Effects of 2-Amino-4-hydroxy-6-carboxy-7,8-dihydropteridine and 2-Amino-4-hydroxy-6-formyl-7,8-dihydropteridine on Growth of *Escherichia coli*¹

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2-Amino-4-hydroxy-6-carboxy-7,8-dihydropteridine inhibited growth of *Escherichia coli* B, while 2-amino-4-hydroxy-6-carboxypteridine did not affect the growth. The half inhibition was achieved with the inhibitor concentration of $2.2 \times 10^{-4}M$. The inhibition could be reversed by 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine. However, when the carboxydihydropteridine was absent in the culture medium, the formyldihydropteridine blocked the microbial growth with an inhibitory power about equal to the carboxydihydropteridine.

In previous experiment (1) with a cell-free system of *Brassica pekinensis* Rupr, it was found that 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine blocked strongly enzymatic formation of dihydropteroic acid from 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine, 2-amino-4-hydroxy-6-(D-erythro-1', 2', 3'-trihydroxy propyl)-7,8-dihydropteridine and GMP, but no inhibition was observed when the pyrophosphate ester of the hydroxymethyldihydropteridine was used as a substrate. The inhibition was reversed by increasing the concentration of the hydroxymethyldihydropteridine supplied to the reaction medium. Recently, it has been established that the step blocked by the carboxydihydropteridine in biosynthetic pathway of dihydropteroic acid is pyrophosphorylating reaction of the hydroxymethyldihydropteridine by action with ATP, and the inhibitor competes with the substrate at this step (2), (3). The present communication demonstrates inhibition of growth of *Escherichia coli* B by 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine

and reversal of the inhibition by 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine.

EXPERIMENTAL

1. Materials

2-Amino-4-hydroxy-6-carboxypteridine sodium salt (4) and 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine (5) were prepared by the published methods. Powdered meat extract was purchased from Mikuni Chemical Industries Ltd.; highly purified agar from Difco Laboratories; and peptone from Kyokutô Pharmaceutical Industries Ltd.

2. Organism

E. coli B was maintained on an agar slant (pH 7.0) consisting of 1 % meat extract, 1 % peptone, 0.5 % NaCl, 1.4 % agar and distilled water. All cultivations were carried out at 30° and all media were sterilized by autoclaving at 15 pounds pressure for 15 minutes.

3. Growth Experiments

Double strength medium of *E. coli* B

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(pH 7.2) contained per liter: Na_2HPO_4 , 12 g; KH_2PO_4 , 6 g; NH_4Cl , 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mg; glucose, 4 g; CaCl_2 , 66.6 mg; and distilled water. This medium (2 ml) was added to sterile test tubes (14×170 mm) including 1.6 ml of distilled water, followed by autoclaving. The pteridine compounds were dissolved in sterilized distilled water and added aseptically to the assay tubes just prior to inoculation, by which the final volumes of the media were filled up to 4 ml. 2-Amino-4-hydroxy-6-carboxypteridine was reduced with $\text{Na}_2\text{S}_2\text{O}_4$ (25 mg/0.4 ml) to the corresponding 7,8-dihydropteridine, and used directly to the growth tests*1. All assay tubes were inoculated with one drop of the cell suspension cultured at 30° for 24 hours on the slanted liquid medium (4 ml) made by 2-fold dilution of the double strength medium with distilled water. The assay tubes were placed at an angle of about 10 degrees, and incubated at 30° for 20 hours. Growth was monitored by determining optical absorbancy at 660 m μ .

4. Determination of Inhibitor Concentration Required for Half Inhibition of *E. coli* Growth, and of Modes of Inhibitory Action

It has been proposed empirically by Tamiya *et al.* (12) that inhibitory modes of the bacterial growth by antibiotics are well represented by the equation

$$H = \frac{G^n}{\phi^n + G^n}$$

, where G is the concentration of antibiotic, ϕ is the concentration of antibiotic needed for half inhibition of the microbial growth, n is the positive number specific for antibiotic used and H is the relative inhibition value ranging between one to zero unit. The logarithmic form of this equation is

$$\log \left(\frac{H}{1-H} \right) = n \log G - n \log \phi$$

On plotting $\log \left(\frac{H}{1-H} \right)$ against $\log G$, a straight line with a slope = n should be

obtained, cutting the horizontal axis at $\log \phi$. Straight lines illustrated in Fig. 2 were obtained by treatment of the values given in Fig. 1 and Table 1 according to this method. The concentrations of the pteridines required for the half inhibition of *E. coli* growth or for the half reversal of the inhibition were calculated from the intercepts of the respective straight lines on the horizontal axis, and n values for the respective pteridines were compared.

RESULTS

As shown in Fig. 1, 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine inhibited growth of *E. coli* B, but the oxidized form of this pteridine was ineffective as a growth inhibitor in the concentrations tested. The inhibition commenced at 2.0×10^{-5} M of the carboxy-dihydropteridine and was complete at 2.0×10^{-3} M. Fifty percent inhibition was obtained at the inhibitor concentration of 2.2×10^{-4} M (see Fig. 2). The growth inhibition induced by the carboxy-dihydropteridine of 10^{-3} M could be reversed by increasing the amount of 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine supplied in the culture medium, as shown in Table 1. The reversal was more conspicuous when the concentration of the formyldihydro-

FIG. 1 Inhibition of *Escherichia coli* growth with 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine

*1 NOTE: Excess $\text{Na}_2\text{S}_2\text{O}_4$ was not removed from the carboxy-dihydropteridine solution added to the assay medium (final concentration: 25 mg of $\text{Na}_2\text{S}_2\text{O}_4$ per 4 ml), since the reducer could stabilize the dihydropteridine. When the media were devoid of the agent, the reduced pteridine was allowed to oxidize rapidly to the carboxypteridine during cultivation of *E. coli*. The controls involving $\text{Na}_2\text{S}_2\text{O}_4$ but not the dihydropteridine were run parallel with all experiments.

TABLE 1

Effects of 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine on *Escherichia coli* growth in the presence or absence of 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine

Concentration of 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine (mM)	Growth inhibition of <i>E. coli</i> (%)	
	In the absence of PtH ₂ COOH*	In the presence of PtH ₂ COOH*
0.04	0	94
0.10	9.5	91
0.20	37	84
0.25	47	—
0.40	—	68

PtH₂COOH*: 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine.

FIG. 2 Tamiya plot of growth inhibition of *Escherichia coli* by 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine and 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine

G , pteridine concentration (M); H , inhibition degree of *E. coli* growth, H at 100% inhibition is expressed as one unit and H at zero% inhibition is as zero unit.

Straight line 1, 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine; straight line 2, 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine; straight line 3, 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine in the presence of 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine (1 mM).

pteridine was beyond 10^{-4} M. Fifty percent reversal was estimated with the formyldihydropteridine content at 7.6×10^{-4} M (see Fig. 2). However, when the carboxydihydropteridine was absent in the culture medium, the formyldihydropteridine acted rather as a strong inhibitor on the microbial growth. The inhibitory power was approximately equal to that of the carboxydihydropteridine. Fifty percent inhibition was given at 2.6×10^{-4} M of

the formyldihydropteridine (see Fig. 2). Data presented in Fig. 1 and Table 1 were plotted according to the method of Tamiya *et al.* (12). As shown in Fig. 2, the plottings for the growth inhibition by the carboxydihydropteridine and by the formyldihydropteridine gave a straight line of a slope = 2.0 and a straight line of a slope = 2.1, respectively. This finding indicates that modes of the inhibitory actions by these pteridines are mutually very similar. Also, a straight line of a slope = -1.2 was obtained by the same plotting for the reversal by the formyldihydropteridine of the growth inhibition induced by the carboxydihydropteridine.

DISCUSSION

Biological functions of folate coenzymes have been recognized to correlate profoundly with cell division. Therefore, reduction in the coenzyme levels *in vivo* caused by blockage of their biosynthesis would elicit a severe growth inhibition of living organisms. It has been established that 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine inhibits dihydrofolic acid synthesis from GTP by specific attacking an enzymatic reaction whereby 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine is pyrophosphorylated by action with ATP in the presence of Mg^{2+} (1-3). The oxidized form of the inhibitor has been found to be ineffective on the pyrophosphorylating step (2). The present study reveals that the carboxydihydropteridine can hinder strongly growth of *E. coli*, but its oxidized pteridine does not exert any effect on the growth. These findings have led to a conclusion that the carboxydihydropteridine blocks the bacterial growth by antagonizing utilization of the hydroxymethyldihydropteridine for biosynthesis of dihydrofolic acid.

Very recently, it has been demonstrated that 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine can be transformed enzymatically to 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine, an established precursor for dihydrofolic acid (6). The transformation requires NADPH as an essential cofactor. However, recent enzymatic investigations (7-9) have shown that the formyldihydropteridine is not

involved as an intermediate in the main biosynthetic pathway of dihydrofolic acid from GTP. Biological significance of the transformation must be clarified. An hypothesis to account for this discrepancy has been proposed previously by the present authors (6,10) that the formyldihydropteridine, but not its oxidized compound, might be a direct product from dihydrofolic acid in folate catabolism, and could be metabolized into the hydroxymethyldihydropteridine and into 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine. The former conversion might provide a salvage route^{*2} for reutilization of the catabolic product, whereby living organisms can be protected against a futile wastage of their metabolites. The latter conversion might have another kind of biological significance from a view point of regulation of folate coenzyme levels *in vivo*, because the carboxydihydropteridine can act as a potent antagonist against dihydrofolic acid formation *in vitro* (1-3) and *in vivo* as shown in the present work. Overproduction of the reduced folate coenzymes exceeding their metabolic needs might cause to promote their degradation, allowing to elevate the level *in vivo* of the inhibitor. The rate of dihydrofolic acid biosynthesis can be reduced at the pyrophosphorylation of the hydroxymethyldihydropteridine to maintain the physiological levels of the coenzymes in the cells. This mechanism can be characterized as a kind of feed-back inhibitions by the product of folate catabolism.

Findings obtained from the present study concerning the effects of 2-amino-4-hydroxy-6-formyl-7,8-dihydropteridine on growth of *E. coli* are in general agreement with the proposed hypothesis. Inhibition of *E. coli* growth by 2-amino-4-hydroxy-6-carboxy-7,8-dihydropteridine can be more efficiently reversed by the formyldihydropteridine. The similar effect is observed with the same microbe inhibited by 2-amino-4-hydroxy-6-hydroxymethylpteridine (11). These observations suggest that the salvage route to convert the formyldihydropteridine into the hydroxymethyldihydropteridine can function dominantly when drop in

levels of folate coenzymes *in vivo* resulted by blockage of the main biosynthetic pathway of dihydrofolic acid. The microbe under the normal growth can obtain mainly folate coenzymes through the main biosynthetic pathway from GTP, and the formyldihydropteridine supplied may be metabolized to the carboxydihydropteridine in the cells. This conversion is likely to proceed under the reduced levels of the pteridines, but not under their oxidized levels, since as shown in the present work, the oxidized form of the carboxydihydropteridine fails to affect the microbial growth. Other possibility to be conceivable with the growth inhibition by the formyldihydropteridine remains that this substance can affect immediately formation of the enzymes constructing the biosynthetic pathway of dihydrofolic acid. This mechanism has a nature of repression by the product of folate catabolism. This must await clarification.

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^{*2} NOTE: The salvage route is trivially named in this report for the metabolic pathway whereby catabolites of folate compounds can be converted to the intermediates of dihydrofolic acid biosynthesis.