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GENERATION OF ONE-CARBON UNITS IN METHIONINE-SUPPLEMENTED *EUGLENA* CELLS*

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Abstract—The possible effect of L-methionine supplements on the folate metabolism of division-synchronized *Euglena gracilis* (strain Z) cells has been examined. Cells receiving 1 mM L-methionine for four cell cycles were examined for folate derivatives, prior to and during cell division. Before cell division, methionine-supplemented cells contained less formylfolate but more methylfolate than unsupplemented cells. During division, both types of folates were present in lower concentrations in the supplemented cells. Growth in methionine for 10 and 34 hr also increased the levels of free aspartate, threonine, serine, cysteine and methionine relative to the controls. Methionine-supplemented cells contained *ca* 50% of the 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) activity per cell of unsupplemented control cultures and specific enzyme activity was reduced *ca* 90%. Supplemented cells contained almost twice as much serine hydroxymethyltransferase (EC 2.1.2.1) activity per cell but comparable levels of glycollate dehydrogenase. Growth in methionine also reduced the incorporation of formate-[^{14}C] into serine, RNA, DNA, adenine and protein methionine. In contrast, incorporation of glycine-[2-^{14}C] and serine-[3-^{14}C] into folate-related products was not greatly altered by this treatment. Levels of radioactivity in these products suggested that formate was a more important C_1 unit source than glycine or serine when growth occurred in unsupplemented medium. It is concluded that methionine reduces formylfolate production by an effect on the cellular levels of formyltetrahydrofolate synthetase.

INTRODUCTION

In our earlier *Euglena* studies [1] evidence was presented for generation of C_1 units via the 10-formyltetrahydrofolate synthetase reaction (EC 6.3.4.3). In division-synchronized cultures, this reaction appeared to be closely related to glycollate oxidation and formate-[^{14}C] was rapidly incorporated into serine and methionine. There is also evidence that *Euglena*, in common with other organisms, can generate methylenetetrahydrofolate from serine [1] and glycine [2]. These latter substrates may have more importance in folate metabolism when growth conditions restrict the production of formylfolates but there is still very little known about the control of these pathways in photosynthetic tissues.

In contrast there is evidence that methionine and its metabolic products regulate folate metabolism in heterotrophs [3]. In such cases, control is commonly exerted on methylenetetrahydrofolate reductase [4–6] but in other species, oxidation of 10-HCOH$_4$PteGlu [7] and net folate biosynthesis [8] are altered. There are reports [9–11] that L-methionine inhibits various folate-dependent enzymes from non-photosynthetic plant tissues. However, the physiological significance of these observations has still to be fully assessed.

The present studies have examined possible effects of exogenous methionine on the C_1 metabolism of division-synchronized *Euglena* cultures. Cells receiving a 1 mM L-methionine supplement contained less formyltetrahydrofolates and the specific activity of 10-formyltetrahydrofolate synthetase was decreased by more than 90%. The supplemented cultures also had decreased ability to incorporate formate-[^{14}C] into various products of folate metabolism.

RESULTS

Folate and amino acid pool sizes in methionine-supplemented cells

When division-synchronized *Euglena* cultures (14 hr light: 10 hr dark) were transferred to media containing 1 mM L-methionine, slightly fewer (*ca* 25%) divisions occurred during the ensuing dark periods. Despite this slower growth rate, the supplemented cells maintained a synchronous growth pattern and accumulated methionine from the culture medium.

Culture in the presence of methionine reduced the concentration of highly conjugated formylfolates (Table 1). These derivatives accounted for *ca* 80 and 60% of total cellular folates in the control and supplemented cultures, respectively. Prior to cell division (10 hr of light phase), the total recovered folate was comparable but methylfolate levels were higher in the supplemented cells. Changes in folate pool sizes were also observed as cell divisions commenced during the dark phase of

* The abbreviations used for derivatives of folic acid are those suggested by the IUPAC–IUB Commission listed in (1967) *Biochem. J.* **102**, 15, e.g. 10-HCOH$_4$PteGlu = N^{10}-formyltetrahydropteroylmonoglutamate. AdoMet = *S*-adenosylmethionine.

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Table 1. Concentrations of folate derivatives after growth in L-methionine-supplemented media

Derivative	Folate concentrations (ng PteGlu equivalents/10^7 cells) Light phase Control	Light phase L-Met	Dark phase Control	Dark phase L-Met
$HCOH_4PteGlu_{1-2}$	3.2	2.2	2.2	1.1
$HCOH_4PteGlu_n$	90.3	68.7	86.0	31.9
$5\text{-}MeH_4PteGlu$	1.2	1.5	1.1	0.6
$5\text{-}MeH_4PteGlu_n$	9.3	15.9	26.8	5.7
$5\text{-}HCOH_4PteGlu_3$ / $5\text{-}MeH_4PteGlu_3$	12.3	16.1	7.1	12.9
Total folate recovered	116.3	104.4	123.2	52.2

Cells were grown synchronously to a density of 3×10^3 cells/ml. At the start of the next light phase, L-methionine (1 mM) was added. Extracts for folate analyses were prepared after 10 hr of the light phase and after 1 hr of the dark phase during the fourth cell cycle. Extracts were chromatographed on DEAE-cellulose before and after γ-glutamylcarboxypeptidase treatment. Assays were performed in triplicate using *L. casei*.

Table 2. Free amino acid concentrations during growth in methionine supplemented medium

Free amino acid	Amino acid pool size (μmol/10^8 cells) 10 hr culture Control	10 hr culture L-Met	34 hr culture Control	34 hr culture L-Met
Aspartate	5.2	10.4	6.9	27.9
Threonine	2.1	3.0	3.1	4.1
Serine	9.3	12.8	10.9	16.8
Glutamate	7.2	9.8	20.0	32.5
Glycine	21.9	21.7	27.4	39.0
Alanine	21.1	25.8	31.6	38.0
Half Cystine	1.8	4.8	4.7	5.5
Methionine*	nd	3.1	nd	7.5

* Including methionine sulphoxide.

Cells were grown with aeration in continuous light to a density of *ca* 3×10^5 cells per ml. L-Methionine, final concentration, 1 mM was then added and culture in light was continued for 10 and 34 hr, respectively. Data are for amino acids related to folate and methionine metabolism or where changes greater than 50% of the controls were observed. Values given are averages of 3 separate analyses. nd = Not detected by amino acid analyser.

growth. Cells receiving methionine contained much less total folate and both formyl- and methylpolyglutamate derivatives were present at lower concentrations.

The methionine supplement also changed the pool sizes of several free amino acids (Table 2). Culture under a 14 hr light:10 hr dark regime or in continuous light gave similar results. Of particular note were increases in aspartate, threonine and serine. Methionine was also detectable in the supplemented cells and by 10 hr these cultures contained levels of cysteine/cystine that were more than double those of the controls.

Effects of methionine on enzyme levels and on the metabolism of C_1 unit precursors

The above analyses suggest that methionine-grown cells may produce less formylfolates or conversely that these growth conditions favour their more rapid utilization. In addition, the greater levels of methylpolyglutamate in the supplemented cells are inconsistent with a

Table 3. Levels of glycollate dehydrogenase and key folate-dependent enzymes in methionine-supplemented and unsupplemented cells

Enzyme	Product formed/mg protein Control	Product formed/mg protein L-Met	Product formed/10^7 cells Control	Product formed/10^7 cells L-Met
Glycollate dehydrogenase	1.8	1.4	1.7	2.1
Serine hydroxymethyltransferase	23	30	37	62
Formyltetrahydrofolate synthetase	270	24	1260	320

Cells were harvested at 10 hr of the light phase (4th cell cycle) of culture in media containing 1 mM L-methionine or lacking this supplement. The dehydrogenase is expressed in enzyme units as defined in Experimental. The other enzymes are expressed in nmol product formed/hr. Data are the averages of 3 separate determinations.

control of folate metabolism at the methylenetetrahydrofolate reductase step [5, 6]. To examine some of these possibilities, cultures receiving methionine were examined for various enzymes and for ability to utilize three potential sources of C_1 units.

Growth in the presence of methionine had little effect on glycollate dehydrogenase levels, expressed on a per cell or protein basis (Table 3). However, comparable data for serine hydroxymethyltransferase and 10-formyltetrahydrofolate synthetase revealed more striking differences. The amounts of transferase activity on a per cell basis were *ca* 70% higher than those of control cultures. The sp. act. of this enzyme was 30% higher in the supplemented cells. Formyltetrahydrofolate synthetase activities were reduced by growth in methionine; activity per 10^7 cells being only 25% of the controls and specific

Table 4. Metabolism of formate-[^{14}C] by methionine-supplemented and control cultures

Fraction	^{14}C incorporated (dpm/1.8 × 10^5 cells) Control: no supplement	1 mM L-Met supplemented
Free amino acids		
Glycine	12.1 (101)	nd
Serine	63.0 (495)	57.3 (348)
Methionine	nd	340 (1450)
Threonine	nd	16.9 (18)
Others	120.1	73.1
Protein amino acids		
Serine	560 (50)	300 (25)
Methionine	750 (178)	92 (26)
Others	79	5.6
RNA	165	65
DNA	95.5	47
Adenine	251 (837)	119 (202)
Sugars	234	32.3
Organic acids	332	158
Acetone solubles	498	132
Total ^{14}C recovered	3159	1438

Cells harvested at 10 hr of the light phase (3rd cell cycle) were centrifuged and resuspended in unsupplemented medium before 30 min equilibration in the dark at 25°C. Formate-[^{14}C], 10 μCi (sp. act. 50 μCi/μmol) was then added and incubation in the dark continued for a 30 min feeding period. Data in parentheses are sp. act. (dpm/μmol × 10^{-3}). nd = Not detected.

Table 5. Metabolism of glycine-[2-^{14}C] and serine-[3-^{14}C]

Fraction	^{14}C incorporated (dpm/1.8 × 10^5 cells) Glycine-[2-^{14}C] Control	L-Met	Serine-[3-^{14}C] Control	L-Met
Free amino acids				
Glycine	12.8 (111)	17.6 (143)	3.1 (23)	1.3 (14)
Serine	7.3 (62)	8.5 (52)	24 (132)	29.3 (211)
Methionine	nd	12.8 (51)	nd	45.3 (205)
Others	8.1	8.0	11.0	9.8
Protein amino acids				
Serine	42.7 (3.6)	35.9 (3.6)	85.3 (7)	60 (5)
Methionine	13.7 (3.6	1.7 (0.5)	20 (5)	3 (0.7)
Glycine	61.3 (2.6)	76 (4)	4 (0.2)	3 (0.1)
Others	16.3	5.6	33.7	13.3
RNA	18.5	24.4	21.3	21.0
DNA	13.8	15.4	10.2	12
Adenine	6.5	6.8	13.5	7.1
Sugars	32.9	10.5	17	14
Organic acids	13.3	17.9	23	24
Acetone solubles	41	99	66	95
Total ^{14}C recovered	288.2	340.1	332.1	338.1

Cells were harvested and preincubated in the dark as in Table 4. 10 μCi of glycine-[2-^{14}C] (57.0 μCi/μmol) and 10 μCi of serine-[3-^{14}C] (56 μCi/μmol), respectively, were then supplied for 30 min. Data in parentheses are sp. act. (dpm/μmol × 10^{-3}). nd = Not detected.

enzyme activity being only 9% (Table 3) of the control value.

To determine whether these differences in enzyme levels affected the generation of C_1 units *in vivo*, methionine-grown cells were incubated with formate-[^{14}C], glycine-[2-^{14}C] and serine-[3-^{14}C], respectively. The supplemented cells incorporated less than half the formate carbon of the controls (Table 4). The total incorporations (free + protein) of ^{14}C into methionine and serine were also significantly lower in the supplemented cells despite some trapping of label in the enlarged free methionine pool. Other major products, including DNA, RNA and adenine were less radioactive in the treated cells. The data tend to support the contention that methionine growth reduces the generation of formylfolate. However, the high sp. act. of free methionine in such cells (Table 4) argues against a severe restriction in the flow of C_1 units between formyl- and methylfolate. To assess this latter situation, two sources of methylenefolate, viz. glycine-[2-^{14}C] and serine-[3-^{14}C], were supplied to control and methionine-grown cells (Table 5).

Both substrates labelled methionine, adenine and the nucleic acid fractions but carbon flow to these products was below that observed in the formate feedings (Table 4). Furthermore, the total incorporation of the label into methionine (free + protein) was not decreased in the methionine-grown cells. For serine, *ca* twice as much C-3 entered methionine when the cells received the supplement. As in the formate experiments, trapping of ^{14}C was observed in the enlarged free methionine pool and as a consequence, the specific radioactivities of protein methionine were lowered (Table 5).

DISCUSSION

In previous work [1] we suggested that the formyltetrahydrofolate synthetase reaction may be the principal route for generation of metabolically important folates in *Euglena*. The results of the present studies also support this conclusion and further suggest that methionine or its metabolic products may modulate formylfolate biosynthesis. For example, the levels of this enzyme in unsupplemented cells were significantly higher (Table 3) than those of serine hydroxymethyltransferase. This difference is particularly noteworthy as the majority of heterotrophic species generate C_1 units from serine [12]. Formylfolates were also the principal folates of *Euglena* cells (Table 1) and formate-[^{14}C] was a better precursor of folate-related products than either glycine-[2-^{14}C] or serine-[3-^{14}C] (Tables 4 and 5).

There is also evidence [1] that the substrate for the synthetase reaction may arise from the metabolism of glycollate. In this regard, the origins of C_1 units in light-grown *Euglena* cells could be distinct from those used by heterotrophs. It is also conceivable that this principal reaction for C_1 unit generation is finely regulated in growing *Euglena* cultures. It was noted earlier [1] that a product of glycollate oxidation may be necessary for maintenance of high synthetase levels. It now appears likely that adequate supplies of methionine may directly or indirectly curtail 10-$HCOH_4PteGlu_n$ production via possible repression of the synthetase (Tables 1 and 3). There have been no previous reports of such control by methionine but a similar overall effect is achieved in mammalian cells [7]. Krebs *et al.* [7] showed that methionine supplementation of liver cells promoted the oxidation of C_1 units. They suggested that AdoMet, produced by the treated cells, inhibited methylenetetrahydrofolate reductase which in turn would increase the concentrations of methylene- and formylfolates. The latter derivatives are normally present at low concentration in liver cells, a situation preventing their oxidation via 10-formyltetrahydrofolate dehydrogenase. However, when dietary methionine is available in excess, the 10-$HCOH_4PteGlu_n$ concentration rises above the K_m value for the dehydrogenase and C_1 units are oxidized to CO_2.

The situation in methionine-supplemented *Euglena* cells is clearly different. Methionine decreased formylfolate concentrations (Table 1) and, during the light phase of growth, methylfolate levels were elevated. This latter

observation argues against an inhibition of methylenetetrahydrofolate reductase and this is supported by the glycine and serine feeding experiments (Table 5), where flow of ^{14}C to methionine was not reduced. We have not examined the effect of AdoMet on the methylenetetrahydrofolate reductase of *Euglena*. However, analyses of cell extracts showed comparable or only *ca* 20% higher levels of AdoMet in methionine grown cells.

Another feature of methionine-supplemented *Euglena* cells was their increased serine hydroxymethyltransferase level (Table 3). The increased ability of these cells to form methylenefolate might explain why more serine carbon entered the free and protein pools of methionine. It is not clear why the hydroxymethyltransferase was more active in the treated cells. Serine appears to serve as an alternative source of methylenetetrahydrofolate in *Euglena* and as such, could assume greater importance when formate activation is curtailed. This increased involvement of serine in folate metabolism could be enhanced if methionine effectively conserved tetrahydrofolate for other pathways. The work of Codd and Merrett [13, 14] and of Murray *et al.* [15] shows that serine hydroxymethyltransferase has importance in the glycollate pathway of *Euglena*. There is however no detailed information on factors controlling methylenetetrahydrofolate production form this source or the extent of possible equilibrium between such folate and that arising from formate and glycine. These aspects of folate metabolism warrant study in autotrophic species where methylenetetrafolate can arise from more than one source.

Methionine supplements also changed the concentrations of amino acids (Table 2) metabolically related to aspartate and methionine. There is now good evidence that methionine and AdoMet exert control over the pathway leading to homocysteine [16]. In some plants [17, 18] this allows diversion of methionine precursors into the threonine pathway. Preliminary homoserine feeding experiments suggest that this may occur in *Euglena* cells supplied with an exogenous source of methionine.

EXPERIMENTAL

Materials. Sodium formate-[^{14}C], glycine-[2-^{14}C] and serine-[3-^{14}C] were purchased from Amersham-Searle, Des Plaines, Illinois. Other chemicals were obtained from Sigma, St. Louis and from Fisher Scientific, Edmonton. Micro-organisms were obtained from the American Type Culture Collection.

Culture of Euglena. *E. gracilis* Klebs (strain Z) ATCC 12716 was grown autotrophically on a mineral salts [19] medium (pH 6.8) which had been modified as described previously [20]. The cultures were division-synchronized at 25° by imposing light:dark cycles of 14:10 hr [21]. The light intensity was 3500 lx, provided by cool white fluorescent lamps. Moistened sterile air (600–700 ml/min) was continuously passed through the cultures. Culture densities were measured with a haemocytometer after HCHO fixation. Cultures with initial densities of *ca* 3×10^3 cells/ml were in some cases, supplemented with Millipore-filtered L-methionine soln to give a final concn of 1 mM. All such supplements were made at the end of the 10 hr dark period.

Extraction and chromatography of folate derivatives. Cell-free extracts were prepared [20] and subjected to DEAE-cellulose chromatography [22] prior to assay for folates. The standard folate assay procedure of ref. [23] was employed with *Lactobacillus casei* ATCC 8081. Polyglutamylfolates were assayed after treatment of the extracts with γ-glutamylcarboxypeptidase [24].

Enzyme studies. Cells (5×10^4–1×10^5/ml) were harvested by centrifugation at 10 hr of the 4th cell cycle following culture in the presence or absence of L-methionine. The cellular pellet was resuspended in 2 ml 5 mM K-Pi buffer (pH 6.9) containing 5 mM mercaptoethanol and sonicated [20] at 2°. After centrifugation (18000 *g* for 20 min) to remove cellular debris, the extracts were passed through columns (1 × 5 cm) of Sephadex G-15. The resulting desalted prepn was used as a source of enzyme. Formyltetrahydrofolate synthetase (EC 6.3.4.3), serine hydroxymethyltransferase (EC 2.1.2.1) and glycollate dehydrogenase were assayed by standard methods [25–27]. One unit of the latter enzyme was taken as the amount causing a decrease in *A* of 0.01/min at 590 nm.

^{14}C *Feeding experiments.* After growth in the presence or absence of L-methionine, cells were harvested at 10 hr of the third cycle by centrifugation. The cells were washed twice by resuspension in 5 ml of unsupplemented growth medium. After final resuspension in 5 ml of growth medium the cultures were placed in the dark for 30 min at 25°. The labelled substrate (10 μCi, glycine-[2-^{14}C], sp. act. 57 μCi/μmol; 10 μCi serine-[3-^{14}C], sp. act. 56 μCi/mol; or 10 μCi, formate-[^{14}C], sp. act. 50 μCi/μmol) was then added, followed by incubation in the dark for 30 min. The cells were then centrifuged and extracted with 5 ml 20% TCA. Protein and nucleic acid fractions were isolated by the method of ref. [28]. Amino acids were isolated using an amino acid analyser equipped with stream division [29]. Radioactivities were measured by liquid scintillation counting [30] with efficiencies of *ca* 70%.

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