One-Carbon Metabolism in *Neurospora crassa* Wild-Type and in Mutants Partially Deficient in Serine Hydroxymethyltransferase

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1. The concentrations of folate-dependent enzymes in *Neurospora crassa* Lindegren A wild type (FGSC no. 853), *Ser-1* mutant, strain H605a (FGSC no. 118), and *for* mutant, strain C-24 (FGSC no. 9), were compared during exponential growth on defined minimal media. Both mutants were partially lacking in serine hydroxymethyltransferase, but contained higher concentrations of 10-formyltetrahydrofolate synthetase than did the wild type. Mycelia of the mutants contained higher concentrations of these enzymes when growth media were supplemented with 1 mM-glycine. In the wild-type, this glycine supplement also increased the specific activities of 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methylenetetrahydrofolate reductase. 2. During growth, total folate and polyglutamyl folate concentrations were greatest in the wild-type. Methylfolates were not detected in mutant *Ser-1*, and were only present in the *for* mutant after growth in glycine-supplemented media. Exogenous glycine increased folate concentration threefold in the wild type, mainly owing to increases in unsubstituted polyglutamyl derivatives. 3. Feeding experiments using ^{14}C -labelled substrates showed that C_1 units were generated from formate, glycine and serine in the wild type. Greater incorporations of ^{14}C occurred when mycelia were cultured in glycine-supplemented media. Formate and serine were precursors of C_1 units in the mutants, but the ability to cleave glycine was slight or lacking.

The metabolism of serine via the serine hydroxymethyltransferase (EC 2.1.2.1) reaction is generally recognized as the principal route for synthesis of C_1 units in biological systems (Blakley, 1969). However, many organisms can utilize glycine as a source of methylenetetrahydrofolate ($5,10\text{-CH}_2\text{-H}_4\text{PteGlu}$)‡ (Blakley, 1969; Kikuchi, 1973; Newman *et al.*, 1974; Meedel & Pizer, 1974). Further, the general occurrence of 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) implies that C_1 units may also be generated at the formyl level of oxidation (Blakley, 1969).

Mutants, particularly of *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas* AM1, have aided the elucidation of inter-relationships between pathways and products of C_1 metabolism (Katzen & Buchanan, 1965; Smith, 1971; Greene *et al.*, 1973; Silber & Mansouri, 1971; Stauffer *et al.*, 1974; Quayle, 1972). Similarly, studies of methionine auxotrophs of *Saccharomyces cerevisiae* and *Neurospora crassa* have provided basic information on the con-

trol of methionine biosynthesis (Cherest *et al.*, 1969; Selhub *et al.*, 1969; Kerr & Flavin, 1970; Selhub *et al.*, 1971; Burton & Metzenberg, 1975). Bacterial mutants have been used to determine the nature of control mechanisms affecting serine hydroxymethyltransferase (Mansouri *et al.*, 1972; Miller & Newman, 1973; Stauffer *et al.*, 1974; Greene & Radovich, 1975). In contrast there have been few studies (Combepine *et al.*, 1975; Burton & Metzenberg, 1975) of formate-requiring mutants, although clearly such strains could be useful in assessing the importance of the 10-formyltetrahydrofolate synthetase reaction.

In *N. crassa*, Harrold & Fling (1952) reported the basic growth characteristics of two mutants that require formate or formaldehyde for growth. The formate requirement of their C-24 mutant could be replaced by adenine and methionine, but not by serine or glycine. It follows that this *for* mutant may lack serine hydroxymethyltransferase and the glycine-cleavage reaction (Kikuchi, 1973), and consequently generates C_1 units principally as $10\text{-HCO-H}_4\text{PteGlu}$. In the *Ser-1* mutant of *N. crassa*, growth and serine hydroxymethyltransferase concentration are increased by culture in minimal medium supplemented with glycine and formate (Cossins *et al.*, 1973). Additions of glycine to cultures of *N. crassa* wild type not only affect this and other enzymes of C_1 metabolism but alter the composition of the folate

‡ Abbreviations: the recommendations of the IUPAC-IUB Commission are followed in designation of pteroylglutamic acid and its derivatives, e.g. $10\text{-HCO-H}_4\text{PteGlu}$, N^{10} -formyltetrahydropteroylmonoglutamate, $5,10\text{-CH}_2\text{-H}_4\text{PteGlu}$, N^5,N^{10} -methylenetetrahydropteroylmonoglutamate, and $5\text{-CH}_3\text{-H}_4\text{PteGlu}$, N^5 -methyltetrahydropteroylmonoglutamate [*Biochem. J.* (1967) 102, 19–20].

pool (Cossins *et al.*, 1975). It was suggested that glycine may participate in C_1 metabolism as a source of 5,10- CH_2 - H_4 PteGlu. If glycine is largely derived from serine in *N. crassa* it follows that mutants deficient in serine hydroxymethyltransferase may lack this source of C_1 units. In the present work we have therefore examined the effects of exogenous glycine on the C_1 metabolism of the wild type, *Ser-1* and for mutants. Although this supplement affected folate metabolism in all three strains, the incorporation of [2- ^{14}C]glycine into products of C_1 metabolism was less extensive in the mutants. Part of this work has appeared as a congress report (Combepine *et al.*, 1975).

Materials and Methods

Materials

Chemicals. Folic acid and tetrahydrofolic acid were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Amino acids, nitrogenous bases and nucleosides were supplied by ICN Pharmaceuticals Inc., Cleveland, OH, U.S.A. All other chemicals were obtained from Fisher Scientific Co., Edmonton, Alberta, Canada. 5-[Me - ^{14}C] CH_3 - H_4 PteGlu, sodium [^{14}C]formate, L-[3- ^{14}C]serine, [1- ^{14}C]glycine and [2- ^{14}C]glycine were purchased from Amersham/Searle, Chicago, IL, U.S.A. The 5-[Me - ^{14}C] CH_3 - H_4 PteGlu was dissolved in 0.5% potassium ascorbate solution (pH 6.0) to give a final specific radioactivity of 1 μ Ci/16 nmol per 0.2 ml of solution. Sodium [^{14}C]formate was dissolved in sterile demineralized water to give a final specific radioactivity of 1 μ Ci/17.7 nmol per 0.1 ml of solution and L-[3- ^{14}C]serine was similarly dissolved to give a final specific radioactivity of 1 μ Ci/3.5 nmol per 0.1 ml of solution. Labelled glycine from the supplier was dissolved in demineralized water to give final specific radioactivities of 1 μ Ci/17.5 nmol and 1 μ Ci/16.6 nmol per 0.1 ml of solution for [1- ^{14}C]glycine and [2- ^{14}C]glycine respectively.

Methods

Culture of *Neurospora crassa*. *Neurospora crassa*, Lindegren A wild type (FGSC no. 853), was maintained in slope culture on defined minimal medium (Vogel, 1964) containing 2% (w/v) sucrose as carbon source and 2% (w/v) agar. A formate-requiring mutant, strain C-24 (FGSC no. 9), was maintained on the same medium supplemented with 10 mM-sodium formate. The minimal medium was supplemented with 10 mM-L-serine for growth of the serine-glycine auxotroph (*Ser-1*) strain H605a, FGSC no. 118. For production of conidia, 500 ml batches of the above media in 1-litre Fernbach flasks were inoculated and incubated in darkness at 30°C for 2 days. This was

followed by a 3-day period of aeration in light (Davis & DeSerres, 1970). Conidiospores were harvested by washing the cultures with sterile water, followed by filtration through four layers of fine cheesecloth. After washing three times in sterile water, the spores were used to inoculate 500 ml samples of fresh liquid minimal medium (Vogel, 1964) contained in 1-litre bottles. For liquid culture of the *for* and *Ser-1* mutants this medium was supplemented with 10 mM-sodium formate and 10 mM-L-serine respectively. In other experiments such media were also supplemented with 1 mM-glycine as described in the Results section. In all cases the initial spore concentration was 10^6 /ml. The cultures were vigorously aerated with sterile air and maintained at 25°C for periods up to 27 h. Under these conditions mycelial growth was exponential 18 h after inoculation (Cossins *et al.*, 1973). Mycelial samples were harvested at intervals by rapid filtration and dry weights were determined after washing with acetone (Davis & Harold, 1962).

Enzyme studies. After harvesting, mycelial samples (1–2 g fresh wt.) were ground at 2°C in 15 ml of 0.2 M Tris/HCl buffer containing 10 mM-KCl, 10 mM-EDTA 1 mM-MgCl₂ and 0.1 mM-dithiothreitol (pH 7.4). The extracts were centrifuged (15000g for 20 min at 2°C), then desalted by passage through columns (1.2 cm $\times$ 18 cm) of Sephadex G-15 (Pharmacia, Montreal, Canada). Enzyme activities were determined at 30°C by the following standard procedures. Formyltetrahydrofolate synthetase was measured by the method of Hiatt (1965), serine hydroxymethyltransferase by the method of Taylor & Weissbach (1965) and 5,10-methylenetetrahydrofolate reductase (EC 1.1.1.68) as described by Dickerman & Weissbach (1964). Methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) was assayed spectrophotometrically (Ramasastry & Blakley, 1964) by using mycelial extracts that had not been desalted. Protein was determined colorimetrically (Lowry *et al.*, 1951) with crystalline egg albumin (ICN Pharmaceuticals Inc.) as a reference standard.

The intracellular localization of serine hydroxymethyltransferase was examined by fractionating mycelial extracts as described by Kuntzel & Noll (1967).

Extraction and assay of folate derivatives. Freshly harvested conidia and mycelial samples (approx. 1.5 g fresh wt.) were suspended in 10 ml of 10% (w/v) potassium ascorbate (pH 6.0), placed in a boiling-water bath for 10 min, and then rapidly cooled in an ice/water bath. After homogenization and centrifugation (18000g for 20 min at 2°C) the supernatants were stored at -28°C. Folate derivatives present in these extracts were separated by DEAE-cellulose column chromatography (Sotobayashi *et al.*, 1966; Roos & Cossins, 1971). The column effluent was collected in the presence of ascorbate (Lor & Cossins, 1972) and assayed for folates by using *Lactobacillus*

casei (A.T.C.C. 7469) and *Pediococcus cerevisiae* (A.T.C.C. 8081) as described by Bakerman (1961). Folate concentrations were calculated by using standard reference curves constructed with authentic PteGlu for *L. casei* and with 5-HCO-H₄PteGlu for *P. cerevisiae* (Roos & Cossins, 1971). Individual folate derivatives were identified by using the basic criteria described previously (Sengupta & Cossins, 1971; Roos & Cossins, 1971). Polyglutamyl folates were assayed as described above, after treatment with a plant γ -glutamate carboxypeptidase (EC 3.4.12.10; Roos & Cossins, 1971; Lor & Cossins, 1972).

Extraction and analysis of free and protein amino acids. Free amino acids were extracted from samples (1.5 g fresh wt.) of mycelium by boiling for 15 min in 10 ml of water. After homogenization and centrifugation (10000g for 20 min) the extract was added to a column (1.2 cm $\times$ 6 cm) of Dowex 50W (X8; H⁺ form) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) resulting in retention of the amino acids. The column was washed with 40 ml of 6M-HCl, and the effluent dried *in vacuo* at 40°C on a flash evaporator. The residue was redissolved in 0.2M-sodium citrate buffer (pH 2.2) and analysed in an amino acid analyser (Beckman Instruments, model 121) as described by Clandinin & Cossins (1972). Insoluble material present in the mycelial homogenate (see above) was hydrolysed with 6M-HCl at 110°C for 16 h in the presence of N₂. Protein amino acids were then recovered by ion-exchange chromatography and fractionated by using the amino acid analyser.

Feeding experiments. Mycelia were harvested after 22 h growth at 25°C in the presence (1 mM) or absence of exogenous glycine. Samples (100 ml) of the culture were harvested, washed with unsupplemented Vogel's (1964) medium and then resuspended in 100 ml of this medium. Half of this suspension was used for determination of dry weights and the remainder for incubation with labelled substrates. Specific conditions for these feeding experiments are given in the appropriate Tables. The feeding period was terminated by rapid filtration, washing with ice-cold Vogel's (1964) medium and immersion in a boiling-water bath for 15 min. Free and protein amino acids were extracted and recovered by using the amino acid analyser as described above. The free amino acid extract contained [¹⁴C]adenine, which was identified by t.l.c. on silica-gel plates (20 cm $\times$ 20 cm) (Eastman Chromagram Sheet 6060; Eastman Kodak Co., Rochester, NY, U.S.A.) with butan-1-ol/acetic acid/water (12:3:5, by vol.) as solvent and by co-chromatography with authentic adenine on the amino acid analyser. Adenine concentrations were determined by measuring extinction at 260 nm and calculated by reference to standard adenine curves.

Radioactive samples (up to 500 μ l) were counted in a liquid-scintillation counter as described by Clandinin & Cossins (1972).

Results

Growth and enzymes of C₁ metabolism after culture in minimal and supplemented media

In Table 1, mycelial growth of the wild type, *Ser-1* and *for* mutants are compared 22 h after inoculation of minimal and supplemented media. The *Ser-1* mutant showed some growth in minimal medium; this was enhanced when serine, glycine and formate were added, but was greatest when 10 mM-L-serine and 1 mM-glycine were both supplied. In contrast, glycine failed to support the growth of the *for* mutant, and serine was relatively ineffectual when compared with media supplemented with formate. Growth of this mutant was also increased when 2 mM-adenine, -glycine and -glyoxylate were supplied with the formate supplement.

The specific activities of four key enzymes of C₁ metabolism were also compared during the first 24 h of mycelial growth (Fig. 1). In all cases the concentrations of these enzymes varied over this time-period and, except for the reductase, were different in the wild type and mutants, particularly during the first 18 h of growth. In this latter respect, both

Table 1. *Effect of media supplements on growth of Ser-1 and for mutants*

Freshly harvested conidia were used to inoculate (10⁶ conidia/ml) 500 ml samples of Vogel's (1964) defined liquid medium containing a 10 mM supplement where indicated. After growth for 22 h at 25°C, mycelia were harvested and dry weights were determined. Values are the means of duplicate experiments which did not differ by more than 10%. Under these conditions the wild type (FGSC no. 853) yielded 225 mg dry wt./100 ml of culture in unsupplemented medium and 150 mg dry wt./100 ml of 1 mM-glycine-supplemented culture.

Strain	Supplement (10 mM)	Growth (mg dry wt./ 100 ml of culture)
<i>Ser-1</i>	None	83
	L-Serine	276
	Glycine	140
	L-Serine + glycine (10 mM)	331
	Formate	104
<i>for</i>	None	75
	Formate	205
	Adenine	157
	L-Methionine	117
	L-Serine	130
	Glycine	69
	Glyoxylate	59
	Formate + adenine (2 mM)	315
	Formate + L-methionine (2 mM)	218
	Formate + glycine (2 mM)	304
	Formate + glyoxylate (2 mM)	268

Fig. 1. Changes in the specific activities of major folate-dependent enzymes during growth

Mycelia of the wild type (○), *Ser-1* mutant (Δ) and *for* mutant (□) were cultured at 25°C in Vogel's (1964) liquid medium. The medium was supplemented with 10 mM-L-serine or 10 mM-sodium formate for growth of *Ser-1* and *for* mutants respectively. Cell-free extracts were prepared at the times indicated and assayed for (a) 10-HCO-H₄PteGlu synthetase, (b) 5,10-CH₂-H₄PteGlu dehydrogenase, (c) 5,10-CH₂-H₄PteGlu reductase and (d) serine hydroxymethyltransferase. Zero-time values were obtained by assaying extracts of freshly harvested conidiospores. Data are the means of triplicate determinations.

mutants contained greater amounts of formyltetrahydrofolate synthetase and methylenetetrahydrofolate dehydrogenase, but were partially deficient in serine hydroxymethyltransferase. When mycelial extracts were prepared in iso-osmotic buffer (0.44 M-sucrose containing 0.1 M-NH₄Cl, 10 mM-MgCl₂ and 10 mM-Tris/HCl, final pH 7.6) and fractionated by

centrifugation (Kuntzel & Noll, 1967), serine hydroxymethyltransferase was partially associated with mitochondrial protein. In this respect, the specific enzyme activities in the wild-type and *for* mutant were 12.5 and 100.6 nmol of product/h per mg of protein respectively. In the former strain, serine hydroxymethyltransferase was mostly (99%) associated with

Table 2. *Specific activities of folate-dependent enzymes after growth in the presence and absence of 1 mM-glycine*

Mycelia were grown in Vogel's (1964) liquid medium for 22 h at 25°C as described in the Materials and Methods section. A supplement of 1 mM-glycine was made before inoculation where indicated. Media for growth of the *Ser-1* and *for* mutants were further supplemented with 10 mM-L-serine and 10 mM-formate respectively. The results are means $\pm$ S.E.M. of three determinations.

Enzyme and media supplement	Activity (nmol of product formed/min per mg of protein)		
	Wild type	<i>Ser-1</i>	<i>for</i>
10-HCO-H ₄ PteGlu synthetase			
Vogel's medium	1.2 $\pm$ 0.04	6.8 $\pm$ 0.09	8.5 $\pm$ 0.71
Vogel's medium + 1 mM-glycine	5.1 $\pm$ 0.14	5.0 $\pm$ 0.09	11.3 $\pm$ 0.38
5,10-CH ₂ -H ₄ PteGlu dehydrogenase			
Vogel's medium	1.9 $\pm$ 0.09	1.8 $\pm$ 0.05	4.5 $\pm$ 0.14
Vogel's medium + 1 mM-glycine	2.9 $\pm$ 0.09	2.5 $\pm$ 0.05	2.4 $\pm$ 0.10
Serine hydroxymethyltransferase			
Vogel's medium	0.76 $\pm$ 0.036	0.48 $\pm$ 0.075	0.092 $\pm$ 0.0019
Vogel's medium + 1 mM-glycine	0.98 $\pm$ 0.023	0.74 $\pm$ 0.028	0.27 $\pm$ 0.014
5,10-CH ₂ -H ₄ PteGlu reductase			
Vogel's medium	0.091 $\pm$ 0.0045	0.089 $\pm$ 0.0042	0.18 $\pm$ 0.0094
Vogel's medium + 1 mM-glycine	0.13 $\pm$ 0.0052	0.091 $\pm$ 0.0042	0.23 $\pm$ 0.008

Table 3. *Changes in total folate concentration during exponential growth of the wild type, Ser-1 and for mutants*

Mycelia were grown in Vogel's (1964) liquid medium at 25°C as in Table 2. Folates were extracted, hydrolysed with γ -glutamate carboxypeptidase and assayed with *L. casei* as described in the Materials and Methods section. The difference in folate concentration before and after enzyme treatment is taken as a measure of polyglutamyl folate content. The zero-time analyses were carried out on freshly harvested conidiospores. Data are expressed in ng of PteGlu equivalents/mg dry wt. and are the means $\pm$ S.E.M., with the number of separate assays in parentheses.

Time (h)	Wild type		<i>Ser-1</i> mutant		<i>for</i> mutant	
	Polyglutamyl folate	Total folate	Polyglutamyl folate	Total folate	Polyglutamyl folate	Total folate
0	6.9 $\pm$ 0.41 (3)	7.8 $\pm$ 0.33 (5)	3.8 $\pm$ 0.06 (3)	4.5 $\pm$ 0.59 (5)	7.8 $\pm$ 0.54 (3)	10.8 $\pm$ 0.81 (4)
20	36.0 $\pm$ 1.82 (3)	48.0 $\pm$ 1.95 (4)	10.3 $\pm$ 0.42 (3)	13.8 $\pm$ 0.79 (5)	11.5 $\pm$ 0.20 (3)	21.0 $\pm$ 1.23 (5)
22	22.5 $\pm$ 0.81 (3)	31.0 $\pm$ 0.88 (4)	11.3 $\pm$ 0.89 (3)	15.3 $\pm$ 0.94 (5)	13.0 $\pm$ 1.06 (3)	21.0 $\pm$ 0.41 (5)
24	28.5 $\pm$ 0.75 (3)	37.0 $\pm$ 1.99 (4)	14.8 $\pm$ 0.39 (3)	18.8 $\pm$ 0.38 (5)	14.6 $\pm$ 1.21 (3)	23.6 $\pm$ 0.92 (5)
26	28.5 $\pm$ 2.37 (3)	38.0 $\pm$ 3.04 (4)	13.5 $\pm$ 0.36 (3)	17.0 $\pm$ 0.65 (5)	9.3 $\pm$ 0.39 (3)	16.3 $\pm$ 1.04 (5)

the 27000g supernatant, but this activity was extremely low (21% of extractable activity) in the mutant.

The concentrations of all four enzymes were increased when growth occurred in media supplemented with 1 mM-glycine (Table 2), the only exceptions being a decrease in the amount of methylenetetrahydrofolate dehydrogenase in the *for* mutant and the lack of effect on the reductase concentrations of mutant *Ser-1*.

Folate and amino acid pool sizes during growth

Germination and mycelial growth were accompanied, in all three strains, by different amounts of folate synthesis. For example, preliminary assays revealed that 42, 32 and 23.3 μ g of PteGlu equivalents were present after 24 h growth per g dry wt., in the wild type, *Ser-1* and *for* cultures respectively. During

the period of exponential growth (20–26 h) the concentration of total folates in each strain (Table 3) remained relatively stable and the derivatives were principally polyglutamyl. Greatest folate concentrations were found in mycelia of the wild type.

When extracts of 22 h-old mycelia were treated with carboxypeptidase and chromatographed on DEAE-cellulose, it was clear (Table 4) that the composition of the folate pool in the mutants was different from that of the wild type. For example, methylfolates were not detected in *Ser-1* or *for* mutants when grown in media supplemented with L-serine and formate respectively. Under such conditions both mutants contained principally formyl and unsubstituted folates. The *Ser-1* mutant also contained two unidentified polyglutamyl derivatives that were not detected in the other two strains.

Table 4. Concentrations of the principal folate derivatives after growth in the presence and absence of glycine

Mycelia were harvested after growth for 22 h at 25°C. Folate derivatives were extracted and hydrolysed with γ -glutamate carboxypeptidase before column chromatography on DEAE-cellulose. Derivatives were assayed with *L. casei* and data are expressed in ng of PteGlu equivalents/mg dry wt. of mycelium for a single analysis. Other analyses showed variations of approx. $\pm 10\%$. Other details were as in Table 2. n.d., Not detected.

Derivative	Media	Activity					
		Wild type		<i>Ser-1</i>		<i>for</i>	
		Vogel's	Vogel's+glycine	Vogel's	Vogel's+glycine	Vogel's	Vogel's+glycine
10-HCO-H ₄ PteGlu ₁₊₂		7.5	2.6	10.8	5.0	11.9	2.3
5-HCO-H ₄ PteGlu ₁₊₂		9.0	1.9	2.0	6.5	2.4	9.9
5-CH ₃ -H ₄ PteGlu ₁₊₂		7.8	10.6	n.d.	n.d.	n.d.	1.3
H ₄ PteGlu		1.3	61.5	13.5	2.6	5.0	7.3
Unidentified conjugated		n.d.	n.d.	7.6	8.1	n.d.	n.d.
Total folate							
Before hydrolysis		8.5	27.0	10.3	8.2	8.0	7.5
After hydrolysis		25.7	76.6	33.9	22.2	19.4	20.7

Table 5. Concentration of free amino acids related to C₁ metabolism during exponential growth

Mycelia were grown in Vogel's (1964) liquid medium at 25°C as in Table 2. Data for zero time are for freshly harvested conidiospores.

		Concentrations (nmol/mg dry wt. of mycelium)															
Amino acid	Time (h)	...	Wild type					<i>Ser-1</i> mutant					<i>for</i> mutant				
			0	20	22	24	26	0	20	22	24	26	0	20	22	24	26
Histidine			8	27	6	1	3	9	15	15	12	12	10	19	23	21	22
Serine			24	28	26	21	15	19	444	307	196	156	47	128	113	94	89
Glycine			3	13	13	11	6	15	35	25	19	20	9	5	4	3	3
Methionine			6	1	2	2	1	11	5	4	3	4	27	5	5	6	7
Total free amino acids			120	618	383	291	179	318	975	747	578	600	372	531	587	420	532

When the growth media were supplemented with 1 mM-glycine, folate concentrations were altered (Table 4,) the greatest effect being noted in the wild type. In this case, the supplement gave a threefold increase in total folate content, which was largely due to increased synthesis of polyglutamyl derivatives that were largely unsubstituted. In mutant *Ser-1*, glycine did not alter total folate content, but the ratio of formyl to unsubstituted derivatives was changed. In the *for* mutant, mycelia grown in glycine-supplemented medium contained methylfolates, but as in mutant *Ser-1*, this treatment did not increase total folate concentration.

The concentrations of free histidine, serine and glycine tended to decline during growth in the wild type and in mutant *Ser-1* (Table 5), but in the latter, pool sizes were larger. In the *for* mutant, the amounts of histidine, serine and methionine were higher than in the wild type, but the glycine pool was smaller.

Effect of glycine supplements on metabolism of C₁-unit precursors

As exogenous glycine affected folate pool sizes and the concentrations of key folate-dependent enzymes, it is conceivable that this supplement may increase the turnover of C₁ units. This possibility was examined by ¹⁴C-feeding experiments (Tables 6–8). In the wild type (Table 6), glycine was readily incorporated into adenine, serine and methionine. C-2 of this acid was more extensively incorporated into these products than C-1, and this difference was generally greatest when the cultures had received exogenous glycine. From these data it follows that glycine was partially utilized as a source of C₁ units and that these were important in methionine biosynthesis. In contrast, [¹⁴C]formate was not incorporated into free or protein methionine, but labelling of serine and adenine was increased by the glycine supplement (Table 6).

Table 6. Incorporation of glycine, formate and serine into products of C₁ metabolism from *N. crassa* wild type

Mycelia were grown in Vogel's (1964) liquid medium, supplemented with 1 mM-glycine as indicated. After harvesting and resuspension in unsupplemented medium (see the Materials and Methods section), 50 ml samples were incubated with 10 μ Ci of [1-¹⁴C]glycine (175 nmol), [2-¹⁴C]glycine (166 nmol), sodium [¹⁴C]formate (177 nmol) or L-[¹⁴C]serine (35 nmol) respectively. The cultures were incubated at 25°C with shaking. The [¹⁴C]formate feeding was terminated after 5 min. Glycine and serine feedings were terminated after 30 min. n.d., Not detected.

Medium ...	10 ⁻³ × Sp. radioactivity (d.p.m./ μ mol)					
	Unsupplemented		+1 mM-glycine		Unsupplemented [¹⁴ C]Formate	+1 mM-glycine [¹⁴ C]Formate
	[1- ¹⁴ C]Glycine	[2- ¹⁴ C]Glycine	[1- ¹⁴ C]Glycine	[2- ¹⁴ C]Glycine		
Free amino acids						
Serine	17	21	73	129	1.5	1.9
Glycine	37	41	87	144	n.d.	n.d.
Methionine	34	34	188	1020	n.d.	n.d.
Protein amino acids						
Serine	1.8	5	12	18	n.d.	n.d.
Glycine	5.6	11	16	20	n.d.	n.d.
Methionine	0.1	0.7	0.3	3	n.d.	n.d.
Adenine	4	8	9	18	15	33
Total uptake (d.p.m./mg dry wt.)	12350		33750		787	550
						5350

Table 7. Incorporation of glycine, formate and serine into products of C₁ metabolism for *N. crassa*, for mutant

Mycelia of the *for* mutant were cultured at 25°C for 22 h in Vogel's (1964) medium containing 10 mM-sodium formate. A supplement of 1 mM-glycine was made as indicated. ¹⁴C-labelled substrates were supplied and the feeding periods were terminated as described in Table 6. n.d., Not detected.

Media ...	10 ⁻³ × Sp. radioactivity (d.p.m./ μ mol)					
	Unsupplemented		+1 mM-Glycine		Unsupplemented [¹⁴ C]Formate	+1 mM-glycine [¹⁴ C]Formate
	[1- ¹⁴ C]Glycine	[2- ¹⁴ C]Glycine	[1- ¹⁴ C]Glycine	[2- ¹⁴ C]Glycine		
Free amino acids						
Serine	1.3	3.8	13	15	0.4	0.1
Glycine	8.1	20.4	81	100	n.d.	n.d.
Methionine	2.4	5.3	22	25	n.d.	11
Protein amino acids						
Serine	1.9	1.6	5.5	3.1	n.d.	n.d.
Glycine	5.3	3.8	21	13	n.d.	n.d.
Methionine	n.d.	n.d.	0.4	1	n.d.	n.d.
Adenine	n.d.	2	1.4	1.4	2.3	7.5
Total uptake (d.p.m./mg dry wt.)	3810		15900		2200	1375
						13000

Table 8. *Incorporation of glycine, formate and serine into products of C₁ metabolism from N. crassa, Ser-1 mutant*

Mycelia of the *Ser-1* mutant were cultured at 25°C for 22h in Vogel's (1964) liquid medium containing 10 mM-L-serine. A supplement of 1 mM-glycine was made as indicated. ¹⁴C-labelled glycine, formate and serine were supplied and the feeding periods were terminated as described in Table 6. n.d., Not detected.

Media	...	10 ⁻³ × Sp. radioactivities (d.p.m./μmol)					
		Unsupplemented		+1 mM-Glycine		Unsupplemented [¹⁴ C]Formate	+1 mM-glycine [¹⁴ C]Formate
		[1- ¹⁴ C]Glycine	[2- ¹⁴ C]Glycine	[1- ¹⁴ C]Glycine	[2- ¹⁴ C]Glycine		
Free amino acids	Serine	2	2.1	1	1	1	0.3
	Glycine	14	13	6	4	n.d.	n.d.
	Methionine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Protein amino acids	Serine	0.3	0.4	0.4	0.4	n.d.	n.d.
	Glycine	1.1	2.1	1.4	1.4	n.d.	n.d.
	Methionine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Adenine		2	2.2	2	3	5.3	4.8
	Total uptake (d.p.m./mg dry wt.)		4875		2875	790	550
							1625

Considering the relatively small amounts of ¹⁴C uptake in these experiments and its initial specific radioactivity, it is clear that formate was an effective precursor of adenine and serine in this wild-type strain. These latter products were also labelled after [3-¹⁴C]serine feeding (Table 6); in addition, ¹⁴C was detected in free and protein glycine.

The mutants had less ability to utilize the labelled compounds and glycine supplements had less effect on the specific radioactivities of major products (Tables 7 and 8). Data for glycine feeding show that this compound was not a major source of C₁ units in either mutant. As expected, the *for* mutant incorporated [¹⁴C]formate into adenine, and this was greater when the mycelia received exogenous glycine. A more pronounced effect of this treatment was, however, the substantial incorporation of formate carbon into free methionine. Despite low amounts of serine hydroxymethyltransferase (Fig. 1), the *for* mutant had some ability to convert C-3 of serine into methionine and adenine.

Discussion

The data for folate-dependent enzymes (Table 2), folate derivatives (Table 4) and the ability to utilize formate, glycine and serine (Table 6) indicate that *N. crassa* wild type generates C₁ units at the formyl and hydroxymethyl levels of oxidation. In this respect, the 10-HCO-H₄PteGlu synthetase reaction may have most importance in purine-ring biosynthesis, as formate was a better precursor of adenine than was serine or glycine (Table 6), despite the fact that [2-¹⁴C]glycine would be expected to contribute more ¹⁴C (Blakley, 1969). In this respect, *Neurospora* may be like some bacteria, where control of this synthetase is clearly related to its prime role in purine synthesis (Albrechet & Hutchinson, 1964).

In the *for* mutant, the 10-HCO-H₄PteGlu synthetase reaction appears to be the principal route for biosynthesis of C₁ units. Besides containing greater concentrations of this enzyme, this mutant had greater amounts of enzymes that catalyse reduction of the substituent group (Table 2). This would tend to compensate for inability to generate sufficient 5,10-CH₂-H₄PteGlu from serine or glycine (Table 7). The *Ser-1* mutant was partially deficient in serine hydroxymethyltransferase, but the concentrations of the other three folate-dependent enzymes were comparable with those in the wild type (Table 2). However, this mutant lacked the ability to synthesize C₁ units from glycine (Table 8).

The finding that serine hydroxymethyltransferase is associated with soluble and particulate fractions in *Neurospora* argues for some compartmentation of CH₂-H₄PteGlu formation. The *for* mutant was essentially lacking in the soluble enzyme, and it follows that the incorporation of [3-¹⁴C]serine into

methionine by this strain (Table 7) was mainly catalysed by the mitochondrial enzyme. As most of the serine hydroxymethyltransferase in the wild type was non-particulate, the *for* mutant may be severely limited in ability to generate glycine from serine. Some support for this view comes from the finding (Table 5) that this mutant contained less free glycine than the wild type, but more free serine. Also, when supplemented with formate and glycine, this mutant produced more mycelium. In this situation more glycine uptake and metabolism occurred (Table 7), but C-2 was not extensively utilized as a source of C₁ units.

In the wild type, supplementing the minimal medium with 1 mM-glycine altered the concentrations of key folate-dependent enzymes (Table 2), the concentration of unsubstituted polyglutamyl folate (Table 4) and increased the ability to incorporate C-2 of glycine into products of C₁ metabolism (Table 6). The glycine-cleavage reaction in bacteria is induced by exogenous glycine (Kikuchi, 1973) and data in Table 6 suggest that this may occur in *Neurospora*. Although glycine might also have a direct effect on the synthesis of serine hydroxymethyltransferase (Botsford & Parks, 1969; Cossins *et al.*, 1973; Burton & Metzenberg, 1975; Cossins *et al.*, 1975), there is growing evidence that this enzyme is concertedly regulated by several effectors (Stauffer *et al.*, 1974; Greene & Radovich, 1975; Burton & Metzenberg, 1975). In *Salmonella* it is clear that these may include folates (Stauffer *et al.*, 1974). Thus glycine, by inducing the cleavage reaction and therefore augmenting production of methylenefolate, could indirectly affect turnover of C₁ units in *N. crassa*.

The relatively large accumulation of unsubstituted folate in glycine-supplemented cultures (Table 4) may arise from CH₂-H₄PteGlu_n, which would dissociate into H₄PteGlu_n under our conditions of extraction and chromatography (Blakley, 1969). This polyglutamyl folate could be important in glycine cleavage, as the *Ser-1* mutant, which appeared to lack this reaction (Table 8), did not accumulate this folate after growth in glycine (Table 4). In *Neurospora* there is evidence that methyl polyglutamyl folates are important in controlling methionine biosynthesis (Selhub *et al.*, 1971). In this case, CH₃-H₄PteGlu_n, by activating cystathionine γ -synthase, is instrumental in synchronizing the converging pathways of methionine biosynthesis (Selhub *et al.*, 1971). Conceivably, other polyglutamyl folates could have roles in activating related pathways of C₁ metabolism. From the present studies it is logical to conclude that glycine-supplemented cultures had an increased turnover of C₁ units at the formyl, hydroxymethyl and methyl levels of oxidation. It would be of interest to determine whether this general stimulation of folate metabolism occurs in other micro-organisms.

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