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ONE-CARBON METABOLISM IN SYNCHRONIZED CULTURES OF *EUGLENA GRACILIS**

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Key Word Index—*Euglena gracilis* (strain *Z*); Euglenales; synchronized cultures, folate derivatives; cell cycle; enzymes of C-1 metabolism.

Abstract—The levels of folate derivatives in division synchronized cultures of *Euglena gracilis* Klebs (strain *Z*) increased rapidly on a per cell basis during illumination while cell number remained constant whereas in darkness cell numbers were approximately doubled and the concentrations of the derivatives per cell declined. The principal derivatives were 5-methyl- and 5-formyltetrahydropteroyltriglutamates and more highly conjugated methyl and formyl derivatives were major components of the folate pool at all stages of the cell cycle. The levels of 10-formyltetrahydrofolate synthetase, serine hydroxymethyltransferase, and 5-methyltetrahydrofolate: homocysteine transmethylase when expressed on a per cell basis increased during illumination and declined during the dark phase of culture. The levels of 5,10-methylenetetrahydrofolate reductase were lowest during cell division and highest when cell numbers were constant.

INTRODUCTION

IN AN appropriate light–dark regime the cell cycle of *Euglena gracilis* may be readily synchronized.[1,2] Under these conditions the cell number doubles during each dark period but remains constant during illumination. Biochemical studies[3–5] of such cultures have shown that the levels of protein, polysaccharides, pigments and RNA double during each light period in a linear fashion whereas the DNA content of the cells increases in a stepwise manner. Clearly net synthesis of these constituents appears to be related to the onset of cell division. As many of these syntheses would be directly or indirectly dependent on C-1 metabolism it follows that fluctuations in the levels of folate derivatives and key enzymes of folate metabolism should occur during the cell cycle of *Euglena*.

To our knowledge there have been no detailed studies of C-1 metabolism in a division synchronized organism. Such studies are of further interest in *Euglena* as recent investigations of the glycollate pathway[6,7] in this organism clearly imply that folate derivatives participate in the metabolism of carbon fixed in photosynthesis.

The present studies have therefore characterized the folate derivatives of *E. gracilis* and measurements of folate pool size have been made during the cell cycle. The levels of four key enzymes of C-1 metabolism have also been related to cell number during synchronized culture.

* The abbreviations used for folic acid and its derivatives are those suggested by the IUPAC-IUB Commission as listed in the *Biochem. J.* **102,** 15 (1967): e.g. 10-HCO-H_4PteGlu = N^{10}-formyltetrahydropteroylmonoglutamate.

[1] J. R. COOK, *Plant Cell Physiol.* **2,** 199 (1961).
[2] J. R. COOK and T. W. JAMES, *Exptl Cell Res.* **21,** 583 (1960).
[3] J. R. COOK, *Biol. Bull.* **121,** 277 (1961).
[4] L. N. EDMUNDS, *Science* **145,** 266 (1964).
[5] L. N. EDMUNDS, *J. Cell Comp. Physiol.* **66,** 159 (1965).
[6] D. R. MURRAY, J. GIOVANELLI and R. M. SMILLIE, *Austral. J. Biol. Sci.* **24,** 23 (1971).
[7] G. A. CODD and M. J. MERRETT, *Plant Physiol.* **47,** 640 (1971).

RESULTS

Synchronized Culture and Changes in Folate Pool Size During the Cell Cycle:

Figure 1 illustrates the light-induced division synchrony of *E. gracilis* when grown autotrophically for 5 cell cyles in a 14:10 hr light–dark regime at 25°. The data for cell numbers shows that synchronous cell division only occurred during the dark periods and resulted in an approximate doubling of cell numbers. Calculations of synchronization index[8] gave average values of 0·65. Cell divisions were generally detected shortly after commencement of each dark period but in a few instances occurred 1–2 hr before. The resulting growth pattern is similar to that reported by Edmunds[9] for this organism.

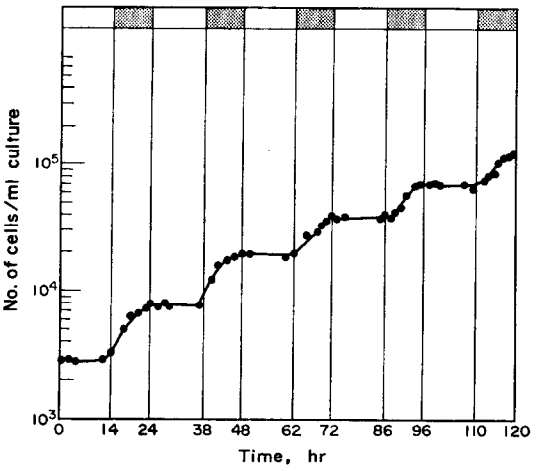

FIG. 1. SYNCHRONOUS INCREASE IN CELL NUMBER IN CULTURES OF *Euglena gracilis* KLEBS *Z*. GROWTH CONDITIONS ARE DESCRIBED IN THE TEXT.

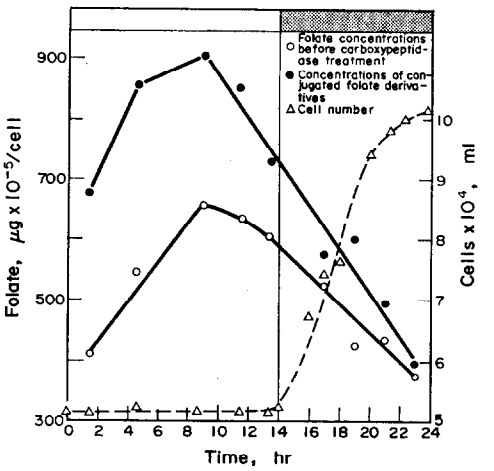

FIG. 2. CHANGES IN FOLATE CONCENTRATION DURING THE DIVISION CYCLE OF SYNCHRONIZED *Euglena* CULTURES.

In order to examine variations in folate levels during the cell cycle, samples of such cultures were withdrawn at intervals and assays involving *Lactobacillus casei* were carried out before and after treatment of the extracts with γ-glutamylcarboxypeptidase. The results of these studies are summarized in Fig. 2. It is clear that the levels of conjugated and unconjugated derivatives increased on a per cell basis during the first 9 hr of the light period. Following this, folate levels declined to the values in the dark period which were approximately half of the maximal values observed in the light. A large proportion of the folate pool consisted of highly conjugated derivatives and this proportion remained relatively constant throuhgout the cell cycle.

Chromatography of Individual Folate Derivatives

To investigate the nature of the folate derivatives in *Euglena*, ascorbate extracts were subjected to chromatography on DEAE-cellulose before and after treatment with γ-glutamylcarboxypeptidase. Eight individual compounds (*a–h*) were identified on the basis

[8] O. H. SCHERBAUM, *J. Protozool.* **6** (suppl.), 17 (1959).
[9] L. N. EDMUNDS, *J. Cell. Comp. Physiol.* **66**, 147 (1965).

of criteria used by Roos and Cossins.[10] These included the ability of the compound to support the growth of *L. casei* and *Pediococcus cerevisiae*, co-chromatography with authentic derivatives and a consideration of chromatographic behaviour after treatment with γ-glutamylcarboxypeptidase.

Before carboxypeptidase treatment, peaks *f*, *g* and *h* were present in relatively large amounts and supported the growth of *L. casei*, but not that of *P. cerevisiae*. Differential microbiological assay of these compounds after carboxypeptidase treatment revealed that peak *f* supported the growth of both assay bacteria but peak *g* still supported only the growth of *L. casei*. Peaks *f* and *g* occurred at positions in the elution sequence which were essentially identical to those of authentic 5-HCO-H_4PteGlu$_3$ and 5-CH_3-H_4PteGlu$_3$.[11] On the basis of chromatographic behaviour and differential growth response these two peaks are tentatively identified as 5-HCO-H_4PteGlu$_3$ and 5-CH_3-H_4PteGlu$_3$ respectively. Peak *h* after enzyme treatment supported the growth of *P. cerevisiae*, which considering its position of elution, suggests that it contained possibly more than one highly conjugated folate derivative. Other derivatives including *a*, 10-HCO-H_4PteGlu, *b*, 10-HCO-H_4PteGlu$_2$, *c*, 5-HCO-H_4PteGlu, and *d*, 5-CH_3-H_4PteGlu were present in smaller quantities. Chromatography of extracts after carboxypeptidase treatment gave these four compounds together with *e*, 5-HCO-H_4PteGlu$_2$, indicating that the highly conjugated derivatives were both formyl and methyl compounds of H_4PteGlu$_n$. A derivative *a'* supporting the growth of both assay organisms was also detected in early fractions from the column before 10-HCO-H_4PteGlu, after carboxypeptidase treatment.

TABLE 1. LEVELS OF FOLATE DERIVATIVES IN SYNCHRONOUS CULTURE OF *Euglena gracilis* AT DIFFERENT STAGES OF THE DIVISION CYCLE

Folgate derivatives (pg/10^5 cells)	Light phase 2 hr Before γ-GCP*	Light phase 2 hr After γ-GCP	Light phase 10 hr Before γ-GCP	Light phase 10 hr After γ-GCP	Dark phase 4 hr Before γ-GCP	Dark phase 4 hr After γ-GCP
Formyl derivatives	188	605	209	728	214	443
Methyl derivatives	106	115	172	188	55	192
Unidentified derivatives						
Fractions 30–38	n.d.	116	n.d.	143	n.d.	103
Peak *h*	80	n.d.	57	n.d.	104	n.d.
Totals	374	835	438	1060	373	738

n.d.—not detected.
* γ-Glutamylcarboxypeptidase.

Changes in Formyl and Methyl Derivatives During the Cell Cycle

Before carboxypeptidase treatment (Table 1) the overall levels of methyl derivatives on a per cell basis increased in the light period and decreased during the dark period. Hydrolysis of the extracts using pea cotyledon γ-glutamylcarboxypeptidase before chromatography gave increases in the levels of formyl derivatives. Such conjugated folates were present at lower concentrations during the dark phase of culture.

[10] A. J. ROOS and E. A. COSSINS, *Biochem. J.* **125**, 17 (1971).
[11] E. A. COSSINS and S. P. J. SHAH, *Phytochem.* **11**, 587 (1972).

Enzyme Studies

The levels of four folate enzymes were examined using cell-free extracts prepared at different times during the cell cycle (Fig. 3). It is clear that 10-HCO-H_4PteGlu synthetase, serine hydroxymethyltransferase and 5-CH_3-H_4PteGlu: homocysteine transmethylase activities varied over the period examined in a somewhat similar manner. In general, the levels of these enzymes per cell increased approximately 2 fold during the light phase and decreased during the dark phase of the cycle. In contrast the levels of 5,10-CH_2=H_4PteGlu reductase decreased from maximal values achieved at the beginning of illumination to only 50% of this value by the end of the light phase. During the dark phase, the levels of this enzyme rose continuously.

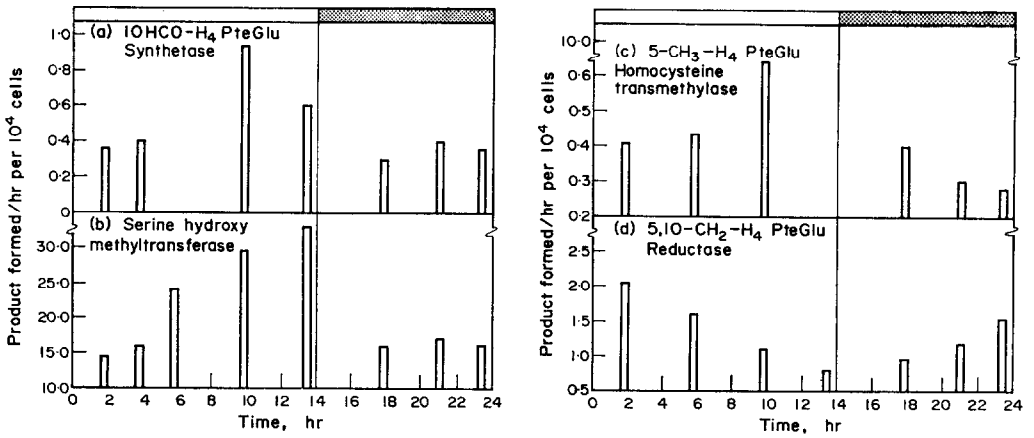

FIG. 3. LEVELS OF FOLATE DEPENDENT ENZYMES DURING THE CELL CYCLE OF *Euglena*.
The activity of the synthetase is expressed as mμmol product formed/hr/10^4 cells; activities of other enzymes are expressed as pmol product formed/hr/10^4 cells.

DISCUSSION

It is clear from the present analyses that the major folates in *Euglena* are formyl and methyl derivatives of $H_4PteGlu_n$. Before carboxypeptidase treatment these were principally 5-CH_3-H_4PteGlu$_3$ and 5-HCO-H_4PteGlu$_3$ while after such treatment the levels of both types of derivatives were increased substantially (Table 1). The role of 5-CH_3-H_4PteGlu$_3$ in the methionine synthesis of this organism is clear from recent studies[12] of the substrate specificity of 5-CH_3-H_4PteGlu$_3$: homocysteine transmethylase. The present investigations, although showing that some folate-dependent enzymes can utilize H_4PteGlu, nevertheless suggest that other reactions of C-1 metabolism may also be preferentially mediated by highly conjugated derivatives.

As various folate derivatives are directly involved in the syntheses of purines, pyrimidines, certain amino acids and proteins[13] it is not surprizing that a net folate synthesis occurred in the cells during illumination (Fig. 2). The general increases in folate pool size (Fig. 2) and turnover of C-1 units as suggested by the enzyme studies (Fig. 3), presumably reflect an increased demand for C-1 units to support synthesis of constituents like RNA and DNA which are both formed predominantly in the light phase of growth.[5] Variations

[12] L. MILNER and H. WEISSBACH, *Arch. Biochem. Biophys.* **132**, 170 (1969).
[13] R. L. BLAKLEY, *The Biochemistry of Folic Acid and Related Pteridines*, p. 219, Elsevier, New York (1969).

in folate pool size during growth also occur in random cultures of *Saccharomyces*.[14,15] As in *Euglena*, much of the increase in folate content could be accounted for by net synthesis of formyl and methyl derivatives of $H_4PteGlu_n$. In *Chlorella ellipsoidea* fluctuations in folate content during growth have been reported[16] but the extraction and assay procedures employed may have resulted in extensive degradation of labile derivatives and comparisons with the present data are therefore not possible.

The changes in folate enzymes levels (Fig. 3) which accompany the cell cycle are in some respects similar to data for related enzymes in random cultures of *L. casei*.[17] In this organism the levels of 10-HCO-H_4PteGlu synthetase, H_2PteGlu reductase and 5,10-CH_2=H_4PteGlu dehydrogenase were affected by the stage and rate of growth; with highest levels being encountered during the logarithmic phase. Experiments with chloramphenicol and actinomycin D supported the suggestion that these enzymes were rapidly synthesized by actively dividing cells. The rapid increases in 10-HCO-H_4PteGlu synthetase, serine hydroxymethyltransferase and the transmethylase (Fig. 3) clearly accompany the net synthesis of folates in *Euglena*, and conceivably catalyze a rapid turnover of C-1 units at this stage of the cell cycle. The levels of 5,10-CH_2=H_4PteGlu reductase were however found to decrease in such cells (Fig. 3) despite the finding (Table 1) that methylated folates were being rapidly synthesized. This suggests that the activity or synthesis of this enzyme may be strictly regulated in *Euglena*. Mechanisms for such regulation have been described for *Saccharomyces*[14,15] and *Escherichia coli*[18] where methyl group biogenesis is controlled by end product inhibition and enzyme repression respectively.

Recent studies of the glycollate pathway in *Euglena*[6] and *C. pyrenoidosa*[19] suggest that C-1 units required in serine synthesis are derived from glyoxylate or glycine. In this connection C-1 units could enter the folate pool of illuminated cells at either the formyl or hydroxymethyl levels of oxidation by a pathway closely related to photosynthesis. If the flow of photosynthetic carbon through the glycollate pathway is appreciable in *Euglena*, it follows that the partial reactions of this sequence could represent a major route for generation of C-1 units. The interrelationships between the glycollate pathway and C-1 metabolism in *E. gracilis* are currently being investigated.

EXPERIMENTAL

Organism and culture. Euglena gracilis Klebs (strain *Z*) was obtained from the American Type Culture Collection, Rockville, Maryland (ATCC 12716). Initially the organism was maintained axenically on agar slants consisting of 1·0% Difco Bacto-Agar and 1·0% Bacto-Peptone. Transfers were made monthly. Autotraphic cultures were grown axenically in 4 l. flasks containing 3·8 l. of Cramer and Myers'[20] pH 6·8 medium. The culture flask was placed on a magnetic stirrer and a siphoning device was attached. Moistened sterile air was bubbled through the culture (600–700 ml/min) using a fine sintered disc. At the start of each experiment the medium was inoculated aseptically to give an initial concentration of *ca.* 3×10^3 cells/ml using a subculture which has been grown synchronously for 5–7 days. All cultures on solid and liquid media were maintained at 25° with a 14:10 hr light:dark cycle. The light intensity of approximately 3500 lx was provided by 'cool white' fluorescent lamps. Cell counts were made with a Coulter counter Model B.

Extraction and chromatography of folate derivatives. For analysis of total folates 300 ml of culture (5×10^4–10×10^4 cells/ml) were withdrawn at different stages of the cell cycle, and the cells were harvested by centrifugation at 2000 *g* for 5 min. The cells were then washed once in 1% K ascorbate buffer (pH 6·0) followed by resuspension in 2 ml of this buffer. The cell suspension was immediately heated to 100° for 10

[14] G. Combepine, E. A. Cossins and K. L. Lor, *FEBS Letters* **14**, 49 (1971).
[15] K. L. Lor and E. A. Cossins, *Biochem. J.* **130** (1972) in press.
[16] Y. Morimura, *Plant Cell Physiol.* **1**, 63 (1959).
[17] O. Hara and R. Silber, *J. Biol. Chem.* **244**, 1988 (1969).
[18] R. T. Taylor, H. Dickerman and H. Weissbach, *Arch. Biochem. Biophys.* **117**, 405 (1966).
[19] J. M. Lord and M. J. Merrett, *Biochem. J.* **117**, 929 (1970).
[20] M. Cramer and J. Myers, *Arch. Mikrobiol.* **17**, 384 (1952).

min and after cooling to 4° was sonicated at 2° (3 pulses of power output 15 sec each at 2°, Fisher Ultrasonic Generator, Model BPO, Blackstone Ultrasonics Inc., Sheffield, Pa., U.S.A.). The extract was finally centrifuged at 10 000 *g* for 20 min and the supernatants were diluted 2 × with 1% K ascorbate (pH 6·0). Extracts which were subsequently fractionated by DEAE-cellulose[21] were prepared from 800–1000 ml aliquots of culture.

Microbiological assay of folate derivatives. Folates were assayed using *L. casei* (ATCC 7469) and *P. cerevisiae* (ATCC 8081).[22] Growth was measured by titration of the lactic acid produced after 70 hr incubation at 37°, using authentic PteGlu and 5-HCO-H_4PteGlu for *L. casei* and *P. cerevisiae* respectively for calibration.[10] Folate derivatives were assayed with *L. casei* after the cell-free extracts had been incubated with the γ-glutamylcarboxypeptidase from 3-day-old pea cotyledons.[10]

Enzyme studies. At different stages of the cell cycle 600 ml aliquots of the culture were withdrawn and the cells were harvested by centrifugation. The sedimented cells were suspended in 2 ml of 0·05 M K phosphate buffer (pH 6·9) containing 5 mM 2-mercaptoethanol and sonicated as described above. After centrifugation at 18 000 *g* for 20 min the supernatant was retained for subsequent enzyme assays. Formyltetrahydrofolate synthetase activity was measured as described by Hiatt.[23] Serine hydroxymethyltransferase was assayed by the method of Taylor and Weissbach[24] and 5,10-methylenetetrahydrofolate reductase according to Dickerman and Weissbach.[25] Methyltetrahydrofolate: homocysteine methyltransferase was assayed by the method of Dodd and Cossins.[26] Protein was determined colorimetrically[27] using crystalline egg albumin as a reference standard.

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[21] H. Sotobayashi, F. Rosen and C. A. Nichol, *Biochemistry* **5**, 3878 (1966).
[22] H. A. Bakerman, *Analyt. Biochem.* **2**, 558 (1961).
[23] A. J. Hiatt, *Plant Physiol.* **40**, 184 (1965).
[24] R. T. Taylor and H. Weissbach, *Analyt. Biochem.* **13**, 80 (1965).
[25] H. Dickerman and H. Weissbach, *Biochem. Biophys. Res. Commun.* **16**, 593 (1964).
[26] W. A. Dodd and E. A. Cossins, *Biochim. Biophys. Acta* **201**, 461 (1970).
[27] O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).