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Methionine Synthesis in *Neurospora crassa*

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Horowitz and co-workers (Horowitz, 1947; Teas, Horowitz & Fling, 1948; Fling & Horowitz, 1951) have determined the pathway of methionine synthesis in *Neurospora crassa*, from genetic analysis of methionine-requiring mutant strains; it involves conversion of cysteine, via cystathionine, into homocysteine, which is methylated to methionine. A similar scheme has been suggested for methionine synthesis in *Escherichia coli* (Simmonds, 1948; Davis & Mingioli, 1950).

The involvement of vitamin B_{12} in the last step, the methylation of homocysteine, has been clearly indicated in *E. coli* (Davis & Mingioli, 1950; Gibson & Woods, 1960) as well as in the vitamin-B_{12}-requiring protozoan *Ochromonas malhamensis* (Johnson, Holdsworth, Potter & Kon, 1957; Dalal, Rege & Sreenivasan, 1961). As vitamin B_{12} is not known to be involved in the metabolism of *N. crassa*, it was considered of interest to study the biosynthesis of methionine from homocysteine in this organism.

EXPERIMENTAL

Cell growth. Mycelial pads of *N. crassa* were grown in 50 ml. or 250 ml. Erlenmeyer flasks on 10 ml. or 50 ml. respectively of the medium employed for choline assay (Horowitz & Beadle, 1943), either as such, for the wild strain, or supplemented with 1 μg. of choline/ml. for the cholineless mutant strain no. 34486. The flasks were inoculated with spore suspensions of the required strain in sterile distilled water, and incubated for 60 hr. at 30°.

Grown mycelial pads were washed thrice with ice-cold distilled water and resuspended in the incubation system for experiments carried out with resting cells.

Preparation of cell-free extracts. The mycelial pads were disintegrated in a Potter–Elvehjem glass homogenizer in ice-cold 0·2 M-potassium phosphate buffer, pH 7·4, and then subjected to alternate freezing and thawing five times, the latter procedure accompanied by shaking with glass beads. The lysed cell suspension was centrifuged at 5000*g* for 15 min. to obtain the cell-free extract.

Synthesis of methionine. The incubation system employed in the study of methionine synthesis from homocysteine, by resting cells and cell-free extracts, was essentially that used for similar studies in *E. coli* (Guest, Helleiner, Cross & Woods, 1960). It contained: 0·01 M-DL-homocysteine, 0·01 M-C_1 donor indicated, 0·1 M-sodium succinate and the enzyme source, either resting cells or cell-free extract as indicated, in 0·1 M-potassium phosphate buffer, pH 7·4. Incubations were carried out at 37° for 4 hr.

Blanks for each experiment included a zero-hour control with the complete system, a mixture incubated for the experimental period without the precursors, homocysteine and C_1 donor.

At the end of the incubation period, the reaction was stopped by steaming for 10 min. and methionine estimated microbiologically in the clear supernatant with *Lactobacillus fermenti* as the test organism (Barton-Wright, 1952). DL-Methionine was used as standard and the results were also expressed in terms of DL-methionine. The presence of aminopterin at concentrations equivalent to those in the samples of reaction mixture to be assayed had no effect on the response of the organism to methionine.

The results, in each case, represent a minimum of four independent determinations.

Determination of protein. Protein was estimated in the cells and cell-free extracts by determining the total nitrogen, as well as non-protein nitrogen after deproteinization by 10% trichloroacetic acid, by Kjeldahl micro-digestion and direct nesslerization as described by Umbreit, Burris & Stauffer (1957) and multiplying the difference between the total and non-protein nitrogen values by 6·25.

RESULTS

Experiments with resting cells. Washed mycelial pads of wild-type *N. crassa* synthesized a small amount of methionine from homocysteine on incubation for 4 hr. at 37° in 0·1 M-phosphate buffer, pH 7·4, in the presence of succinate. Addition of serine increased synthesis considerably. Table 1

Table 1. *Effect of* C_1 *donors on methionine synthesis by* Neurospora crassa (*wild*)

The reaction mixture contained: 0·01 M-DL-homocysteine, 0·01 M-C_1 donor, 0·1 M-sodium succinate, 0·1 M-potassium phosphate buffer (pH 7·4) and mycelial pad (40–50 mg. dry wt.) in a total volume of 25 ml. Incubations were carried out for 4 hr. at 37°. In this and in all other Tables the values are given ± S.E.M.

C_1 donor	Methionine synthesized (μm-moles/mg. of protein)
Nil	3·4 ± 0·8
Formate	66·9 ± 9·6
Formaldehyde	3·6 ± 0·5
DL-Serine	48·5 ± 10·8
Glycine	30·2 ± 9·7
Methanol	11·3 ± 0·6
Choline	14·2 ± 0·4
Betaine	30·1 ± 9·0

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gives the relative efficiency of various C_1 donors in influencing methionine synthesis. Formate was the most effective and was used in all subsequent experiments. Equimolar concentrations of DL-homocystine could not replace homocysteine as methionine precursor in the above system.

Resting cells of the cholineless mutant of *N. crassa* were also tested for methionine synthesis from homocysteine, by using serine, formate or glycine, the C_1 donors found to be most efficient with the wild type. Synthesis in all cases was less than that obtained with the wild type (Table 2).

Addition of pteroylglutamate (1 μg./ml.) to the incubation system did not affect methionine synthesis by mycelia of wild type *N. crassa* (Table 3). However, the inhibition of methionine synthesis by aminopterin demonstrates the involvement of folic acid derivatives in this reaction. Addition of leucovorin (1 μg./ml.) did not reverse this inhibition.

Experiments with cell-free extracts. Table 4 gives a comparison of alumina grinding and the freeze–thaw method, as procedures for preparation of cell-free extracts capable of methionine synthesis. The latter method was more effective, as there was less loss of activity during rupture, and the percentage of activity in the cell-free extract was also greater (around 80 % as compared with about 60 % with alumina grinding). The cell-free extracts used in subsequent experiments were therefore prepared by the freeze–thaw method.

Table 2. *Effect of* C_1 *donors on methionine synthesized by* Neurospora crassa *(cholineless)*

Details were as in Table 1.

C_1 donor	Methionine synthesized (μm-moles/mg. of protein)
Nil	3·2±0·5
Formate	19·0±7·0
DL-Serine	20·7±9·2
Glycine	13·8±3·5

Table 3. *Effect of folic acid derivatives on methionine synthesis*

Details were essentially as in Table 1, with 0·01 M-formate as the C_1 donor. Mycelial pad (25–35 mg. dry wt.) was incubated in a total volume of 10 ml.

Additions	Methionine synthesized (μm-moles/mg. of protein)
Nil	66·9±9·6
Pteroylglutamate (10 μg.)	62·2±6·3
Leucovorin (10 μg.)	69·6±7·0
Aminopterin (10 μg.)	43·4±2·4
Aminopterin + leucovorin	47·3±5·3

Table 4. *Solubilization of methionine-synthesizing system*

The procedures for rupture of cells are described in the text. Other details were as in Table 3. Cell material used as the enzyme source was equivalent to 25–35 mg. dry wt. of mycelium.

	Methionine synthesized (μm-moles/mg. of protein in lysed cells) by cells ruptured by	
	Alumina grinding	Freeze–thaw
Lysed cells	32·1±1·4	38·2±1·0
Cell-free extract	19·7±1·8	31·6±1·0
Cell debris	13·0±0·9	5·5±1·1

Table 5. *Restoration of activity of Dowex-2* (Cl^- *form)-treated cell-free extract*

The enzyme preparation was a cell-free extract equivalent to 12–15 mg. dry wt. of cells in a total volume of 4 ml. of reaction mixture. The cofactor mixture consisted of: ATP, 20 μmoles; DPN, 2 μmoles; $MgSO_4$, 10 μmoles; leucovorin, 400 μg. Other details were as in Table 3.

Addition	Methionine synthesized (μm-moles/mg. of protein)	
	Untreated extract	Dowex-2 (Cl^- form)-treated extract
Nil	57·8±2·8	39·8±3·0
Heat-inactivated cell-free extract of *N. crassa*	54·8±3·3	48·2±2·5
Heat-inactivated cell-free extract of *O. malhamensis*	59·4±3·6	47·4±2·4
Cofactor mixture	55·7±1·8	46·4±1·5

The methionine-synthesizing capacity of the cell-free extract containing 5–6 mg. of protein/ml. was reduced by treatment with 20 % (w/v) Dowex 2 (Cl^- form; 20 mesh) in the cold for 20 min. with occasional shaking. Addition of untreated cell-free extract, inactivated by heating at 65° for 10 min., partially restored the activity. Similar restoration was obtained by addition of a heat-inactivated cell-free extract of *O. malhamensis*. A cofactor mixture, containing ATP, DPN, magnesium sulphate and leucovorin, also reactivated the Dowex-treated cell-free extract to nearly the same extent (Table 5).

DISCUSSION

The requirement of exogeneous C_1 sources for methionine synthesis by resting cells of *N. crassa* is demonstrated by the large increase in synthesis obtained on addition of various C_1 donors (Table 1). The almost equivalent effect of formate and serine in bringing about this increase indicates the

capacity of the organism to utilize C_1 fragments, at both the formyl and hydroxymethyl levels of oxidation, for methionine synthesis. The non-utilization of formaldehyde, which normally functions as a C_1 donor through the intermediate formation of hydroxymethyltetrahydropteroylglutamate (Huennekens & Osborn, 1959) is probably due to its inhibitory effect. The comparatively lower synthesis of methionine obtained with *N. crassa* (cholineless), irrespective of the C_1 donor used (Table 2), explains the mutant's observed requirement of methionine for growth in the absence of choline (Horowitz & Beadle, 1943).

Swendseid & Nyc (1958) have shown that *N. crassa* has a relatively high concentration of folic acid derivatives, accounting for the lack of effect of added pteroylglutamate on methionine synthesis by resting cells (Table 3). Pteroylglutamate might not be converted by the cells into the metabolically active forms of folic acid, which are probably tetrahydro derivatives of pteroylglutamate. The inhibition of growth of wild-type *N. crassa* by aminopterin (Swendseid & Nyc, 1958) is reflected in a decreased methionine synthesis in the presence of this folic acid antagonist. The inability of leucovorin to reverse the effect might be due to some unidentified types of folic acid derivatives functioning as intermediates.

The essential similarity of cofactor requirements of the methionine-synthesizing systems in *O. malhamensis* (Dalal *et al.* 1961) and *N. crassa* is demonstrated by the ability of heat-inactivated extracts of *O. malhamensis* to replace the *N. crassa* extract as a cofactor source (Table 5).

SUMMARY

1. Resting cells of both wild-type and the mutant strain no. 34486 of *Neurospora crassa* form methionine from homocysteine. Formate is the most effective of the various C_1 precursors examined.

2. Added pteroylglutamate has no effect on methionine synthesis by resting cells of the wild-type organism. Leucovorin cannot restore the decrease in synthesis caused by aminopterin.

3. The methionine synthesis obtained by cell-free extracts of *Neurospora crassa* (wild) is decreased by Dowex 2 (Cl^- form) treatment. Addition of heat-inactivated cell-free extracts of either *Neurospora crassa* or *Ochromonas malhamensis*, or a cofactor mixture consisting of adenosine triphosphate, diphosphopyridine nucleotide, magnesium sulphate and leucovorin, partially restores the activity.

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