Effect of Pyrimethamine on Folic Acid Metabolism in Streptococcus faecalis and Escherichia coli

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Pyrimethamine (Daraprim (Burroughs Wellcome); 2,4-diamino-5-p-chlorophenyl-6-ethylpyrimidine) is used in the treatment and prevention of malaria (1). When used in combination with one of the sulfonamides, it is also effective in the treatment of toxoplasmosis (2) and coccidiosis (3). Pyrimethamine is one of a series of 2,4-diaminopyrimidines which have been shown to be antagonists of folic acid and citrovorum factor (formyltetrahydrofolic acid) for the growth of Lactobacillus casei (4). In Streptococcus faecalis the inhibition by pyrimethamine is reversed only at low concentrations by folic acid; citrovorum factor was much more effective as a reversing agent (4). Thus growth inhibition reversal studies would presumably classify pyrimethamine as an antifolic acid drug similar to amethopterin. However, it was believed that a study of the action of pyrimethamine on a system divorced from growth was desirable in order to understand more fully its mechanism of action. The ultimate aim of these studies will be to determine the means by which resistance may develop to the action of the antifolic acid drugs.

The experiments described in this paper were carried out with washed-cell suspensions and cell-free extracts of S. faecalis. It has been demonstrated that pyrimethamine is a potent inhibitor of the conversion of folic acid to citrovorum factor in these systems. The inhibition has been found to be of the non-competitive type and to involve the endogenous conversion of folic acid to citrovorum factor rather than the assimilation of folic acid by the cells. In addition, the effect of pyrimethamine on the synthesis of folic acid from p-aminobenzoic acid has been investigated.

EXPERIMENTAL

Methods

Folic Acid to Citrovorum Factor Reaction Systems-The whole-cell system was similar to that described by Nichol (5) for the study of the action of amethopterin. Cells of S. faecalis (ATCC 8043) were grown overnight in Difco folic acid medium that contained 0.5 mg. of folic acid per ml. After the cells were harvested and washed they were incubated at 37° for 3 hours without aeration in the following medium: 0.1 gm. of MgSO₄, 1.5 gm. of KH₂PO₄, 3.5 gm. of Na₂HPO₄, 2.0 gm. of glucose, and 1000 ml. of distilled water. This depletion of the cells was necessary to avoid errors in assay due to the occurrence of citrovorum factor-like material in the control systems. The concentrations of the reactants with two exceptions were as given by Nichol (5). Only 50 to 100 mg. of folic acid per ml. (100-fold less than that used by Nichol) were necessary for optimal citrovorum factor production in this system. Furthermore, magnesium was added because it was found to double the yield of this factor. Optimal citrovorum factor production occurs with 1 mg. of MgCl₂·6 H₂O per ml and this concentration has been used throughout.

Cell-free extracts of S. faecalis were prepared from 20 l. of cells grown in Difco folic acid medium that contained 0.5 mg. of folic acid per ml. The yield of washed cells from this volume amounted to 18.5 gm. wet weight. They were suspended in 10 ml. of 0.1 M phosphate buffer, pH 6.5, and sonicated at maximum power in the Raytheon 10 kc. sonic oscillator for 1 hour at 6°. The extracts were clarified by several centrifugations at 20,000 X g. By the sulfosalicylic acid method (6), with crystalline bovine albumin as standard, the extracts contained 5.4 mg. of protein per ml. Unlike the system of Nichol (5), TPNH (7-10) has been used here instead of DPN and glucose. ATP, MgCl₂, and sodium formate showed little or no stimulatory effect on citrovorum factor production, so they were omitted. The factors essential for citrovorum factor synthesis are folic acid, extract, ascorbic acid, and TPNH.

After incubation the reaction systems were heated at 120° for 30 minutes to convert the product to citrovorum factor (5). This was assayed with Pediococcus cerevisiae (ATCC 8081) grown in Difco CF assay medium. It was necessary to run a standard curve along with each assay, as the growth response of P. cerevisiae to citrovorum factor is variable. dL-Calcium leucovorin (Lederle) was used as the standard, and the results have been expressed in terms of this diastereoisomer.

p-Aminobenzoic Acid to Folic Acid Reaction System—This system is based on that used by Nimmo-Smith et al. (11) for a study of the action of the sulfonamides on folic acid synthesis. Escherichia coli M48-34, which requires p-aminobenzoic acid for growth, was grown overnight in medium "A" of Lascelles and Woods (12) supplemented with 5 mg. of this vitamin per ml. The cells were depleted of stored folic acid by incubation in the salts-glucose medium described above for a similar treatment of S. faecalis cells. Depletion of the cells results in a higher production of folic acid. The components of the reaction system with one exception were the same as those given by Lascelles and Woods (12). l Glutamic acid was found to be unnecessary for folic acid synthesis when S. faecalis was used as assay organism. The "folic acid" formed was assayed with a strain of S. faecalis which had been rendered approximately 1000-fold resistant to pyrimethamine. Difco folic acid assay medium was employed for the assays.

1 Kindly furnished by Dr. B. D. Davis.
* Unpublished experiment of R. C. Wood.
RESULTS

Effect of Pyrimethamine on Biosynthesis of Citrovorum Factor—The effect of pyrimethamine on the conversion of folic acid to citrovorum factor by whole cells and by cell-free extracts of S. faecalis is shown by the data in Table I. Approximately 10 mg./ml. of pyrimethamine per ml. were required to inhibit citrovorum factor synthesis by the cells and extracts by 50 per cent. This concentration is of the same order of magnitude as that required to inhibit the growth of S. faecalis by 50 per cent (13). Folic acid was capable of counteracting the effects of low (2 mg. per ml.) concentrations of pyrimethamine; however, citrovorum factor production normally was not restored completely to that of the control despite an excess (100 mg. per ml.) of folic acid. This suggested that pyrimethamine may be acting as a noncompetitive inhibitor. A Lineweaver and Burk (14) type plot of the effect of pyrimethamine on citrovorum factor synthesis by the whole cells is shown in Fig. 1. A pair of parallel curves without a common intercept was obtained.

**Table I**

Effect of pyrimethamine on biosynthesis of citrovorum factor by whole-cells and by cell-free extract of *Streptococcus faecalis*

<table>
<thead>
<tr>
<th>Pyrimethamine concentration (mg./ml.)</th>
<th><em>CF</em> produced (mg./mg. cells)</th>
<th>Pyrimethamine concentration (mg./ml.)</th>
<th><em>CF</em> produced (mg./mg. protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>85.0</td>
<td>0.0</td>
<td>3.7</td>
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<tr>
<td>0.5</td>
<td>94.5</td>
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<td>2.4</td>
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<tr>
<td>5.0</td>
<td>55.0</td>
<td>3.1</td>
<td>2.0</td>
</tr>
<tr>
<td>50.0</td>
<td>25.0</td>
<td>12.5</td>
<td>1.3</td>
</tr>
<tr>
<td>500.0</td>
<td>16.5</td>
<td>50.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* The complete whole-cell system consisted of washed, N-depleted bacterial cells (2 mg. dry weight); folic acid (100 mg. per ml.); glucose (0.2 per cent); sodium formate (0.01 m); MgCl₂·6H₂O (0.006 m); and phosphate buffer (0.1 m, pH 6.5) in a final volume of 2.0 ml. The systems were incubated at 37°C for 2 hours in the Dubnoff metabolic shaking incubator under an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide.

† The cell-free systems comprised extract (5.4 mg. proteins); folic acid (2 μg. per ml.); TPNH (0.00014 m); ascorbic acid (0.0014 m); and phosphate buffer (0.1 m, pH 6.5) in a final volume of 2.0 ml. The systems were incubated as above but for 3 hours.

‡ CF, citrovorum factor.

**Fig. 1.** The effect of pyrimethamine on the conversion of folic acid to citrovorum factor by cells of *Streptococcus faecalis*. Upper curve, 2.5 mg. pyrimethamine per ml.; lower curve, control.
methamine on the synthesis of citrovorum factor in the above system might have reflected interference with the uptake of folic acid by the cells, the following experiment was designed to determine the effect of the drug on the conversion of endogenous folic acid to citrovorum factor.

Washed cells of *S. faecalis* were allowed to take up folic acid (15) in the presence and in the absence of pyrimethamine. The cells were washed with phosphate buffer and the cells from each of the three uptake systems (see Table II) were divided into three equal parts. One portion of cells was assayed for the amount of folic acid taken up (the intracellular folic acid), and the other two portions were added to the usual citrovorum factor synthesis system. One of these systems served as control, while the other contained the same relative concentration of pyrimethamine as the folic acid uptake system. No additional folic acid was added. Hence the citrovorum factor was synthesized exclusively from endogenous folic acid. The results of this experiment (Table II) demonstrated that a concentration of 20 µg. of pyrimethamine per ml. was without effect upon the uptake of folic acid, but inhibited the conversion of endogenous folic acid to citrovorum factor by 58 per cent.

**Effect of Stored Pyrimethamine on Synthesis of Citrovorum Factor by *S. faecalis***—When pyrimethamine-2-C¹⁴ is incubated with washed suspensions of *S. faecalis*, a certain proportion of the drug is taken up and stored by the cells (15). It was observed that when the cells were exposed to pyrimethamine and then washed several times with buffer, there was an appreciable inhibition of the subsequent synthesis of citrovorum factor, presumably due to the residual stored drug. This is shown in Table III. Glucose was effective in partially counteracting the inhibition by pyrimethamine of the subsequent synthesis of citrovorum factor, presumably due to the ability of glucose to inhibit the uptake of the drug (15). In contrast, folic acid was incapable of preventing the subsequent inhibition of citrovorum factor synthesis by stored pyrimethamine.

**Effect of Pyrimethamine on Biosynthesis of Folic Acid**—Although it was found that pyrimethamine is a potent inhibitor of the conversion of folic acid to citrovorum factor, there remained the possibility that the site of action of the drug might be broad enough to include also the synthesis of folic acid. Accordingly, the effect of pyrimethamine on the synthesis of folic acid from p-aminobenzoic acid by cells of *E. coli* was investigated. As shown in Table IV, approximately 5 µg. of pyrimethamine per ml. were required to inhibit by 80 per cent the synthesis of folic acid in the *E. coli* system. This is about 500-fold more than is required to inhibit the conversion of folic acid to citrovorum factor.

**Discussion**

The fact that parallel curves are obtained when the effect of pyrimethamine on the conversion of folic acid to citrovorum factor is plotted according to the method of Lineweaver and Burk (Fig. 1), indicates that pyrimethamine, like amethopterin (10), is a noncompetitive inhibitor of citrovorum factor synthesis. The same conclusion regarding pyrimethamine action was reached by Doctor (16), who used chick liver supernatants to study this reaction.

Cook and Jacobs (17) observed that residual activity against *Toxoplasma* persisted for several weeks after administration of pyrimethamine to monkey kidney cells. These results were interpreted to mean that pyrimethamine may be stored by certain cells for prolonged periods of time. The present results with bacterial cells (Table III) would seem to agree with this interpretation. It is interesting to note that despite the inhibitory effect of glucose on the uptake and therefore the storage of pyrimethamine, there is still sufficient absorption of the drug to inhibit significantly the subsequent synthesis of citrovorum factor (Table III).

The biosynthesis of folic acid from p-aminobenzoic acid may be even more insensitive to pyrimethamine than indicated by the results presented in Table IV. The inhibition by 5 µg. of pyrimethamine per ml. may be only apparent and may merely reflect the effect of the drug on the growth of the strain of *S. faecalis* used for the assay of the folic acid synthesized. Similarly, Davidson et al. (18) found that the growth of a p-aminobenzoic acid-requiring mutant of *E. coli* is not inhibited by 10 µg. of amethopterin per ml. It appears reasonably certain that the primary site of action of pyrimethamine is the conversion of folic acid to citrovorum factor, and not the synthesis of folic acid. The marked differences between pyrimethamine and amethopterin in chemotherapeutic effects appear to depend, therefore, on differences in uptake mechanisms (13) rather than on differences in locus of action.

**Summary**

The conversion of folic acid to citrovorum factor by cells and by extracts of *Streptococcus faecalis* is inhibited noncompetitively by a concentration of pyrimethamine which is of the same order of magnitude as that required to inhibit the growth of this bacterium. At a concentration of pyrimethamine which inhibits appreciably the conversion of endogenous folic acid to citrovorum factor, there is no effect upon the assimilation of folic acid. After exposure of *Streptococcus faecalis* cells to pyrimethamine, the cells retain sufficient drug through repeated washes to inhibit a subsequent synthesis of citrovorum factor. This effect is not counteracted by folic acid, but is diminished by glucose which is known to cause the release of pyrimethamine from the cells.

The effect of pyrimethamine on the biosynthesis of folic acid by *Escherichia coli* cells was investigated, and it was found that there is no significant inhibition of this reaction by the drug.

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### Table IV

**Effect of pyrimethamine on biosynthesis of folic acid by cells of *Escherichia coli***

<table>
<thead>
<tr>
<th>Pyrimethamine concentration (µg./ml.)</th>
<th>FA* produced (mg./mg. dry cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>8.5</td>
</tr>
<tr>
<td>0.05</td>
<td>8.5</td>
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<tr>
<td>0.5</td>
<td>6.0</td>
</tr>
<tr>
<td>5.0</td>
<td>4.3</td>
</tr>
</tbody>
</table>

*FA, folic acid.*
REFERENCES

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