Recovery from Vitamin B-12-Induced Unbalanced Growth

THE SHORTENED CELL CYCLE AND THE DEOXYRIBONUCLEOSIDE TRIPHOSPHATE POOLS

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The deoxyribonucleoside triphosphate pools are undetectable in vitamin B-12-deficient cells of *Euglena gracilis*, but appear rapidly after the replenishment with the vitamin. They reach a maximum size that is about 6 times that of normal exponentially growing cells, but decrease to almost zero as the cells divide. The pools expand again during the post-replenishment shortened cell cycle. However, the expansion takes place during rather than before the resumption of DNA synthesis. The maximum sizes reached are still larger than in normal cells. By using the protein-synthesis inhibitor cycloheximide and determining the pool size, we found that vitamin-deficient cells apparently accumulate a large amount of ribonucleoside triphosphate reductase apoenzyme, which lacks the vitamin B-12 coenzyme. We showed that the production of the deoxyribonucleoside triphosphates is not closely coupled to DNA synthesis under our experimental conditions, and that the concentration of the deoxyribonucleoside triphosphate pools per unit of DNA synthesized is almost constant for all stages of growth examined.

Euglena cell growth and cell division are normally co-ordinated or coupled. The mechanism of this coupling can be demonstrated by attempting to dissociate the two processes. We have stalled and practically arrested division by using the vitamin B-12-deficiency system and reported the effect of this on growth and division, and have shown that these vitamin B-12-deficient cells are stalled in S-phase, but are able to continue growth as evidenced by increases in cell volume, protein and RNA content, chloroplast number and the amount of chlorophyll (Carell *et al.*, 1970; Carell, 1974). These abnormally large cells are unable to complete the replication of their DNA at normal rates, but would immediately do this at normal rates on replenishment with vitamin B-12 (recovering cells). This is then followed by a presumptive G₂-phase and the cells undergo two relatively highly synchronous divisions (Johnston & Carrell, 1973; Goetz *et al.*, 1974; Carell, 1974).

Mitchison (1971) proposed a model that portrays the cell cycle as composed of two loosely coupled cycles, the 'growth cycle' and the 'DNA-division cycle'. For the yeast cell, events in the DNA-division cycle would include the duplication and segregation of the nuclear plaque, bud emergence, DNA replication, nuclear migration and division, cytokinesis and cell-wall separation (Mitchison, 1971; Hartwell *et al.*, 1974; Hartwell, 1974; Byers & Goetsch, 1975),

whereas the growth cycle would include the synthesis of most macromolecules and most of the cell wall. The co-ordinating mechanisms that couple the growth cycle to the DNA-division cycle must prevent imbalance between the two cycles. The vitamin B-12 deficiency in *Euglena* results in cell gigantism (Epstein & Weiss, 1960) that would result from continued growth in the absence of division, which is dependent on DNA duplication.

Preliminary results (Carell & Goetz, 1976) have shown that the dNTP (deoxyribonucleoside triphosphate) pools are very small or non-existent in the vitamin B-12-deficient cells and that the pools expand very rapidly on the addition of vitamin B-12. In mammalian cells, the decrease in ribonucleoside diphosphates is tightly controlled by dNTP through a complex allosteric regulation (Reichard *et al.*, 1961; Moore & Hurlbert, 1966), as well as by the cell-cycle-related appearance of ribonucleoside diphosphate reductase activity (Nordenskjöld *et al.*, 1970). Normally, only very low amounts of dNTP accumulate in cells. The sizes and fluctuations of these pools have been extensively studied to determine their possible role in the regulation of cellular DNA synthesis (Nordenskjöld *et al.*, 1970; Skoog *et al.*, 1973; Walters *et al.*, 1973; Bjursell & Reichard, 1973).

In the present paper, we report the results of other experiments dealing with the pool sizes during the recovery from unbalanced growth. We also examine the relationship between the dNTP pools and the shortened cell cycle. In addition, we show the effects of cycloheximide on the initial increase in pool

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size and the effect of hydroxyurea on the resumption of DNA synthesis. We also propose a model to account for the recoupling of the growth cycle and the DNA-synthesis-cell-division cycle.

Experimental

Growth conditions and determinations

Euglena gracilis. Klebs strain Z (Pringsheim) was grown as previously described (Carell, 1969). Growth measurements in terms of cell number and production of vitamin B-12 deficiency, criteria of deficiency condition, have been described (Carell, 1969).

Recovery from vitamin B-12 deficiency was performed as described previously (Johnston & Carell, 1973). Cells containing an average chloroplast number of around 30–35 were used as vitamin B-12-deficient cells. In the replenishment experiments, 10 µg of vitamin B-12/litre was added to vitamin B-12-deficient cells.

Chemicals and reagents

The DNA (calf thymus) and the dNTP species were from Sigma Chemical Co., St. Louis, MO, U.S.A., d[³H]ATP and d[³H]CTP were from New England Nuclear Corp., Boston, MA, U.S.A. DNA polymerase (*Micrococcus luteus*) was from Miles Laboratories, Elkhart, IN, U.S.A. Spectrafluor scintillation fluid was from Amersham/Searle, Des Plaines, IL, U.S.A.

Assay of DNA

Samples containing about 20 × 10⁶ cells were taken in triplicate. The cells were centrifuged (1000 g, 5 min), washed with 1 mM-NaCl, and killed by the addition of 5% (w/v) trichloroacetic acid. The cells were then extracted several times with acetone to remove the chlorophyll. The DNA was determined by the method of Burton (1955) and the amount of DNA was calculated from a standard curve prepared by using calf thymus DNA (A grade; Calbiochem, Los Angeles, CA, U.S.A.).

Inhibitors

Cycloheximide (Sigma) was used at a concentration of 15 mg/ml. This concentration has been shown to inhibit more than 90% of the protein synthesis in recovering cells (Goetz *et al.*, 1974). Hydroxyurea (Calbiochem) was used at a concentration of 50 mM. This concentration has been shown to inhibit cell division in recovering *Euglena* (Carell, 1974).

Extraction and assay of the dNTP pools

The dNTP pools were extracted by the method of Bagnara & Finch (1972) modified as follows. Samples containing about 40 × 10⁶ cells were cooled in ice and centrifuged (2000 g, 5 min). The supernatant was discarded and 6 ml of 0.4 M-HClO₄ was added; the sample was mixed and placed in ice for 20 min to extract the dNTP species. The sample was then centrifuged (8000 g, 5 min) and 5 ml of the supernatant was removed. To this was added 2.5 ml of 0.8 M-KOH in 0.16 M-K₂HPO₄. The precipitate was removed by centrifugation (180000 g, 5 min). The supernatant was collected and its pH was adjusted to 6.8–7.3 by addition of 1 or 2 drops of 2.5 M-HCl.

The assay method was modified from that of Skoog *et al.* (1973). The assay mixture contained the following in a final volume of 0.3 ml: Tris/HCl buffer, pH 7.4, 20 µmol; MgCl₂, 1 µmol; 2-mercaptoethanol, 0.3 µmol; dATP, dCTP, dGTP and dTTP, 50 pmol each; DNA (calf thymus), 5 µg; cell extract and/or water to 0.28 ml; DNA polymerase, 0.02 ml [1 unit (1 µmol/min)]. d[³H]CTP was used in the assay of dATP only, and d[³H]ATP was used in the assay of the other three dNTP species. The dNTP that was being assayed was omitted from the incubation mixture. The reaction mixture was incubated at 37°C for 90 min, which was found to be optimal under our conditions.

At the end of the reaction, 1 drop of 1% bovine serum albumin was added. The tubes were placed in ice and 1 ml of 10% trichloroacetic acid in 0.1 M-sodium pyrophosphate was added. The precipitate was collected on Reeve Angel 9AH-34 glass-fibre filters and washed with 5% trichloroacetic acid in 0.01 M-sodium pyrophosphate. The filters were dried, then placed in Spectrafluor scintillation fluid and counted for radioactivity in a Beckman LS-250 liquid-scintillation counter. The number of pmol present was calculated from standard curves.

Since the specific radioactivity of the radioactive nucleoside triphosphate in the reaction mixture will decrease depending on the amount of the corresponding unlabelled nucleoside triphosphate present in the reaction mixture, we developed the following equations to correct for this condition:

$$\frac{50x}{50+y} = n_1; \quad \frac{50y}{50+x} = n_2 \quad (1)$$

$$\frac{50u}{50+x} = n_3 \quad (2)$$

$$\frac{50v}{50+x} = n_4 \quad (3)$$

where n_1 , n_2 , n_3 and n_4 refer to apparent pmol in the extract and x , y , u and v refer to actual pmol of

dATP, dCTP, dGTP and dTTP respectively in the extract.

All samples were checked for the presence, in the extract, of inhibitors of the polymerization reaction by mixing the extracts with the complete incubation mixture. No inhibitory effect was found in any sample.

Results

Changes in cell number and DNA concentration during recovery from vitamin B-12 deficiency

The data in Fig. 1 are similar to those published previously (Carell, 1974), and are presented here to correlate the time relationship between the events of DNA synthesis, the resumption of cell divisions and the dNTP pools.

Kinetics of the deoxyribonucleoside triphosphate pools in recovering cells

In a preliminary report (Carell & Goetz, 1976), we showed that the dNTP pools are zero in vitamin B-12-deficient cells and that the pools become very

large after vitamin B-12 replenishment. As shown in Figs. 1 and 2, the sizes of the pools decrease drastically as the cells began to divide. The pools start to increase during the second S-phase. As in the first post-replenishment burst of cytokinesis, the dNTP pools decrease as the cells start to divide again. By this time, the synchrony of cell division is being lost.

dNTP pool sizes during various stages of growth and its relation to DNA synthesis

Since a vitamin B-12-dependent ribonucleoside triphosphate reductase (EC 1.17.4.2) has been described in *E. gracilis* (Gleason & Hogenkamp, 1972) and because of the large increase in pool size on replenishment with vitamin B-12, it was decided first to determine the dNTP pool sizes of exponentially growing and plateau-stage normal *Euglena* with sufficient vitamin B-12. The data in Table 1 indicate the absence of any detectable dNTP pools in

Fig. 1. Kinetics of change in the DNA concentration and in the cell number/ml of *Euglena* culture replenished with vitamin B-12

Cells were grown in a medium containing a suboptimal concentration of vitamin B-12 until they reached advanced deficiency. Vitamin B-12 was then added to the culture to the normal concentration and samples were taken at intervals to determine (a) cell number by using the Coulter Counter and (b) DNA concentration by the diphenylamine method (Burton, 1955) with calf thymus DNA as a standard. For details see the text.

Fig. 2. Time course of the deoxyribonucleoside triphosphate pools in vitamin B-12-deficient and -replenished *Euglena* cells

Euglena cells were grown to vitamin B-12 deficiency. Samples were taken from these cells (zero time) and vitamin B-12 was then added to the culture and samples were taken at the indicated intervals. The dNTP pools were extracted and from these samples by the method of Bagnara & Finch (1972), frozen and assayed later by the method of Skoog *et al.* (1973). Details are given in the text. (a) dATP; (b) dCTP; (c) dGTP; (d) dTTP.

Table 1. *dNTP pool sizes during various stages of growth of normal Euglena cells*

Pools were extracted from cells grown with sufficient vitamin B-12 at various stages of growth. The extraction and assay is as described in the Experimental section.

Stage	Deoxyribonucleoside triphosphate pools (pmol/10 ⁶ cells)			
	dATP	dCTP	dGTP	dTTP
Exponential	24	20	12	32
Plateau	0	0	0	0

Table 2. *Average pool size during the post-replenishment DNA synthesis periods, first S- and second S-phase*
Average pool size (pmol/10⁶ cells) was calculated from Fig. 2 by using the 1–2 h interval for the first S-phase and the 6–9 h interval for the second S-phase.

	dATP	dCTP	dGTP	dTTP
First S-phase	130	110	35	160
Second S-phase	35	56	16	80

the plateau-stage cells and small pools in the exponentially growing normal cells.

Table 2 shows the average dNTP pool size in cells after replenishment with vitamin B-12, resumption and completion of DNA synthesis (first S-phase), and the subsequent DNA-synthesis phase (second S-phase). Fig. 1 shows that cells take about 3 h to double their number during the second wave of cytokinesis, compared with about 2 h in the first wave, indicating a slight loss of division synchrony. This loss of synchrony does not account for the much smaller pool sizes during the second S-phase. The average exponential S-phase pool size (Table 2) is much smaller still. Thus it appears that vitamin B-12-replenished cells have abnormally large pools during their recovery from vitamin B-12 deficiency. Table 3 shows the dNTP pool sizes per unit of DNA synthesized per 10⁶ cells. The ratio is largest during the completion of the blocked S-phase. It decreases by an average of 30% during the second S-phase, and by 60% in exponentially growing cells.

It should be noted that the size of the cells, as determined by either the Coulter counter (results not shown) or by the amount of protein per cell (Johnston & Carell, 1973) is also decreasing. Cells in the blocked S-phase are about twice as large and contain about twice as much protein as during the second S-phase. These same cells are about 4 times as large and contain 4 times as much protein as exponentially growing cells. Therefore, on these bases, the amount of the dNTP per unit of DNA in the cell is relatively constant, as shown in Table 4.

Table 3. *dNTP pool sizes per µg of DNA synthesized normalized for 10⁶ cells*

The pool sizes from Table 2 were divided by the average amount of DNA synthesized during the 1–2 h period of the first S-phase and the 6–9 h interval of the second S-phase. Data from which values for exponentially growing cells were calculated are not shown here.

	dATP	dCTP	dGTP	dTTP
First S-phase	13	11	3.5	16
Second S-phase	4.5	7.3	2.1	10.4
Exponentially growing cells	3.6	3.0	1.8	4.8

Table 4. *Concentration of the dNTP species per µg of DNA synthesized normalized for 10⁶ cells*

These concentrations were calculated by using approximate volume estimates of cells obtained from measurements by Coulter counter. Vitamin B-12-deficient cells were 4 times the volume of exponentially growing cells, and cells in the second S-phase were twice the volume of exponentially growing cells.

	dATP	dCTP	dGTP	dTTP
First S-phase	3.25	2.75	0.9	4
Second S-phase	2.25	3.65	1.05	5.2
Exponentially growing cells	3.6	3.0	1.8	4.8

Effect of cycloheximide on the size of the dNTP pools in recovering cells

It was essential to determine whether or not the vitamin B-12-dependent apoenzyme required for the formation of the dNTP is present in vitamin B-12-deficient cells lacking the vitamin B-12 coenzyme. We have shown (Goetz *et al.*, 1974) that vitamin B-12-deficient cells can synthesize DNA when protein synthesis is inhibited with cycloheximide. Using cycloheximide again to test for the formation of the dNTP species, we find that, during the first hour, cycloheximide-treated vitamin B-12-replenished cells synthesize 75% of the amount of DNA synthesized by non-treated vitamin B-12-replenished cells (Goetz *et al.*, 1974). This amount of DNA synthesis will allow a substantial amount of pool turnover to occur. Therefore we may conclude that during the first hour cycloheximide does not cause a substantial increase in the size of the dNTP pools. The results of Table 5 indicate that the size of the dNTP pools in cycloheximide-treated vitamin B-12-replenished cells follows approximately the same kinetics of expansion as in non-treated vitamin B-12-replenished cells (Fig. 2) during the first hour. The size of the dATP, dCTP and dGTP pools of cycloheximide-treated vitamin B-12-replenished cells is larger after 1 h than those of the control cells, suggesting some accumulation by this time due to the effect of cycloheximide on

Table 5. Onset of synthesis of dNTP species in the presence of cycloheximide in *Euglena* recovering from vitamin B-12 deficiency

Cycloheximide (15 mg/litre) was added to the vitamin B-12-deficient cells 5 min before vitamin B-12 replenishment. Pools were extracted and assayed as described in the Experimental section.

Time (min)	Deoxyribonucleoside triphosphate pools (pmol/10 ⁶ cells)			
	dATP	dCTP	dGTP	dTTP
0	0	0	0	0
15	0	0	5	10
30	59	61	31	112
60	183	162	84	180

Table 6. Effect of hydroxyurea on DNA synthesis and cell division

Vitamin B-12-deficient cells were replenished with vitamin B-12 alone (control) or given vitamin B-12 and hydroxyurea. Details are given in the text.

Time (h)	DNA (μ g/ml)		Growth (10 ⁻⁶ × cells/ml)	
	Control	+Hydroxyurea	Control	+Hydroxyurea
0	64.0	64.0	1.25	1.25
1	78.5	78.0	1.24	1.25
2	92.0	92.0	1.23	1.24
3	98.0	98.5	1.25	1.25
4	99.0	99.0	1.25	1.22
5	102.0	102.0	1.75	1.24
6	—	—	2.25	1.24

DNA synthesis. The dTTP pool is about the same size in both cases.

Effect of hydroxyurea on DNA synthesis and cell division in vitamin B-12-replenished cells

We asked whether or not a vitamin B-12-independent ribonucleotide reductase system was present in *Euglena*, as in most eukaryotes (Gleason & Hogenkamp, 1972). To answer this question, we added hydroxyurea, a specific inhibitor of vitamin B-12-independent ribonucleotide reductases (Elford, 1968), to the vitamin B-12-deficient *Euglena* replenished with the vitamin. The results in Table 6 show that hydroxyurea has no effect on DNA synthesis, at least during the first S-phase. It does, however, inhibit cell division during the first 6 h and even longer (Johnston, 1973).

Discussion

We have shown that the dNTP pools are essentially absent from vitamin B-12-deficient *Euglena*, but

that they appear very rapidly on the addition of vitamin B-12 to these cells (Carell & Goetz, 1976). They remain at abnormally high values during the completion of the first S-phase (Fig. 2).

The pools then decrease drastically and in three cases actually reach zero. Walters *et al.* (1973) has also found in Chinese-hamster cells that the dNTP pools disappear as the cells begin to divide; however, this degradation has not been observed in all cases (Nexo, 1975; Skoog *et al.*, 1973).

The expansion of the pools during the second S-phase is not as great as during the completion of the first S-phase. However, the pools are still 2–3 times the size found in exponentially growing cells (Table 1). The cells at this stage still have twice the amount of protein of exponentially growing normal cells with sufficient vitamin B-12 (Johnston & Carell, 1973). The relative sizes of the four pools are the same as during the first S-phase, with dTTP the largest and dGTP the smallest. The pool sizes that we found during the second S-phase are comparable with those reported for *Tetrahymena* (Nexo, 1975). In *Tetrahymena*, as in *Euglena*, the dTTP pool is always the largest. A similarity between the dNTP pools of *Euglena* and Chinese-hamster cells is that the dGTP pool is always the smallest (Nordenskjöld *et al.*, 1970). This is also the case in human bone-marrow cells (Hoffbrand *et al.*, 1974).

It is noteworthy that the dNTP pools expand during the second S-phase rather than before it, as has been shown in other systems (Skoog *et al.*, 1973; Walters *et al.*, 1973). This would suggest that, in the vitamin B-12-deficiency-reversion system, the production of the dNTP species is limiting DNA synthesis during the beginning of the second S-phase, implying that the initiation of DNA synthesis would have to take place before the expansion of the dNTP pools. A careful examination of Figs. 1 and 2 shows that the pool size reaches a minimum as DNA synthesis (second S-phase) begins. This could possibly be the point of assembly of the initiation complex. Since in other systems the expansion of the dNTP pools occurs before DNA synthesis (Skoog *et al.*, 1973), we therefore suggest that during the recovery from unbalanced growth the expansion of the dNTP pools is out of phase with the beginning of the second S-phase.

It can be argued that the large dNTP pool size is due to large NTP pools causing overshoot. It has been shown by Savageau (1976) that feedback systems are buffered by a factor of $h/(h-g)$, where h is the apparent kinetic order of the reaction with respect to substrate and g is the apparent kinetic order of the reaction with respect to the end product. If intermediate values for these are assumed, e.g. $h = 0.5$ and $g = -2$, then it can be shown that a 6-fold difference in dNTP pool size (e.g. between normal *Euglena* and reverting *Euglena*) would require over a

30-fold difference in the dNTP pool size. This explanation does not appear very probable.

As was stated in the Results section, if one considers the concentration of dNTP pools in the cells and divides this by the amount of DNA synthesized per cell, one can obtain values that are almost constant (Table 4). The production of large amounts of dNTP species may be a homoeostatic mechanism to keep concentration of dNTPs at an optimum for DNA synthesis.

The data in Fig. 2 and Tables 2, 3 and 4 indicate that the pool size during the first S-phase is abnormally large. Several investigators (Murphree *et al.*, 1969; Fujioka & Silber, 1971; Lowdon & Vitols, 1973) have found that, when the S-phase is slowed or blocked, an increase in the activity of the ribonucleoside diphosphate reductase occurs. If we assume that pool size is a function of the enzyme amount, then it can be inferred from Table 5 that vitamin B-12-deficient *Euglena* contain a large amount of the ribonucleoside triphosphate reductase apoenzyme. Vitamin-deficient cells, even in the absence of protein synthesis, are capable of producing large dNTP pools when replenished with vitamin B-12 (Table 5).

Since the production of the dNTP pools is not closely coupled to DNA synthesis, it is possible that the enzymes that synthesize them are produced in the growth cycle rather than in the DNA-division cycle and that the pools themselves can be considered as a link between the two cycles. The growth cycle would continue even though the DNA-division cycle is blocked, and, if our explanation is correct, would result in large amounts of ribonucleotide reductase in vitamin B-12-deficient cells, as inferred above.

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