNA increases shortly afterward. However, the DNA increased only slightly up to the point at which the cells contained 16–18 chloroplasts, then dropped sharply to a minimum value comparable to that found in complete cells at late stationary phase. Thereafter, the DNA increased continuously, reaching a value almost twice its minimum by late deficiency.

The differences in the time course of DNA synthesis, as opposed to those in RNA and protein synthesis, would support our hypothesis that vitamin B_{12} is somehow involved in nuclear DNA replication and has no apparent role in RNA and protein synthesis. The increase in DNA cannot be accounted for in terms of an increase in chloroplast DNA, since the DNA value reaches its minimum level at a chloroplast number of about 22, and attains its maximum value at 36 or more chloroplasts per cell. In addition, chloroplast DNA ordinarily comprises 3% of the total DNA of the *Euglena* cell (15), and an increase of approximately 175% in the DNA between cells containing 22 and 36 chloroplasts per cell could not possibly be accounted for by the increment in the number of chloroplasts and their contribution to the total DNA. This argument is supported by our finding of the increase of DNA per nucleus (Fig. 4).

On the other hand, the chloroplast RNA and protein account for 14 and 38%, respectively, of the total RNA and protein per cell in normal *Euglena* (16). Since the chloroplast number increased by 3–4-fold, the increment in plastid RNA and protein would amount to 40–50% in the RNA and 100–120% in the protein, and could not account for the increase of more than 300% in these two macromolecules per cell.

Our findings would seem contrary to those found by others who reported reduction in the cellular concentration of DNA, RNA, and protein in B_{12}-deficient *Euglena* (17, 18). In *Lactobacillus leichmanii*, Rege and Sreenivasan (19) reported that cells grown in optimum B_{12} medium were richer in DNA than those grown in B_{12} deficient medium. Beck et al. (4) reported a slight reduction of RNA and a decrease in DNA: protein and DNA:RNA ratios under B_{12} deficiency in this bacterium. The disagreement between our results and those reported above could be explained on the basis that in those cases macromolecular concentrations were expressed either per whole culture, per unit dry weight, or as ratios. Also, in the case of *L. leichmanii*, the role of B_{12} may be different than in *Euglena*; for example, the reduction of ribonucleotides to deoxyribonucleotides (20, 21). While our data disagree with Beck's interpretation, they are in agreement with his ratios showing (Table I) a decrease in both DNA:RNA and DNA:protein. This decrease results not from a decrease in the DNA per cell, but rather from a disproportionate increase in the RNA and protein. The cellular concentrations of all three macromolecules are actually increasing during B_{12} deficiency in *Euglena*. Thus our data do not contradict those of other workers; however, they do furnish a new explanation for these altered ratios.

This study on the effect of B_{12} deficiency on the macromolecular composition in *Euglena* differs from any other deficiency studies in this organism. Epstein and Allaway (22) found that either phosphate or sulfate deficiency caused marked reduction in DNA, chlorophyll, chloroplast number, and RNA. However, with zinc deficiency, Wacker (23) found that a decrease in protein and RNA was accompanied by an increase in cell size and a doubling of the DNA content of the cells. We found that vitamin B_1 which is usually added to *Euglena* medium is not required for normal growth of the Z strain (unpublished data and 24).

Most relevant to our results are the findings of Davidson et al. (25) that in megaloblastic bone marrow induced by vitamin B_{12} deficiency the cells contain abnormally high amounts of DNA, and especially of RNA.

Vitamin B_{12} function has been implicated in a variety of reactions related to nucleic acid synthesis (20, 21, 26). The fact that DNA synthesis occurs in B_{12}-deficient *Euglena* rules out the likeli-

hood of the participation of B_{12} in those reactions necessary for the production of DNA precursors or for DNA polymerization.

The implication of vitamin B_{12} in a variety of methylation processes, specifically in the methylation of RNA (27), coupled with our findings that there is an increase in the DNA of deficient *Euglena* in the absence of cell division, gives support to our notion that vitamin B_{12} may act as a methyl donor to the newly synthesized nuclear DNA, specifically to cytosine. Since the methylation of adenine and cytosine residues in DNA of *E. coli* follows polymerization of the DNA (28), it is possible to postulate that methylation of polymerized nuclear DNA may occur in our system. 5'-methylcytosine is absent in the DNA of the chloroplasts, which continue to replicate during B_{12} deficiency, but is present in the DNA of the nucleus (29).

Work is in progress in our laboratory to seek evidence for the role of methylcobalamin in the methylation of DNA of vitamin B_{12}-deficient cells.

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REFERENCES

1. Carell, E. F. 1969. *J. Cell Biol.* **41**:431.
2. Epstein, S. S., J. B. Weiss, D. Causeley, and P. Bush. 1962. *J. Protozool.* **9**:336.
3. Beck, W. S., S. Hook, and B. H. Barnett. 1962a. *Biochim. Biophys. Acta.* **55**:455.
4. Beck, W. S., M. Goulian, and S. Hook. 1962b. *Biochim. Biophys. Acta.* **55**:470.
5. Kempner, E. S., and J. H. Miller. 1965. *Biochim. Biophys. Acta.* **104**:11.
6. Webb, J. M., and J. B. Levy. 1955. *J. Biol. Chem.* **213**:107.
7. Dische, Z. 1955. *In* The Nucleic Acids. E. Chargaff and J. N. Davidson, editors. Academic Press Inc., New York. **1**:285.
8. Lowry, O. M., J. N. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193**:265.
9. Lillie, R. D. 1948. Histopathologic Technic, Blakiston, Division of the McGraw-Hill Book Co., Inc., New York. 89.
10. Richards, O. U. 1963. A. O. Baker Interference Microscope Reference Manual. American Optical Corporation, Southbridge, Mass. 2nd edition.
11. Patau, K. 1952. *Chromosoma.* **5**:341.
12. Pollister, A. W. 1952. *Lab. Invest.* **1**:106.
13. Pogo, B. T. G., I. R. Ubero, and A. O. Pogo. 1966. *Exp. Cell Res.* **42**:58.
14. Shortman, K., and I. R. Lehman. 1964. *J. Biol. Chem.* **239**:2964.
15. Edelman, M., C. A. Cowan, H. T. Epstein, and J. A. Schiff. 1964. *Proc. Nat. Acad. Sci. U.S.A.* **52**:1214.
16. Brawerman, G., A. O. Pogo, and E. Chargaff. 1962. *Biochim. Biophys. Acta.* **55**:326.
17. Soldo, A. T. 1955. *Arch. Biochem. Biophys.* **55**:71.
18. Ventkataraman, S., M. S. Netrawali, and A. Sreenivasan. 1965. *Biochem. J.* **96**:552.
19. Rege, D. V., and A. Sreenivasan. 1954. *J. Biol. Chem.* **210**:373.
20. Beck, W. S., and J. Hardy. 1965. *Proc. Nat. Acad. Sci. U.S.A.* **54**:286.
21. Blakley, R. L., and M. A. Barker. 1964. *Biochem. Biophys. Res. Commun.* **16**:391.
22. Epstein, H. T., and Elizabeth Allaway. 1967. *Biochim. Biophys. Acta.* **142**:195.
23. Wacker, Warren E. C. 1962. *Biochemistry.* **1**:859.
24. Cook, J. R. 1968. *In* The Biology of Euglena. D. E. Buetow, editor. Academic Press Inc., New York. **1**:243.
25. Davidson, J. N., I. Leslie, and J. C. White. 1948. *J. Pathol. Bacteriol.* **60**:1.
26. Lindstrand, K. 1967. *Scand. J. Clin. Lab. Invest.* **19** (Suppl. 95):3.
27. Walerych, W. S., S. Ventkataraman, and B. C. Johnson. 1966. *Biochem. Biophys. Res. Commun.* **23**:368.
28. Lark, C. 1968. *J. Mol. Biol.* **31**:389.
29. Brawerman, G., and J. M. Eisenstadt. 1964. *Biochim. Biophys. Acta.* **91**:477.