Sample locality	Date	No. of specimens	Frequency of genotypes				Allele frequency	
			Ldh-1/*Ldh*-1	*Ldh*-1/*Ldh*-2	*Ldh*-2/*Ldh*-2	X^2	*Ldh*-1	*Ldh*-2
Volano Beach (Adriatic Sea – 100 km south of Venice)	September 1971	476	Obs. 165 Exp. 166.8	234 239.9	77 79.2	0.22	0.592	0.408
	September 1972	421	Obs. 149 Exp. 152. 5	209 201.7	63 66.6	0.53	0.602	0.398
The Gulf of Naples (Tyrrhenian Sea)	September 1972	706	Obs. 286 Exp. 279.3	317 329.5	103 97.1	0.98	0.629	0.371

Our hypotheses were also confirmed by the good fit between the observed and expected gene frequencies (see the Table). The gene frequency was nearly identical in the various populations. This is not surprising in view of the wide range and high rate of migration of *Belone belone* populations living in the Mediterranean Sea.

The foregoing observations show that both Adriatic and Tyrrhenian populations of *Belone belone* contain 2 panmictic homozygous genotypes of LDH isoenzyme (*Ldh*-1/*Ldh*-1 and *Ldh*-2/*Ldh*-2), which through interbreeding, yield a hybrid (*Ldh*-1/*Ldh*-2) displaing intermediate features.

Riassunto. Gli isoenzimi della LDH di *Belone belone* si presentano multipli e polimorfi all'analisi elettroforetica. Nelle popolazioni studiate si mettono in evidenza 3 genotipi diversi, 2 dei quali a se stanti ed 1 con caratteristiche intermedie. L'ibridazione molecolare in vitro ed il confronto fra le frequenze genotipiche osservate e teoriche dimostrano che si tratta di 2 genotipi omozigoti che incrociandosi danno un ibrido con caratteristiche intermedie.

C. Callegarini and D. Ricci

Istituto di Anatomia comparata, via Scienze, 17, I–44100 Ferrara (Italy), 2 April 1973.

Effects of Glycine and Serine on Serine Hydroxymethyltransferase Levels in Logarithmic Cultures of *Neurospora crassa* Wild Type and *Ser-1* mutant

From recent detailed investigations it is clear that the formation and metabolism of one-carbon units in microorganisms is finely regulated. This may be achieved by repression[1–8] of certain key pteroylglutamate[9] dependent enzymes and by feedback inhibition of their activity[8,10–13]. As the serine hydroxymethyltransferase reaction is generally considered to be the major physiological route for synthesis of C-1 units it is conceivable that the formation as well as metabolism of 5,10-CH_2-H_4PteGlu will be controlled in these systems. In *Escherichia coli* 113-3 such control is exerted by exogenous L-methionine[7] which regulates the availability of C-1 units by repression and derepression of serine hydroxymethyltransferase. In an amethopterin-resistant strain of *Streptococcus faecium*[3] high levels of L-serine in the culture medium cause some repression of this enzyme's synthesis. In *Saccharomyces cerevisiae*, where the β-carbon of serine is a direct precursor of the methyl carbon of methionine[14], it is clear that synthesis of 5-CH_3-H_4PteGlu is regulated by this latter amino acid[8,13] and that glycine stimulates formation of serine hydroxymethyltransferase[15].

As part of a continuing study[8,13] of C-1 metabolism in fungi, the present investigation has examined the possible regulation of serine hydroxymethyltransferase by glycine, serine and methionine in *Neurospora crassa* wild type. Comparative studies with a *N. crassa* serine-glycine auxotroph (*Ser-1*), known to be deficient in synthesis of serine from phosphohydroxypyruvate[16], are also reported.

Materials and method. Neurospora crassa Lindegren A (FGSC no. 853) and *Ser-1* mutant strain H605a (FGSC no.118) were cultured aerobically at 25°C in the minimal medium of Westergaard and Mitchell[17] modified to include ammonium citrate[18]. 6 l aliquots of

[1] A. M. Albrecht and D. J. Hutchison, J. Bact. *87*, 792 (1964).
[2] O. Ohara and R. Silber, J. biol. Chem. *244*, 1988 (1969).
[3] A. M. Albrecht, F. K. Pearce and D. J. Hutchison, J. Bact. *95*, 1779 (1968).
[4] J. J. Burchall and G. H. Hitchings, Adv. Enzyme Regul. *6*, 323 (1967).
[5] R. J. Rowbury and D. D. Woods, J. gen. Microbiol. *24*, 129 (1961).
[6] R. Silber and A. Mansouri, Ann. N. Y. Acad. Sci. *186*, 55 (1971).
[7] A. Mansouri, J. B. Decter and R. Silber, J. biol. Chem. *247*, 348 (1972).
[8] K. L. Lor and E. A. Cossins, Biochem. J. *130*, 773 (1972).
[9] Abbreviations for pteroylglutamate derivatives are those of the IUPAC-IUB Commission as listed in Biochem. J. *102*, 15 (1967).
[10] F. R. Dalal and J. S. Gots, Biochem. biophys. Res. Commun. *22*, 340 (1966).
[11] F. R. Dalal and J. S. Gots, J. biol. Chem. *242*, 3636 (1967).
[12] R. T. Taylor, H. Dickerman and H. Weissbach, Biochem. biophys. Res. Commun. *16*, 593 (1964).
[13] G. Combepine, E. A. Cossins and K. L. Lor, FEBS Lett. *14*, 49 (1971).
[14] J. L. Botsford and L. W. Parks, J. Bact. *94*, 966 (1967).
[15] J. L. Botsford and L. W. Parks, J. Bact. *97*, 1176 (1969).
[16] A. Meister, Adv. Enzymol. *16*, 185 (1955).
[17] M. Westergaard and H. K. Mitchell, Am. J. Bot. *34*, 573 (1947).
[18] G. Turian, Nature, Lond. *202*, 1240 (1964).

the media (medium M) were inoculated with freshly harvested conidia to give an initial concentration of 3×10^5 conidia/ml. The culture vessels were vigorously aerated with sterile air for periods up to 25 h. Samples were removed during growth, harvested by rapid filtration and immediately placed in liquid N_2. For dry weight determinations, filtered samples were washed with acetone[19] and dried prior to weighing. In several experiments medium M was supplemented with glycine and L-serine as summarized in the Results.

For enzyme studies the mycelial samples were ground at 2°C, within 24 h of harvesting, in a buffer containing *Tris*-HCl, 0.2 *M*; KCl, 10 m*M*; EDTA,10 m*M*; $MgCl_2$, 1 m*M*; dithiothreitol, 0.1 m*M* (pH 7.4). After centrifugation (15,000 × *g* for 20 min) samples of the extract were passed through columns of Sephadex G-15. Serine hydroxymethyltransferase activity was assayed by the method of TAYLOR and WEISSBACH[20]. Labelled 5,10-CH_2-H_4PteGlu, formed in the reaction, was converted to the formaldehyde-dimedon adduct and assayed for ^{14}C by liquid scintillation counting[21]. Protein was assayed colorimetrically[22]. Specific enzyme activities are expressed as nmoles product formed/min/mg protein under the standard assay conditions.

Results and discussion. From Figure 1 it is clear that the wild type displayed logarithmic growth in unsupplemented medium M, 17–20 h after inoculation. After this period the culture entered the stationary phase. Cultures receiving supplements of serine, glycine and methionine at 0.1 m*M* by comparison, displayed logarthmic growth for longer periods. Changes in the specific activity of serine hydroxymethyltransferase were observed (Table I) when extracts were prepared from such cultures. During culture in medium M the specific activity declined by approximately 40%. In contrast, cultures receiving supplements of glycine had higher enzyme levels and these were increased during growth. Serine at 1 m*M* gave a similar effect. When the medium was supplemented with L-methionine the specific activities were again higher than those of the control. This finding suggests that synthesis of serine hydroxymethyltransferase in *N. crassa* may not be repressed by exogenous L-methionine as occurs[7] in *E. coli* 113-3. This amino acid also failed to decrease enzyme activity in vitro (Table II).

In studies with the Ser-1 mutant (Figure 2) some growth was observed in the absence of glycine or serine. This growth was not appreciably increased by additions

Table I. Specific activity of serine hydroxymethyltransferase after culture of *N. crassa* wild type in supplemented minimal medium

Supplement of minimal medium	Age of culture (h)			
	18	20	22	24
None	1.30	0.96	0.95	0.79
Glycine (1 m*M*)	1.95	3.11	3.13	3.56
Glycine (0.1 m*M*)	1.32	2.20	2.62	2.55
Serine (1 m*M*)	1.28	1.97	2.23	3.84
Serine (0.1 m*M*)	1.59	1.96	1.06	1.66
Methionine (0.1 m*M*)	2.48	2.24	2.20	1.91

Table II. Serine hydroxymethyltransferase of *N. crassa* wild type. Lack of inhibition in vitro by L-methionine

Final concentration of L-methionine in reaction system (m*M*)	Enzyme activity (nmoles product formed/10 min)
0	0.32
0.33	0.31
0.66	0.33
1.65	0.30
3.30	0.31

Cell-free extracts were prepared from mycelia harvested after 24 h of growth at 25°C in the minimal medium M. After gel filtration, enzyme activity was assayed on aliquots containing 18 µg protein using the standard reaction system.

[19] R. H. DAVIS and F. M. HAROLD, *Neurospora* Newslett. *2*, 18 (1962).
[20] R. T. TAYLOR and H. WEISSBACH, Analyt. Biochem. *13*, 80 (1965).
[21] W. A. DODD and E. A. COSSINS, Archs Biochem. Biophys. *133*, 216 (1969).
[22] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, J. biol. Chem. *193*, 265 (1951).

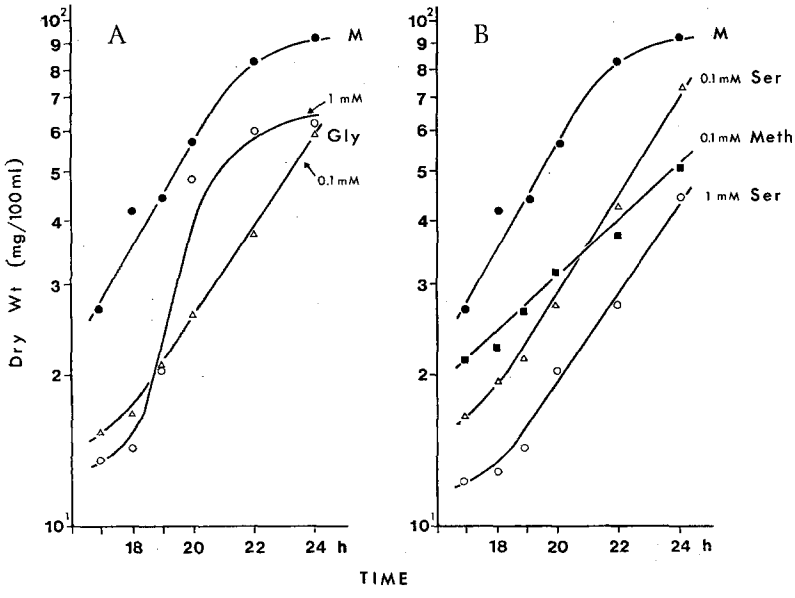

Fig. 1. Growth of *N. crassa* wild type in supplemented minimal media. Cultures were aerated at 25°C in the media indicated. Between 17–24 h samples of mycelia were harvested and dry weights determined.

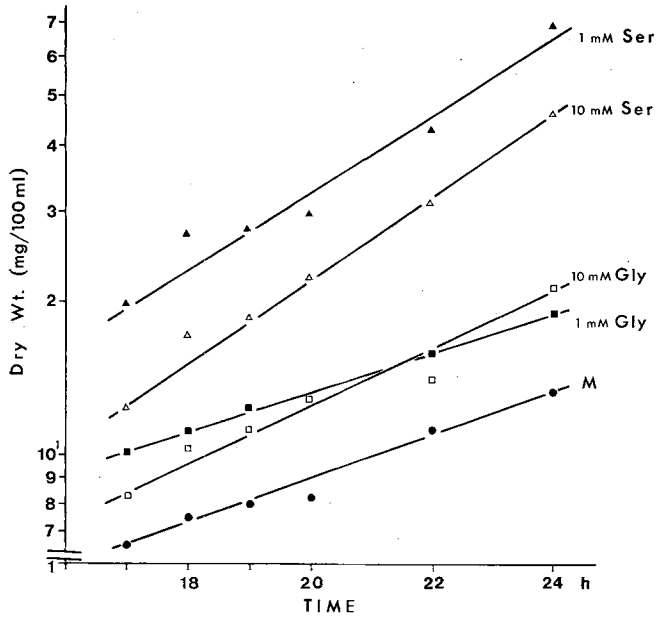

Fig. 2. Effect of media supplements on the growth of *N. crassa Ser-1* mutant. Cultures were treated as in Figure 1.

of glycine (1 and 10 m*M*). Greater increases in dry weight were however observed in the presence of L-serine. As this mutant lacks ability to catalyze the transamination of phosphohydroxypyruvate[16] it follows that sufficient synthesis of serine to support a relatively low rate of growth may occur via the serine hydroxymethyltransferase reaction. Extracts of this mutant were found to contain this enzyme activity. During the period of growth examined in Figure 2 the specific activity rose from 0.9 to 1.2 and from 0.8 to 1.9 in the 10 m*M* serine and unsupplemented cultures respectively. In more detailed studies (Figure 3A and B) cultures were aerated in medium M for 18 h at which time various supplements were made. Addition of 1 m*M* serine resulted in an increased growth rate but glycine at 1 and 10 m*M* failed to give this effect within 7 h. Some stimulation of growth rate was observed when glycine and formate at 10 m*M* were added together (Figure 3B). These treatments affected enzyme levels as summarized in Table III. Enzyme levels in 1 m*M* serine-supplemented cultures were similar to those of the control. In glycine at 10 m*M* the specific activities were above those of the control and this stimulatory effect was further increased when medium M was supplemented with glycine and formate. Formate, when added to medium M alone, did not increase enzyme levels above those observed for glycine at 10 m*M*.

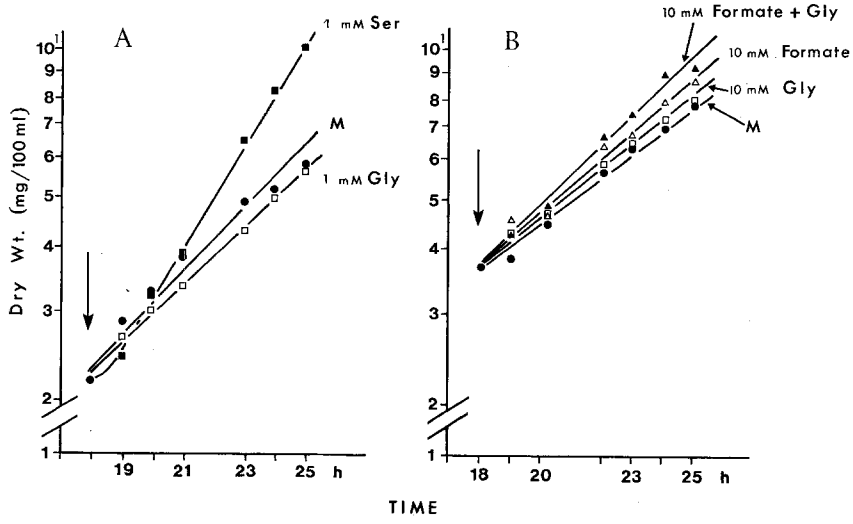

Fig. 3. Growth of *N. crassa Ser-1* mutant following additions of glycine, serine and formate to the minimal medium. Cultures in minimal medium M were aerated at 25 °C for 18 h. Supplements of the amino acids and formate were made as indicated by the arrows. Cultures M (●) did not receive supplement and served as controls.

Table III. Effects of media supplements on the specific activity of serine hydroxymethyltransferase in the *Ser-1* mutant

Time after supplement made (h)	Supplement of medium M				
	None	Serine (1 m*M*)	Glycine (1 m*M*)	Glycine (10 m*M*)	Glycine + formate (10 m*M*)
2	1.16	—	1.15	2.07	1.83
5	—	1.97	1.99	2.56	2.94
7	1.91	2.13	2.32	2.35	4.35

Supplements were made to 18-h-old cultures aerated in minimal medium at 25 °C. Specific enzyme activity before making the supplements was 0.76.

The effects of glycine on the levels of serine hydroxymethyltransferase in both the wild type and *Ser-1* mutant suggest that synthesis of this enzyme may be to some extent regulated by exogenous glycine. This effect would have physiological significance if the serine hydroxymethyltransferase reaction served as a route for serine synthesis in *Neurospora*. Evidence for involvement of this enzyme in serine synthesis is well documented for several organisms including *Saccharomyces*[23,24,15]. In the latter species glycine can serve as the precursor of the β-carbon of serine[25] and may act also as an inducer of serine hydroxymethyltransferase synthesis[15]. From the present studies it would appear that glycine and to a lesser extent serine, can regulate this key enzyme of C-1 metabolism in *N. crassa*. The origins of the C-1 unit required for synthesis of serine from glycine remain to be elucidated. If 5,10-CH_2-H_4PteGlu readily arises from glycine in the *Ser-1* mutant it is difficult to account for the lack of appreciable growth stimulation when glycine was supplied exogenously (Figure 3). On the other hand, if C-1 units for serine synthesis arise via 10-HCO-H_4PteGlu, formate together with glycine would serve as precursors of serine formed in the serine hydroxymethyltransferase reaction. These and related possibilities are currently under more detailed investigation.

Résumé. Dans un milieu minimal la glycine et la sérine, ajoutées en concentrations physiologiques, augmentent le taux de la sérine-hydroxyméthyltransférase durant la croissance logarithmique du *Neurospora crassa*. La glycine influence également la synthèse de cet enzyme chez le mutant *Ser-1* de *Neurospora*. Ainsi la glycine exogène exerce une régulation de la sérine-hydroxyméthyltransférase et cet enzyme joue un rôle possible dans la synthèse de la sérine dans cet organisme en phase logarithmique de croissance.

E. A. COSSINS[26,27], G. COMBEPINE and G. TURIAN

Laboratory of General Microbiology, University of Geneva, CH-1211 Geneva 4 (Switzerland), 9 July 1973.

[23] R. D. SAGERS and I. C. GUNSALUS, J. Bact. *81*, 541 (1961).
[24] E. A. COSSINS and S. K. SINHA, Biochem. J. *101*, 542 (1966).
[25] J. F. DEBORSO and A. O. M. STOPPANI, Biochim. biophys. Acta *148*, 48 (1967).
[26] Permanent address: Department of Botany, University of Alberta, Edmonton, Alberta, Canada. Professeur Invité, Université de Genève 1972-73.
[27] Acknowledgements. This investigation was supported by grant No. 3.540.71 from the Fond National Suisse de la Recherche Scientifique. E.A.C. was the recipient of a National Research Council of Canada travel fellowship. The authors thank Mlle MARTINE GOURJON for excellent technical assistance.

Effect of Wall-Degrading Enzymes on Protoplasts Transaminase Activity

Eliminating plant cell walls in order to obtain large amounts of protoplasts is usually achieved by using enzymes which destroy the skeletal envelope[1]. But such enzymes may alter some of the protoplast properties. It was, therefore, necessary to ascertain such a probable deterioration. Since protoplasts can be produced by slicing walls with no biochemical treatment[2], a comparative study of both kinds of protoplasts might possibly solve this question. In fact, it has been observed[3] that the transaminase activity of protoplasts enzymatically prepared from *Allium* roots was frequently lower than that of protoplasts mechanically obtained. However, such a comparison was not very helpful because the volume and the nature of the cells giving protoplasts and the size of the protoplasts themselves depend to a large extent on the two techniques adopted[4]. To decide whether wall-destroying enzymes really change the protoplasts metabolism during their enzymatical preparation, the effect of these enzymes has to be directly tested on the protoplasts obtained mechanically. The biochemical reactivity of the protoplasts will be analysed by measuring – as done previously[3] – their transaminase activity.

Roots (30 mm $\pm$ 3) of *Allium cepa* were used[5]. After removing the tip (3 mm) and first soaking (5 min) in 20% sucrose solution, they were cut into small sections which were placed immediately (85 min) in a solution of sucrose (20%) and NaCl (1.2%)[2]. On both faces, each thin root section gave protoplasts (approximately $4.5 \times 10^6 \pm 1 \times 10^6$ for 20 g of roots). The protoplasts suspension was immediately used or stored (dark, 25 °C $\pm$ 0.5) in a 20% sucrose solution with Penicillin G (Specia) at 8×10^{-4} *M*. and with or without enzymes mixture. Penicillin was found to be ineffective on cells of higher plants[6] but it stopped any bacterial contamination for at least 16 h. It has to be noted that the enzymes used were not purified and undoubtedly contained many different active species[7]. The incubation (12h), the nature (Macerozyme [pectinases]: UNWIN, Herts; Cellulase ONOZUKA P 1500: All Japan. Biochem.) and the concentration (5%) of the enzymes were chosen identically to those previously adopted for the enzymatical preparation of protoplasts[8]. Because of the optimal pH of the enzymes[9], all the solutions were buffered at pH 5.0 $\pm$ 0.3. Before extracting the enzymes, the protoplasts were washed carefully 4 times with a buffered 20% sucrose solution[5]. Isolating the transaminase[10] was adapted to the protoplasts[3], and the Aspartate-aminotransferase (EC 2.6.1.1) was analysed (incubation: 60 min; 37 °C $\pm$ 0.5) by determining spectrophotocolorimetrically (492 nm) the coloured hydrazones formed after using a pyruvate standard[11].

[1] E. C. COCKING, Ann. Rev. Plant. Physiol. *23*, 29 (1972).
[2] J. A. PLOWE, Protoplasma *12*, 196 (1931); R. PRAT and J. C. ROLAND, C. r. hebd. Séanc. Acad. Sci., Paris *271*, 1862 (1970).
[3] P. E. PILET, Experientia *28*, 638 (1972).
[4] P. E. PILET, in *Protoplastes et fusion de cellules somatiques végétales* (Coll. Intern. CNRS, 1973), vol. 212, p. 99.
[5] P. E. PILET, R. PRAT and J. C. ROLAND, Pl. Cell Physiol. *13*, 297 (1972).
[6] B. PARTHIER, Nature *206*, 783 (1965); M. A. VENIS, Phytochemistry *6*, 799 (1967).
[7] K. SELBY, in *Protoplastes et fusion de cellules somatiques végétales* (Coll. Intern. CNRS, 1973), vol. 212, p. 33.
[8] J. B. POWER and E. C. COCKING, J. exp. Bot. *21*, 24 (1970); T. NAGATA and I. TAKEBE, Planta *92*, 301 (1970).
[9] R. U. SCHENK and A. C. HILDEBRANDT, Phyton, Argentina *26*, 155 (1969).
[10] S. RELTMAN and S. FRANKEL, Am. J. clin. Path. *28*, 56 (1957).
[11] P. E. PILET and M. ATHANASIADES, C. r. hebd. Séanc., Acad. Sci., Paris *262*, 1090 (1966).