ALTERED FOLATE METABOLISM IN A VITAMIN B_{12}-METHIONINE AUXOTROPH

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The biosynthesis of methionine in strains of Escherichia coli is a convergence of two separate syntheses; namely, those of the homocysteine and methyl moieties. Even prior to the complete elucidation of the enzymatic steps and cofactor requirements of methionine synthesis, Wijesundera and Woods (1953; 1960), as well as Cohn et al (1953), recognized that the growth of organisms in media containing L-methionine led to the suppression of methionine synthesis from homocysteine and serine. Rowbury and Woods (1961) found that methionine could repress the conversion of various one-carbon precursors to the methionine methyl group both in vivo and in vitro. Derepression of methyl group synthesis occurred in methionine-free media even prior to resumption of growth of the organisms.

The object of the present study was to determine what effect methionine had on the two individual enzymes involved in the formation of the methionine-methyl groups from $N^{5,10}$-methylene-folate-H_4 and whether a specific locus for autoregulation exists. During the course of this study, a paradoxical inhibition of the growth rate was observed during growth of a methionine-vitamin B_{12} (cyano-B_{12}) auxotroph of E. coli K_{12} in a medium containing sufficient cyano-B_{12} for growth but with suboptimal concentrations of methionine. The following results are an attempt to clarify this observed growth inhibition.

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The organism used in this study was the phage produced mutant, E. coli K_{12}, strain AB 1172 - substrain 2276 (methionine$^-$ or cyano-B_{12}^-, thiamine$^-$) which was generously provided by Dr. Austin L. Taylor of the National Institute of Neurological Diseases and Blindness (Taylor, 1964). The organisms were transferred from tryptone broth agar slants into minimal salts-glucose medium supplemented with thiamine hydrochloride, 0.2 μg/ml, and cyano-B_{12}, 0.01 μg/ml. The minimal media was a half-strength preparation of medium 56 described by Monod et al (1951). The cultures were incubated on a rotary shaker for sixteen hours at 35° C. Appropriate aliquots of the overnight culture were harvested, washed twice with media, and transferred to 1 liter flasks which contained 100 ml minimal media, 1 ml 20% glucose, 1 μg cyano-B_{12} and varying concentrations of L-methionine (0.1-20 μg/ml). Bacterial growth was followed by measurement of turbidity at 600 mμ on Beckman DU spectrophotometer.

For enzyme assays, the cells were collected by centrifugation for 10 minutes at 5000 x g, washed once with 10 ml of 0.1 M Tris HCl buffer, pH 7.4, suspended in 2 ml of the same buffer, and disrupted by exposure to two 15-second periods of sonication with a Brownwill probe sonifier. The fragmented cell suspension was centrifuged at 5000 x g x 10 min. and the clear supernatant fraction used as source of enzyme(s) in the subsequent assays. The protein concentration of the supernatant fraction ranged from 1 - 10 mgm/ml.

The assay of N^5-methylfolate-H_4-homocysteine methyltransferase has been described in a previous publication (Weissbach, et al., 1963). $N^{5,10}$-methylene-folate-H_4 reductase was assayed by the back reaction, the menadione-dependent oxidation of N^5-^{14}C-methyl-folate-H_4 to folate-H_4 and ^{14}C-formaldehyde (Donaldson and Keresztesy, 1961). The latter compound is in direct equillibrium with the immediate product, $N^{5,10}$-methylene-folate-H_4. The assay is based on the retention of the substrate ^{14}C-methyl-N^5-methyl-folate-H_4 on a Dowex 1 chloride column with elution of the ultimate product, ^{14}C-

formaldehyde. A unit of either enzyme's specific activity is defined as 1 mμmole of product formed in 60 min. per mgm protein at 37° C. Protein concentration was determined by the method of Lowry et al (1951).

In order to demonstrate the effect of exogenous L-methionine on the enzymes responsible for the synthesis of the methionine methyl moiety, inocula of cyano-B_{12} grown organisms were subcultured in glucose-salt minimal media containing cyano-B_{12} at a concentration twenty-fold greater than the minimal requirement and graded concentrations of the amino acid. A marked effect of methionine on growth was observed (Fig. 1). The cells grown on

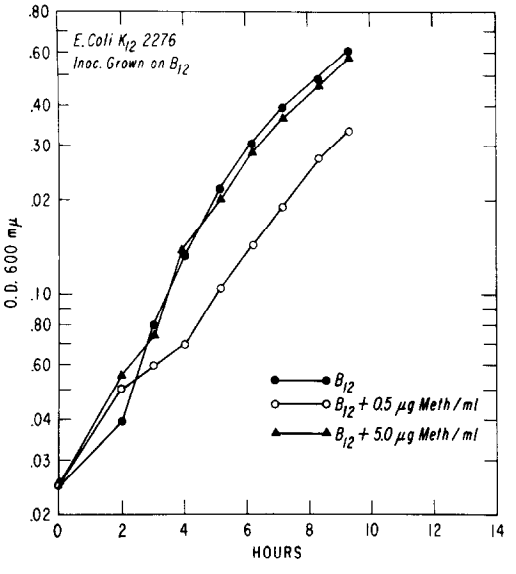

Fig. 1. The inhibition of growth of E. coli K_{12} mutant 2276. The inocula were grown in a glucose-salt minimal media containing cyano-B_{12} (0.01 μg/ml). The subsequent subcultures were grown in the same media with the following additions: ●——●, cyano-B_{12}, 0.01 μg/ml; o——o cyano-B_{12}, 0.01 μg/ml plus L-methionine 0.5 μg/ml; ▲——▲, cyano-B_{12}, 0.01 μg/ml plus L-methionine, 5.0 μg/ml. Growth was estimated as the change in turbidity recorded at 600 mμ; temperature was 35° C.

cyano-B_{12} alone or in combination with methionine at concentrations exceeding 5 μg/ml grew at the same rate and to the same extent. Those cells grown with cyano-B_{12} and an exogenous methionine concentration of 0.5 - 2.5 μg/ml were significantly inhibited. This inhibition became apparent after one to two cell divisions. Further studies revealed that the growth inhibition was

dependent on the time of addition of methionine. If the amino acid was added during the first two hours of growth, inhibition would result, but if added after this interval, no effect was seen. The growth effect was observed when glucose was the carbon source but not with glycerol.

Extracts of cells, grown under the above conditions, were assayed for $N^{5,10}$-methylene-folate-H_4 reductase and N^5-methyl-folate-H_4-homocysteine methyltransferase activities. At levels of exogenous methionine which exceeded 4 μg/ml, there was almost complete suppression of the reductase activity[2] (Fig. 2). However, in the concentration range of 0.5 - 2.5

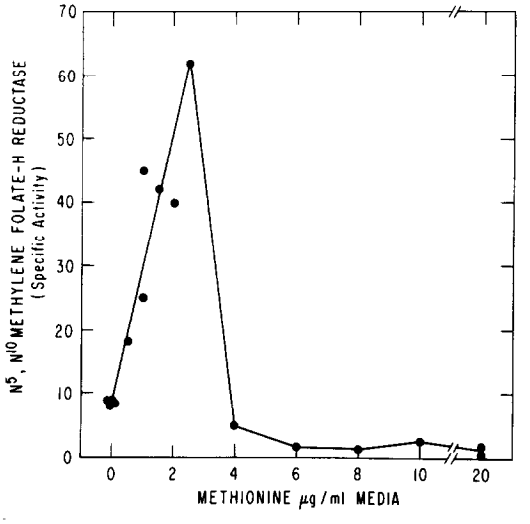

Fig. 2. Changes in specific activity of $N^{5,10}$-methylene-folate-H_4 reductase with varied media methionine concentrations. Conditions of growth and preparation of the extracts are described in the text. The reaction mixtures contained KPO_4, buffer, pH 7.4, 10 μmoles; N^5-^{14}C-labelled methyl-folate-H_4, 20 mμmoles (specific activity 2000 c.p.m./mμmole); flavin adenine dinucleotide, 1 mμmole; menadione bisulfite, 1 mμmole; sodium ascorbate, 5 μmoles; formaldehyde, 5 μmoles; and suitable aliquots of enzyme. Final incubation volume was 0.2 ml. Duration of incubation was 30 min. and temperature was 37° C. The control tubes contained all the reactants except enzyme; the enzyme was then added at the end of the incubation. Following incubation 0.3 ml of ice water was added to all tubes and the contents of the tubes were applied to a Dowex-1-chloride column (0.5 x 3.0 cm). The eluate as well as a 1.5 ml water wash was collected in a vial for liquid scintillation counting (Bray, 1960).

[2] Katzen has reported that a marked derepression of the $N^{5,10}$-methylene-folate-H_4 reductase occurred in E. coli W 113-3 when the cells were grown on cyano-B_{12} alone or with low concentrations of L-methionine (Katzen, 1964).

μg/ml of methionine, there was a five-to-ten fold increase in reductase activity compared to extracts derived from cells grown on cyano-B_{12} alone. There was no evidence of *in vitro* activation of the reductase activity by

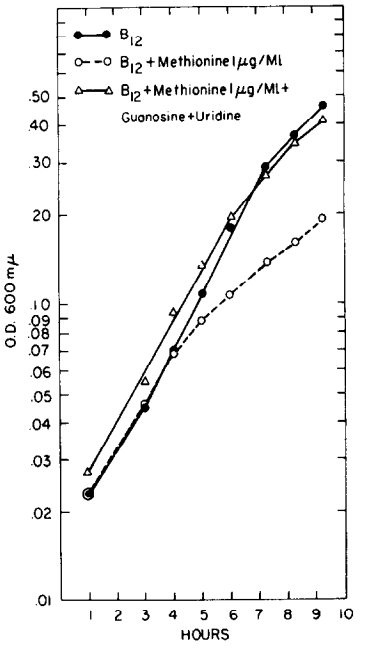

Fig. 3. Protective effect of nucleosides on growth inhibition observed at "low" methionine concentrations. The culture conditions were the same as described in Fig. 1 with the following additions: ●——●, cyano-B_{12}, 0.01 μg/ml; o- - -o, cyano-B_{12}, 0.01 μg/ml, plus L-methionine, 1 μg/ml; Δ——Δ, cyano-B_{12}, 0.01 μg/ml, L-methionine, 1 μg/ml, guanosine, 0.1 μmole/ml and uridine, 0.1 μmole/ml. Growth was again determined as the change in turbidity recorded at 600 mμ.

addition of methionine. The concentrations of methionine at which growth inhibition was observed were in the same range as those at which increase in reductase activity was observed (between 0.3 - 2.5 μg/ml). In contrast to the alterations in reductase activity, the addition of methionine to the medium led to an incomplete suppression of methyltransferase activity without a comparable increase in specific activity.

The association of growth inhibition with an increased specific activity of $N^{5,10}$-methylene-folate-H_4 reductase suggested that a divergence

of one-carbon units, generated through the serine hydroxymethylase reaction might be occurring. Such an abnormal shunt would increase methionine methyl group synthesis at the expense of purine base and thymidylate synthesis. As a test of this hypothesis, purine and pyrimidine nucleosides, alone or in combination, were added to media containing an inhibitory concentration of methionine. Fig. 3 shows the results of a representative experiment. Addition of guanosine and uridine to the media protected the cells against the growth inhibition. The protective effect, like the inhibition by low concentrations of methionine, occurred only if the nucleosides were added prior to the first or second cell division. Attempts at reversal by addition of exogenous nucleosides after inhibition had occurred were unsuccessful.

Guanosine, alone or in combination with pyrimidine nucleosides or adenosine, was most effective in protection against the growth inhibition. Deoxyguanosine was almost as effective as guanosine, while adenosine, alone or in combination with pyrimidine nucleosides, was only slightly protective. Pyrimidine nucleosides alone or in combination with other pyrimidine nucleosides, either were ineffective or actually led to an intensification of the growth inhibition.

The present report describes an experimental situation in which a shunt of one-carbon units towards methionine synthesis results in a relative purine deficiency. $N^{5,10}$-methylene-folate-H_4 reductase appears to be the sensitive enzyme under these conditions. It is markedly stimulated by low levels of methionine in the medium and almost completely repressed when higher levels of the amino acid are present. It is tempting to speculate that the metabolism of $N^{5,10}$-methylene-folate-H_4 is under stringent metabolic control as this compound provides one-carbon units for purine base, thymidylate and methionine biosyntheses.

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