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Methionine Synthesis in *Ochromonas malhamensis*

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The vitamin B_{12} requirement of the protozoan *Ochromonas malhamensis* is spared by methionine. The observation that glycine, in the presence of a trace of vitamin B_{12}, but not in its absence, could give a growth response equivalent to that given by methionine, and could also reverse inhibition produced by ethionine, has been interpreted by Johnson, Holdsworth, Potter & Kon (1957) to indicate the involvement of vitamin B_{12} in methionine synthesis in this organism.

Working with resting cells and cell-free extracts of mutants of *Escherichia coli*, Woods and co-workers have demonstrated more directly that the addition of vitamin B_{12} enhances the synthesis of methionine from homocysteine (Gibson & Woods, 1960; Guest, Helleiner, Cross & Woods, 1960). The involvement of folic acid derivatives in this reaction has also been demonstrated (Szulmajster & Woods, 1960; Kisliuk & Woods, 1960) with extracts of acetone-dried cells of an *E. coli* mutant requiring either serine or glycine for growth. The presence of vitamin B_{12} was essential in the growth medium to confer on the extracts the ability to utilize tetrahydropteroylglutamate as a carrier of C_1 units derived from formaldehyde or the β-C of serine (Kisliuk & Woods, 1960).

The present work relates to certain observations on the characterization of the methionine-synthesizing enzyme in *O. malhamensis*.

EXPERIMENTAL

Cell growth. O. malhamensis cells were grown in 250 ml. Erlenmeyer flasks in 100 ml. of the defined medium employed by Johnson *et al.* (1957), containing 0·4 μg. of vitamin B_{12} or 0·6 g. of DL-methionine/l. as indicated. Each flask was inoculated with 0·5 ml. of a 5- to 7-day culture maintained on a medium of similar composition, fortified with either vitamin B_{12} or methionine, and incubated for 5 days at 29° in a Perspex water bath illuminated by a pair of fluorescent tubes placed at a distance of 1 ft. under the bath.

The complex medium used contained (in g./l.): glucose, 10; peptone, 5; proteolysed liver extract, 2; the pH was 5·5.

The cells were harvested by centrifuging, washed twice and resuspended in ice-cold 0·2M-potassium phosphate buffer, pH 7·0, in experiments with resting cells, or in distilled water when cell-free extracts were prepared.

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Extraction and purification of enzyme system. Cell-free extracts were obtained by repeated alternate freezing and thawing, and subsequent centrifuging at 5000*g* for 15 min. under aseptic conditions. Microscopic examination and lack of growth response on transfer confirmed absence of viable cells.

Proteins from the extracts were fractionated by ammonium sulphate precipitation and dialysis to remove the salt. All operations were carried out at 0–5°.

Synthesis of methionine. Methionine synthesis was followed in the reaction system containing basically: 0·01M-DL-homocysteine, 0·01M-glycine, 0·1M-potassium phosphate buffer, pH 7·0, and the enzyme preparation in a total volume of 5 ml. Incubation was at 30° under aseptic conditions. Each experiment included a zero-time control with the complete system and a mixture incubated for the experimental period without precursors.

At the end of the incubation period, the reaction was stopped by steaming for 10 min. Methionine was estimated microbiologically, in the clear supernatant by using *Lactobacillus fermenti* (Barton-Wright, 1952). DL-Methionine was used as standard and the results were expressed in terms of DL-methionine. The results in each case represent a minimum of at least four independent determinations.

Incorporation of activity from radioactive glycine into methionine. The incubation system was similar to the one used above except that 0·01M-[2-^{14}C]glycine (activity; $1{\cdot}25 \times 10^5$ counts/min.) was used.

Methionine was isolated by a modification of the chromatographic method of Moore & Stein (1951, 1954). Inactive glycine and methionine (1 mg. each) were added as carriers to a sample of the clear supernatant which was adsorbed on Dowex 50 (200 mesh; Na^+ form) columns (16 mm. × 30 cm.) and eluted with 200 ml. of 0·1M-citrate buffer, pH 3·1, followed by 200 ml. of 0·2M-citrate buffer, pH 4·25. Methionine, free from glycine, was eluted out in the fraction between 230 and 250 ml.

Activity of the isolated methionine was determined directly at infinite thinness on glass planchets with the Tracerlab-SC-16 windowless flow-gas counter in conjunction with the Tracerlab-SC-51 autoscaler.

Methionine was determined microbiologically in a separate sample of the supernatant as described above.

Estimation of protein. Protein was computed from total and non-protein nitrogen values determined by micro-digestion and direct nesslerization as described by Umbreit, Burris & Stauffer (1957). Protein was estimated in the ammonium sulphate precipitates with the Folin–Ciocalteu reagent (Layne, 1957).

Determination of folic acid derivatives. The folic acid derivatives were liberated from cell-free extracts by overnight autolysis in 0·1M-phosphate buffer, pH 7·4, under toluene at 37° (Pfander, Dietrich, Monson, Harper &

Elvehjem, 1952; Mitbander & Sreenivasan, 1955). Folic acid activity was assayed with *Streptococcus faecalis* R turbidimetrically, by the modified medium of Mitbander & Sreenivasan (1954). *Leuconostoc citrovorum* factor activity was assayed with *L. citrovorum* (ATCC no. 8081) as the test organism, leucovorin as the standard and the basal medium of Sauberlich & Baumann (1948).

RESULTS

Methionine synthesis by resting cells

Washed cells of *Ochromonas*, grown in complex medium and incubated at 30° in 0·1 M-potassium phosphate buffer, pH 5·5, synthesized methionine from homocysteine and glycine. A rapid methionine synthesis occurred up to 10 hr., after which the rate fell off (Fig. 1). Aseptic conditions were maintained throughout harvesting and incubation procedures. The optimum pH for the reaction was 7·0 (Fig. 2) and this was used in all subsequent experiments.

Effect of C_1 *donors.* Glycine was the most effective of the C_1 donors tested with resting cells followed by serine, choline, methanol and betaine (Table 1). Formaldehyde and formate were ineffective. Glycine was therefore employed as a C_1 donor in all subsequent experiments. [2-^{14}C]Glycine was incorporated into methionine by the resting cells (see Table 3).

Experiments with cell-free extracts

Table 2 shows the distribution of methionine-synthesizing ability in the cytoplasm and the residue of lysed cells grown in natural medium or synthetic medium containing vitamin B_{12}. In both cases, about 80% of the activity was in the cell-free extract. Alternate freezing and thawing was repeated five times in these experiments.

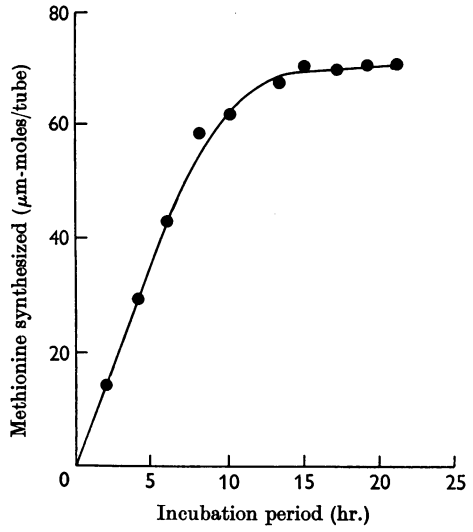

Fig. 1. Effect of period of incubation on methionine synthesis. The reaction mixture contained: DL-homocysteine, 0·01 M; glycine, 0·01 M; phosphate buffer (pH 5·5), 0·1 M; cells, 15–20 mg. dry wt.; total volume, 5 ml. The temperature of incubation was 30°.

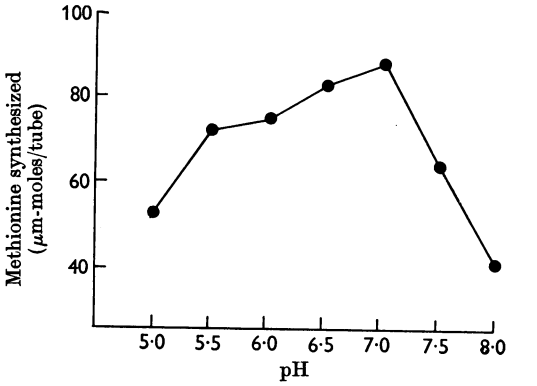

Fig. 2. Effect of pH on methionine synthesis. Details of the reaction system were as in Fig. 1. Period of incubation was 18 hr.

Table 1. *Effect of* C_1 *donors on methionine synthesis*

The reaction mixture contained: DL-homocysteine, 0·01 M; C_1 donor, 0·01 M; phosphate buffer (pH 7·0), 0·1 M; cells, 15–20 mg. dry wt.; total volume, 4 ml. Incubations were carried out for 18 hr. at 30°. 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	10·1 ± 1·2
DL-Serine	26·1 ± 1·5
Glycine	29·2 ± 2·0
Formaldehyde	9·0 ± 0·3
Methanol	17·3 ± 1·4
Choline	19·0 ± 1·3
Betaine	15·1 ± 0·3
Formate	8·2 ± 1·1

Table 2. *Intracellular distribution of methionine-synthesizing ability*

The compositions of the media are described in the text. Details were essentially as in Table 1, with 0·01 M-glycine as the C_1 donor. Cell material used as the enzyme source was equivalent to 15–20 mg. dry wt. of cells.

	Methionine synthesized (μm-moles/mg. of protein in cells) by organism grown in	
	Complex medium	Defined medium
Cells	31·3 ± 1·8	26·6 ± 1·0
Lysed cells	25·8 ± 1·6	20·7 ± 1·3
Cell-free extract	19·9 ± 1·1	17·9 ± 2·5
Cell debris	6·1 ± 0·9	5·2 ± 1·2

Radioactivity incorporation. Methionine synthesis by cell-free extracts was also demonstrated by the incorporation of radioactivity into methionine on incubation with homocysteine and [2-^{14}C]-glycine (Table 3).

Dowex 2 treatment. To deplete it of cofactors, the cell-free extract containing 3–4 mg. of protein/ml. was treated with 20 % (w/v) of Dowex 2 (Cl^- form; 20 mesh) in the cold for 20 min. with occasional shaking. Methionine synthesis was reduced by nearly 50 % after this treatment (Table 4). Addition of cell-free extract, previously heated to 65° for 10 min. to inactivate the enzyme system, restored activity. The activity of the untreated cell-free extract was also slightly increased by the addition of heated cell-free extract.

Effect of cofactors. The ability of a cofactor mixture, containing ATP, DPN, magnesium sulphate, leucovorin and vitamin B_{12}, to replace the heat-inactivated cell-free extract was next tested. The mixture increased methionine synthesis by both the untreated and Dowex-treated cell-free extracts, though to a lesser extent in the former case. Omission of each of the components in turn showed that the activity of this mixture was attributable to ATP (Table 4). AMP and succinate at the same concentration did not replace ATP in the above system. Sodium fluoride (0·6 μM) either by itself or in the presence of ATP had no effect, indicating a lack of adenosine-triphosphatase activity, which was subsequently confirmed by direct assay (Novikoff, Hecht, Podber & Ryan, 1952) of the cell-free extract.

Tetrahydropteroylglutamate is needed for methionine synthesis by enzyme preparations from sheep liver (Nakao & Greenberg, 1958) and from *E. coli* (Kisliuk & Woods, 1960). The activity of heated extracts in increasing methionine synthesis by the preparations from *O. malhamensis* might be due to the presence of folic acid derivatives, and attempts were therefore made to isolate these.

Dowex 2 (Cl^- form) treatment reduced the activity of the cell-free extract for *S. faecalis* measured as pteroylglutamate from 6·1 to 1·6 μmg./mg. dry wt. The *Leuconostoc citrovorum* factor activity, expressed as leucovorin, was reduced from 2·0 to 0·4 μmg./mg. dry wt.

The cell-free extract (20 ml., containing 3–4 mg. of protein/ml.) was adsorbed on Dowex 2 (Cl^- form; 50–100 mesh) columns (12 mm. × 15 cm.) and eluted with M-formic acid. The 15–25 ml. fraction accounted for most of the *S. faecalis* activity adsorbed on the column. Addition of this fraction, after freeze-drying and resuspension in 5 ml. of water, to the incubation system containing the Dowex-treated cell-free extract, ATP, homocysteine and glycine, did not, however, increase methionine synthesis.

Ammonium sulphate fractionation of cell-free extract

Partial purification of the methionine-synthesizing enzyme system was attempted by ammonium sulphate fractionation. The dialysed, total protein precipitate obtained by saturation of the cell-free extract with ammonium sulphate formed negligible

Table 3. *Incorporation of activity from* [2-^{14}C]-*glycine into resting cells and cell-free extracts of* Ochromonas malhamensis

Resting cells, 12 mg. dry wt., and cell-free extract equivalent to 16 mg. dry wt. of cells were used per tube. [2-^{14}C]Glycine (0·01 M; $1·25 \times 10^5$ counts/min.) was the C_1 donor. Other details were as in Table 1 and text.

	Total activity in methionine (counts/min.)	Specific activity of methionine (μc/μmole)
Cells	15 950	1·87
	20 636	2·52
Cell-free extract	8 484	2·06
	9 815	2·47

Table 4. *Effect of cofactor additions on methionine-synthesizing ability of cell-free extracts of* Ochromonas malhamensis

The enzyme preparation was a cell-free extract equivalent to 15–20 mg. dry wt. of cells. Other details were as in Table 2. The composition of the cofactor mixture was: ATP, 20 μmoles; DPN, 2 μmoles; $MgSO_4$, 10 μmoles; leucovorin, 400 μg.; vitamin B_{12}, 0·1 μg.

Additions	Methionine synthesized (μm-moles/mg. of protein): Untreated extract	Dowex 2 (Cl^- form)-treated extract
Nil	40·3±2·5	20·9±2·1
Heat-inactivated cell-free extract	48·3±4·3	45·8±3·1
Cofactor mixture	49·2±1·4	36·2±1·4
Cofactor mixture without vitamin B_{12}	46·5±2·2	34·7±1·2
Cofactor mixture without leucovorin	48·1±2·5	37·4±1·9
Cofactor mixture without $MgSO_4$	48·1±2·8	36·3±1·8
Cofactor mixture without DPN	47·6±3·1	36·9±0·8
Cofactor mixture without ATP	46·3±2·1	23·4±1·9

amounts of methionine ($3{\cdot}2 \pm 1{\cdot}2$ μm-moles/mg. of protein) on incubation for 6 hr. in the usual system with homocysteine and glycine as precursors. On addition of heat-inactivated cell-free extract, however, synthesis was considerably enhanced ($25{\cdot}9 \pm 2{\cdot}6$ μm-moles/mg. of protein).

On fractionation of the protein of the cell-free extract by adding ammonium sulphate, the 25–50% fraction was found to possess maximum methionine-synthesizing activity (Table 5). Addition of heat-inactivated cell-free extract increased synthesis substantially. However, the addition of a cofactor mixture similar to that used previously failed to increase the synthesis either in the presence or absence of the heat-inactivated cell-free extract (Table 5). On dialysis the heated cell-free extract lost almost 50% of its activity, which was restored by the cofactor mixture. The single omission of components of this mixture showed that ATP, DPN and magnesium sulphate were all necessary for maximum synthesis (Table 6).

The 50–75% fraction could also synthesize methionine from precursors (6 μm-moles/mg. of protein in 6 hr.) and this was increased on addition of heat-inactivated cell-free extract to 43·5 μm-moles/mg. of protein. The 0–25 and 75–100% fractions possessed negligible activity.

Relationship with vitamin B_{12}. Since vitamin B_{12} has been implicated from growth studies in methionine synthesis in this organism (Johnson *et al.* 1957) methionine synthesis by cells grown in the absence of the vitamin was studied. No net synthesis of methionine could be demonstrated by the whole cells or by the cell-free extracts. Addition of vitamin B_{12} (0·02 μg./ml.) had no effect. Preincubation of harvested cells for periods up to 3 hr. in fresh medium containing vitamin B_{12} (0·4 μg./l.) also failed to confer methionine-synthesizing ability on the cells.

However, on addition of even trace amounts of vitamin B_{12} (0·04 μg./l.) to the growth medium containing optimum concentrations of methionine for growth, both resting cells and cell-free extracts could synthesize methionine at levels comparable to that of cells grown in optimum concentrations of vitamin B_{12}.

These results indicated that the transformation of vitamin B_{12} into an active complex was an essential prerequisite to its functioning in the methionine-synthesizing system. The effect of an extract of cells (equivalent to 5 mg. dry wt. of cells/ml. of reaction mixture) grown in the presence of the vitamin was therefore studied after inactivating the enzymes by heating at 65° for 10 min. This addition to the extracts of cells grown in a methionine-containing medium free of vitamin B_{12} showed net synthesis of $12{\cdot}9 \pm 0{\cdot}7$ μm-moles of methionine/mg. of protein.

The coenzyme form of vitamin B_{12} (Barker, Weissbach & Smyth, 1958; Weissbach, Toohey & Barker, 1959), dimethylbenzimidazole–vitamin B_{12} coenzyme, at a concentration of 0·5 μM in the incubation system did not replace the heat-inactivated extract of cells, grown in vitamin B_{12}-containing medium, in the above system.

Table 5. *Effect of various additions on methionine synthesis by a partially purified enzyme preparation*

The enzyme preparation was a 25–50% ammonium sulphate precipitate of the cell-free extract equivalent to 0·7–0·9 mg. of protein/tube. The incubation period was 6 hr. The composition of the cofactor mixture was as in Table 4. Other details were as in Table 2.

Additions	Methionine synthesized (μm-moles/mg. of protein)
Nil	$16{\cdot}2 \pm 1{\cdot}5$
Cofactor mixture	$16{\cdot}1 \pm 2{\cdot}6$
Heated cell-free extract	$67{\cdot}1 \pm 2{\cdot}5$
Heated cell-free extract + cofactor mixture	$62{\cdot}5 \pm 2{\cdot}4$
Dialysed, heated cell-free extract	$31{\cdot}8 \pm 3{\cdot}0$
Dialysed heated cell-free extract + cofactor mixture	$66{\cdot}1 \pm 3{\cdot}5$

Table 6. *Effect of omission of individual components of the cofactor mixture on methionine synthesis by a partially purified enzyme preparation*

Dialysed, heated cell-free extract and cofactor mixture (cf. Table 4) were added to the reaction mixture. Other details were as in Table 5.

Omissions	Methionine synthesized (μm-moles/mg. of protein)
Nil	$66{\cdot}1 \pm 3{\cdot}5$
ATP	$35{\cdot}7 \pm 3{\cdot}8$
DPN	$52{\cdot}5 \pm 5{\cdot}6$
$MgSO_4$	$35{\cdot}1 \pm 2{\cdot}6$
Leucovorin	$69{\cdot}9 \pm 4{\cdot}8$
Vitamin B_{12}	$69{\cdot}7 \pm 6{\cdot}2$

DISCUSSION

The relatively high amount of methionine synthesis obtained in the presence of glycine or serine as C_1 donors (Table 1) indicates the need of exogenous C_1 sources for substantial methionine synthesis by resting cells of *O. malhamensis* and is in general agreement with results obtained in growth studies of Johnson *et al.* (1957).

Cofactor additions, of which ATP was found to be the only active component, restore activity of Dowex-2-treated cell-free extract only partially (Table 4), indicating the presence of other necessary cofactors in the cell-free extract.

The heated cell-free extract, required for optimum synthesis by the 25–50% ammonium sulphate fraction (Table 5), was replaced by dialysed heated cell-free extract in conjunction with an

extraneous cofactor mixture whose effect was subsequently traced to ATP, DPN and Mg^{2+} ions (Table 6). An unidentified, non-dialysable component, present in the cell-free extract, was apparently required for optimum synthesis since the cofactor mixture was ineffective in the absence of the dialysed extract (Table 5).

The inability of resting cells or cell-free extracts of *O. malhamensis* to synthesize methionine except in the presence of at least trace quantities of vitamin B_{12} in the growth medium, indirectly demonstrated that the vitamin is required for methionine synthesis.

Resting cells or cell-free extracts of the organism grown in methionine apparently cannot convert vitamin B_{12} into its active coenzyme form, unlike the cell-free extracts of the methionine–vitamin-B_{12}-requiring mutant of *E. coli* (Guest *et al.* 1960).

A coenzyme form of vitamin B_{12}, isolated from *Clostridium tetanomorphum* (Weissbach *et al.* 1959), which is active in the conversion of glutamate into β-methylaspartate in cell-free extracts of the micro-organism (Barker *et al.* 1958), propionate into succinate in *Propionibacterium* species (Stadtman, Overath, Eggerer & Lynen, 1960) and, reportedly, in amino acid incorporation into protein by microsomal pH 5 enzyme system (Mehta, Wagle & Johnson, 1959), did not, at the concentration tested, endow cell-free extracts of methionine-grown cells with the ability to synthesize methionine. Recent reports (Kisliuk, 1960; Takeyama & Buchanan, 1960) show that this factor is also not the active coenzyme form of vitamin B_{12} for methionine synthesis in *E. coli*. However, the activation by heated extracts of cells grown in a vitamin-B_{12}-containing medium seems to indicate that the coenzyme form of vitamin B_{12} in this system is not a heat-labile protein as is the vitamin B_{12} coenzyme active for methionine synthesis in *E. coli* (Guest, 1959; Kisliuk, 1960). Alternatively, vitamin B_{12} may be present in the heat-inactivated extract in a form that, unlike cyanocobalamin, is readily convertible into the active coenzyme form by the system.

SUMMARY

1. Both resting cells and cell-free extracts of *Ochromonas malhamensis* grown in a vitamin-B_{12}-containing medium are capable of methionine synthesis. Optimum conditions of pH and reaction time, as well as the relative contribution of various precursors in the formation of the methyl moiety, have been ascertained.

2. The decrease of methionine-synthesizing activity of the cell-free extract caused by Dowex 2 (Cl^- form) treatment was restored completely on addition of heat-inactivated cell-free extract and partially by adenosine triphosphate.

3. The fraction obtained by 25–50 % saturation with ammonium sulphate of the cell-free extract caused optimum synthesis of methionine in the presence of heat-inactivated cell-free extract or dialysed, heat-inactivated cell-free extract together with a cofactor mixture whose active components were adenosine triphosphate, diphosphopyridine nucleotide and magnesium sulphate.

4. Cell-free extracts of the micro-organism grown in a methionine-containing medium in the absence of vitamin B_{12} only synthesized methionine in the presence of heated extracts of cells grown with the vitamin. Dimethylbenzimidazole–vitamin B_{12} coenzyme did not replace the heated extract.

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