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INTERRELATIONSHIPS BETWEEN ONE-CARBON METABOLISM AND THE GLYCOLATE PATHWAY IN DIVISION SYNCHRONIZED CULTURES OF EUGLENA GRACILIS

by

KIM-LOON LOR

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled INTERRELATIONSHIPS BETWEEN ONE-CARBON METABOLISM AND THE GLYCOLATE PATHWAY IN DIVISION SYNCHRONIZED CULTURES OF *EUGLENA GRACILIS* submitted by KIM-LOON LOR in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Biochemistry.

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External Examiner

DATE October 2nd 1974

ABSTRACT

Synchronized growth of *Euglena gracilis* Klebs (Strain Z) was achieved in an aerated inorganic salts medium at 25°C employing a 14:10 hr light-dark cycle. The levels of pteroylglutamate derivatives were determined throughout the cell cycle by microbiological assay using *Lactobacillus casei* (ATCC 7469) and *Tetrahymena pyriformis* (ATCC 8081). The levels of pteroylglutamate derivatives increased rapidly on a per cell basis during illumination while cell numbers remained constant whereas in darkness cell numbers were approximately doubled and the concentration of the derivatives per cell declined. Analysis of the pteroylglutamate pool by DEAE-cellulose column chromatography revealed that formyl- and methyl-pteroylglutamates were rapidly synthesized during the light period. In contrast, no net synthesis of these compounds was detected during the dark period. Enzyme studies using cell-free extracts show that the levels of 10-formyltetrahydrofolate synthetase, serine hydroxymethyltransferase and 5-methyltetrahydrofolate: homocysteine transmethylase increased on a per cell basis during the light phase and decreased during the dark phase. In contrast the levels of 5,10-methylenetetrahydrofolate reductase decreased during the light phase but increased as the cells divided.

When cells were cultured in high CO_2 (5% in air) for four cell cycle, the pool of formyl pteroylglutamates was markedly decreased but an accumulation of methyl derivatives occurred. This treatment appeared to cause repression of glycolate dehydrogenase and 10-formyl-tetrahydrofolate synthetase but gave increases in the levels of serine

hydroxymethyltransferase. These effects were reversed when such cells were subsequently cultured in low CO_2 (0.03% in air). Culture in the presence of α-hydroxy-2-pyridinemethane sulfonate also reduced formyl pteroylglutamate pool size, the levels of glycolate dehydrogenase and 10-formyltetrahydrofolate synthetase.

The effect of high CO_2 concentration on 10-formyltetrahydrofolate synthetase was further examined *in vivo* by incubating the cells with sodium [^{14}C]formate in the presence of high and low CO_2. Such feeding experiments indicated that cells cultured for four cell cycles in high CO_2 had less ability to incorporate [^{14}C]formate into serine than cells cultured in air but were capable of producing larger amounts of $^{14}CO_2$. Incorporation of [^{14}C]formate into glycine, alanine, glutamic acid and sugars, was also affected by high CO_2 treatments. An enzyme, catalyzing the production of formic acid from C-2 of glyoxylate was found to be present in cells cultured under both conditions.

It is concluded that one-carbon units, principally at the formyl level of oxidation, are produced from glycolate in *Euglena*. Operation of the glycolate pathway and formation of these one-carbon units appear to be regulated by the concentration of CO_2 available to the cells. Exogenous L-methionine also regulated synthesis of formylpteroylglutamates conceivably by its effect on 10-formyltetrahydrofolate synthetase levels. Under these latter conditions, the serine hydroxymethyltransferase reaction appeared to have more importance in the generation of one-carbon units. The significance of these control mechanisms and the interrelationships between pteroylglutamate-mediated one-carbon metabolism and the glycolate pathway in *Euglena* are discussed.

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I sincerely wish to thank Dr. Edwin A. Cossins for his assistance, encouragement during this study and for help in the preparation of this thesis and subsequent publications.

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LIST OF ABBREVIATIONS

*10-HCO-H_4PteGlu	:	10-formyltetrahydropteroylglutamate
PteGlu	:	pteroylmonoglutamic acid
$PteGlu_n$	:	pteroylpolyglutamic acid (indeterminate number of glutamic acid moieties)
SAM	:	S-adenosyl-L-methionine
α-HPMS	:	α-hydroxy-2-pyridinemethane sulfonate
EC	:	Enzyme Commission
ATCC	:	American Type Culture Collection
DEAE-cellulose	:	diethylaminoethyl-cellulose
t-RNA	:	transfer RNA
μCi	:	microcurie
cpm	:	counts per minute
ATP	:	adenosine triphosphate
NAD(P)	:	nicotinamide adenine dinucleotide (phosphate)
PALP	:	pyridoxal-5'-phosphate

*The abbreviations used for pteroylglutamic acid and its derivatives are those suggested by the IUPAC-IUB Commission as listed in the *Biochemical Journal* 102: 15 (1967).

All other abbreviations are those commonly used in biochemical literature.

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INTRODUCTION

Derivatives of H_4PteGlu, a reduced form of PteGlu or folic acid, act as coenzymes in many metabolic reactions (Blakley, 1969). These coenzymes are primarily concerned with the transfer of one-carbon units at the oxidation levels of formate, formaldehyde and methanol and in transforming these one-carbon units from one oxidation state to another. In biological materials these derivatives may be present as such or as conjugated forms having various numbers of glutamic acid residues attached in γ-peptide linkage.

Pteroylglutamate derivatives are now commonly assayed microbiologically using *Lactobacillus casei*, *Streptococcus faecalis* R and *Pediococcus cerevisiae* (Bakerman, 1961) and can conveniently be isolated by DEAE-cellulose chromatography (Silverman *et al.*, 1961; Sotobayashi *et al.*, 1966). The occurrence of these derivatives has now been demonstrated in a wide variety of biological systems. In *Saccharomyces cerevisiae*, a large proportion of the derivatives are conjugated and contain more than three glutamate residues (Schertel *et al.*, 1965; Lor and Cossins, 1972). The major compounds were 5-methyl derivatives with lesser amounts of 5- and 10-HCO-H_4PteGlu. In most animal tissues (Cropper and Scott, 1966; Noronha and Aboobaker, 1963) and higher plants (Roos *et al.*, 1968; Shah *et al.*, 1970; Roos and Cossins, 1971; Rohringer *et al.*, 1969) 5-CH_3-H_4PteGlu and its conjugated derivatives are commonly the principal components of the pteroylglutamate pool. There was no detailed information on the occurrence of such derivatives in algae, until the present work (Lor and Cossins, 1973) revealed that in *Euglena*

gracilis, highly conjugated methyl and formyl derivatives were major components of the pteroylglutamate pool at all stages of the cell cycle.

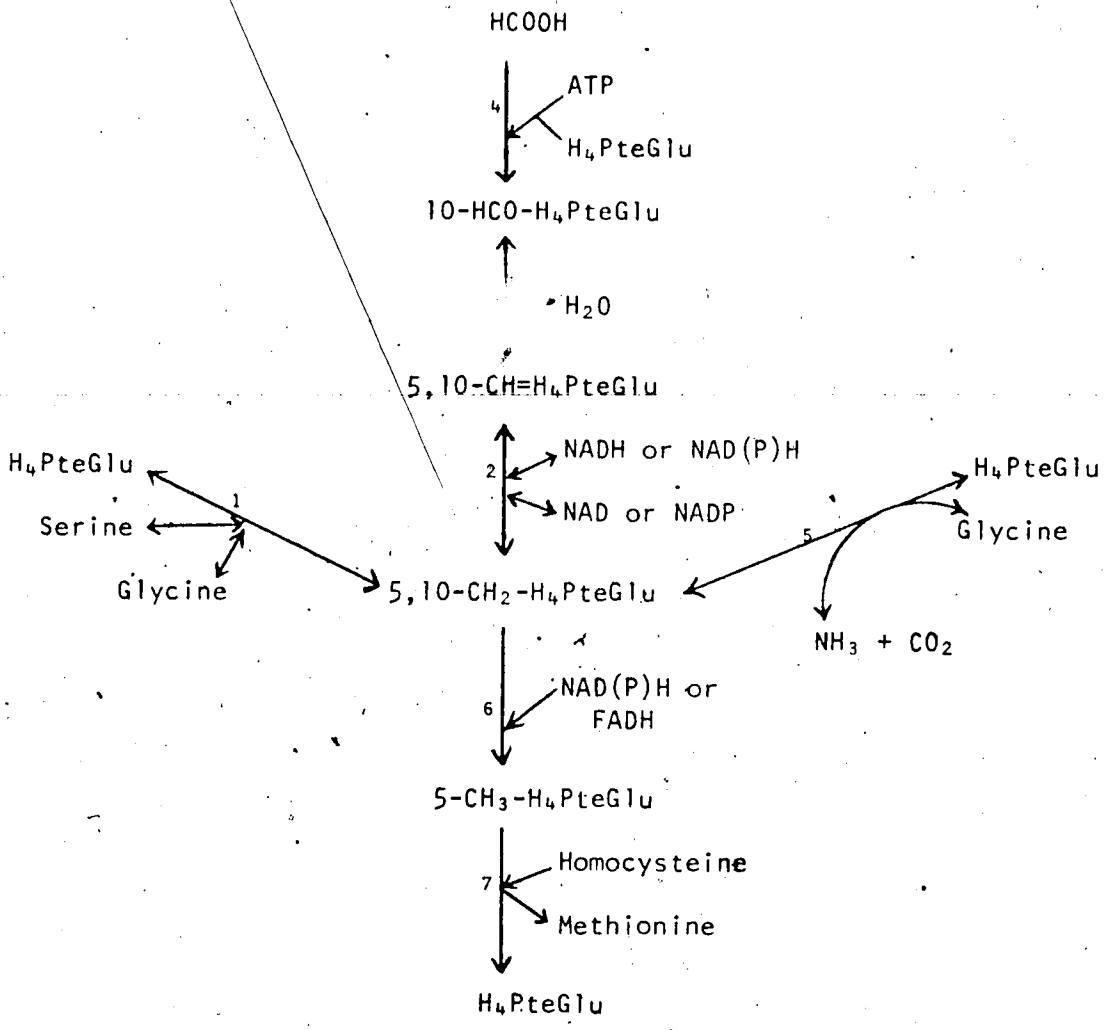
Source of one-carbon units for the pteroylglutamate pool

Scheme 1 summarizes the major reactions now known to be involved in oxidation and reduction of the activated one-carbon units within the pteroylglutamate pool. It is generally believed that the major biological sources of such units are serine, formate and glycine.

Serine hydroxymethyltransferase (Scheme 1, reaction 1) which catalyzes the reversible formation of 5,10-CH_2-H_4PteGlu and glycine from serine and H_4PteGlu is thought to be the first reaction in the synthesis of the majority of one-carbon units in animals. This enzyme has been detected in bacteria (Wright, 1955), plants (Cossins and Sinha, 1966; Clandinin and Cossins, 1972), and in animal tissues (Blakley, 1954; Chan and Schirch, 1973). As a result of this reaction the ß-carbon of serine may enter the pteroylglutamate pool at the hydroxymethyl level of oxidation. Formyltetrahydrofolate synthetase (Scheme 1, reaction 4) has also been detected in a large number of microorganisms, plants and animals (Blakley, 1969). The normal physiological role of this enzyme is generally believed to be the synthesis of metabolically active one-carbon units from formate (Greenberg *et al.*, 1955; Whiteley *et al.*, 1958) in a reaction requiring ATP (Blakley, 1969). Several bacterial species which utilize purines for growth (Rabinowitz and Pricer, 1962; Whiteley *et al.*, 1959) have high levels of this synthetase. In other bacterial species, this activity is low or not detectable (Whiteley *et al.*, 1959; Albrecht and Hutchinson, 1964). It should be emphasized that the actual importance of this enzyme in

SCHEME 1. The major reactions for production of one-carbon units and interconversion of H_4PteGlu derivatives

Reaction No.	Trivial Name of Enzyme	Systematic Name of Enzyme	E.C. Number
1	Serine hydroxymethyltransferase	L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase	2.1.2.1
2	5,10-methylenetetrahydrofolate dehydrogenase	5,10-methylenetetrahydrofolate: NADP oxidoreductase	1.5.1.5
3	Methenyltetrahydrofolate cyclohydrolase	5,10-methenyltetrahydrofolate 5-hydrolase	3.5.4.9
4	10-formyltetrahydrofolate synthetase	Formate:tetrahydrofolate ligase (ADP)	6.3.4.3
5	Glycine decarboxylase or glycine splitting enzyme		
6	5,10-methylenetetrahydrofolate reductase	5,10-methylenetetrahydrofolate: NADP oxidoreductase	1.1.1.68
7	5-methyltetrahydrofolate: homocysteine methyltransferase		2.1.99


HCOOH
4
ATP
H_4PteGlu
10-HCO-H_4PteGlu
H_2O
5,10-CH=H_4PteGlu
2
NADH or NAD(P)H
NAD or NADP
H_4PteGlu
Serine
1
Glycine
5,10-CH_2-H_4PteGlu
5
H_4PteGlu
Glycine
NH_3 + CO_2
6
NAD(P)H or
FADH
5-CH_3-H_4PteGlu
7
Homocysteine
Methionine
H_4PteGlu

metabolic generation of active one-carbon units has not really been clearly established, particularly as 10-HCO-H_4PteGlu can be readily generated by oxidation of 5,10-CH_2-H_4PteGlu in the 5,10-methylenetetrahydrofolate dehydrogenase reaction (Scheme 1, reaction 2).

In *Escherichia coli*, *Peptococcus glycinophilus* and mammalian liver, glycine molecules are cleaved in a reaction (Scheme 1, reaction 5) involving H_4PteGlu and PALP to yield CO_2, NH_3 and 5,10-CH_2-H_4PteGlu (Kawosaki *et al.*, 1966; Sato *et al.*, 1969; Motokawa and Kikuchi, 1971; Yoshida and Kikuchi, 1970; Yoshida and Kikuchi, 1971; Klein and Sagers, 1966a,b). There is some evidence that such a splitting reaction may also occur in plants (Sinha and Cossins, 1964; Cossins and Sinha, 1966; McConnell, 1964; Clandinin and Cossins, 1972). In this connection the reaction has been implicated in the glycolate pathway (Tolbert and Yamazaki, 1969; Bruin *et al.*, 1970) and in photorespiration (Kisaki and Tolbert, 1970). The possible significance of this reaction in plant metabolism will be described in a later section of this Introduction.

In early basic studies of one-carbon metabolism it was also shown that several other compounds could serve as sources of one-carbon units. These included the methyl groups of choline, acetone, dimethylglycine and sarcosine (Seigel and Lafaye, 1950; Mitoma and Greenberg, 1952; Sakami, 1949; Siekevitz and Greenberg, 1950; Sakami, 1950; MacKenzie, 1950; MacKenzie and Abeles, 1956; MacKenzie and Frisell, 1958); the formimino groups of formiminoglutamic acid and formiminoglycine (Tabor and Wyngarden, 1959; Rabinowitz and Pricer, 1956).

Metabolic interconversion of H_4PteGlu derivatives

Derivatives of H_4PteGlu are known to be freely interconverted

through several enzyme-mediated reactions. The key reactions involved are summarized in Scheme 1.

In the presence of NADP and 5,10-methylene-H_4PteGlu dehydrogenase, 5,10-CH_2-H_4PteGlu, formed from free formaldehyde, C-3 of serine or C-2 of glycine, may be oxidized to 5,10-CH=H_4PteGlu (Scheme 1, reaction 2) (Osborne and Huennekens, 1957; Uyeda and Rabinowitz, 1967a; Wong and Cossins, 1966; Cossins *et al.*, 1970). Partial purification of 5,10-CH_2-H_4PteGlu dehydrogenase from yeast (Ramasastri and Blakley, 1964), *Escherichia coli* (Donaldson *et al.*, 1965), calf thymus (Yeh and Greenberg, 1965), *Salmonella thyphimurium* (Dalal and Gots, 1967) and pea seedlings (Cossins *et al.*, 1970) have been described and some properties of this enzyme have been investigated. Hydration of 5,10-CH=H_4PteGlu usually occurs to 10-HCO-H_4PteGlu (Scheme 1, reaction 3) by action of cyclohydrolase (Rabinowitz and Pricer, 1956; Tabor and Rabinowitz, 1956; Tabor and Wyngarden, 1959).

Reduction of 5,10-CH_2-H_4PteGlu to 5-CH_3-H_4PteGlu (Scheme 1, reaction 6) in the presence of reduced pyridine nucleotides is a key reaction in the biogenesis of methyl groups. The equilibrium of this reaction, catalyzed by 5,10-CH_2-H_4PteGlu reductase, strongly favors the formation of 5-CH_3-H_4PteGlu (Katzen and Buchanan, 1965). The reverse reaction can be greatly accelerated by electron accepting compounds such as menadione (Donaldson and Keresztesy, 1962). Under physiological conditions, the reaction is practically irreversible and recycling of H_4PteGlu can only take place as a result of a homocysteine-dependent transmethylation reaction (Scheme 1, reaction 7; Herbert and Zalvsky, 1962). The reductase has been detected in the livers of various vertebrates (Katzen and Buchanan, 1965; Donaldson and Keresztesy, 1962;

Kisliuk, 1963; Kutzbach and Stokstad, 1967), and in certain bacteria (Hatch *et al.*, 1961; Cathou and Buchanan, 1963; Kisliuk, 1963). When highly purified the enzyme displays a specific requirement for FADH (Guest *et al.*, 1964; Foster *et al.*, 1964), NADH only serving as a reductant when present with FAD and lipoamide dehydrogenase (Katzen and Buchanan, 1965). In this regard, it is generally agreed that FAD is bound to the enzyme *in vivo*.

5-HCO-H_4PteGlu cyclodehydrase, catalyzing the synthesis of 5-HCO-H_4PteGlu from 5,10-CH=H_4PteGlu, although present in several species (Peters and Greenberg, 1957; Greenberg *et al.*, 1965; Kay *et al.*, 1960) has not been so extensively studied. The interconvertibility of 5-HCO-H_4PteGlu and 10-HCO-H_4PteGlu by a mutase has been reported by Greenberg (1954) and investigated further by Kay *et al.* (1960). 5-HCNH-H_4PteGlu, formed by metabolism of purines (Rabinowitz and Pricer, 1956) and histidine (Borek and Waelsch, 1953), is readily deaminated and converted to 5,10-CH=H_4PteGlu by the enzyme formiminotetrahydrofolate cyclodeaminase. The latter has been purified from *Clostridium cylindrosporum* (Rabinowitz and Pricer, 1956; Uyeda and Rabinowitz, 1967b) and mammalian liver (Tabor and Rabinowitz, 1956; Tabor and Wyngarden, 1959) but has not been studied in plants. The H_4PteGlu derivatives formed by these enzymic interconversions are commonly involved in supporting the syntheses of a wide variety of cellular constituents as noted in the next section of this Introduction.

Metabolic functions of common H_4PteGlu derivatives

One-carbon units donated by various pteroylglutamate derivatives play key roles in the synthesis of certain amino acids as well as

contributing less directly in the methylation of a wide variety of cellular constituents. In purine ring biosynthesis, the H_4PteGlu derivatives play an important role as donors of carbons 2 and 8 (Buchanan and Hartman, 1959). H_4PteGlu derivatives are also involved in the introduction of one-carbon units in the synthesis of thymine, 5-hydroxymethylcytosine and 5-methyluridine (Whittaker and Blakley, 1961; Flaks and Cohen, 1959; Maley, 1962).

Participation of H_4PteGlu and 5,10-CH_2-H_4PteGlu in freely reversible interconversions of serine and glycine catalyzed by serine hydroxymethyltransferase have been demonstrated in a wide variety of organisms (Blakley, 1969) and has been noted earlier in this Introduction. 5-CH_3-H_4PteGlu or its polyglutamate derivatives are instrumental in the biosynthesis of methionine from homocysteine in a reaction catalyzed by 5-CH_3-H_4PteGlu:homocysteine methyltransferases. Two distinct systems for this have been described, one involving vitamin B_{12} and the other proceeding without this vitamin. In the vitamin B_{12} system, 5-CH_3-H_4PteGlu tri- or monoglutamate are effective methyl donors to homocysteine. Besides vitamin B_{12}, the reaction also has requirement for catalytic amounts of SAM:

$$5\text{-}CH_3\text{-}H_4PteGlu \text{ - tri- (mono-) glutamate} + \text{homocysteine} \xrightarrow[\text{SAM}]{\text{vit. } B_{12}} H_4PteGlu + \text{methionine}$$

In the non-vitamin B_{12} system, only glutamyl conjugates of 5-CH_3-H_4PteGlu are effective substrates. Vitamin B_{12} and SAM are not required:

$$5\text{-}CH_3\text{-}H_4PteGlu_{(n>1)} + \text{homocysteine} \rightarrow H_4PteGlu + \text{methionine}$$

The vitamin B_{12} system has been observed in mammalian liver extracts (Sakami and Ukstins, 1961; Mangum and Scrimgeour, 1962) but both systems have been observed in *Escherichia coli*, *Aerobacter aerogenes* and *Salmonella typhimurium* (Woods *et al.*, 1965; Morningstar and Kisliuk, 1965). A non-vitamin B_{12} transmethylase has been detected in extracts of *Euglena gracilis* (Milner and Weissbach, 1969) which is somewhat surprizing considering the vitamin B_{12} requirement for growth of this species. In work with germinating pea cotyledons, Dodd and Cossins (1969, 1970) showed that a homocysteine-dependent transmethylase with a high affinity for 5-CH_3-H_4PteGlu had importance in the *de novo* synthesis of methionine. Methionine arising from these reactions may be converted to S-adenosylmethionine by ATP:L-methionine S-adenosyltransferase (E.C. 2.5.1.6) (Mudd, 1960; Dodd and Cossins, 1970; Cantoni, 1965). SAM then functions as an important source of methyl groups in a wide variety of transmethylation reactions including methylation of t-RNA (Mandel and Borek, 1961), DNA (Gold and Hurwitz, 1961), lipids (Bremer and Greenberg, 1961) as well as biosynthesis of lignin, pectin, chlorophyll and quinones (Byerrum *et al.*, 1954; Sato *et al.*, 1958; Radmer and Bogorad, 1967; Threlfall *et al.*, 1967).

In work on the pathway of histidine biosynthesis in microorganisms, it has been shown that 10-HCO-H_4PteGlu plays a key role by donating one-carbon units for C-2 of the imidazole ring (Meister, 1965). 10-HCO-H_4PteGlu is also an important formyl donor in the formylation of methionyl-transfer ribonucleic acid (*met*-$tRNA_f$) (Dickerman *et al.*, 1967), a reaction of importance in the initiation of polypeptide synthesis.

Possible relationships between one-carbon metabolism and photosynthetic carbon metabolism

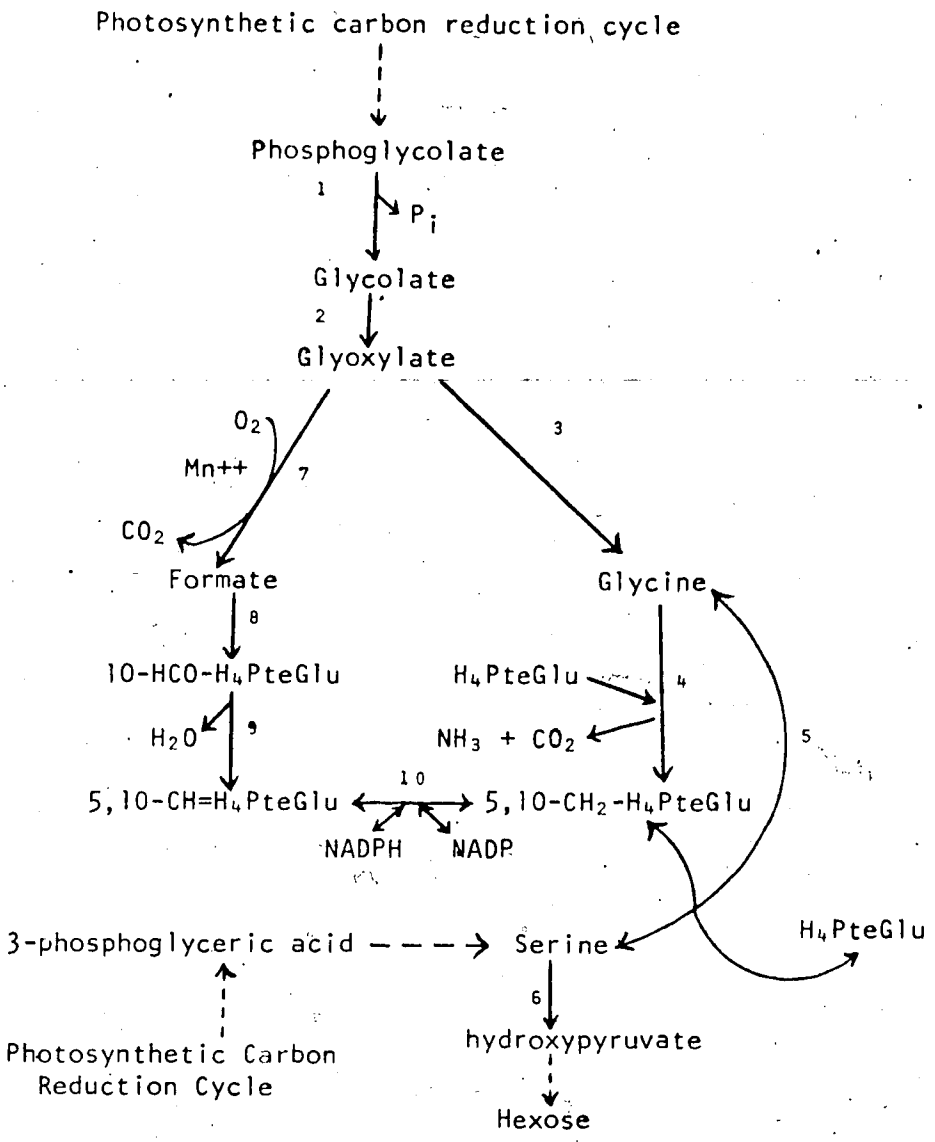
Glycolic acid is a ubiquitous product of carbon dioxide fixation via the photosynthetic carbon reduction cycle (Tolbert, 1963). Details of the metabolic fate of this compound in photosynthetic tissues were sought initially in higher plants. It has now been established that the glycolate pathway (Scheme 2) is an important route in higher plants for flow of photosynthetically fixed CO_2 into hexoses (Tolbert and Yamazaki, 1969).

Recent investigations have shown that the glycolate pathway functions in algae and that glycolate can either be metabolized via the pathway or released from the cell depending on the growth conditions. The glycolate oxidizing enzyme in algae fails to utilize oxygen as the electron acceptor and has been designated glycolate dehydrogenase to distinguish it from the glycolate oxidase of higher plants. The natural electron acceptor for algal glycolate dehydrogenase is still unknown (Merrett and Lord, 1973). Studies of higher plants have shown that the enzymes of glycolate pathway are mainly localized in the peroxisomes with some exceptions which are associated with the chloroplastic and cytoplasmic fractions (Yamazaki and Tolbert, 1970). There is evidence indicating that the glycolate dehydrogenase of *Euglena* (Lord and Merrett, 1971; Graves *et al.*, 1971a,b) and of *Chlorella* (Codd *et al.*, 1972) are localized in microbodies distinct from chloroplasts or mitochondria.

These investigations of the glycolate pathway in higher plants and algae tend to imply that pteroylglutamate derivatives participate in the metabolism of carbon recently fixed in photosynthesis. It is generally

SCHEME 2. Possible relationships between one-carbon metabolism and photosynthesis

Reaction No.	Common name of enzyme	Systematic name of enzyme	E.C. Number
1	Phosphoglycolate phosphatase	Phosphoglycolate phosphohydrolase	3.1.3.18
2	Glycolate oxidase	Glycolate:O_2 oxidoreductase	1.1.3.1
3	Serine-glyoxylate aminotransferase or glutamate-glyoxylate aminotransferase	Glycine:α-oxoglutarate aminotransferase	2.6.1.4
4	Glycine decarboxylase		
5	Serine hydroxymethyltransferase	L-Serine:tetrahydrofolate-5,10-hydroxymethyltransferase	2.1.2.1
6	Serine-glyoxylate aminotransferase		
7	Glyoxylate decarboxylase		
8	10-Formyltetrahydrofolate synthetase	Formate:tetrahydrofolate ligase (ADP)	6.3.4.3
9	5,10-Methenyltetrahydrofolate	5,10-Methenyltetrahydrofolate-5-hydrolase	3.5.4.9
10	5,10-Methylenetetrahydrofolate dehydrogenase	5,10-Methylenetetrahydrofolate: NADP oxidoreductase	1.5.1.5


Photosynthetic carbon reduction cycle
Phosphoglycolate
1
P_i
Glycolate
2
Glyoxylate
O_2
Mn++
7
3
CO_2
Formate
Glycine
8
10-HCO-H_4PteGlu
H_4PteGlu
4
H_2O
9
NH_3 + CO_2
5
10
5,10-CH=H_4PteGlu
5,10-CH_2-H_4PteGlu
NADPH
NADP
3-phosphoglyceric acid
Serine
H_4PteGlu
6
Photosynthetic Carbon Reduction Cycle
hydroxypyruvate
Hexose

claimed that an essential step of the pathway is an overall conversion of glycine molecules to serine and CO_2. Tolbert and Yamazaki (1969) have shown that glycine-^{14}C and other early intermediates of the glycolate pathway can be metabolized to serine by photosynthesizing plant tissues. The intramolecular distribution of ^{14}C in serine produced from this and related precursors suggested that such conversion involved two distinct reactions, decarboxylation of glycine and synthesis of serine (Rabson *et al.*, 1962; Wang and Waygood, 1962; Wang and Burris, 1963; Sinha and Cossins, 1964; Miflin *et al.*, 1966; Bruin *et al.*, 1970). Studies of the glycine to serine conversion by spinach preparations (Kisaki *et al.*, 1971) resulted in the proposal that glycine is first split to form 5,10-CH_2-H_4PteGlu, CO_2 and ammonia by glycine decarboxylase. A second molecule of glycine would then condense with 5,10-CH_2-H_4PteGlu to form serine in the serine hydroxymethyltransferase reaction. Clearly, therefore, 5,10-CH_2-H_4PteGlu produced in the glycine decarboxylase reaction may exchange with the general one-carbon pool or be utilized, apparently preferentially, in the biosynthesis of serine.

The glycine decarboxylase reaction is also thought to be the major reaction producing CO_2 evolved in photorespiration (Tolbert, 1963; Tolbert *et al.*, 1968; Tolbert and Yamazaki, 1969; Kisaki and Tolbert, 1970; Bruin *et al.*, 1970), a light dependent process which encompasses the uptake of O_2 and release of CO_2 associated with photosynthesis. Alternative pathways of glycolate metabolism, besides the conversion of glycine to serine, could also result in the production of CO_2 and synthesis of one-carbon units. For example, envelope-free spinach chloroplasts have been shown to carry out the oxidative decarboxylation of glyoxylate to yield 1 mol each of CO_2 and formate (Zelitch, 1972a).

Such formate could conceivably enter the one-carbon pool as a result of 10-HCO-H_4PteGlu synthetase activity. The amount of CO_2 released in this decarboxylation is, according to Zelitch (1972b) more than sufficient to account for the observed rates of CO_2 released in photorespiration than that accompanying the synthesis of serine from glycine in higher plants.

In algal species, glycine-serine interconversion is well established for *Chlorella pyrenoidosa* (Lord and Merrett, 1970), *Chlamydomonus* (Bruin *et al.*, 1970) and *Euglena graailis* (Codd and Merrett, 1971). On the other hand, glycine decarboxylase and the enzymatic decarboxylation of glyoxylate in such organisms have not yet been studied. Although enzymes such as 10-HCO-H_4PteGlu synthetase and serine hydroxymethyltransferase have recently been detected in extracts of *Euglena gracilis* cells (Murray *et al.*, 1971; Lor and Cossins, 1973), it is not yet known whether glyoxylate or glycine is the major C_2-unit from which one-carbon units are derived during operation of the glycolate pathway.

Activated one-carbon groups can also be formed from the carbon 3 of serine by serine hydroxymethyltransferase. Such serine could arise within another cellular compartment from glycolate pathway or form 3-phosphoglycerate, synthesized by operation of the photosynthetic carbon reduction cycle. The significance of the latter route for serine synthesis has not been evaluated in higher plants or algae particularly under conditions where the glycolate pathway would be inoperative due to repression of glycolate dehydrogenase. Repression of glycolate dehydrogenase by high CO_2 has been well documented in *Chlamydomonas* and *Euglena* (Nelson and Tolbert, 1969; Codd *et al.*, 1969).

Regulation of one-carbon metabolism

Most information on regulation of one-carbon metabolism comes from detailed studies of bacterial species. Such studies have shown that one-carbon metabolism is finely regulated through control of several pteroylglutamate-dependent enzymes. Furthermore, it is now clear that various aspects of one-carbon metabolism, including the biosynthesis of purines, thymidylate and methyl groups, may be independently regulated. In this connection, formyltetrahydrofolate synthetase, a key enzyme in purine biosynthesis, is induced by formate and histidine in *Micrococcus aerogenes* (Whiteley, 1967), but is repressed by purines in *Streptococcus faecalis* (Albrecht and Hutchinson, 1964) and by PteGlu in *Lactobacillus casei* (Ohara and Silber, 1969). In *Escherichia coli*, where one-carbon units for purine synthesis are mainly derived from serine, it was found (Taylor *et al.*, 1966) that ATP, ITP and GTP inhibited the activity of 5,10-CH_2-H_4PteGlu dehydrogenase. In contrast, this enzyme is repressed by serine in an amethopterin-resistant strain of *Streptococcus faecalis* (Albrecht *et al.*, 1966). Repression of dihydrofolate dehydrogenase by thymidine in wild-type and trimethoprim-resistant strains of *E. coli* (Burchall and Hitchings, 1967) may have physiological importance in the regulation of the reactions which link one-carbon metabolism and thymidylate biosynthesis. Regulation of methyl-group biosynthesis is relatively well documented. For example, in *E. coli* the biosynthesis of methionine, and therefore also of SAM, appears to be regulated through repression of 5,10-CH_2-H_4PteGlu reductase by methionine (Rowbury and Wood, 1961). In *Saccharomyces cerevisiae*, 5,10-CH_2-H_4PteGlu reductase and 5-CH_3-H_4PteGlu: homocysteine transmethylase are respectively inhibited and repressed by L-methionine (Lor and Cossins, 1972). Very

little information on the regulation of one-carbon metabolism has been reported in photosynthetic tissues. However, product inhibition of 5-CH_3-H_4PteGlu;homocysteine transmethylase by methionine (Dodd and Cossins, 1970) and inhibition of glycine decarboxylase by methionine and 5-CH_3-H_4PteGlu (Clandinin, 1973) have been reported for one higher plant species.

Present investigation

It is clear from the above review of the literature pertaining to various aspects of one-carbon metabolism that the possible metabolic role of pteroylglutamate derivatives in photosynthetic systems have, to date, been mainly studied *in vitro* and consequently the physiological significance of these roles *in vivo* has not been completely evaluated. As mentioned earlier extensive studies of the glycolate pathway and photorespiration have pointed to an involvement of pteroylglutamate derivatives in these areas of plant metabolism. Elucidation of the possible relationships between one-carbon metabolism and photosynthetic fixed carbon would be of particular interest in a photosynthetic microorganism maintained in defined media. *Euglena gracilis* appeasr to be fairly unique among unicellular photosynthetic organisms, in that it can be maintained in division synchronized culture, both photoautotrophically and heterotrophically. In addition, there have been a number of detailed investigations on biochemical changes during the cell cycle (Cook, 1961; Edmunds, 1965a,b; Codd and Merrett, 1971a,b) some of which implicate one-carbon metabolism. Preliminary experiments in this area by the author characterized the pteroylglutamate derivatives of *E. gracilis* and measurements of pteroylglutamate pool size were made during the cell

cycle.

In view of the evidence that glycolate dehydrogenase, a first enzyme in the glycolate pathway, is repressed by high concentrations of CO_2, further studies were undertaken to examine the effect of these conditions on pteroylglutamate pool size and the activity of key enzymes of one-carbon metabolism. Using this system, an evaluation of the contribution made by photosynthetic intermediates to the one-carbon pool was attempted. In addition, the possible regulation of this source of one-carbon units by CO_2 concentration was considered. The present studies have shown that considerable alterations in pool size occurs when CO_2 concentrations are altered. Parallel enzyme studies demonstrated that high concentrations of CO_2 repressed the synthesis of glycolate dehydrogenase and formyltetrahydrofolate synthetase. Under such conditions, the major route for the generation of one-carbon units appeared to involve serine hydroxymethyltransferase. Experiments using [^{14}C]formate supported the hypothesis that in *E. gracilis* the generation and subsequent metabolism of one-carbon units is regulated by the concentration of CO_2 available to the cells.

MATERIALS AND METHODS

Materials

Chemicals. α-Hydroxy-2-pyridinemethane sulfonate was purchased from Terochem Laboratories Ltd., Edmonton, Alberta, Canada. [^{14}C]Formate [2-^{14}C]PteGlu acid, [*methyl*-^{14}C]-5-CH_3-H_4PteGlu, [1-^{14}C]glyoxylate, [2-^{14}C]glyoxylate and L-[3-^{14}C]serine were purchased from Amersham-Searle Corporation, Des Plaines, Illinois, U.S.A. Other chemicals, of the highest quality commercially available, were purchased from Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A., Sigma Chemical Company, St. Louis, Mo., U.S.A., and Fisher Scientific Co., Edmonton, Alberta, Canada. PteGlu acid and tetrahydrofolic acid were purchased exclusively from Sigma Chemical Company. Scintillation grade 2,5-diphenyloxazole (PPO) and 1,4-*bis*-[4-methyl-5-phenyloxazol-2-yl)-benzene (dimethyl-POPOP) were purchased from Nuclear-Chicago, Des Plaines, Illinois, U.S.A. Cylinders of air containing 5.0 ± 0.1% CO_2 were purchased from Matheson of Canada, Ltd.

Preparation of Euglena culture media

The inorganic salt medium (pH 6.8) of Cramer and Meyers (1952) was used with some modification. The composition of this medium is shown in Table 1. The vitamin B_{12} solution was sterilized separately and added to the sterile medium after it had cooled. All sterilizations were preformed in standard steam autoclave at 15 lbs. psi. The media were maintained at this pressure for 15 minutes.

TABLE 1. Composition of the culture medium for *Euglena**.

Component	mg/liter
$(NH_4)_2HPO_4$	1000
KH_2PO_4	1000
$MgSO_4 \cdot 7H_2O$	200
$CaCl_2$	20
$Na_3C_6H_5O_7 \cdot 2H_2O$	800
$Fe_2(SO_4)_3 \cdot 6H_2O$	3
$MnCl_2 \cdot 4H_2O$	1.8
$CoCl_2 \cdot 6H_2O$	1.5
$ZnSO_4 \cdot 7H_2O$	0.4
MoO_3	0.2
$CaSO_4 \cdot 5H_2O$	0.02
Thiamin·HCl	0.2
Vitamin B_{12}	0.0010

*The medium of Cramer and Meyers (1952) was modified by raising the concentrations of vitamin B_{12} and thiamin·HCl from 0.005 mg/liter and 0.1 mg/liter respectively.

Solid media for maintenance of Euglena

5 g of Difco Bacto-Agar and 5 g Bacto-Peptone (Difco Laboratories, Detroit, Michigan, U.S.A.) were suspended in 400 ml of water in a 500 ml Erlenmeyer flask. The flask was plugged with cotton and heated on a boiling water bath until the agar was completely dissolved. The volume was then adjusted to 500 ml with distilled water. A pipetting syringe was used to dispense the agar-peptone solution (10 ml) into fifty 20 x 150 mm culture tubes with screw caps. Following autoclaving for 15 minutes at 15 lbs. psi, they were cooled in a slanted position and stored at 2°C.

Culture of Euglena gracilis

Euglena gracilis Klebs (strain Z) was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. (ATCC 12716). The original culture strain was maintained axenically on agar slants and transferred monthly. Autotrophic cultures were grown axenically in 4 l flasks containing 3.8 l of Cramer and Meyers (1952) modified inorganic salts medium (pH 6.8). The culture flask was placed on a magnetic stirrer and a siphoning device was attached. Moistened sterile air or 5% CO_2 in air was bubled through the culture using a fine sintered disc at the rate of 600-700 ml/min as measured with a Rotameter gas flowmeter (Roger Gilmont Instruments, Inc., N.Y.). Air circulation was maintained by a small Neptune Dyna Pump (Fisher Scientific Co. Ltd., Canada). The air flow was saturated with distilled water, and sterilized by passage through a series of two sterile glass-wool filters. 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 (0.03% CO_2 in air). All cultures on solid and liquid media were maintained in a growth chamber at 25°C with a 14:10 hr light:dark cycle. The light intensity of approximately 3500 lux was provided by 'cool white' fluorescent lamps.

Measurement of cell number

Growth of *Euglena* cells and degree of synchronization were monitored by measurement of cell numbers. At intervals of 2 - 3 hr throughout the experiments, samples (10 ml) were removed by a siphoning device. Four drops of 37% formaldehyde solution were added to fix the cells. Cell counts were made within 48 hr after collecting the samples. Before determination of cell number, 0.25 ml of 0.5 M NaCl was added to each 10 ml of *Euglena* culture to render it 0.9% (w/v) saline (Edmunds, 1965a) and the cells were counted in a Coulter counter Model B with the settings of lower threshold 10, upper threshold 100, 1/amplification 4 and 1/aperture current 1/2. Dilution of samples was made with 0.9% (w/v) saline to give cell concentrations within the range of 3 - 10 x 10^3/0.5 ml of sample.

Extraction of pteroylglutamate derivatives

Pteroylglutamate derivatives were extracted from *Euglena* cells by the method of Bird *et al.* (1965), with slight modifications. For analysis of total pteroylglutamates, 300 ml of culture (5×10^4 - 1×10^5 cells/ml) were withdrawn at different stages of the cell cycle, and the cells were harvested by centrifugation at 4,000 *g* for 10 min in a Servall Refrigerated Automatic Centrifuge operated at 2°C. 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°C in a water bath for 10 min and after cooling rapidly to 4°C was sonicated at 2°C (10 pulses of maximum power output, 15 sec each at 2°C, Fisher Ultrasonic Generator, Model BPO, Blackstone Ultrasonics Inc., Sheffield, Pa., U.S.A.). Cell debris and denatured protein were removed by centrifugation at 18,000 x *g* for 20 min. The supernatant was diluted two-fold using 1% (w/v) K ascorbate (pH 6.0).

Microbiological assay

Pteroylglutamates were measured by the 'aseptic plus ascorbate' method of Bakerman (1961). *Lactobacillus casei* (ATCC 7469) and *Pediococcus cerevisiae* (ATCC 8081), purchased from the American Type Culture Collection, Rockville, Maryland, U.S.A., were used as the assay bacteria. As these bacteria require pteroylglutamates for growth in defined media, the level of these compounds could be determined by measurement of growth (Jukes and Stokstad, 1948; Freed, 1966). Bacterial growth was measured by titration of the lactic acid produced after 72 hr incubation at 37°C (Roos *et al.*, 1968). Reference curves were constructed using authentic PteGlu for *L. casei* and 5-HCO-H_4PteGlu for *P. cerevisiae*.

Assay of polyglutamyl derivatives

Because pteroylglutamyl derivatives with more than three glutamyl residues do not support the growth of either *L. casei* or *P. cerevisiae*, their assay involved prior hydrolysis using a γ-glutamyl carboxypeptidase (Blakley, 1969). A γ-glutamyl carboxypeptidase from 3-day-old pea cotyledons (Roos and Cossins, 1971) was routinely used in these studies. The pea cotyledon hydrolase was prepared as described by Roos and Cossins (1971). The reaction mixture included 0.1 M sodium acetate

buffer (pH 4.5) containing 1.0% (w/v) K ascorbate. Enzyme activities were routinely checked by using Difco-Bacto yeast extract as substrate (Roos and Cossins, 1971). Enzyme activities were confirmed in all cases by including reaction systems containing boiled enzyme.

Chromatography of pteroylglutamate derivatives

Pteroylglutamate derivatives were separated by DEAE-cellulose column chromatography (Roos and Cossins, 1971). Aliquots of extracts, containing approximately 0.5 µg of pteroylglutamates, were applied to the DEAE-cellulose columns (20 cm x 1.8 cm) and the derivatives were eluted by using a continuous concentration gradient of potassium phosphate buffer (pH 6.0) in the presence of ascorbate (Roos and Cossins, 1971). Fractions of 3 ml were either assayed immediately with *L. casei* and *P. cerevisiae* or were stored in a frozen state until required. Individual derivatives were identified on the basis of criteria used earlier (Roos and Cossins, 1971; Sengupta and Cossins, 1971).

Sodium [^{14}C]formate feeding experiments

Euglena cells, grown in the presence of air and 5% CO_2 in air, were harvested during the 4th cell cycle and resuspended in 15 ml of fresh inorganic culture media in large culture tubes. The tubes were aerated with air or 5% CO_2 in air and illuminated from above (2000 ft.c) with a mercury lamp for a 3 hr equilibration period at 25°C. Following this, 10 µCi of sodium [^{14}C]formate (59 µCi/µmol) were added to the cultures and incubation was continued for up to 5 minutes. Evolved $^{14}CO_2$ was absorbed in 15 ml of 20% KOH solution contained in a tube connected to the outlet air line of the culture tube. The cells were harvested after the required feeding period, and washed twice with cold sterile

demineralized water to remove excess [^{14}C]formate. The cells were then suspended in 2 ml of 1.5 N perchloric acid and sonicated for 2 min at 4°C. After centrifugation at 18,000 x *g* for 10 min, the residue was washed with about 3 ml of ice cold demineralized water. The pH of the combined supernatants was adjusted to 6.3 by addition of solid $KHCO_3$. After a further centrifugation at 18,000 x *g* for 10 min, the supernatants were fractionated into amino acid, organic acid and sugar fractions by use of ion exchange resins (Canvin and Beevers, 1961; Cossins and Beevers, 1963).

Acid hydrolysis of insoluble residue

The samples of the insoluble residue obtained above were suspended in 3 ml 6N HCl. Hydrolysis of such samples was carried out in sealed evacuated tubes at 145°C for 4½ hr. The hydrolysate was filtered and dried *in vacuo* at 40°C on a Buchler flash-evaporator. The dry residue was then redissolved in 10 ml of distilled water and again brought to dryness. The process was repeated until the hydrolysate was acid-free. Protein amino acids present in the hydrolysate were then recovered by ion exchange chromatography using Dowex resin in H^+ form as described below.

Analysis of amino acid pools

Levels of free amino acids in *Euglena* extracts were determined using a Beckman Automatic Amino Acid Analyzer, Model 121. For such analyses, synchronized cultures were harvested at the 4th cell cycle by centrifugation, washed and resuspended in 2 ml of demineralized water. The suspensions were then sonicated and heated to 95°C for 10 min. Denatured protein and cellular debris were removed by centrifugation,

and the supernatants were passed through columns (1 x 6 cm) of Dowex 50W-X8 (H^+ form) which were subsequently washed by 50 ml of demineralized water followed by 50 ml of 4N HCl. The 4N HCl effluent containing the amino acids was collected, dried *in vacuo* at 40°C, and finally re-dissolved in 2 ml of 0.2 M citrate buffer (pH 2.2). Aliquots of this amino acid extract were analyzed using UR-30 and PA-35 spherical resins (Beckman Instruments Inc., California, U.S.A.). The eluting buffer for separation of the neutral and acidic amino acids was 0.20 M citrate at pH 3.22 and 4.25. The basic amino acids were eluted from PA-35 resin using 0.35 M citrate (pH 5.25). The pH values of all buffers were measured at 22°C and elution was carried out at 53°C, at a flow rate of 70 ml/hr.

For determination of radioactive amino acids, the 4N HCl effluent from the Dowex 50W-X8 H^+ column was dried *in vacuo* at 40°C and re-dissolved in 1 - 2 ml of 0.2 M citrate buffer (pH 2.2). Aliquots of this were subjected to amino acid analysis. The amino acids were eluted by the buffers mentioned above but in this case the effluent was collected in fractions of 1.9 ml using a fraction collector. The elution pattern of the amino acids was determined by using an authentic amino acid mixture as reference. The collected fractions were reacted with ninhydrin in the fractions collected to confirm the elution sequence of different amino acids. For further confirmation, the amino acid extracts were co-chromatographed with authentic amino acid and analyzed by the same method.

Counting of radioactive samples

Radioactivity was measured in a liquid scintillation counter

(Nuclear Chicago Corp., Unilux II model). Aliquots (0.1 - 0.5 ml) of the labelled samples were counted in 15 ml of fluor containing 12 g of PPO and 0.5 g of POPOP to each liter of a mixture of dioxane:anisole: dimethoxyethane (6:1:1 by volume). A counting efficiency of approximately 75% was obtained as determined by a ^{14}C-toluene internal standard.

$^{14}CO_2$ absorbed in KOH solution and radioactive areas on thin layer chromatograms detected by autoradiography were counted in 15 ml of a toluene counting solution containing 12 g of PPO and 0.5 g POPOP per liter of toluene at similar efficiencies.

Degradation of [^{14}C]serine

Samples of [^{14}C]serine isolated in [^{14}C]formate feeding experiments by the amino acid analyzer were degraded by periodate oxidation. This method yielded carbon dioxide from the carboxyl group and formate and formaldehyde from the 2 and 3 positions of serine respectively (Sakami, 1950).

$$\begin{array}{lll} (3)\ CH_2OH & & (3)\ HCHO \\ \quad | & \xrightarrow[\text{oxidation}]{\text{periodate}} & \\ (2)\ CHNH_2 & & (2)\ HCOOH \\ \quad | & & \\ (1)\ COOH & & (1)\ CO_2 \end{array}$$

The products of the periodate oxidation were recovered by the modified method of Sinha (1964). The degradation apparatus consisted of a 50 ml round bottom flask connected to a short water condenser with a side arm to allow aeration and introduction of reactants in solution. A 2 ml sample of [^{14}C]serine was placed in the reaction flask with 4 ml of 0.5 M phosphate buffer (pH 5.2). Carbon dioxide-free air was passed through the apparatus for 10 min to ensure complete removal of CO_2. Then 3 ml of 0.5 M sodium periodate were introduced from the side arm and

carbon dioxide-free air passed through for a further hour. Carbon dioxide produced from the carboxyl group of serine was absorbed in a 15 x 0.5 cm Vigreaux column containing 10% KOH. The reaction flask was then rapidly cooled to 2°C and the pH raised to 8 by addition of 1N NaOH solution. Formaldehyde derived from the 3 position of serine was distilled off after addition of 1 µmole of formaldehyde as carrier. The distillate was collected in an ice bath.

The reaction flask was again cooled in ice and the contents adjusted to pH 2 by addition of 20% phosphoric acid. The acidified solution was distilled once again and formic acid, derived from the 2 position of serine, was collected in a receiver cooled to 2°C.

Degradation of commercial samples of [3-^{14}C]serine showed that greater than 95% of the label was collected as formaldehyde.

Experiments involving cell-free extracts

Cells (5.0 - 10.0 x 10^4/ml) were harvested at different stages of cell cycle and washed as described earlier. The cells were suspended in 5 mM potassium phosphate buffer (pH 6.9) containing 5 mM 2-mercaptoethanol, except in assay of glycolate dehydrogenase, and sonicated for 5 min at 4°C. After centrifugation at 18,000 x *g* for 20 min, the supernatant was either dialyzed against 5 mM potassium phosphate buffer at 2°C for 12 hr, or passed through a column of Sephadex G-15 (1 x 5 cm). The resulting desalted protein solution was assayed for the following enzyme activities.

(a) *10-HCO-H_4PteGlu synthetase*

The reaction system for this assay (Hiatt, 1965) contained 100 µmol triethanolamine buffer (pH 8.0), 150 µmol Tris formate (pH 8.0), 2.5

µmol $MgCl_2$, 200 µmol KCl, 4 µmol DL-H_4PteGlu (Sigma Chemical Company, U.S.A.), 2 µmol of ATP and cell-free extract in a total volume of 1 ml. The control systems contained all of these components with the exception of ATP. The reaction mixture was incubated at 30°C for 10 min. The reaction was stopped by adding 1 ml of 1N HCl and the tubes allowed to stand for 10 min. Denatured protein was removed by centrifugation. Under these conditions, the 10-HCO-H_4PteGlu formed in the reaction was converted to 5,10-CH=H_4PteGlu. The latter compound was estimated spectrophotometrically at 355 mµ (E_m = 22,000).

(b) *Serine hydroxymethyltransferase*

The isotopic method of Taylor and Weissbach (1965) was used in this assay. Radioactivity in the one-carbon unit of 5,10-CH_2-H_4PteGlu produced in the reaction, was trapped with carrier formaldehyde and converted to a dimedon addition product. The reaction system contained 30 µmol phosphate buffer (pH 8.5), 1.0 µmol H_4PteGlu in 1.0 M 2-mercaptoethanol, 0.1 µmol pyridoxal-5'-phosphate, 0.1 µCi of L-[3-^{14}C]serine (48 µCi/µmol) and cell-free extract in a total of 0.7 ml. All components except serine were first incubated for 5 min at 30°C. Reactions initiated by addition of the substrate were terminated 15 min later by addition of 0.3 ml of 1.0 M sodium acetate (pH 4.5), 20 µl of 1.0 M formaldehyde and 0.3 ml of 0.4 M dimedon (in 50% ethanol) in succession. The reaction vessels were then heated for 5 min in a boiling water bath to accelerate formation of the HCHO-dimedon derivative. After cooling for 5 min in an ice bath the dimedon compound was extracted by vigorous shaking with 3 ml of toluene at room temperature. The aqueous and toluene phases were separated by centrifugation, the toluene phase being removed for measurement of ^{14}C.

(c) *5-CH_3-H_4PteGlu:homocysteine transmethylase*

An isotopic assay (Dodd and Cossins, 1970) was used to measure the activity of this enzyme. The standard 0.5 ml assay mixture consisted of 1 µmol of L-homocysteine, freshly prepared from the thiolactone (Sigma Chemical Company, Missouri, U.S.A.), 0.1 µCi of [*methyl*-^{14}C]-5-CH_3-H_4-PteGlu (1 µCi/0.016 µmol), 50 µmol of potassium phosphate buffer (pH 6.9) and cell-free extract. Control systems contained all of these components with the exception of the homocysteine. The mixture was incubated at 30°C for 30 min and the reaction terminated by rapid cooling in an ice bath. The cooled reaction mixture was placed on a column (0.5 x 2.5 cm) of Dowex 1-X10 resin in the Cl^- form. The column was eluted with six washings each of 0.2 ml distilled water. Under these conditions the labelled substrate was retained by the Cl^- column while labelled methionine was quantitatively eluted and collected in a scintillation vial for counting. The amount of methionine produced was calculated from the specific radioactivity of the 5-CH_3-H_4PteGlu.

(d) *5,10-CH_2-H_4PteGlu reductase*

The enzyme was assayed by the menadione-dependent oxidation of 5-[*methyl*-^{14}C]-CH_3-H_4PteGlu to H_4PteGlu and [^{14}C]formaldehyde (Dickerman and Weissbach, 1964). The latter compound being in equilibrium with the immediate product, 5,10-CH_2-H_4PteGlu. The reaction system for this assay contained 1.0 µmol potassium phosphate buffer (pH 7.4), 5 mµmol FAD, 5 mµmol manadione, 5 µmol formaldehyde, 0.1 µCi of [*methyl*-^{14}C]-5-CH_3-H_4PteGlu (1 µCi/.016 µmol) and cell-free extract in the total volume of 0.40 ml. Control systems contained all of these components with the exception of the cell-free extract. The reaction system was incubated at 30°C for 30 min and the reaction was stopped by rapid cooling in an

ice bath. The cooled reaction mixture was immediately placed on a column (0.5 x 2.5 cm) of Dowex AG1-X10 resin (Cl^- form). The column was eluted with three washings each of 0.5 ml distilled water. Under these conditions, 5-$^{14}CH_3$-H_4PteGlu was retained by the column and the product, [^{14}C]formaldehyde, was eluted and collected in a scintillation vial for counting. The amount of [^{14}C]formaldehyde formed was calculated from the specific radioactivity of the 5-CH_3-H_4PteGlu.

(e) *Glycolate dehydrogenase*

This enzyme was assayed by the method of Zelitch and Day (1968) which utilzes 2,6-dichlorophenolindophenol as hydrogen acceptor. The complete reaction system contained 100 µmol of potassium phosphate buffer (pH 7.0), 30 µmol potassium glycolate, 0.08 µmol 2,6-dichlorophenolindophenol and cell-free extract, in a total volume of 3 ml. After mixing the solution, the cuvette was covered with a layer of toluene. The reaction system without glycolate served as a control in each experiment. The change in absorbancy was measured in a Beckman DB recording spectrophotometer (Beckman Instruments Inc., Palo Alto, California, U.S.A.) at 590 mµ at 25°C. One unit of activity was taken as the amount of enzyme causing a decrease in absorbancy of 0.01 per min.

(f) *Enzymic decarboxylation of glyoxylate*

The method of Zelitch (1972a) was modified to include use of conventional Warburg flasks. The main compartment contained 100 µmol potassium phosphate buffer (pH 7.5), 10 µmol $MnCl_2$, 0.1 µCi of [1-^{14}C] glyoxylate (1 µCi/0.131 µmol), 350 units of bovine liver catalase (Sigma Chemical Company, Missouri, U.S.A.) and cell-free extract in a total volume of 1.7 ml. The side arm contained 0.5 ml 2N HCl and the center well contained a fluted filter paper moistened with 0.1 ml of 20%

KOH to trap CO_2. The reaction was carried out at 30°C in a shaking water bath and terminated by addition of the acid from the side arm. After shaking for a further 30 min, the $^{14}CO_2$ trapped in KOH was measured with a scintillation counter. Reaction systems contained boiled enzymes and served as controls in each assay. Formate, the other reaction product, was identified by thin layer chromatography on 20 x 20 cm Silica Gel GF plates using ethanol:NH_4OH:water (80:4:16 v/v/v) as solvent system. The Rf values of formate and glyoxylate were found to be 0.55 and 0.34 respectively.

(g) *Formic dehydrogenase*

In this assay, the main compartment of the Warburg flask contained 50 µg of NAD, 2 µmol of sodium [^{14}C]formate (0.1 µCi) and cell-free extract in a total volume of 1.2 ml. Sodium [^{14}C]formate was added after a 10 min equilibration period at 30°C. The $^{14}CO_2$, evolved from the enzymic decarboxylation of formate, was absorbed on a fluted filter paper moistened with 0.1 ml of 20% KOH in the center well. The reaction was terminated by addition of 0.5 ml of 2N HCl from the side arm. The $^{14}CO_2$ absorbed during the reaction was assayed for ^{14}C after an additional shaking period of 30 min.

Estimation of protein

The protein content of cell-free extracts was estimated colorimetrically using the method of Lowry *et al.* (1951). Crystalline egg albumin was used as a reference standard. All determinations were made in duplicate.

RESULTS

Synchronous growth of Euglena gracilis

Euglena gracilis (strain Z) was grown autotrophically as described in the Materials and Methods, using a 14:10 hr light-dark regime at 25°C. The initial inoculum was obtained from a sub-culture grown under the same conditions for 5 to 7 days. It has been reported (Edmunds, 1965a; Codd and Merrett, 1971a) that under such conditions, synchronous growth of *Euglena* can be induced. Figure 1 illustrates the light-induced division synchrony of *E. gracilis* monitored for 5 cell cycles in the present work. 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. Cell divisions were generally detected shortly after commencement of each dark period but in a few instances occurred 1 - 2 hr before. In an attempt to estimate the degree of synchrony shown by these cultures, data for cell number from 5 cell cycles were examined by the following equation (Scherbaums, 1959):

$$SI = \frac{n - n_0}{n_0} \left(1 - \frac{t}{gt}\right)$$

Where SI is the synchronization index, N_0 is the cell number before synchronous division, n is the cell number after synchronous division, t is the duration of the fission time and gt is the generation time. Calculation of such synchronization index gave average values of 0.65. This value and the resulting growth pattern are similar to those reported by Edmunds (1965a) for this organism. It is apparent, therefore, that cell division in *E. gracilis* under the present conditions can be

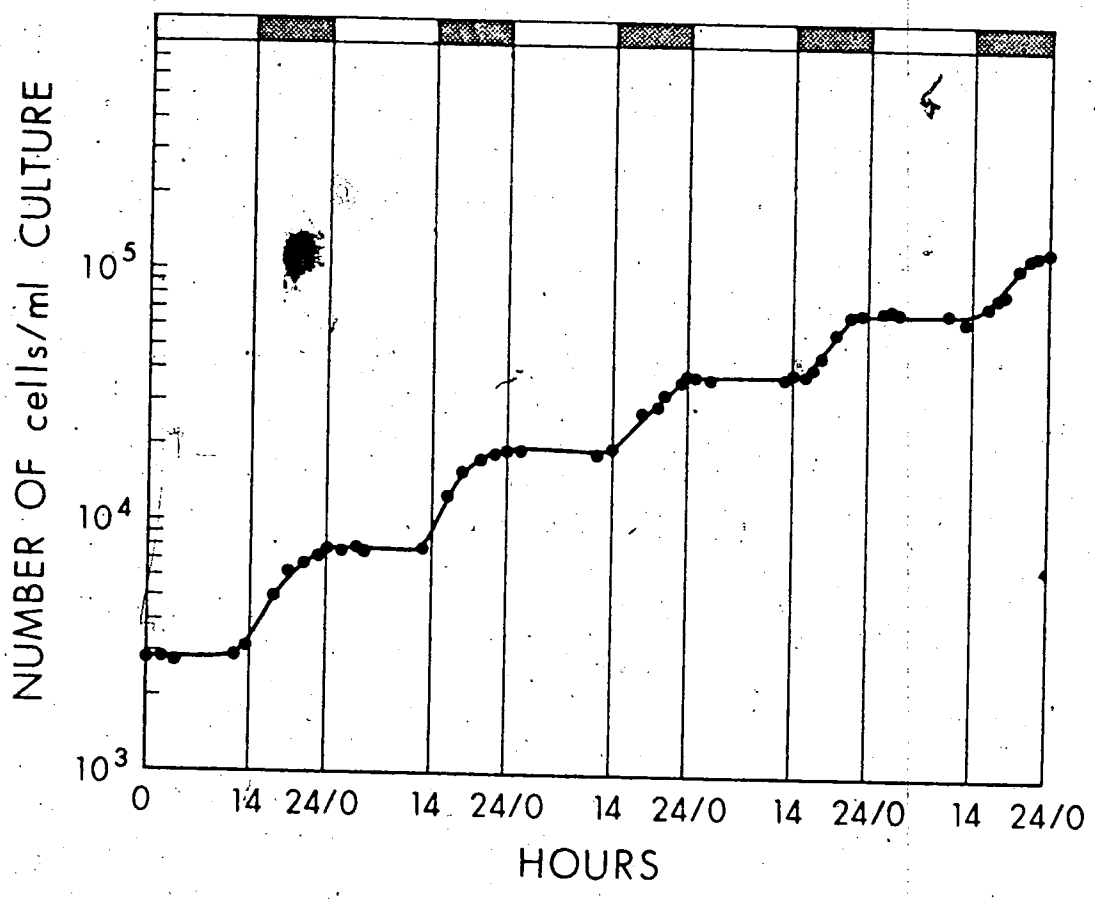


FIGURE 1. *Synchronous increase in cell number in cultures of Euglena gracilis.* Cells were cultured with aeration (0.03% CO_2 in air) in the autotrophic culture medium of Cramer and Myers. Cultures were maintained at 25°C and received a 14:10 hr light:dark cycle. Cell numbers were measured in a Coulter counter Model B.

sufficiently well synchronized to provide a good system for studies of one-carbon metabolism during various stages of the cell cycle.

Changes in pteroylglutamate pool size during the cell cycle.

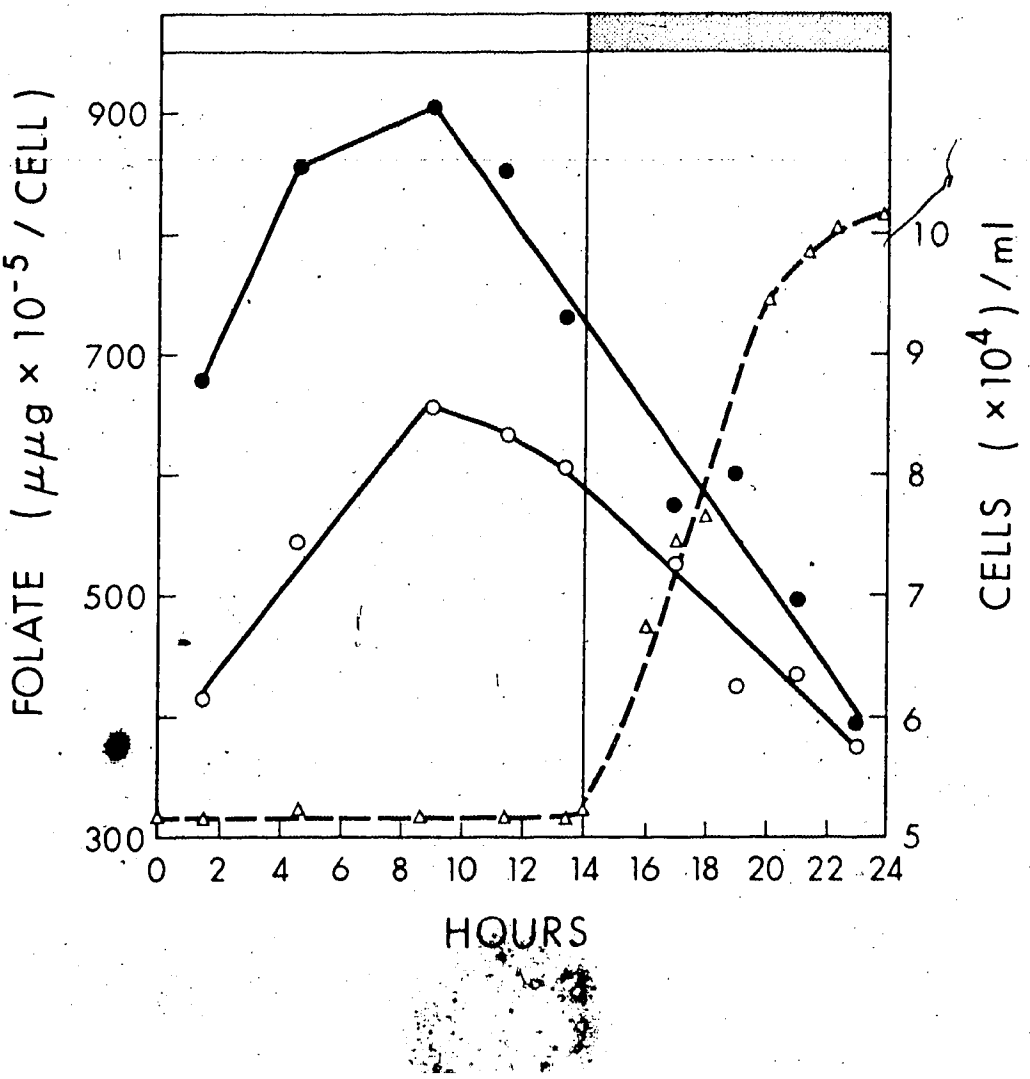
Biochemical studies of synchronized cultures of *E. gracilis* (Cook, 1961; Edmunds, 1964; Edmunds, 1965b) have shown that the levels of protein, polysaccharides, pigments and RNA double during each light phase in a linear fashion whereas the DNA content of the cells increases in a stepwise manner. Clearly 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 one-carbon metabolism it follows that fluctuations in the levels of pteroylglutamates should occur during the cell cycle of this organism. In order to examine variations in pteroylglutamate levels during the cell cycle, samples of the cultures were withdrawn at intervals and ascorbate extracts were prepared as described in the Materials and Methods. The levels of pteroylglutamates were then assayed microbiologically employing *L. casei* before and after treatment of extracts with γ-glutamyl carboxypeptidase isolated from 3-day-old pea cotyledons. The data obtained before γ-glutamyl carboxypeptidase treatment represents the levels of pteroylglutamates which contain no more than three glutamyl residues. Data obtained after such peptidase treatment represents levels of highly conjugated pteroylglutamates. The results of these assays are summarized in Figure 2. It is clear that levels of conjugated and unconjugated derivatives increased on a per cell basis during the first 9 hr of the light phase. Following this, pteroylglutamate levels declined to the values in the dark phase which were approximately half of

FIGURE 2

CHANGES IN PTEROYLGLUTAMATE CONCENTRATION DURING THE DIVISION CYCLE OF SYNCHRONIZED *EUGLENA* CULTURES

Cells were grown with aeration (0.03% CO_2 in air) as described in the Materials and Methods. Pteroylglutamate extracts were prepared at different stages of the cell cycle. Pteroylglutamate concentrations were determined with *L. casei* and expressed as μμg of PteGlu/10^5 cells before and after pea cotyledon γ-glutamyl carboxypeptidase treatment. Concentrations of conjugated derivatives (●) were calculated by subtracting the values obtained before carboxypeptidase treatment (o) from total values obtained after enzyme treatment.

Dotted line - cell number/ml culture.


FOLATE (μμg x 10⁻⁵ / CELL)
900
700
500
300
0 2 4 6 8 10 12 14 16 18 20 22 24
HOURS
CELLS (x10⁴) / ml
10
9
8
7
6
5

the maximal values observed in the light. A large proportion of the pteroylglutamate pool consisted of highly conjugated derivatives and this proportion remained relatively constant throughout the cell cycle.

Chromatography of pteroylglutamate derivatives extracted from E. gracilis

To investigate the nature of the pteroylglutamate derivatives in *E. gracilis*, ascorbate extracts were subjected to chromatography on DEAE-cellulose before and after treatment with γ-glutamyl carboxypeptidase. Fractions collected from the columns were subjected to differential microbiological assay using *L. casei* and *P. cerevisiae*.

Recoveries of pteroylglutamates from the columns ranged from 85-99% in these analyses and the individual derivatives were identified on the basis of criteria used by Roos and Cossins (1971). These included the ability of the compound to support the growth of *L. casei* and *P. cerevisiae*, co-chromatography with authentic derivatives and a consideration of chromatographic behaviour after treatment with γ-glutamyl carboxypeptidase.

The typical differential assay of individual pteroylglutamates present in extracts of *E. gracilis* is illustrated in Figure 3A and B. Before carboxypeptidase treatment (Fig. 3A), seven individual compounds (a-h) were detectable. Peaks a, b, c and d were identified on the basis of criteria described above as 10-HCO-H_4PteGlu, 10-HCO-H_4PteGlu$_2$, 5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu respectively. These derivatives were present in small quantities. 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

FIGURE 3

CHROMATOGRAPHY AND DIFFERENTIAL ASSAY OF PTEROYLGLUTAMATES FROM *EUGLENA*

Extracts of cells before (A) and after (B) treatment with pea cotyledon γ-glutamyl carboxylpeptidase were subjected to chromatography on DEAE-cellulose followed by assay using *L. casei* (●) and *P. cerevisiae* (o).

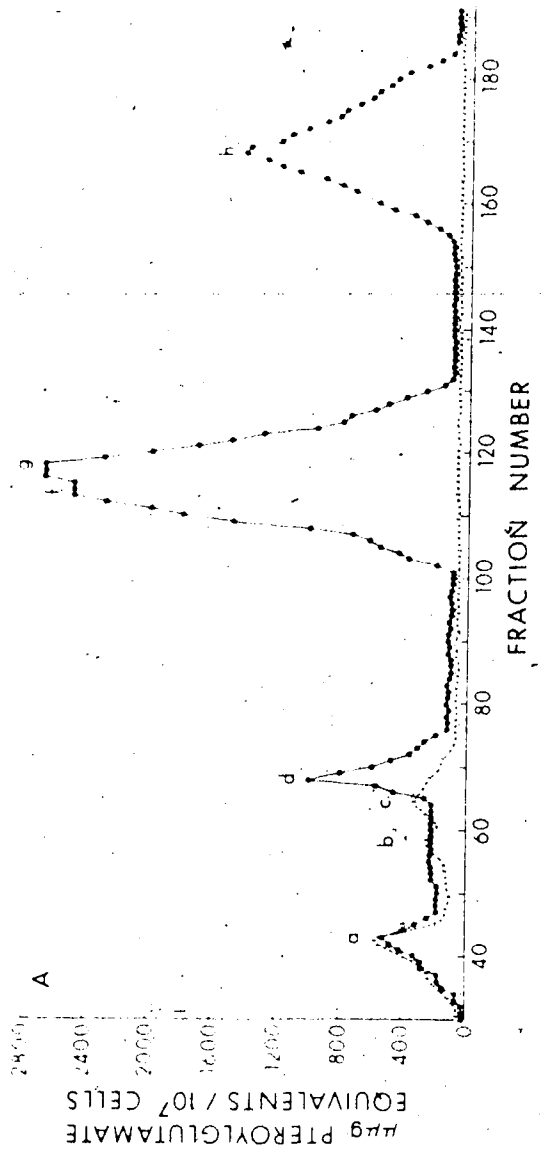
The derivatives shown are:

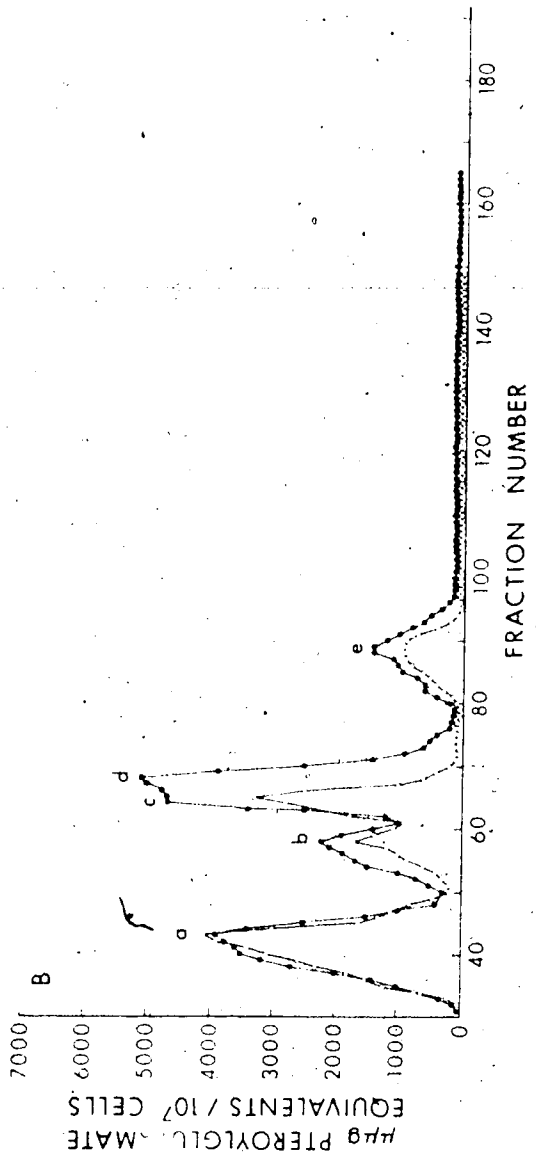
(a), 10-HCO-H_4PteGlu; (b), 10-HCO-H_4PteGlu$_2$;

(c), 5-HCO-H_4PteGlu; (d), 5-CH_3-H_4PteGlu;

(e), 5-HCO-H_4PteGlu$_2$; (f) 5-HCO-H_4PteGlu$_3$;

(g), 5-CH_3-H_4PteGlu$_3$; (h) unidentified conjugated derivatives. No growth response was obtained before fraction 30 or after fraction 190. The data are in PteGlu equivalents for *L. casei* and 5-HCO-H_4PteGlu equivalents for *P. cerevisiae*.


A
a
b, c
d
e
f
g
h
0
400
800
1200
1600
2000
2400
40
60
80
100
120
140
160
180
FRACTION NUMBER
μμg PTEROYLGLUTAMATE
EQUIVALENTS / 10⁷ CELLS


B
a
b
c
d
e
0
1000
2000
3000
4000
5000
6000
7000
40
60
80
100
120
140
160
180
FRACTION NUMBER
μμg PTEROYLGLU MATE
EQUIVALENTS / 10⁷ CELLS

L. casei. Peaks f and g occurred at positions in the elution sequence which were identical to those of authentic 5-HCO-H_4PteGlu$_3$ and 5-CH_3-H_4PteGlu$_3$ (Cossins and Shah, 1971). On the basis of chromatographic behaviour and differential growth response these two peaks were 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 pteroylglutamate derivative.

Carboxypeptidase treatment of extracts before chromatography resulted in large increases in the levels of peaks a, b, c, d and e which was identified as 5-HCO-H_4PteGlu$_2$. These results indicate that the highly conjugated derivatives were both formyl and methyl derivatives of H_4PteGlu. Although not shown in Figure 3, a trace amount of H_4PteGlu, located in fractions 75-78, was detected in some cases.

It should be emphasized that these analyses failed to reveal the presence of H_2PteGlu, PteGlu, 5,10-CH_2-H_4PteGlu, 5,10-CH=H_4PteGlu and 5-HCNH-H_4PteGlu, derivatives which have also been implicated in one-carbon metabolism (Blakley, 1969). Their occurrence in *E. gracilis* cannot be ruled out by the present data however as 5,10-CH_2-H_4PteGlu, 5,10-CH=H_4PteGlu and 5-HCNH-H_4PteGlu are unstable under these conditions of extraction and isolation.

Changes in formyl and methyl derivatives during the cell cycle

The studies described above show that variations occur in pteroylglutamate pool size during the cell cycle. It was of interest to examine further changes in individual pteroylglutamates during the cell cycle especially those derivatives known to be directly involved in the

synthesis of purines, pyrimidines, certain amino acids and proteins. For such an examination, extracts prepared from cells at different stages of the cell cycle were subjected to DEAE-cellulose column chromatography before and after treatment with the γ-glutamyl carboxypeptidase. The results are shown in Table 2. It is clear from the data that before carboxypeptidase treatment, the overall levels of methyl derivatives on a per cell basis increased in the light phase and decreased during the dark phase. There was no dramatic changes in the total levels of unconjugated formyl derivatives during the light phases examined. These overall levels of formyl derivatives were, however, lower on a per cell basis when the cells were dividing during the dark phase of culture. Enzyme hydrolysis of the extracts before chromatography resulted in increases in the levels of the principal formyl derivatives. Such formyl conjugated derivatives increased during the light phase but were present at lower levels in the dark phase of culture.

Changes in the levels of pteroylglutamate-dependent enzymes during the cell cycle

The results of previous experiments indicate that net synthesis of formyl and methyl derivatives of $H_4PteGlu_n$ occurred prior to cell division. Conceivably such syntheses would be accompanied by rapid increases in the activities of pteroylglutamate-dependent enzymes. To examine this possibility the levels of 10-HCO-H_4PteGlu synthetase, serine hydroxymethyltransferase, 5-CH_3-H_4PteGlu:homocysteine transmethylase and 5,10-CH_2-H_4PteGlu reductase were examined using cell-free extracts prepared at different times during the cell cycle. The results are shown in Figure 4. It is clear that 10-HCO-H_4PteGlu synthetase

TABLE 2. Levels of pteroylglutamate derivatives in division synchronized cultures of *Euglena gracilis* at different stages of the cell cycle.

Derivative (μμg/10^7 cells)	Light phase				Dark phase	
	2 hr		10 hr		4 hr	
	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP
10-HCO-H_4PteGlu	1051	31372	2785	37501	1375	25301
10-HCO-H_4PteGlu$_2$	3105	16884	1190	20531	2075	8424
5-HCO-H_4PteGlu	1443	12892	2592	15727	1425	14029
5-CH_3-H_4PteGlu	575	11498	2452	18754	2325	19190
5-HCO-H_4PteGlu$_2$	*n.d.*	10917	*n.d.*	13382	*n.d.*	6819
5-HCO-H_4PteGlu$_3$ + 5-CH_3-H_4PteGlu$_3$	23189	*n.d.*	29046	*n.d.*	19625	*n.d.*
Peak g	8031	*n.d.*	5733	*n.d.*	10395	*n.d.*
Total	37394	83558	43798	105895	37220	73763

n.d. - no detectable growth response with *L. casei*.

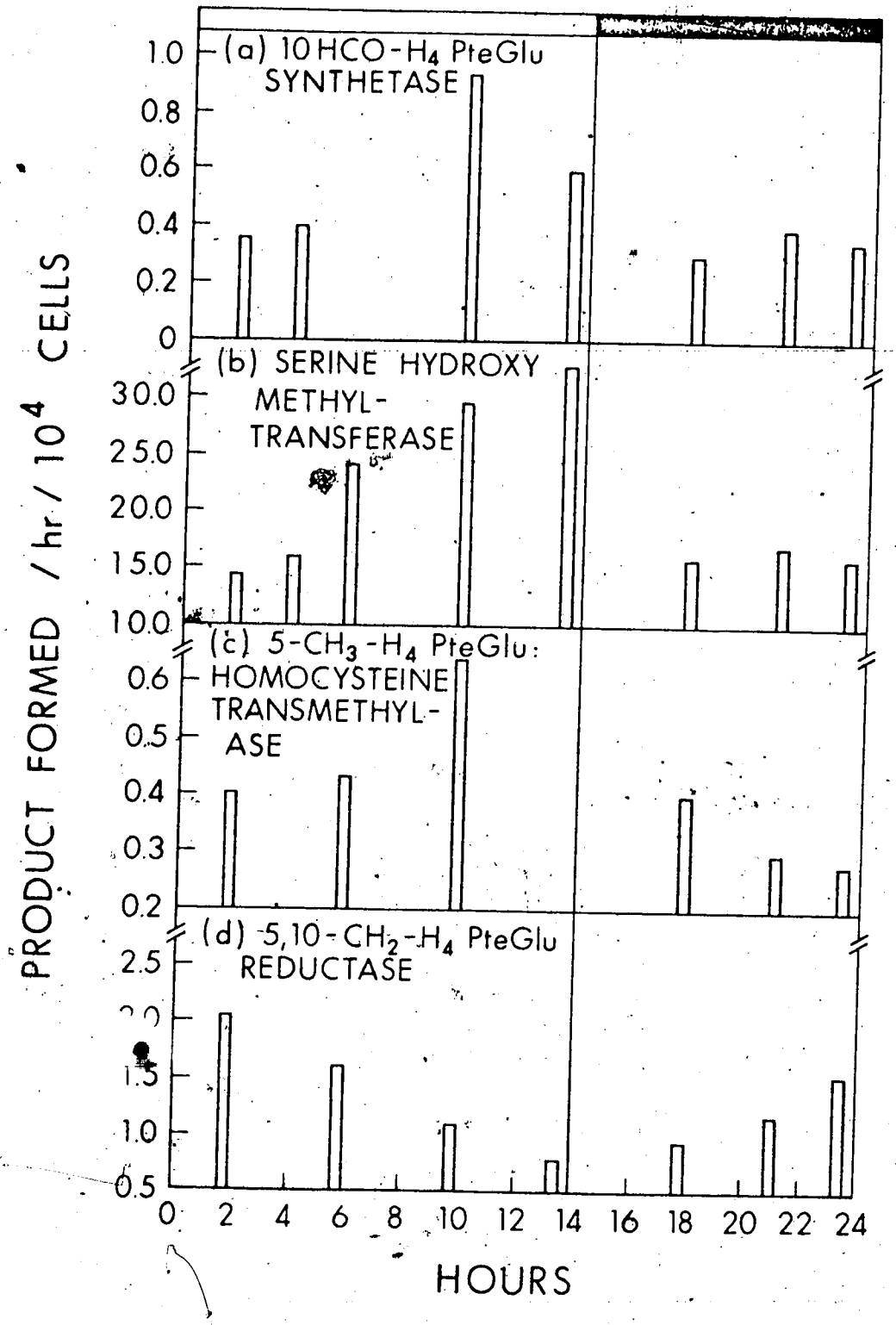
Cells were grown with aeration (0.03% CO_2 in air) as described in the Materials and Methods. Pteroylglutamate extracts prepared at different stages of the cell cycle were chromatographed on DEAE-cellulose before and after treatment with pea cotyledon γ-glutamyl carboxypeptidase (γ-GCP). The assay organism was *L. casei*. Results are expressed as μμg of PteGlu/10^7 cells.

FIGURE 4

LEVELS OF PTEROYLGLUTAMATE-DEPENDENT ENZYMES

DURING THE CELL CYCLE OF *EUGLENA*

Cell-free extracts prepared at different times during the cell cycle were used as a source of the enzymes. Enzyme activities were measured as described in the Materials and Methods. All assays were carried out in duplicate and values shown are averages of two separate experiments. The activity of the synthetase is expressed as mμmol product formed/hr/10^4 cells; activities of other enzymes are expressed as μμmol product formed/hr/10^4 cells.


(a) 10 HCO-H4 PteGlu SYNTHETASE
(b) SERINE HYDROXY METHYL-TRANSFERASE
(c) 5-CH3-H4 PteGlu: HOMOCYSTEINE TRANSMETHYL-ASE
(d) 5,10-CH2-H4 PteGlu REDUCTASE
PRODUCT FORMED / hr / 10^4 CELLS
HOURS
0
2
4
6
8
10
12
14
16
18
20
22
24

(Fig. 4a), serine hydroxymethyltransferase (Fig. 4b) and 5-CH_3-H_4PteGlu: homocysteine transmethylase (Fig. 4c) 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 cell cycle. In contrast, the levels of 5,10-CH_2-H_4PteGlu reductase (Fig. 4d) 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.

The specific activities of these enzymes are shown in Figure 5. No appreciable changes in specific enzyme activity were noted for 10-HCO-H_4PteGlu synthetase, serine hydroxymethyltransferase and 5-CH_3-H_4PteGlu transmethylase during the light phase. This indicates that enzyme synthesis in each case closely paralleled the rate of synthesis of total soluble protein. During the dark phase the specific enzyme activities of 10-HCO-H_4PteGlu synthetase and 5-CH_3-H_4PteGlu transmethylase were, however, decreased to some extent. In contrast, the specific enzyme activity of 5,10-CH_2-H_4PteGlu reductase decreased during the light phase then rose during the dark period as was observed in Figure 4 where enzyme activity is expressed on a per cell basis.

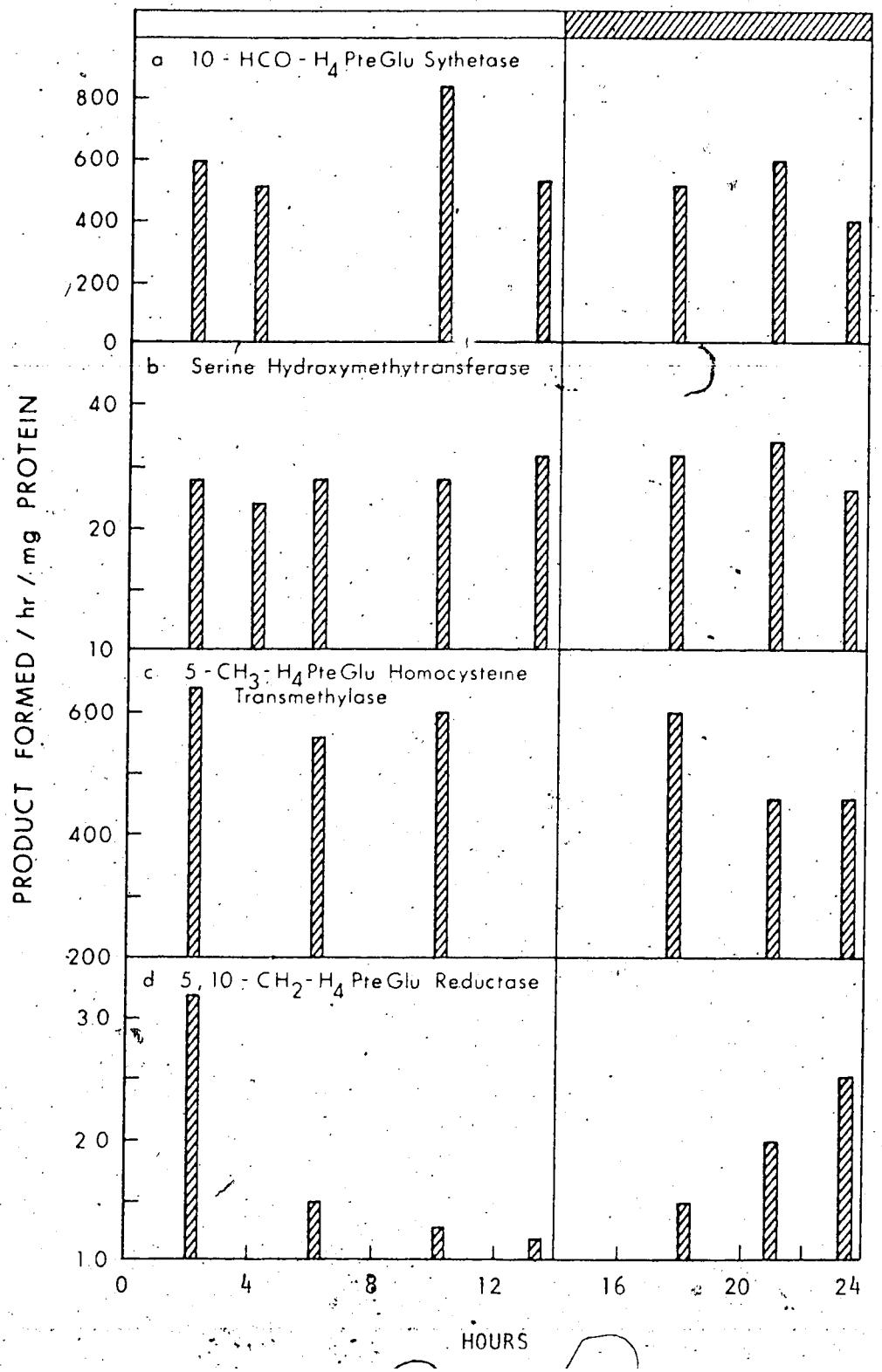
Effects of 5% CO_2, α-hydroxy-2-pyridinemethane sulfonate and L-methionine on synchronous growth of E. gracilis

As outlined in the Introduction, one-carbon units required in serine synthesis in *Euglena* might be derived from glycolate. Since it has been reported that glycolate dehydrogenase in this organism is repressed and inhibited by high concentration of CO_2 and α-HPMS

FIGURE 5

SPECIFIC ACTIVITIES OF PTEROYLGLUTAMATE-DEPENDENT ENZYMES DURING THE CELL CYCLE OF *EUGLENA*

Experimental details are as in Fig. 4. The specific enzyme activity of 5-CH_3-H_4PteGlu:homocysteine transmethylase is expressed as μμmol product formed/hr/mg protein; specific activities of other enzymes are expressed as mμmol product formed/hr/mg protein.


a 10 - HCO - H_4 PteGlu Sythetase
800
600
400
200
0
b Serine Hydroxymethytransferase
40
20
10
c 5 - CH_3 - H_4 PteGlu Homocysteine Transmethylase
600
400
200
d 5, 10 - CH_2 - H_4 PteGlu Reductase
3.0
2.0
1.0
0
4
8
12
16
20
24
HOURS
PRODUCT FORMED / hr / mg PROTEIN

respectively (Codd *et al.*, 1969; Codd and Merrett, 1971b), it follows that under such conditions the metabolism of one-carbon units might also be altered. Further investigations were therefore undertaken to elucidate the possible effects of these treatments on the one-carbon metabolism of *Euglena* cells. In addition, as L-methionine has been shown to have a regulatory role on one-carbon metabolism in different microorganisms, the possible regulation of one-carbon metabolism by this amino acid was also examined.

Before any attempts were made to study such effects of CO_2, α-HPMS and L-methionine, the effects of these treatments on growth were first studied. In these studies, cells were inoculated and grown synchronously in the presence of air and 5% CO_2 in air respectively, as described in the Materials and Methods. Cells were also cultured in the presence of air with supplements of 5 mM α-HPMS or 1 mM of L-methionine. Aliquots of cultures were withdrawn at different times during the cell cycle and cell counts were made with a Coulter counter. The results are shown in Figure 6. It is clear from the results that in the presence of high CO_2(air + 5% CO_2) and L-methionine the cells still showed cell divisions restricted to the dark phase. However, the amount of growth, as reflected by cell numbers was approximately 25% more than control culture in the presence of high CO_2 while in L-methionine, growth was inhibited by approximately 25%. Cells grown in the presence of α-HPMS failed to divide during the dark phase.

Effect of α-HPMS on pteroylglutamate contents

α-HPMS is known to be a competitive inhibitor of glycolate dehydrogenase (Zelitch, 1957) and has been widely used in studies of the

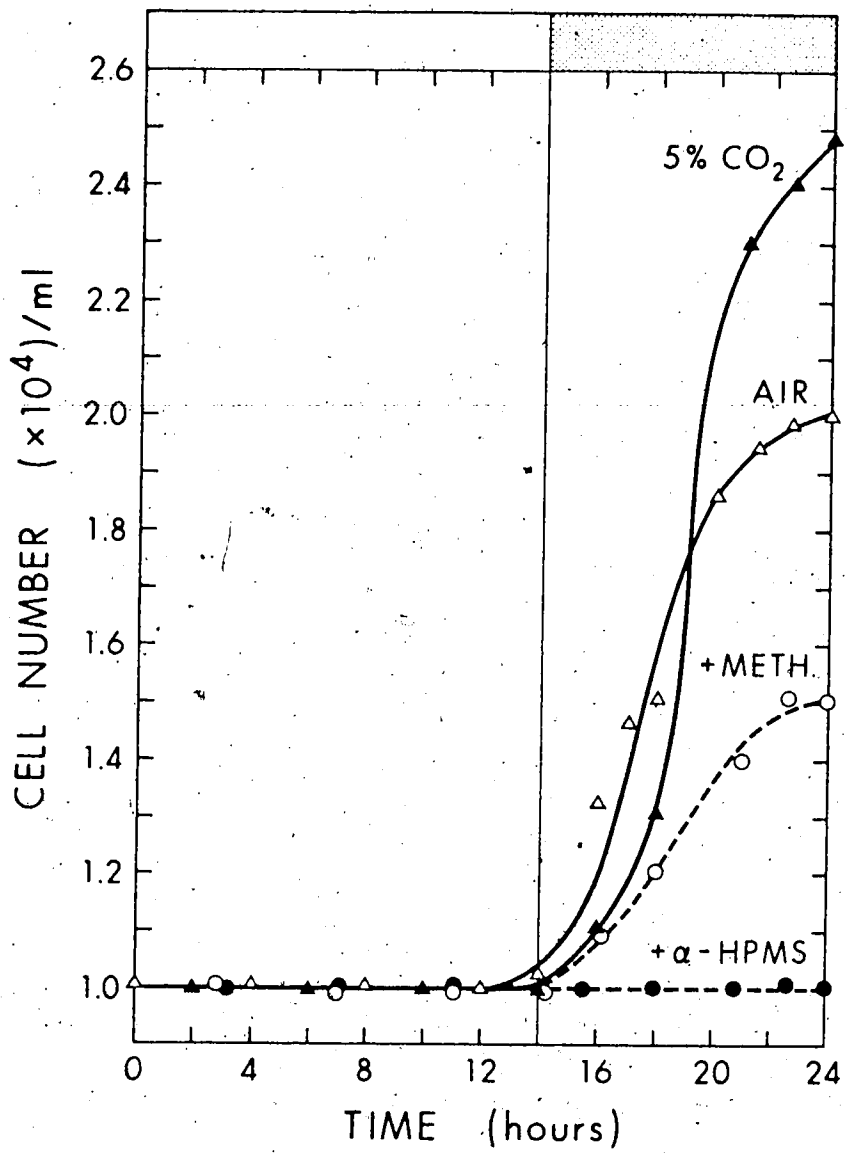


FIGURE 6. EFFECTS OF 5% CO_2, α-HPMS AND L-METHIONINE ON SYNCHRONOUS GROWTH OF [illegible]

Cells were inoculated and grown synchronously in the presence of air and 5% CO_2 in air respectively as described in the Materials and Methods. Cells were also grown in the presence of air with supplements of 5mM α-HPMS and 1mM of L-methionine respectively. The results shown for the air, methionine and 5% CO_2 cultures were obtained during the 4th cell cycle. Cell counts for the α-HPMS culture were obtained during the 1st cell cycle, the inhibitor being added at zero time.

photosynthetic origin and metabolism of glycolate. Accumulation of glycolate following the administration of α-HPMS has been observed during photosynthesis in leaves of higher plants (Zelitch, 1965; Hess and Tolbert, 1966) and in culture of *Chlorella pyrenoidosa* (Lord and Merrett, 1969) and *E. gracilis* (Codd and Merrett, 1971b). If the partial reactions of glycolate pathway represent a major route for generation of one-carbon units, it follows that administration of this inhibitor, should affect the levels of pteroylglutamate derivatives which derive their one-carbon moieties from glycolate. To examine this possibility, *E. gracilis* was grown synchronously as described in the Materials and Methods. When the cell density had reached 5×10^4 cells/ml culture, 5 mM of α-HPMS was added aseptically at the start of a light phase. Pteroylglutamate extracts were prepared from cells harvested after 10 hr of the light phase and after 1 hr of the dark phase. Such extracts were examined after DEAE-cellulose column chromatography with and without a γ-glutamyl carboxypeptidase treatment. As some isomerization of 10-HCO-H_4PteGlu and 5-HCO-H_4PteGlu could occur during this extraction and isolation (Blakley, 1969) the data are given in Table 3 according to the substituent group of the principal derivatives. It is clear that before γ-glutamyl carboxypeptidase treatment, α-HPMS treated cells contained slightly higher concentrations of formyl mono- and diglutamyl derivatives. However, the total concentrations of 5-HCO-H_4PteGlu$_3$ and 5-CH_3-H_4PteGlu$_3$ were largely reduced by the presence of α-HPMS. As growth of the cells was completely inhibited by α-HPMS (Fig. 6) a greater utilization of these pteroylglutamates in the presence of α-HPMS was unlikely. These results rather indicate that pteroylglutamate synthesis might be reduced by α-HPMS; furthermore, under these conditions, there appeared to be

TABLE 3. Effect of α-HPMS on the concentrations of pteroylglutamate derivatives in synchronous cultures of *E. gracilis*.

	Pteroylglutamate concentration (μμg/10^7 cells)							
	Light phase; 10 hr				Dark phase; 1 hr			
	Air		+ α-HPMS		Air		+ α-HPMS	
Derivative	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP
5-HCO-H_4PteGlu$_{1-2}$	3270	105940	4666	48810	1352	77841	2295	56624
5-CH_3-H_4PteGlu	805	10624	1071	9874	789	17856	778	3672
5-HCO-H_4PteGlu$_3$ + 5-CH_3-H_4PteGlu$_3$	20562	*n.d.*	9802	*n.d.*	6480	*n.d.*	2304	*n.d.*
Total	24637	116564	15539	58684	8621	95706	5377	60296

n.d. - not detected.

Cells were grown synchronously as described in the Materials and Methods. At a cell density of 5 x 10^4 cells/ml culture, α-HPMS (5 mM) was added aseptically at the start of a light phase. Pteroylglutamate extracts were prepared from cells harvested after 10 hr of light phase and after 1 hr of the dark phase. The extracts were then chromatographed on DEAE-cellulose before and after pea cotyledon γ-glutamyl carboxypeptidase (γ-GCP) treatment. The results are expressed as μμg of PteGlu/10^7 cells. The assay organism was *L. casei*.

depletion of conjugated derivatives possibly to maintain levels of less conjugated derivatives. This possibility is substantiated by the data obtained after γ-glutamyl carboxypeptidase treatment (Table 3). In the light phase, a decrease of approximately 50% in the conjugated formylated derivatives occurred in the presence of α-HPMS. The concentration of methyl derivatives was not however appreciably affected. During the dark phase, however, only 27% of the total formylated derivatives were decreased in contrast to the concentrations of methyl derivatives which were drastically reduced by this hydroxysulfonate.

Effect of α-HPMS on glycolate dehydrogenase, serine hydroxymethyltransferase and 10-HCO-H_4PteGlu synthetase

The levels of these three enzymes were determined by using dialyzed extracts of cells cultured in the presence and absence of α-HPMS (5 mM). In all cases cultures of *E. gracilis* and the addition of α-HPMS was as described earlier. Extracts for enzyme studies were prepared after 10 hr of the light phase. Specific enzyme activities and activity on a per cell basis are shown in Table 4. Cells grown in the presence of α-HPMS contained much lower levels of glycolate dehydrogenase and 10-HCO-H_4PteGlu synthetase on the basis of the protein and cell numbers. In contrast, the levels of serine hydroxymethyltransferase were not appreciably altered by the presence of this inhibitor.

Effect of CO_2 on the concentrations of pteroylglutamates

In these experiments, *E. gracilis* was grown synchronously with air as described in the Materials and Methods. When the cell density had reached 5 x 10^4 cells/ml culture, air supplemented with 5% CO_2 was supplied at the beginning of the light phase. Cells were then harvested

TABLE 4. Effects of α-HPMS on glycolate dehydrogenase, serine hydroxymethyltransferase and 10-HCO-H_4PteGlu synthetase activities.

Enzyme	Enzyme activity			
	Activity/mg protein		Activity/cell	
	Control	α-HPMS	Control	α-HPMS
Glycolate dehydrogenase (enzyme units)*	2.66	0.76	2.0×10^{-7}	0.6×10^{-7}
Serine hydroxymethyltransferase (mμmol HCHO formed/hr)	18.6	17.0	49.8×10^{-7}	45.5×10^{-7}
10-HCO-H_4PteGlu synthetase (mμmol 10-HCO-H_4PteGlu formed/hr)	304	93	1.6×10^{-4}	0.6×10^{-4}

* As defined in the Materials and Methods.

Experimental details are as in Table 3. Cell-free extracts prepared at 10 hr of light period were used as a source of the enzymes. The activities indicated were assayed as described in the Materials and Methods.

at different times during the cell cycle and ascorbate extracts were prepared as described earlier. The resulting extracts were assayed for pteroylglutamates both before and after γ-glutamyl carboxypeptidase treatments. The results are shown in Table 5. The results show that the concentrations of free and conjugated pteroylglutamates in both cultures increased during the first 12 hr of the cell cycle and then decreased during the dark phase. However, when the concentration of pteroylglutamates in the two cultures are compared, it is clear that high CO_2 only increased the total pool size of pteroylglutamates to a small extent. Extracts of the 7-hour samples were further analyzed by DEAE-cellulose column chromatography to determine whether the high CO_2 treatment might alter the concentrations of individual derivatives. The results of the further analyses are shown in Table 6. It is clear from these data that the concentrations of formyl derivatives were not affected by treatment with 5% CO_2. However, the concentration of methyl derivatives was affected by this treatment. Clearly more methyl polyglutamates were present in the cells receiving 5% CO_2 in air. An effect of CO_2 on pteroylglutamate synthesis therefore appeared to be established within seven hours of initiating this treatment. As the principal derivatives in *Euglena* are known to be readily interconvertible, further more pronounced effects on pteroylglutamate synthesis might be observed with longer periods of treatment. Subsequent experiments were therefore carried out on cells which received the CO_2 treatment for more than one cell cycle.

In these experiments, the media were inoculated aseptically to give an initial concentration of *ca.* 3×10^3 cells/ml and grown synchronously with either low (0.03% in air) or high CO_2 (5% in air). Extracts were

TABLE 5. Effect of high CO_2 (5% CO_2 in air) on pteroylglutamate pool size at different stages of the first cell cycle.

Time (hr)		Air		5% CO_2 in air	
		Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP
Light phase	0	80	173	79	173
	2	83	250	81	262
	4	98	300	111	330
	6	104	310	123	370
	7	133	398	146	420
	12	143	405	164	475
Dark phase	15	120	260	163	380
	23	88	190	102	230

[a] Pteroylglutamate extracts were prepared from cells grown in high CO_2 (5% CO_2 in air) and low CO_2 (0.03% CO_2 in air) during the 1st cell cycle after the start of the experiment. Pteroylglutamate concentrations were determined with *L. casei* and are expressed as μμg of PteGlu/10^4 cells before and after pea cotyledon γ-glutamyl carboxypeptidase (γ-GCP).

TABLE 6. Concentrations of individual pteroylglutamate derivatives during growth in high and low CO_2 - First cell cycle.

Derivative	Pteroylglutamate concentration (μμg/10^7 cells)			
	Air		5% CO_2 in air	
	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP
$HCO\text{-}H_4PteGlu_{1-2}$	4100	98772	4656	91220
$5\text{-}CH_3\text{-}H_4PteGlu$	2080	25228	1303	36670
$5\text{-}HCO\text{-}H_4PteGlu_3$ } $5\text{-}CH_3\text{-}H_4PteGlu_3$	36780	*n.d.*	38300	*n.d.*
Total	42960	124000	44259	127890

n.d. - not detected.

Pteroylglutamate extracts were prepared from cells harvested after 7 hr of growth in the presence of high and low CO_2. The extracts were assayed as in Table 3. Data are expressed as μμg of PteGlu/10^7 cells. The assay organism was *L. casei*.

prepared at different stages of the 4th cell cycle. The results of pteroylglutamate analysis of these extracts both before and after γ-glutamyl carboxypeptidase treatments are shown in Table 7. Again, high CO_2 did not alter the total pteroylglutamate pool size at different stages of the cell cycle. However, when extracts prepared at 10 hr and 15 hr of the 4th cell cycle, were subjected to DEAE cellulose column chromatography (Table 8), it was clear that in high CO_2, the pool of formyl pteroylglutamates was markedly decreased but an accumulation of methyl derivatives occurred. These results suggest that CO_2 concentration may in some way regulate the production of one-carbon units at the formyl and methyl levels of oxidation.

Effects of CO_2 on glycolate dehydrogenase, serine hydroxymethyltransferase and 10-HCO-H$_4$PteGlu synthetase

The possible role of CO_2 concentration in one-carbon metabolism was further examined in enzyme studies to determine whether alteration in the level of enzymes of glycolate metabolism and one-carbon metabolism could contribute to the observed differences in pteroylglutamates. In all experiments cell-free extracts were prepared from cells harvested during the light phase, the four cell cycles after starting the high CO_2 treatment (air + 5% CO_2).

Glycolate dehydrogenase activity of extracts from cells grown in the high and low CO_2 are shown in Figure 7. Levels of this enzyme on a protein and cell number basis are given in Table 9. In agreement with results obtained for *Chlamydomonas reinhardii* (Nelson and Tolbert, 1969) and random cultures of *E. gracilis* (Codd *et al.*, 1969; Codd and Merrett, 1971b), the present data show that glycolate dehydrogenase activity

TABLE 7. Effect of high CO_2 (5% CO_2 in air) on pteroylglutamate pool size at different stages of the fourth cell cycle.

Time (hr)		Air		5% CO_2 in air	
		Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP
Light phase	2	76	195	69	180
	5	94	205	90	222
	8½	103	227	116	264
	13½	117	288	115	282
Dark phase	15	116	263	93	196
	24	73	158	60	150

Cells were grown synchronously with either low (0.03% CO_2 in air) or high CO_2 (5% CO_2 in air). Pteroylglutamate extracts were prepared at different stages of the 4th cell cycle. Pteroylglutamate concentrations were determined with *L. casei* and expressed as μμg of PteGlu/10^4 cells before and after pea cotyledon γ-glutamyl carboxypeptidase (γ-GCP).

TABLE 8. Concentrations of individual pteroylglutamate derivatives during growth in high and low CO_2 - fourth cell cycle.

	Pteroylglutamate concentration (μμg/10^7 cells)							
	Light phase; 10 hr				Dark phase; 1 hr			
	Air		5% CO_2 in air		Air		5% CO_2 in air	
Derivative	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP
HCO-H_4PteGlu$_{1-2}$	3400	155710	1890	13130	1350	77840	580	65730
5-CH_3-H_4PteGlu	790	13990	560	50690	390	17860	360	49310
5-HCO-H_4PteGlu$_3$ } 5-CH_3-H_4PteGlu$_3$	20560	*n.d.*	29940	*n.d.*	6480	*n.d.*	7140	*n.d.*
Total	24750	169700	32390	163820	8620	95700	8080	115040

Cells were harvested after 10 hr of the light phase and after 1 hr of the dark phase during the 4th cell cycle. Growth was in the presence of high and low CO_2 respectively. Pteroylglutamate extracts were assayed as in Table 3. Pteroylglutamate concentrations were determined by assay with *L. casei*.

n.d. - not detected.

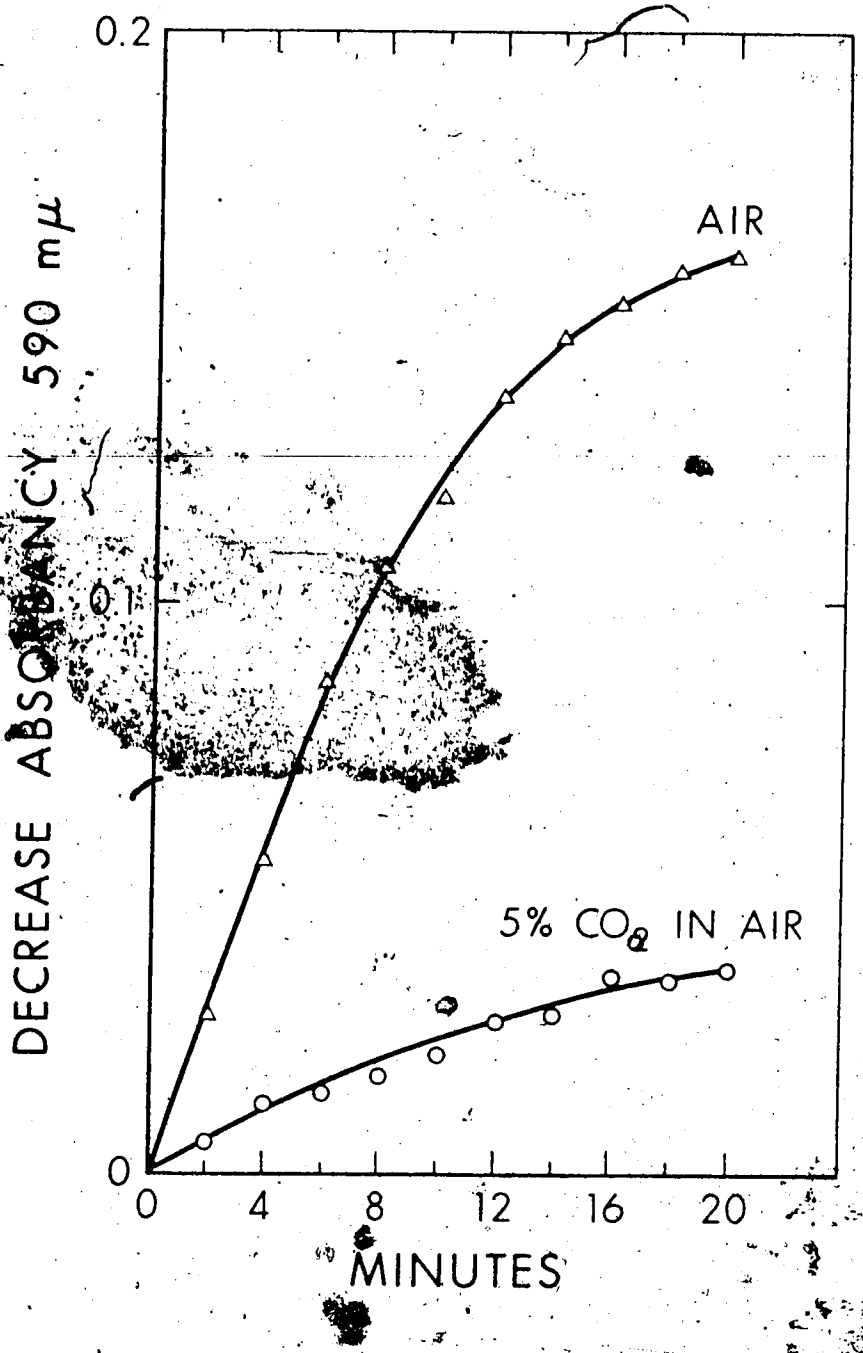


FIGURE 7. GLYCOLATE DEHYDROGENASE ACTIVITY OF [illegible] GROWN IN HIGH AND LOW CO.

Cells were cultured in high and low CO. respectively for four [illegible] cycles. Cell-free extracts were prepared after 10 hr of growth in the light phase. Glycolate dehydrogenase activity was measured as described in the Materials and Methods.

TABLE 9. Levels of glycolate dehydrogenase after growth in high and low CO_2

Culture conditions	Enzyme activity	
	units/mg protein	units/10^7 cells
Air	2.66	2.00
5% CO_2 in air	0.35	0.22

Experimental details are as in Fig. 7. Enzyme units are defined in the Materials and Methods.

was approximately 8 times higher in air-grown cells than in cells receiving high CO_2. These results imply that high concentrations of CO_2 cause repression of glycolate dehydrogenase in this organism.

The effects of CO_2 concentration on the levels of serine hydroxymethyltransferase and 10-HCO-H_4PteGlu synthetase, were also examined during the light phase of the 4th cell cycle (Fig. 8, Table 10). High CO_2 treatment increased the levels of serine hydroxymethyltransferase but decreased in the amounts of 10-HCO-H_4PteGlu synthetase per cell. These effects were apparent throughout the light phase examined.

Levels of glycolate dehydrogenase, serine hydroxymethyltransferase and 10-HCO-H_4PteGlu synthetase upon transfer from high CO_2 to low CO_2

The results of previous experiments (Figs. 7, 8 and Tables 9, 10) suggest that in high CO_2 the synthesis of glycolate dehydrogenase and 10-HCO-H_4PteGlu synthetase were repressed but in contrast the levels of serine hydroxymethyltransferase were increased. In order to determine whether these effects were readily reversible, the following experiments were undertaken. Cultures which had been grown synchronously with high CO_2 for 3 cell cycles were transferred to low CO_2 at the start of the 4th cell cycle. Extracts of these cells were prepared as before at various stages of ensuing light phase. Control cultures were maintained in the high CO_2 throughout the 4th cell cycle. The results are shown in Figure 9.

The effects of high CO_2 on these enzymes were indeed readily reversed on transfer to low CO_2. During the first 2 hr after transfer from high CO_2 to low CO_2, levels of glycolate dehydrogenase (Fig. 9c) and 10-HCO-H_4PteGlu synthetase (Fig. 9a) were either extremely low or

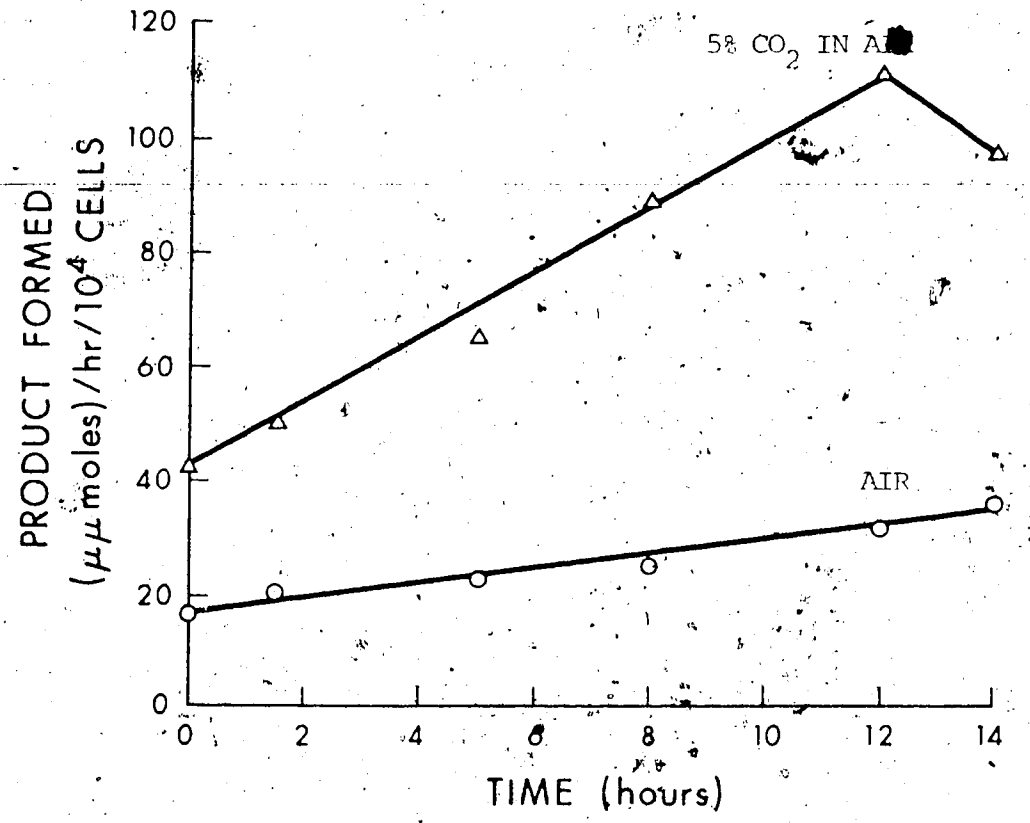


FIGURE 8. EFFECT OF HIGH AND LOW CO_2 TREATMENT ON SERINE HYDROXYMETHYLTRANSFERASE ACTIVITY.

Cells were cultured in high and low CO_2 respectively for 4 cell cycle. Cell-free extracts were prepared from cells harvested during the light period of the fourth cell cycle. Cell-free extracts were assayed using [3-^{14}C]serine as described in the Materials and Methods. Assays were carried out in duplicate and averaged. Enzyme activity is expressed as μmol product formed/hr/10^4 cells.

TABLE 10. Effect of high and low CO_2 treatment on 10-HCO-H_4PteGlu synthetase activity

Time (hr)	Enzyme activity			
	Air		5% CO_2 in air	
1½	0.27	*255*	*n.d.*	*n.d.*
5	0.31	*270*	0.03	*19*
8	0.66	*290*	0.05	*20*
12	0.74	*280*	0.07	*23*

n.d. - not detected.

Extracts were prepared from cells harvested during the light phase of the fourth cell cycle as in Fig. 8. Enzyme activities were expressed as mμmol product formed/hr/10^4 cells. Values in italics are mμmol product formed/hr/mg protein.

FIGURE 9

LEVELS OF GLYCOLATE DEHYDROGENASE, SERINE HYDROXYMETHYLTRANSFERASE AND 10-HCO-H_4PteGlu SYNTHETASE UPON TRANSFER FROM HIGH CO_2 TO LOW CO_2

Cultures were initially cultured in the presence of high CO_2 for 3 cell cycles, then transferred to low CO_2 at the start of the 4th cell cycle. Extracts were prepared at various stages of the ensuing light period. Control cultures received the high CO_2 treatment throughout. Enzyme assays were carried out in duplicate and the values averaged.

o---o : cultures transferred to low CO_2

Δ---Δ : control cultures

Specific enzyme activities at 12 hr are as follows:

(a) 230 mμmol/hr/mg protein (transferred culture)

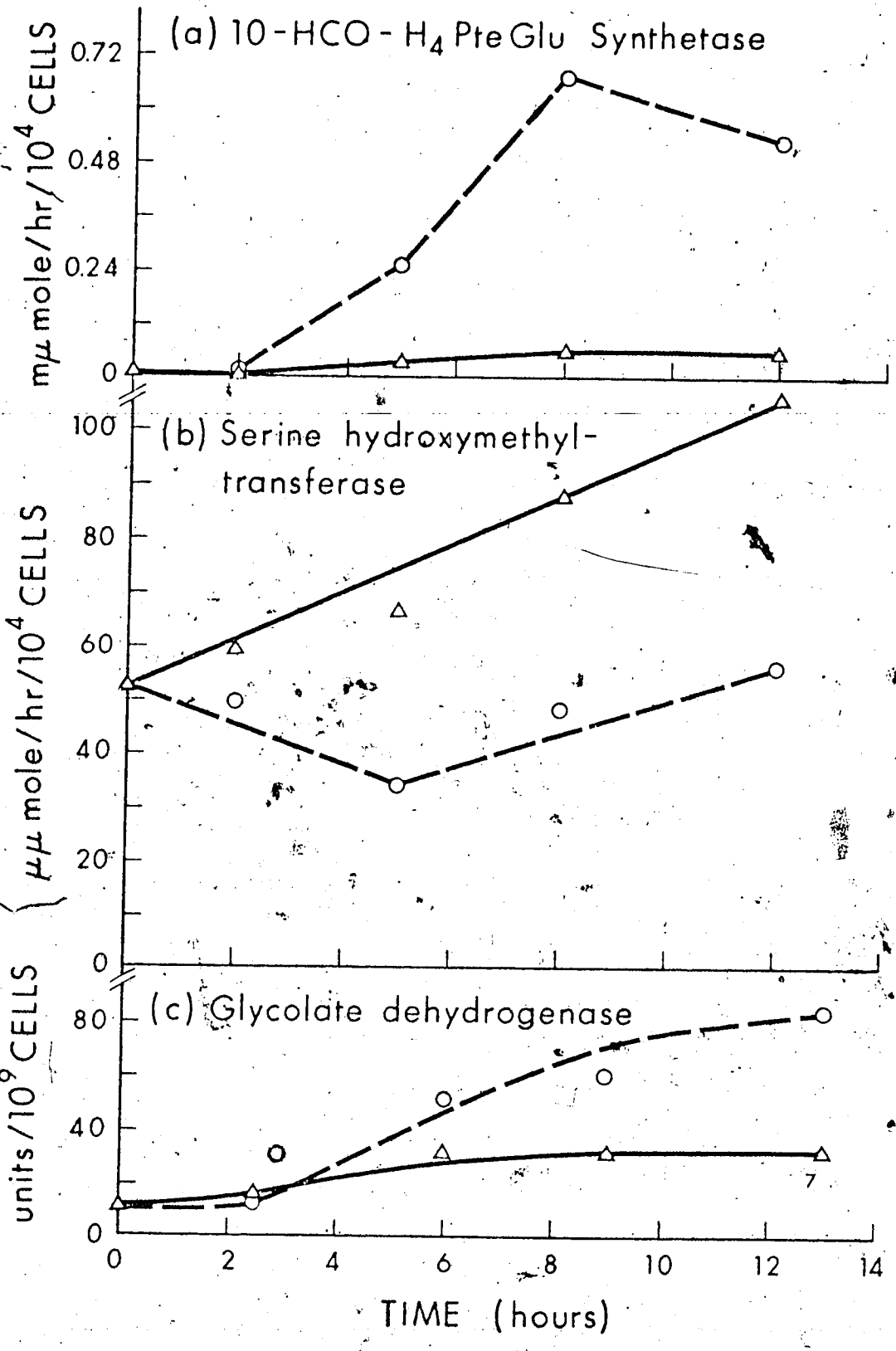
21 mμmol/hr/mg protein (control)

(b) 20 mμmol/hr/mg protein (transferred culture)

46 mμmol/hr/mg protein (control)

(c) 0.28 units/hr/mg protein (control)

1.22 units/hr/mg protein (transferred culture)


(a) 10-HCO-H_4 Pte Glu Synthetase
mμ mole/hr/10^4 CELLS
0.72
0.48
0.24
0
(b) Serine hydroxymethyl-transferase
μμ mole/hr/10^4 CELLS
100
80
60
40
20
0
(c) Glycolate dehydrogenase
units/10^9 CELLS
80
40
0
0
2
4
6
8
10
12
14
TIME (hours)

undetectable as was the case for the cells in high CO_2. However, after longer periods, the levels of these two enzymes increased at a higher rate and by 13 hr had reached levels where glycolate dehydrogenase was approximately three times higher than the control and 10-HCO-H_4PteGlu synthetase was approximately 10 times higher. Serine hydroxymethyltransferase activity (Fig. 9b) decreased during the first 5 hr after transfer and then rose slightly. In contrast, the levels of this enzyme in cells remaining in the high CO_2 increased linearly during the light phase and were higher at all stages than those of the transferred cells.

Effect of CO_2 on the concentrations of free and protein amino acids in Euglena.

Pteroylglutamate derivatives have been shown (Blakley, 1969) to participate in the synthesis and metabolism of certain amino acids. Considering the effects of high CO_2 on pteroylglutamate levels and key enzymes of one-carbon metabolism, it is possible that the pools of related amino acids may also be changed under these conditions. The concentrations of the free and protein amino acids of cells cultured in high and low CO_2 were therefore compared in further experiments (Tables 11 and 12).

Although the presence of high CO_2 increased the total amino acid pools by only 30% (Tables 11 and 12), the concentrations of several individual amino acids were appreciably changed by this treatment. For example, the high CO_2-grown cells contained relatively high concentrations of free aspartic acid, serine, glutamic acid, glycine and alanine (Table 11). Higher concentrations of glutamic acid, glycine and alanine

TABLE II. Levels of free amino acids after growth in high and low CO_2.

Amino acid (mμmol/10^7 cells)	Air	5% CO_2 in air
Lysine	26	28
Histidine	14	13
Arginine	299	283
Aspartic	5	23
Threonine	Trace	Trace
Serine	18	56
Glutamic	23	65
Proline	Trace	Trace
Glycine	8	16
Alanine	90	143
Half cysteine	29	28
Valine	16	27
Methionine	*n.d.*	*n.d.*
Isoleucine	5	4
Leucine	7	8
Total	540	694

Cells were harvested after 10 hr of the light phase (4th cell cycle). The data are average values of three separate analyses.

TABLE 12. Levels of protein amino acids after growth in high and low CO_2.

Amino acid (μmol/10^7 cells)	Air	5% CO_2 in air
Lysine	8.0	12.4
Histidine	2.5	3.1
Arginine	7.0	8.8
Aspartic acid	18.3	21.8
Threonine	9.5	9.9
Serine	8.9	8.6
Glutamic acid	14.5	21.8
Proline	9.5	9.2
Glycine	13.2	21.2
Alanine	12.8	24.5
Valine	9.4	15.2
Methionine	1.3	2.0
Isoleucine	4.8	7.9
Leucine	11.8	15.9
Tyrosine	2.4	5.3
Phenylalanine	1.1	2.0
Total	135.0	189.6

Cells were grown and harvested as in Table 11.

were also present in the protein hydrolyzates of such cells (Table 12).

Sodium [^{14}C]formate feeding experiments

The results of the pteroylglutamate analyses and enzyme studies suggest that in the presence of high CO_2, the decreases in formyl pteroylglutamate pool size was mainly due to a decrease formation of one-carbon units at the formyl level of oxidation as a result of the repression of 10-HCO-H_4PteGlu synthetases. Under these conditions one-carbon units required to support pteroylglutamate-dependent syntheses could conceivably be generated principally at the hydroxymethyl level as suggested by the increases in serine hydroxymethyltransferase associated with the high CO_2 treatment. To test the validity of this suggestion, sodium [^{14}C]formate was supplied to CO_2 treated cells and an analysis of the major products formed was carried out.

The distribution of ^{14}C in various cellular fractions following [^{14}C]formate feeding to high and low CO_2 pretreated cells is shown in Table 13. After a 2 min incubation with [^{14}C]formate, the total uptake of ^{14}C by the high CO_2-grown cells was only 46% of that shown by the low CO_2-grown cells. When the feeding time was extended to 5 min, the total uptake of ^{14}C of high CO_2-grown cells was 64% of that by low CO_2-grown cells. When comparison is made of the ^{14}C incorporated into various fractions, it is clear that the cells grown in low CO_2 had greater abilities to metabolize [^{14}C]formate. In the low CO_2-grown cells, the sugars and insoluble compounds together accounted for the bulk of the label incorporated in 2 min. As the time of feeding was increased to 5 min, the proportion of label in insoluble compounds rose, accompanied by declines in the proportion of label in free amino acids, organic acids

and sugar. Labelled CO_2 released by the low CO_2-grown cells accounted for only a very small proportion of the total ^{14}C in all cases. In contrast, high CO_2-grown cells released labelled CO_2 which accounted for a major proportion of the ^{14}C. In these cells only a small amount of label was found in the sugars. These results, in agreement with the enzyme studies indicate that, in high CO_2 utilization of formate via 10-HCO-H$_4$PteGlu synthetase was not favored. Under such conditions, formate may be utilized principally via formic dehydrogenase with some fixation of the $^{14}CO_2$ produced. These possibilities were examined in the further experiments.

As amino acids, such as glycine, serine and methionine, are closely related to the metabolism of one-carbon units, the incorporation of [^{14}C]formate into these compounds would point to a flow of carbon from formate through the pteroylglutamate pool to these products. Consequently, the levels of ^{14}C in individual free and protein amino acids were examined in detail (Tables 14, 15). The free pools of serine, glutamic acid, proline, alanine and valine were all labelled in cells which had been grown in high and low CO_2. Methionine, isoleucine and leucine were only labelled in low CO_2-grown cells after 5 min of [^{14}C]formate feeding (Table 14). Of the amino acids labelled, alanine was found to contain the most radioactivity. The amounts of ^{14}C in each amino acid were decreased by the high CO_2 treatment. This effect was most obvious in the shortest feeding experiments. These amino acids, together with others, were labelled in the protein hydrolyzates (Table 15). For example, glycine and aspartic acid were only labelled in low CO_2-grown cells while threonine was only labelled in high CO_2-grown cells. Again, in 2 min feedings, the labels in all the amino acids were reduced by the

TABLE 14. Distribution of radioactivity in individual free amino acids following [^{14}C]formate feeding in the presence of high and low CO_2.

Amino acid	2 min		5 min	
	Air	5% CO_2 in air	Air	5% CO_2 in air
Serine	805	577	729	608
Glutamic acid	1733	714	1474	760
Proline	486	n.d.	239	24
Alanine	3785	790	1881	805
Valine	2280	653	1657	562
Methionine	n.d.	n.d.	289	n.d.
Isoleucine	n.d.	n.d.	320	n.d.
Leucine	n.d.	n.d.	258	n.d.
Total	9089	2734	6847	2977

n.d. - not detected.

Experimental details are as in Table 13. Individual amino acids were separated by using an amino acid analyzer. Data are expressed as cpm incorporated by 3×10^7 cells.

TABLE 15. Distribution of radioactivity in individual protein amino acids following [^{14}C]formate feeding in the presence of high and low CO_2.

Protein amino acid	2 min		5 min	
	Air	5% CO_2 in air	Air	5% CO_2 in air
Aspartic acid	2066	*n.d.*	3268	*n.d.*
Threonine	*n.d.*	880	425	2218
Serine	4418	1804	5350	3633
Glutamic acid	1216	*n.d.*	1125	*n.d.*
Proline	426	*n.d.*	365	*n.d.*
Glycine	2189	*n.d.*	2493	*n.d.*
Alanine	2209	*n.d.*	2994	334
Valine	1762	*n.d.*	2584	745
Methionine	4114	2106	4940	5685
Isoleucine	729	*n.d.*	942	349
Leucine	1053	284	1534	653
Total	20181	5074	26020	13617

n.d. - not detected.

Experimental details are as in Table 13. Individual protein amino acids recovered after acid hydrolysis of the insoluble residue were separated by using amino acid analyzer. Data are expressed in cpm incorporated by 3×10^7 Cells.

presence of high CO_2 while in 5 min feedings most labelled amino acids, except threonine and methionine, contained the higher radioactivity when the cells were grown in low CO_2.

Intramolecular distribution of ^{14}C in serine

There are two major pathways by which serine could be labelled during the [^{14}C]formate feedings. First, [^{14}C]formate after conversion to [^{14}C]formyl pteroylglutamate and reduction could condense with glycine to produce [^{14}C]serine. The ^{14}C would as a result be predominantly in the 3 position of serine. Secondly, [^{14}C]formate might be oxidized to $^{14}CO_2$ by formic dehydrogenase followed by photosynthetic refixation of $^{14}CO_2$. In this case, serine would be predominantly labelled in the 1 position (Rabson *et al.*, 1962).

In order to examine these possibilities, [^{14}C]serine, isolated from protein hydrolysate of cells incubated with [^{14}C]formate for 5 min, was degraded to determine the intramolecular distribution of ^{14}C. The results are shown in Table 16. Serine isolated from the low CO_2-grown cells contained 51% and 41% of the ^{14}C in the 3 and 2 positions respectively, with only 9% of the label in the 1 position. On the other hand, cells grown in the presence of high CO_2 contained [^{14}C]serine which was 60%, 9% and 31% labelled in the 3, 2 and 1 positions, respectively. This suggests that in the presence of high CO_2, synthesis of serine from [^{14}C]formate involved to some extent refixation of $^{14}CO_2$. In the presence of low CO_2, such serine appeared to derive most of its label from the one-carbon pool. Labelling of the [illegible]ition of serine in the latter case suggests that the glycine pool which acts as a precursor of serine was labelled by reverse of the glycine decarboxylation reaction observed in animals (Kawosaki *et al.*, 1966; Sato *et al.*,

TABLE 16. Intramolecular distribution of ^{14}C in protein serine after metabolism of [^{14}C]formate.

	5% CO_2 in air		Air	
	cpm recovered	C^{14} distribution (%)	cpm recovered	C^{14} distribution (%)
COOH	958	31.0	246	9.0
CNH_2	272	9.0	1152	40.0
CH_2OH	1845	60.0	1436	51.0

Protein [^{14}C]serine was isolated from cells incubated with sodium [^{14}C]formate for 5 min in the presence of high and

1969) and in plants (Wang and [illegible] 1962, Colombo and Sinha, 1966; Murray *et al.*, 1971).

Effect of CO_2 on levels of formic dehydrogenase and on the enzymic decarboxylation of glyoxylate

As CO_2 was one of the major products of formate metabolism in *Euglena* grown in high CO_2, the levels of formic dehydrogenase were examined to determine whether these were also affected by the CO_2 treatments.

Assays of formic dehydrogenase activity in dialyzed cell-free extracts showed that additions of NAD or NADP only slightly increased the rate of CO_2 production. A comparison of enzyme activities in cell-free extracts of high CO_2-grown cells and low CO_2-grown cells are shown in Figure 10. Enzyme activity on a per cell basis was much higher in the high CO_2-grown cells than found in the low CO_2-grown cells.

The results of previous experiments indicate close relationships between glycolate dehydrogenase activity and the ability to synthesize 10-HCO-H_4PteGlu. In this synthesis it follows that glyoxylate, a product of glycolate dehydrogenase, might act as the source of formate for the 10-HCO-H_4PteGlu synthetase reaction. An enzymic decarboxylation of glyoxylate yielding CO_2 and formic acid has recently been reported by Zelitch (1972). It was, therefore, of some interest in the present work to determine whether this reaction could be demonstrated in *Euglena* cells which showed decreased glycolate dehydrogenase and 10-HCO-H_4PteGlu synthetase activities.

Assays were carried out under aerobic conditions with illumination. It is clear from Table 17 that the enzymic decarboxylation of glyoxylate

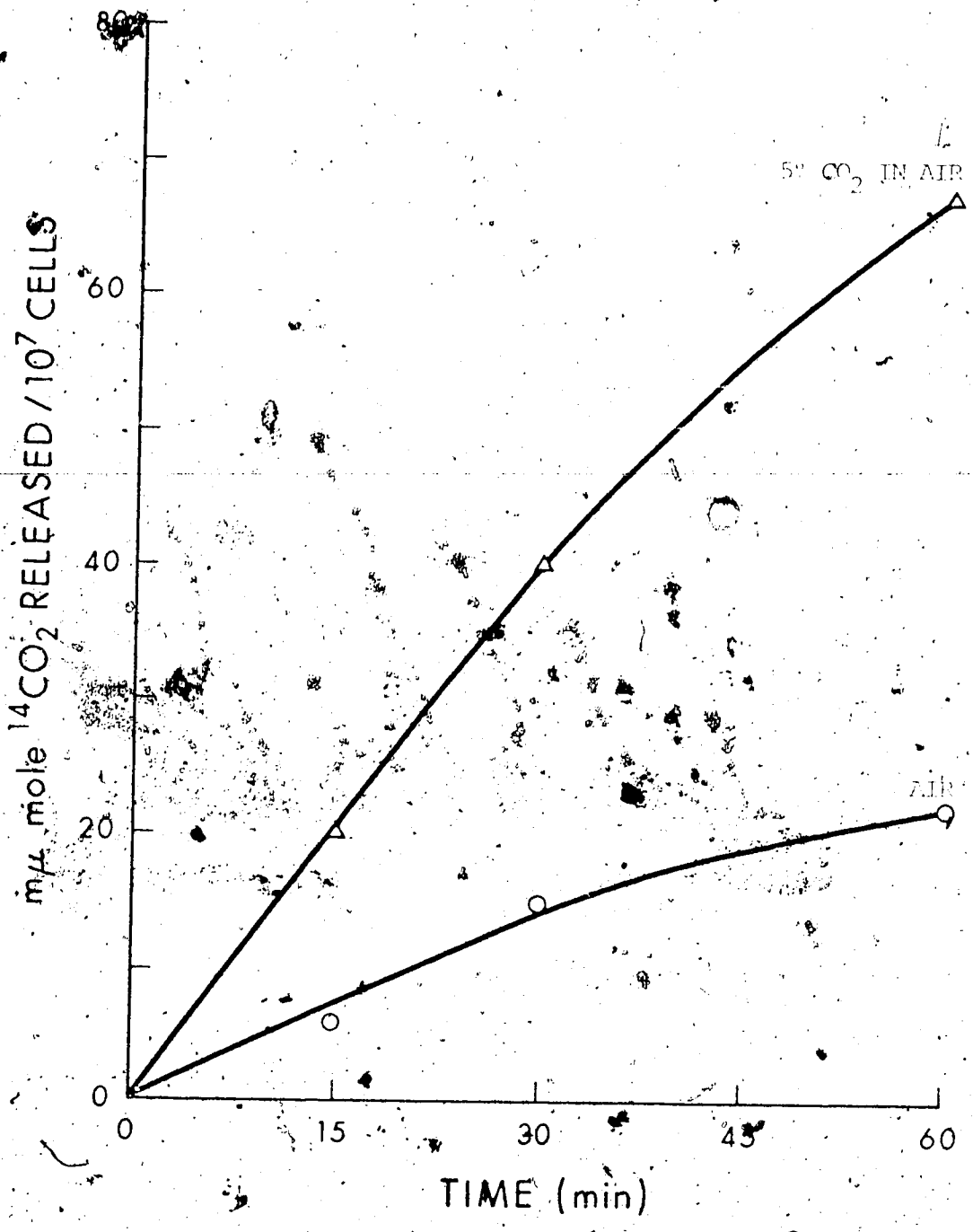


FIGURE 10. EFFECT OF HIGH AND LOW CO_2 TREATMENT ON LEVELS OF FORMIC DEHYDROGENASE

Cell-free extracts were prepared from cells harvested after 10 hr of the 4th cell cycle following culture in high and low CO_2. The complete reaction mixture, total volume 1.2 mls, containing 50 ug NAD, 0.1 uCi Na [^{14}C]formate (2 umol) and cell free extract was incubated at 30°C.

TABLE 17. Requirements for the enzymic decarboxylation of [1-^{14}C]glyoxylate

Reaction mixture	$^{14}CO_2$ produced (μμmol)
Complete	172.0
-Mn	52.0
+ ThPP	183.0
Boiled enzyme	17.0

The complete reaction mixture, total volume 1.7 ml, containing 100 μmol potassium phosphate (pH 7.5), 10 μmol $MnCl_2$, 0.1 μCi [1-^{14}C]glyoxylate (1 μCi/0.131 μmol), and 350 units catalase was incubated with cell-free extract (300 μg of protein) for 1 hr at 30°C.

had an absolute requirement for manganous ions but not for thiamine pyrophosphate. When assays of extracts from low CO_2-grown cells and high CO_2-grown cells were compared, no appreciable differences were observed (Fig. 11). Further studies of this reaction employing [1-^{14}C]glyoxylate and [2-^{14}C]glyoxylate as substrates, followed by analysis of the reaction products (Table 18) indicated that $^{14}CO_2$ arose enzymatically from [1-^{14}C]glyoxylate but not from [2-^{14}C]glyoxylate. The data further showed that formate and CO_2 were produced in equimolar amounts and that the formate was exclusively derived from the aldehyde carbon of glyoxylate.

The effects of L-methionine on the concentration of pteroylglutamate derivatives during the cell cycle

It is clear from the Introduction that pteroylglutamates are involved in the biosynthesis of methionine, a compound of considerable physiological significance as it is a direct precursor of S-adenosyl-methionine. In different organisms, this biosynthesis appears to be regulated through repression or inhibition of pteroylglutamate-mediated enzymes of the methionine biosynthetic pathway. In certain instances (Taylor *et al.*, 1966; Lee and Cossins, 1972) this control is exerted by L-methionine itself. In earlier results (Fig. 6) it was shown that the growth of *Euglena* was in fact affected by exogenous L-methionine. It was, therefore, of interest to examine the possible effect of this amino acid on levels of pteroylglutamate derivatives during the cell cycle.

In these experiments, culture media with and without a supplement of L-methionine (1 mM) were inoculated aseptically to give an initial concentration of *ca.* 3×10^3 cells/ml and grown synchronously with air

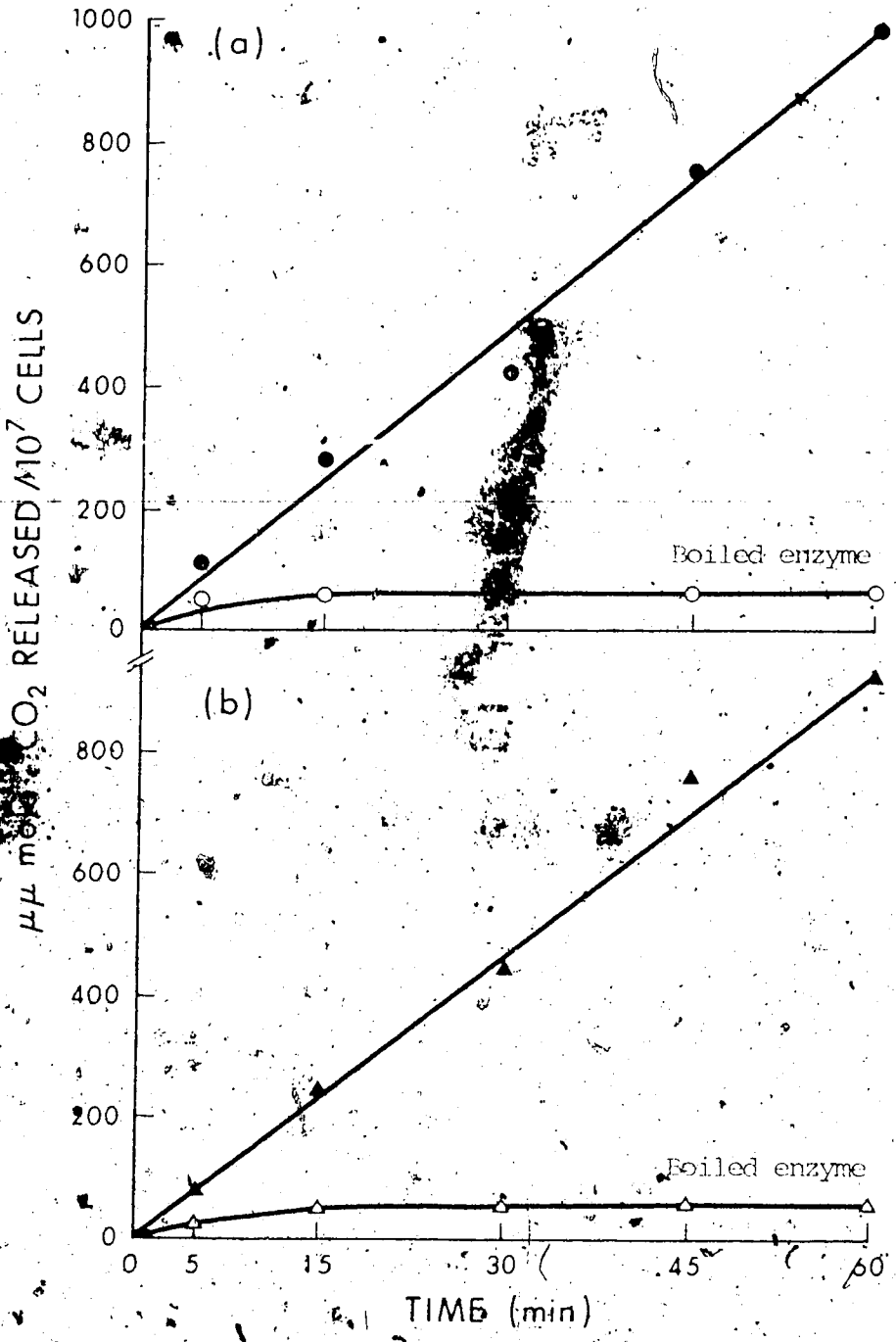


FIGURE 11. EFFECT OF HIGH AND LOW CO_2 TREATMENT ON THE ABILITY OF EXTRACTS TO DECARBOXYLATE GLYOXYLATE

Cell-free extracts were prepared from cells harvested after 10 hr of the 4th cell cycle following culture in (a) low CO_2 and (b) high CO_2. The reaction conditions are as in Table 17.

TABLE 18. Products of the enzymic decarboxylation of glyoxylate

Substrate	Air		5% CO_2 in air	
	$^{14}CO_2$ (μμmol)	[^{14}C]formate (μμmol)	$^{14}CO_2$ (μμmol)	[^{14}C]formate (μμmol)
[1-^{14}C]glyoxylate	6488	*n.d.*	7191	*n.d.*
[2-^{14}C]glyoxylate		7580	48	8142[a]

Cell-free extracts and assay conditions are as in Fig. 11 with the exception that 1.5 mg of cell-free extract protein and 1 μCi [1-^{14}C]glyoxylate (0.131 μmol) or [2-^{14}C]glyoxylate (0.135 μmol) were used in the assays.

n.d. = not detected.

as described earlier. The cells were harvested after 10 hr of the light phase and after 1 hr of the dark phase during the 4th cell cycle. Pteroylglutamate extracts were prepared and then chromatographed on DEAE-cellulose both before and after treatment with γ-glutamyl carboxypeptidase. The results of these assays are shown in Table 19.

In the light phase the concentrations of formyl derivatives, before γ-glutamyl carboxypeptidase treatment, were reduced by approximately 30% when the medium contained L-methionine. In contrast, the concentrations of methyl derivatives were not appreciably changed by this treatment. After γ-glutamyl carboxypeptidase treatment of the extracts, the concentrations of formyl derivatives were again reduced by approximately 25% in the presence of L-methionine. However, there was an increase of 20% in the concentration of conjugated methyl derivatives in the methionine-grown cells.

In the dark phase of the 4th cell cycle the concentration of formyl derivatives before and after carboxypeptidase treatment were drastically reduced in the presence of L-methionine. Similar sharp decreases were apparent for methyl derivatives occurring as polyglutamates in the methionine-grown cells.

Enzyme levels after growth in the presence of L-methionine

The previous analyses suggest that decreases in formyl derivatives associated with methionine feeding may be due to either a greater utilization of formyl derivatives or a decreased production of these derivatives when the cells receive exogenous methionine. If this rationale is correct, differences in enzyme levels, particularly 10-HCO-H_4PteGlu synthetase, may also be associated with this methionine

TABLE 19. Effect of L-methionine (1 mM) on the concentrations of pteroylglutamate derivatives in *E. gracilis*.

Derivative	Pteroylglutamate concentration (μμg/10^7 cells)							
	Light phase; 10 hr				Dark phase; 1 hr			
	- Methionine		+ L-Methionine		- Methionine		+ L-Methionine	
	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP
HCO-H_4PteGlu$_{1-2}$	3166	93460	2197	70962	2217	88226	1121	33118
5-CH_3-H_4PteGlu	1488	10781	1548	17496	1087	27920	650	6355
H_4PteGlu	*Trace*	*Trace*	*Trace*	*n.d.*	*Trace*	*Trace*	*n.d.*	*n.d.*
5-HCO-H_4PteGlu$_3$ + 5-CH_3-H_4PteGlu$_3$	12285	*n.d.*	16092	*n.d.*	7094	*n.d.*	12953	*n.d.*
Total	16939	104241	19837	88458	10398	116146	14724	39473

n.d. - not detected.

Pteroylglutamate extracts were prepared from cells harvested after 10 hr of the light phase and after 1 hr of the dark phase during the 4th cell cycle. Growth was in the presence and absence of L-methionine (1 mM) respectively. Pteroylglutamate extracts were assayed as in Table 3 using *L. casei*. Data are expressed as μμg of PteGlu/10^7 cells.

treatment. The levels of this enzyme, serine hydroxymethyltransferase and glycolate dehydrogenase are shown in Table 20. From the data it is clear that the level of the synthetase was drastically reduced in those cells receiving the L-methionine supplement, while the levels of serine hydroxymethyltransferase were increased but those of glycolate dehydrogenase were not appreciably altered. In this connection, the effects of L-methionine on 10-HCO-H_4PteGlu-synthetase and serine hydroxymethyltransferase are similar to the effects of high CO_2 concentration. This implies that production of one-carbon units at the formyl and methyl levels of oxidation are to some extent regulated by L-methionine, a product of one-carbon metabolism in this organism.

TABLE 20. Effect of L-methionine on the levels of 10-HCO-H_4PteGlu synthetase, serine hydroxymethyltransferase and glycolate dehydrogenase.

Enzyme	Enzyme activity: Product formed/mg protein - Meth	+ Meth	Product formed/cell - Meth	+ Meth
10-HCO-H_4PteGlu synthetase (mμmol product formed/hr)	270	24	1.26×10^{-4}	0.32×10^{-4}
Serine hydroxymethyl-transferase (mμmol product formed/hr)	23	30	37×10^{-7}	62×10^{-7}
Glycolate dehydrogenase (enzyme units)	1.8	1.4	1.7×10^{-7}	2.1×10^{-7}

Cells were harvested after 10 hr of the 4th cell cycle following culture in the presence and absence of L-methionine (1 mM). Cell-free extracts were assayed as described in the Materials and Methods.

DISCUSSION

As outlined in the Introduction, operation of the glycolate pathway in *E. gracilis* is widely recognized as an important route in the metabolism of carbon fixed during photosynthesis. It is also clear that operation of this pathway is finely regulated by various culture conditions including CO_2 concentration. From the present studies, it may be concluded that CO_2 concentration and L-methionine may both regulate such carbon flow by causing repression of a key enzyme of one-carbon metabolism. In the following discussion, the significance of these controls and the interrelationships between the glycolate pathway and pteroylglutamate mediated one-carbon metabolism in *E. gracilis* will be emphasized.

Changes in pteroylglutamate pool size and enzymes of one-carbon metabolism during the cell cycle.

The experimental results in Figure 2 show that the levels of both conjugated and unconjugated pteroylglutamates fluctuated during the cell cycle. It is perhaps not surprising that net pteroylglutamate synthesis occurred during illumination of the cells (Fig. 2, Table 2) as these metabolically important derivatives are known to be both directly and indirectly involved in the syntheses of purines, pyrimidines, certain amino acids and proteins (Blakley, 1969), constituents which are predominantly synthesized during the light phase of synchronized cultures (Edmunds, 1965). These increases in pteroylglutamate pool size (Fig. 2, Table 2) and the turnover of one-carbon units implied by the parallel

enzyme studies (Fig. 4), conceivably reflect an increased demand for one-carbon units to support synthesis of constituents like RNA and DNA which are both formed prior to cell division in this organism (Edmunds, 1965b). Variations in pteroylglutamate pool size during growth have also been observed in random cultures of *Saccharomyces* (Combepine *et al.*, 1971; Lor and Cossins, 1972). As in *Euglena*, much of the increase in pteroylglutamate content could be accounted for by net synthesis of formyl and methyl derivatives of $H_4PteGlu_n$. In *Chlorella ellipsoidea* fluctuations in pteroylglutamate content during growth have been reported (Morimura, 1959) but the extraction and assay procedures employed in this earlier work may have resulted in fairly extensive degradation of labile derivatives so that comparisons with the present data are not possible.

Changes in pteroylglutamate enzyme levels (Fig. 4) which accompanied the cell cycle were in some respects similar to data for related enzymes in random cultures of *L. casei* (Ohara and Silber, 1969). In this bacterium 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 enzyme levels being encountered during the exponential growth phase. Experiments with chloramphenicol and actinomycin D supported the suggestion that these enzymes were rapidly synthesized by the actively dividing cells. In the present studies, the rapid increases in 10-HCO-H_4PteGlu synthetase, serine hydroxymethyltransferase and the transmethylase (Fig. 4) clearly accompanied not only the net synthesis of pteroylglutamate derivatives but also the rise in glycolate dehydrogenase activity (Codd and Merrett, 1971b) and increased photosynthetic capacity (Walther and Edmunds, 1973). All of these changes argue for a rapid turnover of one-carbon units at this stage of

the cell cycle. The levels of 5,10-CH_2-H_4PteGlu reductase were, however, found to decrease during the light phase of growth (Fig. 4d) despite the finding (Table 2) that methylated pteroylglutamates were rapidly synthesized at this stage of the cell cycle. This suggests that the activity or synthesis of this enzyme may be strictly regulated in *Euglena*, as methylated derivatives accumulate. Mechanisms for such regulation have been described for *Saccharomyces* (Combepine *et al.*, 1971; Lor and Cossins, 1972) and for *E. coli* (Taylor *et al.*, 1966), where methyl group biogenesis is controlled by end product inhibition and enzyme repression, respectively. An examination of *E. gracilis* for similar controls of methyl pteroylglutamate biosynthesis would appear to be warranted, particularly at this stage of the cell cycle.

Analyses of the pteroylglutamates of *Euglena* showed that the major derivatives in this organism were formyl and methyl forms 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 2). The role of 5-CH_3-$H_4PteGlu_3$ in the methionine synthesis of this organism is now clear from recent studies (Milner and Weissbach, 1969) of the substrate specificity of 5-CH_3-$H_4PteGlu_3$:homocysteine transmethylase. The present investigation, although showing that some pteroylgutamate-dependent enzymes can utilize H_4PteGlu, nevertheless suggests that other reactions of one-carbon metabolism might be mediated by highly conjugated derivatives.

Regulation of one-carbon metabolism by CO_2 concentration

The results of the present studies indicate that levels of

$HCO\text{-}H_4PteGlu_n$ and $CH_3\text{-}H_4PteGlu_n$ were affected by the level of CO_2 received by the cells. The dramatic decrease in the levels of formylated pteroylglutamates by the high CO_2 treatment (Table 8) was accompanied by an apparent repression of 10-HCO-H_4PteGlu synthetase activity (Table 10). This effect of CO_2 may not be a direct effect on synthesis of the synthetase but it would nevertheless tend to reduce the biosynthesis of formyl derivatives. This reduction in formyl group biosynthesis was, however, accompanied by accumulation of methyl derivatives and by increases in the levels of serine hydroxymethyltransferase. These results suggest that conditions which reduce synthesis of one-carbon units at the formyl level do not prevent their generation at the hydroxymethyl level. This suggestion is further supported by the finding (Fig. 9) that the effect of high CO_2 on 10-HCO-H_4PteGlu synthetase and serine hydroxymethyltransferase were readily reversed when the cells were transferred to low CO_2 conditions. The lack of any significant effect of high CO_2 on formyl pteroylglutamate pool size during the first cell cycle of CO_2 treatment (Table 6) perhaps shows that 10-HCO-H_4PteGlu can be generated by oxidation of 5,10-CH_2-H_4PteGlu, a route of possible importance for generation of formyl groups when their supply via the 10-HCO-H_4PteGlu synthetase reaction is restricted.

The general conclusions based on pteroylglutamate analyses and enzyme studies are also substantiated by the results of the [^{14}C]formate feeding experiments. As the levels of 10-HCO-H_4PteGlu synthetase were decreased in cells receiving high CO_2 (Table 10) it follows that less [^{14}C]formate should enter the pteroylglutamate pool at the formyl level. Incorporation of [^{14}C]formate into the serine pool, particularly in C-3

could be regarded as an indication that a flow of one-carbon units was occurring through the pteroylglutamate pool. As formic dehydrogenase activity was readily detected in *Euglena* (Fig. 10), some refixation of formate carbon via CO_2 could be expected. In this regard only data from short feeding experiments would be meaningful in making conclusions regarding a flow of formate carbon through the pteroylglutamate pool. It is clear from the data presented in Tables 14 and 15 that high CO_2-grown cells had less capacity to incorporate [^{14}C]formate into serine although such cells were also found (Table 11) to contain higher levels of serine in the free amino acid pool. Degradation of [^{14}C]serine produced by cells grown in low CO_2 showed that almost all of the label was equally distributed between C-2 and C-3 (Table 16) which suggests that one-carbon units derived from formate were also incorporated into C-2 of glycine. Although the relatively small pool of free glycine was not labelled in these experiments, label was detected in the protein glycine of these cells. The glycine decarboxylase reaction, if it were reversible in *Euglena*, could account for this labelling of glycine. Its importance, in generation of this amino acid, could well be significant and may represent an aspect of one-carbon metabolism worthy of more detailed study in this organism. [^{14}C]Serine from high CO_2-grown cells had a different intramolecular distribution of ^{14}C. This finding also supports the suggestion that the high CO_2 treatment changed the metabolic fate of [^{14}C]formate. In this connection, it must be noted the greater amounts of $^{14}CO_2$ evolved by such cells (Table 13) and their greater levels of formic dehydrogenase (Fig. 10).

The metabolism of formate by higher plants (*e.g.* Doman and Romanova, 1962; Cossins and Sinha, 1965, Jolbert, 1955) and by

Saccharomyces cerevisiae (Lor and Cossins, 1972) has now been investigated in detail. In photosynthetic tissues the assimilation of formate appears to be partially light-dependent and involves incorporation of formate principally into serine plus some oxidation to CO_2 and subsequent refixation via the carbon reduction cycle (Doman and Romanova, 1962). In *Saccharomyces*, the metabolism of [^{14}C]formate is regulated by L-methionine present in the culture medium (Lor and Cossins, 1972). Doman and Romanova (1962) reported that the presence of atmospheric CO_2 strongly inhibited the assimilation of formic acid vapour by *Phaseolus vulgaris*. The implicit conclusion of these authors was that the apparent inhibition resulted from dilution of the specific radioactivity by atmospheric CO_2. This possibility is, however, unlikely to account for the reduced formate utilization of *Euglena* cells grown in high CO_2 as serine, formed from formate, was mainly labelled in the 3 position despite the fact that such cells had greater abilities to oxidize formate to CO_2. This observation tends to support the contention that decreased utilization of [^{14}C]formate was a result of a partial repression of 10-HCO-H_4PteGlu synthetase by high CO_2 rather than a dilution of formate carbon in an intermediary pool of CO_2.

The glycolate pathway and biosynthesis of formyl pteroylglutamates

As reviewed in the Introduction, the glycolate pathway is known to operate in *E. gracilis*. In this regard Codd and Merrett (1971b) have shown that glycolate dehydrogenase activity per volume of culture increased 4-fold prior to cell division and remained at this level during the dark phase when the cells divided. The present studies show that variations in pteroylglutamate pool size occur during the cell

cycle (Fig. [illegible]) and may be correlated with these changes in glycolate dehydrogenase activity. This implies that a close interrelationship exists between synthesis of one-carbon units and the metabolism of glycolate. Glycolate metabolism in photosynthetic tissues involves oxidation to glyoxylate and subsequent transamination to produce glycine (*e.g.*, Cossins and Sinha, 1965; King and Waygood, 1968; Tolbert *et al.*, 1969) as well as decarboxylation of glyoxylate to formic acid and CO_2 (Zelitch and Ochoa, 1953; Tolbert *et al.*, 1949; Kenten and Mann, 1952). Glycine may also give rise to one-carbon units through the glycine decarboxylase reaction in some plants (Cossins and Sinha, 1966; Clandinin and Cossins, 1972), animals (Sato *et al.*, 1969; Yoshida and Kikuchi, 1970) and bacteria (Klein and Sagers, 1966a,b). In *E. gracilis*, it is not known whether the glycine splitting reaction has any physiological significance in the biosynthesis of one-carbon units within the pteroylglutamate pool. If it was of importance one might expect treatments such as high CO_2 and α-HPMS, where glycolate dehydrogenase levels were decreased (Tables 4 and 9), to cause parallel decreases in the levels of 5-CH_3-H_4PteGlu and possibly also formyl pteroylglutamates. In the present studies, however, methyl derivatives tended to increase or were not affected by such treatments (Tables 3 and 8).

The effects of high CO_2 and α-HPMS treatments on the level of formyl pteroylglutamate derivatives rather suggests that formic acid, rather than glycine, is the major one-carbon source for the synthesis of HCO-H_4PteGlu$_n$. The decreases in HCO-H_4PteGlu$_n$ pool sizes, associated with decreased levels of glycolate dehydrogenase and 10-HCO-H_4PteGlu synthetase in the presence of α-HPMS (Table 4) and the occurrence of an

enzyme catalyzing the production of formate from glyoxylate (Table 17 and Fig. 11) all supports this view. It is interesting to note that production of H_2O_2 by the glycolate dehydrogenase reaction has so far not been established and glycolate dehydrogenase containing microbodies in *Euglena* lack catalase activity (Graves *et al.*, 1971). These observations may exclude from a physiological role the non-enzymic decarboxylation of glyoxylate by H_2O_2 (Zelitch and Ochoa, 1953; Kenten and Mann, 1952) and tend to strengthen the case for an enzymic decarboxylation *in vivo* as a route for generation of one-carbon units.

The results of the present investigation also show that when *E. gracilis* cells, grown in high CO_2 were transferred to low CO_2 , glycolate dehydrogenase appeared to be derepressed. These results are similar to reports for *Chlamydomonas reinhardii* (Nelson and Tolbert, 1969). An apparent repression and derepression of 10-HCO-H_4PteGlu synthetase by high CO_2 and upon transfer to low CO_2 (Table 10, Fig. 9a) followed the pattern of glycolate dehydrogenase. This implies that production of one-carbon units at the formyl level is related to operation of the glycolate pathway. It is, however, not clear whether the level of 10-HCO-H_4PteGlu synthetase is regulated by the availability of formate. Induced synthesis of this enzyme has, in fact, been observed in *Micrococcus aerogenes* (Whiteley, 1967) and would be worth examining in *Euglena*.

The [^{14}C]formate feeding experiments also showed that in the presence of low CO_2, considerable amounts of ^{14}C were incorporated into the sugar fraction (Table 13). This is consistent with operation of the glycolate pathway. Other labelled amino acids observed in these experiments, such as alanine and glutamic acid, were conceivably also

products of [^{14}C]serine metabolism. When the cells were grown in high CO_2, much less [^{14}C]formate was incorporated into sugars. This observation implies that a later reaction in the pathway leading to sugar formation may also be regulated, perhaps indirectly, by CO_2 concentration.

Regulation of formyl pteroylglutamate biosynthesis by L-methionine

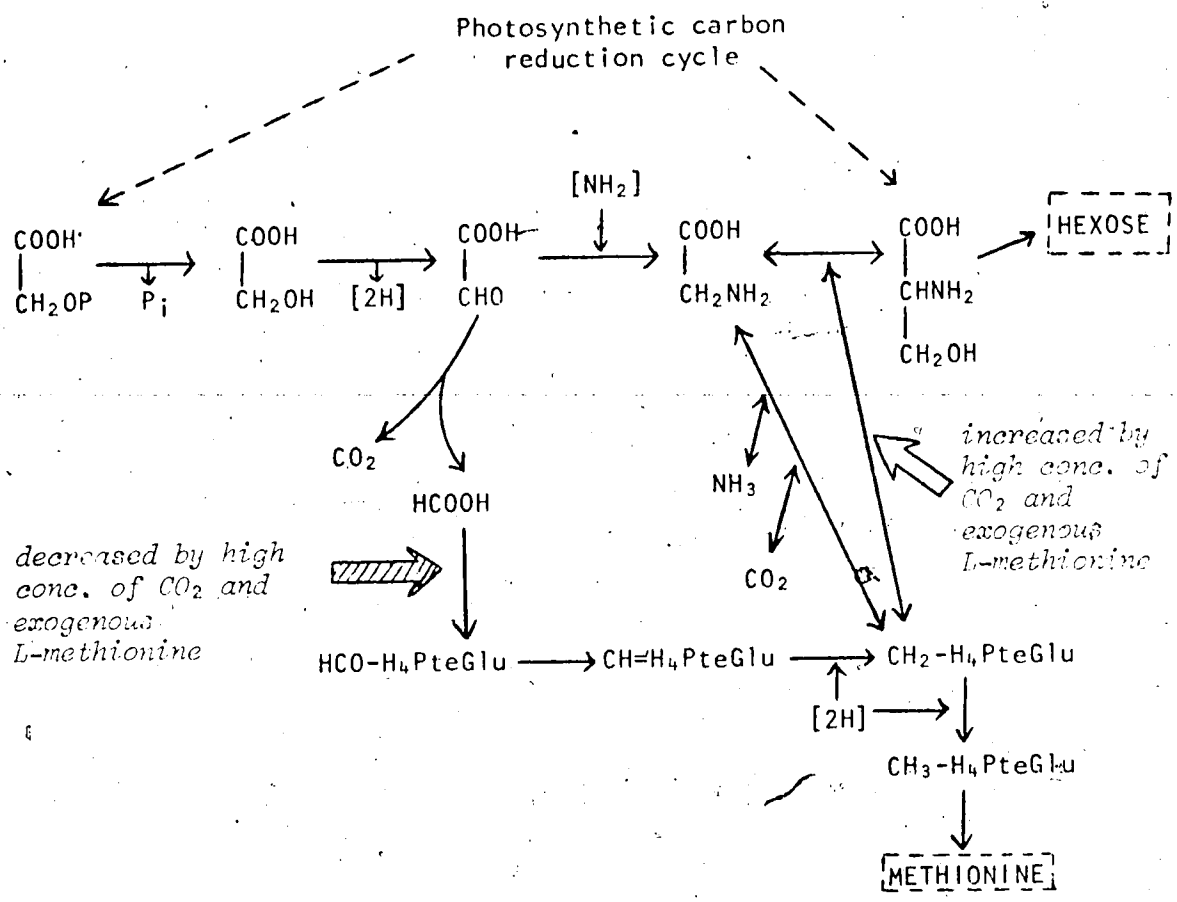
The present investations have also suggested that methionine or its product regulates the biosynthesis of HCO-H_4PteGlu$_n$. Conceivably this could occur through lowering the levels of 10-HCO-H_4PteGlu synthetase (Table 20). This effect in *Euglena* is apparently different from that observed in *Saccharomyces* (Lor and Cossins, 1972), in *E. coli* (Taylor *et al.*, 1966) and in higher plants (Dodd and Cossins, 1970; Clandinin and Cossins, 1973). In *Saccharomyces*, L-methionine decreases the flow of carbon through the methyl-H_4PteGlu pool by inhibition of 5,10-CH_2-H_4PteGlu reductase and by repression of 5-CH_3-H_4PteGlu:homocysteine transmethylase. In *E. coli*, methionine controls one-carbon metabolism by repression of 5,10-CH_2-H_4PteGlu reductase. In the mammalian system, the reductase is not repressed but is inhibited by S-adenosylmethionine. In plants, 5-CH_3-H_4PteGlu: homocysteine transmethylase (Dodd and Cossins, 1970) and glycine decarboxylase (Clandinin and Cossins, 1973) appear to be inhitibed by L-methionine *in vitro*. The apparent repression of 10-HCO-H_4PteGlu synthetase by exogenous methionine in *Euglena* would conceivably conserve H_4PteGlu by decreasing the flow of one-carbon units in the direction of methionine synthesis. Under these conditions one-carbon units required for other syntheses may be derived from serine as

indicated by the accompanied increased levels of serine hydroxymethyltransferase.

Concluding remarks

The results of the present work clearly indicate that a net synthesis of pteroylglutamates occurs in division synchronized cultures of *E. gracilis* prior to cell division. One-carbon units for this synthesis may be derived from glycolate and as such would be produced predominantly at the formyl level of oxidation. Operation of the glycolate pathway and formation of one-carbon units at the formyl level appears to be regulated by the concentration of CO_2 available to the cells and also to some extent by the presence of L-methionine. High concentrations of CO_2 decrease in the levels of glycolate dehydrogenase and 10-HCO-H_4PteGlu synthetase by approximately 95% and consequently HCO-H_4PteGlu$_n$ pool size was decreased by this treatment. L-methionine also appears to regulate synthesis of 10-HCO-H_4PteGlu$_n$ by an effect on the level of 10-HCO-H_4PteGlu synthetase. Under these conditions, the serine hydroxylmethyltransferase reaction assumes a key role in the generation of one-carbon units. These basic conclusions are summarized in Scheme 3.

At present, knowledge of one-carbon metabolism is still incomplete. This is particularly true of information regarding production and utilization of pteroylglutamates and the basic mechanisms which regulate these reactions. The present results have focussed attention on the interrelationships between one-carbon metabolism and glycolate pathway and have drawn attention to mechanisms which could regulate the biosynthesis of one-carbon units under autotrophic conditions. However,



SCHEME 3. Glycolate pathway and synthesis of one-carbon units in *Euglena gracilis*.

the actual mechanisms for regulation of 10-HCO-H_4PteGlu synthetase and serine hydroxymethyltransferase by CO_2 concentration and by L-methionine still remain to be fully elucidated.

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