Observations on the Effects of Methionine and Homocysteine on Growth of a Cholineless Mutant of *Neurospora crassa*

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Besides choline, *Neurospora crassa* strain no. 34486 (a cholineless mutant) can also utilize for growth monomethylethanolamine and dimethylethanolamine, the immediate precursors of choline (Horowitz, Bonner & Houlahan, 1945); this indicates that the block in synthesis of choline from ethanolamine in this mutant precedes monomethylethanolamine (Horowitz, 1946). Methionine is also active (Horowitz & Beadle, 1943) though at much higher concentrations. It was therefore of interest to determine whether homocysteine could, either partially or totally, replace methionine for growth of the mutant, and whether the addition of C_1 donors or pteroylglutamate, whose derivatives are recognized cofactors in transformylation reactions (Huennekens & Osborn, 1959), or both, facilitated the methylation either of monomethylethanolamine to choline or of homocysteine to methionine.

EXPERIMENTAL

Organism and media. Stock cultures of the cholineless mutant no. 34486 were maintained on agar slants of the following composition (J. F. Nyc, personal communication) in g./l.: potassium tartrate, 5; $NaNO_3$, 4; KH_2PO_4, 1; $MgSO_4,7H_2O$, 0·5; NaCl, 0·1; $CaCl_2$, 0·1; glycerol, 20; hydrolysed casein, 0·25; yeast extract, 5; malt extract, 5; agar, 15. The pH was 5·6–5·8. The basal medium used in these experiments was the same as that used for choline assay by Horowitz & Beadle (1943).

Growth studies. Growth studies were carried out in 50 ml. Erlenmeyer flasks containing 10 ml. of medium. After autoclaving at 15 lb. for 15 min. the flasks were cooled and inoculated with 0·2 ml. of a spore suspension in sterile distilled water. They were incubated at 30° for 72 hr. except where stated otherwise. The mycelial pads were then removed, pressed out on filter paper, dried at 80° and weighed to the nearest 0·1 mg. Flasks were kept in duplicate and each experiment was repeated at least twice.

[Me-^{14}C]*Methionine uptake during synthesis.* In experiments with ^{14}C additions, the washed mycelial pads were disintegrated in a Potter–Elvehjem glass homogenizer and made up to 6 ml. with distilled water. Portions (2 ml.) were plated on stainless-steel planchets and dried under an infrared lamp, and the radioactivity was determined directly, with the Tracerlab-SC-16 windowless gas-flow counter in conjunction with a Tracerlab-SC-51 autoscaler. Weight of dried mycelial matter on each planchet was determined to the nearest 0·1 mg.

Determination of free sulphydryl groups. This was by the method of Grunert & Phillips (1951).

RESULTS

Effects of homocysteine on growth. Homocysteine, by itself, failed to replace choline for growth at any concentration from 5 to 400 μg./ml. Addition of DL-serine (50 μg./ml.) or formate (50 μg./ml.) as C_1 donors did not increase growth response over the blank, indicating a lack of methylating ability under the experimental conditions. Addition of

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pteroylglutamate (1 μg./ml.) also made no difference.

The organism could utilize methionine in place of choline to a limited extent, although maximum growth was never reached even with high concentrations. At suboptimum concentrations of DL-methionine (e.g. 20 μg./ml.) increasing concentrations of DL-homocysteine up to 75 μg./ml. markedly increased growth (Fig. 1). Further increase in homocysteine concentration, however, inhibited growth, which fell even below the basal medium blank at sufficiently high concentrations. Similar effects were noticeable at different concentrations of methionine, as well as in experiments where homocysteine solution sterilized by Seitz filtration was added aseptically to previously autoclaved basal medium.

When choline at various concentrations was substituted for methionine (Fig. 2) no stimulation of growth by homocysteine was observed and there was inhibition at higher levels of homocysteine.

The cholineless strain can utilize dimethylethanolamine and, to a lesser extent, monomethylethanolamine for growth (Horowitz *et al.* 1945). Growth either with monomethylethanolamine or with various concentrations of homocysteine in the presence of methionine was not stimulated by either serine or formate or by leucovorin.

Nature of relationship between methionine and homocysteine. The dual effect of homocysteine might be due to a sparing of the methionine requirement at lower concentrations and to competition between the two metabolites at higher concentrations of homocysteine. The utilization of labelled methionine was therefore followed in the presence of various concentrations of homocysteine. Radioactive methionine was added to each flask at suboptimum concentrations (20 μg./ml.) together with various concentrations of homocysteine. Activity per mg. dry weight of mycelium decreased as the concentration of homocysteine increased to 75 μg./ml. and then rose again (Table 1). On the other hand, when methionine was present at greater than optimum concentration (200 μg./ml.) the activity per mg. dry wt. did not show this trend.

Nature of homocysteine inhibition. The inhibitory effect of homocysteine might be attributable to its free –SH group content. However, it was observed that incubation for 18 hr. of the autoclaved basal medium containing homocysteine resulted in complete removal of the –SH groups. The –SH group was, however, unaffected in the absence of the basal medium. This disappearance could be due to (i) auto-oxidation catalysed by the metal ions in the medium, or (ii) chelation of the metal ions by the –SH groups, or both. The trace-element content of the medium (which was added as salts, in mg./l.: B 0·01, Mo 0·02, Fe 0·2, Cu 0·1, Mn 0·02 and Zn 2·0) was therefore raised. This increase of trace elements decreased both the stimulation by homocysteine up to 75 μg./ml. and the inhibition at higher concentrations when suboptimum levels of methionine were present (Fig. 3). At optimum levels of methionine, increased trace-element

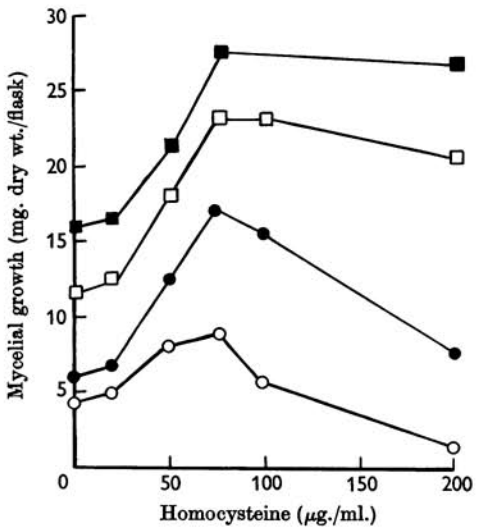

Fig. 1. Effect of various concentrations of homocysteine and methionine on growth of *N. crassa* (cholineless). The basal medium was modified with various concentrations of DL-homocysteine and DL-methionine as indicated and the extent of growth at 30° after 72 hr. was compared. Other details were as described in the text. Concn. of DL-methionine (μg./ml.): ○, 10; ●, 20; □, 40; ■, 80.

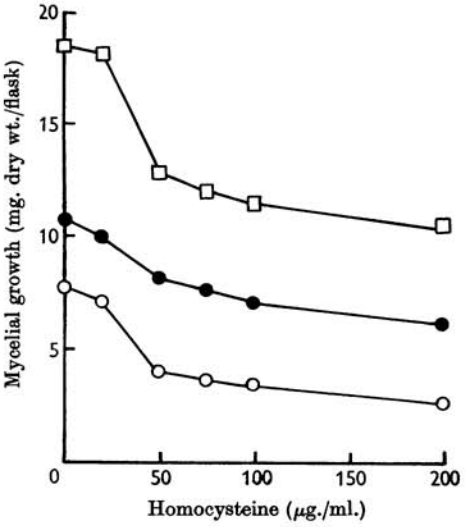

Fig. 2. Effect of various concentrations of homocysteine and choline on growth of *N. crassa* (cholineless). Details were essentially as in Fig. 1. Concn. of choline (μg./ml.): ○, 0·2; ●, 0·5; □, 1·0.

Table 1. *Incorporation of radioactive methionine into* Neurospora crassa *(cholineless) mycelium*

The results represent extent of incorporation of [Me-^{14}C]methionine activity into cell material of *N. crassa* (cholineless) during 72 hr. of growth on basal medium, as affected by various concentrations of homocysteine. Suboptimum methionine and excess of methionine were (per flask) 200 and 2000 μg. of DL-[Me-^{14}C]methionine respectively, and $9{\cdot}2 \times 10^3$ and $9{\cdot}2 \times 10^4$ counts/min. respectively.

Homocysteine (μg./flask)	Suboptimum methionine		Excess of methionine	
	Dry weight of mycelium (mg.)	Activity (counts/min./mg. dry wt.)	Dry weight of mycelium (mg.)	Activity (counts/min./mg. dry wt.)
Nil	7·2	850	12·0	2040
	6·0	940	12·3	2230
200	7·2	830	13·2	2950
	7·8	770	13·2	2910
750	15·3	550	13·8	2430
	14·3	490	16·6	2220
2000	6·9	1030	19·5	2570
	6·9	1000	21·6	2310
4000	2·7	1000	18·3	2690
	3·0	940	18·0	2670

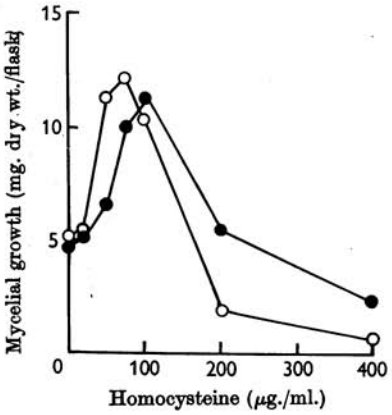

Fig. 3. Effect of concentration of trace elements on growth of *N. crassa* (cholineless). The basal medium was supplemented with 20 μg./ml. of DL-methionine. Other details were as in Fig. 1. ○, Basal trace-element concentration; ●, basal trace-element concentration increased fivefold.

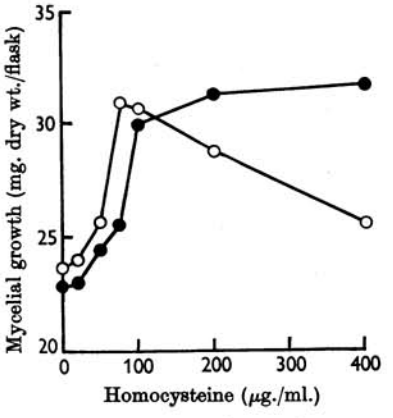

Fig. 4. Effect of concentration of trace elements on growth of *N. crassa* (cholineless). The basal medium was supplemented with 80 μg./ml. of DL-methionine. Other details were as in Fig. 1. ○, Basal trace-element concentration; ●, basal trace-element concentration increased fivefold.

concentrations in the medium completely eliminated the inhibition due to higher concentrations of homocysteine (Fig. 4).

Studies in Neurospora crassa *(wild)*. A similar inhibition should also be exerted on the growth of the wild strain if growth inhibition were due to chelation of metal ions by –SH groups. With the wild-type *N. crassa*, increasing the concentrations of homocysteine inhibited growth either in the presence or absence of methionine or choline (Table 2). This decrease in growth, which was nearly 50 % when homocysteine concentration was 400 μg./ml., could be restored by increasing the trace-element concentration in the basal medium (Table 3).

Table 2. *Effect of homocysteine on growth of* Neurospora crassa *(wild)*

Additions were made to the basal medium as indicated. Other details were essentially as in Fig. 1.

Homocysteine added (μg./ml.)	Mycelial dry matter (mg./flask) with		
	No addition	Methionine (20 μg./ml.)	Choline (0·5 μg./ml.
0	60·0	64·1	60·4
20	63·7	63·1	57·6
50	57·1	60·0	60·5
75	57·2	57·6	53·0
100	46·2	53·9	55·8
200	43·6	43·2	46·9
400	28·3	26·5	36·7

Table 3. *Effect of trace elements on inhibition of growth of* Neurospora crassa *(wild) by homocysteine*

Additions of trace elements and DL-homocysteine (400 μg./ml.) were made as indicated. Other details were essentially as in Fig. 1.

Concn. of trace elements	Mycelial dry matter (mg./flask) Without homocysteine	With homocysteine
Basal	67·3	33·9
4-fold	67·6	47·2
6-fold	61·0	52·6
8-fold	60·1	50·0
10-fold	60·2	57·6
15-fold	62·9	63·9
20-fold	67·7	62·2

DISCUSSION

The inability of homocysteine to support growth of the mutant strain no. 34486 of *N. crassa* in the absence of methionine indicates that it cannot totally replace methionine. This inability is not due to absence of potential C_1 donors since the presence of serine or formate in the basal medium has no effect.

The stimulation of growth produced by homocysteine in the presence of suboptimum concentrations of methionine (Fig. 1) could be due to a sparing effect of homocysteine for methionine. The decrease in radioactivity incorporated/mg. dry wt. of mycelium from radioactive methionine in the presence of homocysteine (Table 1), incorporation being minimal when growth stimulation is maximum, tends to confirm that homocysteine partially replaces methionine, diluting the activity incorporated into the cell material. On the other hand, the increase in growth obtained with homocysteine even in the presence of optimum concentrations of methionine (80 μg./ml.; Fig. 1) indicates that homocysteine does not exhibit a simple sparing effect which would be nullified at optimum methionine concentrations. A similar stimulation is not observed when choline replaces methionine in the medium.

The stimulatory effect of homocysteine in the presence of methionine was not accentuated by the addition of serine or formate and pteroylglutamate. Further, growth obtained with monomethylethanolamine is also not affected by similar additions, indicating that exogenous C_1 donors or folic acid derivatives are not utilized for methylation of monomethylethanolamine to choline, or of homocysteine to methionine. Folic acid derivatives are, however, present in the organism (Swendseid & Nyc, 1958).

Inhibition of growth at homocysteine concentrations above 75 μg./ml. (Fig. 1) could be due to competitive inhibition of methionine utilization by homocysteine, since the degree of inhibition decreases with increasing methionine concentrations. However, even at optimum concentrations of methionine, some inhibition is observable, indicating that competitive inhibition cannot be the sole cause of retardation of growth.

The considerable decrease in inhibition by homocysteine with increasing concentrations of the trace elements in the medium showed that the inhibitory effect may be due, in part at least, to chelation of the trace elements by the –SH group of homocysteine. However, the possibility cannot be precluded that the trace elements act by enhancing auto-oxidation of homocysteine to the inactive homocystine. At optimum concentration of methionine, increased concentrations of trace metals eliminated the inhibitory effect completely (Fig. 4), thus confirming the dual nature of inhibition.

The inhibition by homocysteine of growth of the wild strain, either in the presence or absence of choline or methionine (Table 2), is apparently solely due to the effect of trace elements in the medium (Table 3).

SUMMARY

1. Homocysteine alone failed to support growth of *Neurospora crassa* mutant strain no. 34486. In the presence of suboptimum concentration of methionine, it stimulated growth up to a concentration of 75 μg./ml., beyond which inhibition was observed. These effects were independent of the presence of pteroylglutamic acid or the C_1 donors formate and serine.

2. Stimulation was not observed when choline was substituted for methionine.

3. Homocysteine produced inhibition of growth of wild-type *Neurospora crassa* in the presence or absence of methionine or choline.

4. Increase in concentrations of boron (as borate), molybdenum (as molybdate), Fe^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} ions in the medium eliminated inhibition partially in the cholineless mutant and completely in wild-type *Neurospora crassa*.

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