Biochemical Pharmacology, 1961, Vol. 6, pp. 113–124. Pergamon Press Ltd., Printed in Great Britain.

THE RELATIONSHIP OF CELLULAR PERMEABILITY TO THE DEGREE OF INHIBITION BY AMETHOPTERIN AND PYRIMETHAMINE IN SEVERAL SPECIES OF BACTERIA

R. C. WOOD,* R. FERONE and G. H. HITCHINGS

Burroughs Wellcome and Co. (U.S.A.) Inc.,
Wellcome Research Laboratories, Tuckahoe, New York

(*Received* 16 *July* 1960)

Abstract—As a possible means for determining the influence of cellular permeability on the action of the antifolic acid drugs in bacteria, a comparison has been made of their effects on whole cells and on cell-free extracts of several species. The parameters examined were inhibition of growth, inhibition of folinic acid synthesis in whole cells and inhibition of folinic acid synthesis in cell-free extracts. In *Streptococcus faecalis* the concentrations of amethopterin and pyrimethamine required to inhibit all of these functions are in the order of several millimicrograms per milliliter. In *Escherichia coli* and to a more limited extent in *Lactobacillus arabinosus*, millimicrogram amounts of these drugs will inhibit the synthesis of folinic acid by the extracts, whereas microgram concentrations are required to produce the inhibitions in the whole cells. These results suggest that certain non-folic acid-requiring bacteria such as *E. coli* and *L. arabinosus* are relatively insensitive to the antifolic acid drugs because they apparently are unable to assimilate them with as much facility as are the folic acid-requiring bacteria such as *S. faecalis*. It does not appear that the intracellular enzymes that convert folic acid to folinic acid in the non-exacting bacteria are insensitive to the drugs.

The following results suggest that the uptake by *L. arabinosus* of amethopterin (or aminopterin), but not that of folic acid, is mediated by an active cellular transport system which normally takes up thiamin.

(1) The inhibition of growth produced by amethopterin can be prevented by thiamin or by thiamin plus pyridoxal, but not by comparable concentrations of folic or folinic acids.

(2) In washed-cell suspensions thiamin interferes with the uptake of aminopterin, but not of folic acid. Glucose stimulates the uptake of aminopterin and of folic acid.

(3) In washed-cell suspensions thiamin interferes with the inhibitory action of amethopterin on the synthesis of folinic acid.

For the following reasons the uptake of pyrimethamine by *L. arabinosus* seems to be of a passive, diffusive nature, not involving a thiamin permease system.

(1) The inhibition of growth produced by pyrimethamine cannot be reversed by thiamin or by thiamin plus pyridoxal or by folic or folinic acids.

(2) Thiamin has no effect on the uptake of pyrimethamine by washed-cell suspensions. Glucose inhibits the uptake of pyrimethamine. The drug is taken up equally well by living or dead cells. Its uptake is dependent upon the pH of the system, more drug being absorbed at values closer to its pK_a.

(3) In washed-cell suspensions pyrimethamine inhibits the conversion of folic acid to folinic acid, but this inhibition is not counteracted by thiamin.

The possible exploitation of the high degree of specificity of certain permease systems for the purposes of practical chemotherapy is discussed.

* Present address: Department of Microbiology, School of Medicine, The George Washington University, Washington 5, D.C., U.S.A.

INTRODUCTION

THE work of McGlohon *et al.*[1] and of McGlohon and Bird[2] demonstrated that the growth of certain non-folic acid-requiring micro-organisms, including *Lactobacillus arabinosus*, is inhibited by rather low concentrations of the anti-folic acid drugs, and that this inhibition could not be counteracted by reasonable levels of folic or folinic acids. Since Hendlin *et al.*[3] have shown that the anti-folic acid drugs block the synthesis of folinic acid in *L. arabinosus*, the inability of this factor to counteract growth inhibition by these drugs in *L. arabinosus* seemed anomalous. Studies on the uptake of folic acid, leucovorin, and the anti-folic acid drugs by bacteria[4] have indicated that there is a high degree of specificity with regard to the comparative efficiency with which these compounds are assimilated by various species. For example, *Pediococcus cerevisiae* assimilates leucovorin with ease, but it is unable to take up folic acid unless the latter is supplied at 1000 times the concentration of leucovorin.[5] It seemed possible that leucovorin may be unable to counteract the effects of the anti-folic acid drugs in *L. arabinosus* and in similar organisms because it may be only poorly assimilated by the cells. The following studies resulted from this suggestion and deal with the concentrations of amethopterin and pyrimethamine* required to inhibit growth and folinic acid synthesis by intact cells as compared to cell-free extracts. In addition, the means by which these drugs permeate cells of *L. arabinosus* has been investigated in more detail.

There is a very strict specificity with regard to the utilization of folic acid and its derivatives. Certain micro-organisms which require *p*-aminobenzoic acid for growth are unable to utilize *p*-aminobenzoylglutamic, folic or folinic acids, even though these compounds are end-products of *p*-aminobenzoic acid metabolism. Examples of these organisms are strains of *Clostridium acetobutylicum*,[6] *Acetobacter suboxydans*,[6] *Escherichia coli*,[4, 7–9] *Neurospora crassa*.[10] The inability of bacteria such as certain *E. coli* mutants to utilize folic acid for growth in place of *p*-aminobenzoic acid is paralleled by the finding that this species can incorporate *p*-aminobenzoic acid–carboxyl-^{14}C,[11, 12] but not folic acid-2-^{14}C.[12] *P.cerevisiae* cannot utilize reasonable concentrations of folic acid for growth in place of leucovorin,[13] even though it can convert folic acid to folinic acid.[3, 5] However, it can utilize *high* concentrations of folic acid for growth[13, 14] and can be trained to grow on lower concentrations of folic acid.[14] This training is accompanied by a greatly increased sensitivity to aminopterin and amethopterin.[14]

Folic acid and its derives have been found to be incapable of reversing the growth inhibitory effects of the sulfonamides in a number of micro-organisms. This is despite the fact that the sulfonamides have been clearly shown to interfere with the synthesis of folic acid.[9] Examples of species in which folic acid is incapable of reversing sulfonamide bacteriostasis are *E. coli*,[9, 15–19] *Aerobacter aerogenes*,[16] *Staphylococcus aureus*,[18] and *Diplococcus pneumoniae*.[18] Presumably *p*-aminobenzoic acid could counteract the inhibition by sulfonamides in all of these species. In *Mycobacterium tuberculosis* folic acid was incapable of counteracting the inhibition of growth produced by *p*-aminosalicylic acid, whereas *p*-aminobenzoic acid did effect a reversal.[20] Moreover, folic or folinic acids or their derivatives were unable to counteract the action of the anti-folic acid drugs in *Bacillus subtilis*,[21] *N. crassa*,[22] *Streptococcus pyogenes*,[2] or *E. coli*.[23]

* Pyrimethamine ("Daraprim") is 2:4-diamino-5-*p*-chlorophenyl-6-ethylpyrimidine.

Concentrations of the anti-folic acid drugs which are many times higher than those required to inhibit *Streptococcus faecalis* or *Lactobacillus casei* have been found necessary to inhibit the growth of non-folic acid-requiring organisms such as *E. coli*,[11, 23–27] *A. aerogenes*,[28] *Lactobacillus plantarum*,[25] *N. crassa*,[22] and *Schizosaccharomyces octosporus*,[25] despite the fact that these micro-organisms presumably convert folic acid to folinic acid.

These seemingly contradictory observations made by earlier investigators in the folic acid field, who used intact bacterial cells, could be explained on the basis of permeability differences among the various bacterial species tested. It is the purpose of this paper to report observations which may explain many of the apparent discrepancies.

METHODS

Bacterial strains employed and their cultivation

Streptococcus faecalis (ATCC 8043) was grown in Difco folic acid-assay medium containing either folic acid (1 mμg/ml) or leucovorin, as indicated in the tables *Lactobacillus arabinosus* 17–5 (ATCC 8014) was grown in the semi-synthetic medium of Shiota.[29] "Salts B", included but not described by him, were considered to be anhydrous $MgSO_4$ (100 mg) and 10 mg each of NaCl, $MnSO_4$ and $FeSO_4 \cdot 7H_2O$ per liter of single-strength medium. The complete medium contained *p*-aminobenzoic acid, thiamin, and pyridoxal at 10 mμg, 1 μg and 2μg per ml, respectively. *Escherichia coli* M48–34 (originally from Dr. B. D. Davis) was grown in medium "A" of Lascelles and Woods[9]. Ten milligrams of Na_2HPO_4 per ml were added to this medium in order to bring the pH to 7·2 without further adjustment. To supply the *p*-aminobenzoic acid requirement of this strain of *E. coli*, 5 mμg of the vitamin were added per ml of medium. *Bacillus subtilis* (Merck 3R 8788) was grown in a medium originally described for the growth of *Proteus vulgaris*.[30] Growth was measured turbidimetrically using the Klett–Summerson photoelectric colorimeter with filter no. 66.

Folic acid to folinic acid reaction systems

The preparation of these systems from cells and extracts of *S. faecalis* has been described.[31] Washed-cell suspensions of *L. arabinosus* were prepared from cells harvested from the medium described above. Experience showed, however, that it was necessary to grow the cells in a limiting amount of *p*-aminobenzoic acid (i.e. 0·2 mμg/ml) in order to achieve appreciable synthesis of folinic acid. The details of the reaction systems are given in the tables. *E. coli* M48–34 was also cultured in a limiting concentration of *p*-aminobenzoic acid (less than 6 mμg/ml). Maximum synthesis of folinic acid by washed cells of *E. coli* occurred in the presence of L-glutamic acid; less synthesis occurred with sodium formate, and practically none occurred when both of these substrates were present.

Cell-free extracts of *L. arabinosus* were prepared from cells grown in from 5 to 10 l. of complete Shiota[29] medium. The washed cells (from 20 to 27 g, wet weight) were suspended in about 55 ml of 0·1 M, pH 6·5 Sorensen's phosphate buffer and treated for 1 hr at 6 °C in the Raytheon sonic oscillator (model DF 101) with the addition of approximately 1·5 g of Carborundum powder no. 240. The sonicates were clarified by centrifugation at 35,000 **g** for 20 min in the cold. The sulfosalicylic acid method,[32].

with crystalline bovine albumin as standard, showed the final protein concentration to be approximately 13 mg/ml.

Cell-free extracts of *E. coli* were prepared from cultures grown in Lascelles and Woods[9] medium. They were sonicated for 5 min without the inclusion of Carborundum powder, and then were treated as described above for *L. arabinosus*.

Neither the *L. arabinosus* nor the *E. coli* extracts remained active (with respect to the synthesis of folinic acid) for more than 2 weeks, even when they were kept in a freezer. Extracts of *E. coli*, strain B (either dialysed or undialysed), were of no value for this reaction system because of the high background of folinic acid activity contained in extracts prepared from this wild-type strain.

Following incubation at 37 °C in the Dubnoff metabolic shaking incubator under an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide, the reaction systems were heated at 115 °C for 30 min and assayed for folinic acid with *Pediococcus cerevisiae* (ATCC 8081). (The washed-cell systems were treated with chicken pancreas conjugase, prepared by the method of Doctor and Couch,[33] before they were assayed for folinic acid.) The folinic acid synthesized has been reported in terms of the total, natural form. The washed-cell systems were incubated for 2½ hr in the Dubnoff incubator; the cell-free extract systems were incubated for from 3 to 5 hr.

Sources of the labeled compounds

The aminopterin-2-^{14}C was a gift of the Southern Research Institute, Birmingham, Alabama. Pyrimethamine-2-^{14}C was prepared in this laboratory. For further details on these tracers see Wood and Hitchings[4]. The folic acid was tritiated commercially, and purified by Dr. E. Bresnick of this laboratory.

RESULTS

Comparative effects of amethopterin and pyrimethamine on the growth and synthesis of folinic acid by S. faecalis, L. arabinosus *and* E. coli

It is well known that the folic acid-requiring bacteria, such as *S. faecalis* and *L. casei*, are extremely sensitive to the anti-folic acid drugs. Moreover, it is known that certain other bacteria which have no growth requirement for folic acid are relatively insensitive to the anti-folic acids (for examples see Introduction). *L. arabinosus* seems to occupy an intermediate position in this respect for it is capable of utilizing for growth either relatively high concentrations of folic acid or low concentrations of *p*-aminobenzoic or *p*-aminobenzoylglutamic acids[34] (although growth on folic acid may be a resultant of its prior degradation to *p*-aminobenzoylglutamic acid[35]).

A comparison has been made of the inhibitory effects of amethopterin and pyrimethamine on folinic acid-synthesis by whole cells and by cell-free extracts of a representative folic acid-requiring bacterium (*S. faecalis*), a non-folic acid-utilizing bacterium (*E. coli*) (see Introduction), and the intermediate type micro-organism, *L. arabinosus*. These data are presented in Table 1. The concentration of amethopterin required to inhibit by 50 per cent the production of folinic acid by cell-free extracts of these three species of bacteria ranged between 0·1 to 5 mμg/ml. However, the concentration of this drug needed to effect the same degree of inhibition of folinic acid-synthesis in the intact cells ranged between 0·05 mμg for *S. faecalis* to greater than 1000 mμg for *E. coli*. Similar differences between the intact and the disrupted cells were found with pyrimethamine (Table 1). From these results it may be tentatively

concluded that *E. coli* and *L. arabinosus* are less permeable to these drugs than *S. faecalis*.

The data in Table 2 show that the concentrations of amethopterin and pyrimethamine required to produce 50 per cent inhibition of the growth of the three representative bacteria directly paralleled those concentrations needed to inhibit the synthesis of folinic acid by the intact cells (Table 1). Also, it is shown that there was a

TABLE 1. INHIBITION OF FOLINIC ACID SYNTHESIS BY ANTIFOLIC ACID DRUGS: CELLS *vs.* EXTRACTS

Preparation	Drug	*S. faecalis*	*L. arabinosus*	*E. coli*
(a) Washed cells	Amethopterin	0·05*	30*	>1000*
(b) Extract	Amethopterin	0·1	0·5	5
(a) Washed cells	Pyrimethamine	10	7000	>10,000
(b) Extract	Pyrimethamine	10	45	33

* Concentration, in mμg per ml, of either amethopterin or pyrimethamine required for 50 per cent inhibition of folinic acid biosynthesis.

Reaction systems—(a) Washed-cell preparations. *S. faecalis*: N-depleted bacterial cells (2 mg dry weight), folic acid (100 mμg/ml), glucose (0·02 M), ascorbic acid (0·01 M), sodium formate (0·02 M), $MgCl_2 \cdot 6H_2O$ (0·01 M), and phosphate buffer (0·01 M, pH 6·5) in a final volume of 2·0 ml. *L. arabinosus*: Washed cells, glucose, ascorbic acid, $MgCl_2 \cdot 6H_2O$, sodium formate and phosphate buffer were employed at the same concentrations given for *S. faecalis*. *p*-Aminobenzoylglutamic acid (0·5 μg/ml) replaced folic acid. *E. coli*: Washed cells, glucose, ascorbic acid, $MgCl_2 \cdot 6H_2O$ and phosphate buffer were employed at the same concentrations given for *S. faecalis*. *p*-Aminobenzoic acid (0·5 μg/ml) and L-glutamic acid (0·006 M) were also included.

(b) Cell-free extract preparations. *S. faecalis*: Extract (5 mg protein), folic acid (2 μg/ml), TPNH (0·0003 M), ascorbic acid (0·003 M) and phosphate buffer (0·1 M, pH 6·5) in a final volume of 2·0 ml. *L. arabinosus*: Extract (1·2 mg protein), folic acid (2 μg/ml), $MgCl_2 \cdot 6H_2O$ (0·0025 M), ascorbic acid (0·003 M), sodium formate (0·0074 M), ATP (0·0005 M), TPNH (0·0007 M) and phosphate buffer (0·05 M, pH 6·5) in a final volume of 2·0 ml. *E. coli*: Extract (0·73 mg protein) plus folic acid, $MgCl_2 \cdot 6H_2O$, ascorbic acid, sodium formate, ATP and phosphate buffer at the concentrations given for *L. arabinosus*. Glucose (0·005 M) and TPN (0·0006 M) were also included.

corresponding increase in the amount of leucovorin needed to counteract the inhibition of growth by these concentrations of the anti-folic acid drugs. Thus, growth studies also serve to suggest that amethopterin, pyrimethamine and leucovorin penetrate with progressively less facility into *S. faecalis*, *L. arabinosus*, and *E. coli*, in this order.

A role for thiamin in the uptake of amethopterin by L. arabinosus

McGlohon *et al.*[1] have reported that folic acid and leucovorin are relatively ineffective in preventing the action of aminopterin on the growth of *L. arabinosus*, whereas the combination of thiamin plus pyridoxal is quite effective in this respect. We have repeated their growth experiments—however, using the richer medium of Shiota[29]—and have observed the same type of protection by these vitamins when amethopterin was substituted for aminopterin; as illustrated in Fig. 1, thiamin plus pyridoxal was the most effective combination. Thiamin alone was nearly as effective as the combination, while pyridoxal alone was ineffective. Folic acid or leucovorin was much less active than either the combination of thiamin plus pyridoxal or thiamin alone. Under the same experimental conditions none of these vitamins was active in counteracting the inhibition of growth of *L. arabinosus* produced by pyrimethamine (Fig. 1).

McGlohon *et al.*[1] interpreted the counteracting effect of thiamin and pyridoxal as evidence for the existence of an alternative pathway for the synthesis of polynucleotides "which has a limited dependence on folic acid compounds but is mediated in some way by B_1 and B_6". However, it seemed that there were other possible interpretations for the effects of thiamin. One possibility was that thiamin might interfere with the uptake of amethopterin and aminopterin. Accordingly, the effect of thiamin

TABLE 2. INHIBITION OF GROWTH BY THE ANTIFOLIC ACID DRUGS: EFFECT OF LEUCOVORIN

Drug added	*S. faecalis*		*L. arabinosus*		*E. coli*	
	(a)	(b)	(a)	(b)	(a)	(b)
Amethopterin	0·1	<0·5	30	10,000	50,000	200,000
Pyrimethamine	3	<0·5	500	>10,000	8000	>200,000

(a) Concentration in mμg/ml of either amethopterin or pyrimethamine required to produce 50 per cent inhibition of growth.

(b) Concentration of leucovorin in mμg/ml required to counteract completely the inhibition produced by drug concentration given in column (a).

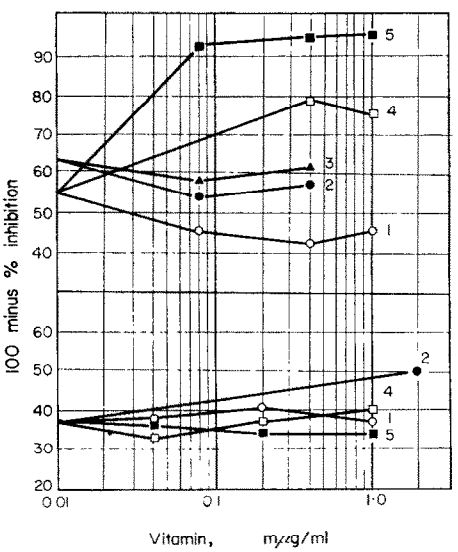

FIG. 1. Capacity of several vitamins to counteract the inhibition of the growth of *L. arabinosus* produced by amethopterin or pyrimethamine. *Upper* half of figure depicts the effects of the vitamins on the inhibition of growth produced by 30 mμg of amethopterin per ml, recorded at 41 hr of growth. *Lower* half depicts the effects of the vitamins on the inhibition produced by 500 mμg of pyrimethamine per ml, recorded at 23 hr of growth. *Curves*: no. 1 = pyridoxal; no. 2 = leucovorin; no. 3 = folic acid; no. 4 = thiamin; and no. 5 = thiamin plus pyridoxal. The medium contained 10 mμg of *p*-aminobenzoic acid per ml.

on the uptake of aminopterin-2-^{14}C by washed suspensions of *L. arabinosus* was investigated, with the results as given in Table 3. Thiamin inhibited markedly the assimilation of aminopterin by *L. arabinosus*. Since Guthrie *et al.*[36] have observed that thiamin and certain of its derivatives, but not folic acid or leucovorin,[21, 36] counteract the inhibitory action of amethopterin on the growth of *Bacillus subtilis*, the effect of

thiamin on the uptake of aminopterin-2-^{14}C by this species was also determined. Again, thiamin interfered with the uptake of aminopterin (Table 3). While our work was in progress Pine[43] reported the same interference by the pyrimidine moiety of thiamin with the uptake of aminopterin by *B. subtilis*.

TABLE 3. FACTORS AFFECTING THE UPTAKE OF FOLIC ACID AND ITS ANTAGONISTS

Uptake systems*	Radioactivity released from cells, counts/min per mg dry cells	
	L. arabinosus	*B. subtilis*
(a) Aminopterin-2-^{14}C	17·0	9·0
+ thiamin	10·0	3·8
+ glucose	202·0	10·8
+ glucose + thiamin	85·0	2·9
(b) Pyrimethamine-2-^{14}C	5·2	
+ thiamine	5·6	
+ glucose	2·3	
+ thiamin + glucose	2·3	
(c) Folic acid-^{3}H	6·1	
+ thiamin	6·5	
+ glucose	29·2	
+ glucose + thiamin	25·2	

* The uptake systems contained (where indicated) in a total volume of 3·0 ml:
(a) Aminopterin-2-^{14}C (3·0 μg; specific activity = 12,333 counts/min per μg) + washed bacterial cells (6·5 mg (*B. subtilis*) or 8·5 mg (*L. arabinosus*) dry weight) + glucose (1·5 per cent) + thiamin (25 μg) + Sørensen's phosphate buffer (0·05 M, pH 6·4).
(b) Pyrimethamine-2-^{14}C (30·0 μg; specific activity = 235 counts/min per μg) + washed cells (8·5 mg dry weight) + glucose (3 per cent) + thiamin (30·0 μg) + phosphate buffer (0·1 M, pH 7·4).
(c) Folic acid-^{3}H (15 μg; specific activity = 3500 counts/min per μg) + washed cells (8·5 mg dry weight) + glucose (3 per cent) + thiamin (30 μg) + phosphate buffer (0·1 M, pH 6·5).
The systems were incubated aerobically for 30 min at 37 °C. Then the cells were washed three times with buffer and resuspended in 1 ml of water. This was followed by heating at 120 °C for 10 min. The cells were removed by centrifugation and the entire supernatant fraction was dried on a planchet and counted at infinite thinness in a gas flow counter.

TABLE 4. INHIBITION OF FOLINIC ACID BIOSYNTHESIS BY THE ANTIFOLIC ACID IN *L. arabinosus*: EFFECT OF THIAMIN

Reaction system*	Folinic acid synthesized, mμg/mg dry cells	Percentage inhibition
Control	99·0	
+ thiamin	106·5	
+ amethopterin	46·5	53·0
+ amethopterin + thiamin	97·5	8·5
+ pyrimethamine	61·5	37·8
+ pyrimethamine + thiamin	66·0	38·0

* The basal reaction system was exactly as given in Table 1 for washed-cell preparations of *L. arabinosus*. Where indicated, amethopterin (20 mμg/ml), pyrimethamine (10 μg/ml) and thiamin (10 μg/ml) were added to the basal reaction system.

Just as thiamin was incapable of counteracting the inhibition of the growth of *L. arabinosus* produced by pyrimethamine (Fig. 1), so also was thiamin without effect on the uptake of pyrimethamine-2-^{14}C by this bacterium (Table 3). Interestingly, thiamin had no effect on the uptake of folic acid-^{3}H (Table 3).

To determine the influence of thiamin on the degree of inhibition of folinic acid synthesis produced by amethopterin and pyrimethamine, cells of *L. arabinosus* were harvested from media lacking thiamin. These cells were then used in the usual folinic acid synthesis system with *p*-aminobenzoylglutamic acid as the limiting substrate. The results of this experiment are presented in Table 4. The addition of thiamin to the reaction system lowered the inhibition by amethopterin from 53 per cent to 8·5 per cent*. There was no effect of thiamin on the inhibition of folinic acid synthesis produced by pyrimethamine.†

It may be concluded from the foregoing experiments that thiamin counteracts the inhibition of the growth of *L. arabinosus* produced by amethopterin by preventing its uptake by the cell. This in turn interferes with the subsequent effect of amethopterin on the intracellular synthesis of folinic acid. It can safely be assumed that amethopterin does not inhibit the growth of *L. arabinosus* by interfering with the uptake of thiamin, as this bacterium can grow readily in the absence of added thiamin.

Influence of pH on the inhibition of L. arabinosus *by pyrimethamine*

During the course of this study it was noted empirically that slight variations in the pH to which the medium had been adjusted prior to sterilization profoundly influenced the subsequent degree of growth inhibition produced by pyrimethamine or

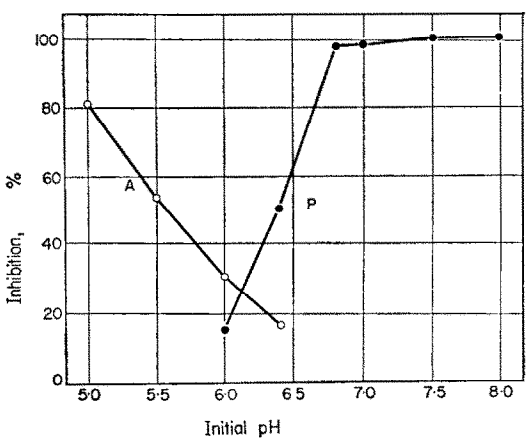

Fig. 2. Effect of the pH to which the growth medium had been adjusted prior to sterilization on the subsequent degree of growth inhibition of *L. arabinosus* produced by amethopterin or pyrimethamine. Curve A depicts the effects of 30 mμg of amethopterin per ml on growth. Curve P depicts the effects of 500 mμg of pyrimethamine per ml on growth. Turbidities were recorded at 18 hr of growth in a medium containing 10 mμg of *p*-aminobenzoic acid per ml.

amethopterin. This effect is illustrated in Fig. 2. A drop in the pH of the medium decreased the inhibition of growth caused by pyrimethamine; it increased the inhibition by amethopterin. It was considered possible that the pH of the medium might influence the cellular penetration of the drugs. This possibility was investigated in

* Pine and Guthrie[38] found that the pyrimidine moiety of thiamin was able to prevent the inhibitory effect of amethopterin on the incorporation of formate-^{14}C by *B. subtilis*.

† In washed-cell experiments with *S. faecalis* thiamin (1 μg/ml) was unable to counteract the inhibition of folinic acid-synthesis produced by either amethopterin (10 mμg/ml) or pyrimethamine (20 mμg/ml) (unpublished observation).

detail only in the case of pyrimethamine because the supply of aminopterin-2-^{14}C had become exhausted by this time.

If the effect of pH on the inhibition of growth by pyrimethamine were actually a reflection of its influence on the uptake of the drug, then the uptake of pyrimethamine by washed cells should be similarly related to the pH of the uptake reaction system. This was tested directly by incubating washed suspensions of *L. arabinosus* with pyrimethamine-2-^{14}C at several pH values. This experiment is depicted in Fig. 3.

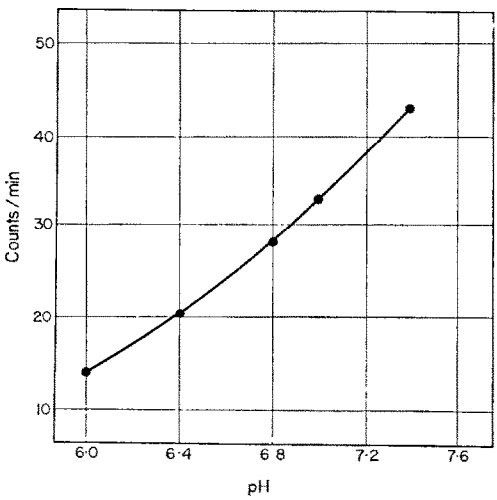

FIG. 3. Effect of pH on the uptake of pyrimethamine-2-^{14}C by *L. arabinosus* cells. The 3·0-ml uptake systems contained 8·5 mg dry weight of washed cells, 30 μg of labeled drug and 0·1 M Sorensen's phosphate buffers of the pH values indicated. The systems were incubated at 37 °C for 20 min and then the cells were washed once with the same buffer in which they had been incubated. The systems were treated then as described in Table 3.

There was a definite rise in the retention of the drug with increasing pH, demonstrating the desired relationship between inhibition and uptake.

The pH of the medium influences the growth-inhibitory action of pyrimethamine on *L. arabinosus*. From this it should follow that the pH should affect in a similar fashion the inhibition by pyrimethamine of the synthesis of folinic acid, as this is presumably the means by which pyrimethamine inhibits this bacterium (see Table 1). The effect of pH on the synthesis of folinic acid by whole cells and by cell-free extracts of *L. arabinosus* was determined, with the results as depicted in Fig. 4. Pyrimethamine produced more inhibition of folinic acid synthesis by whole cells as the pH was raised, which was the same influence pH had on the inhibition of growth by the drug. In cell-free extracts, however, pyrimethamine exerted progressively more inhibition of folinic acid synthesis as the pH dropped.

There was some question as to whether pyrimethamine-2-^{14}C was actually assimilated by *L. arabinosus* cells, for it was found that heat-killed cells retained the same amount of radioactivity as the viable cells. The following experiment was performed to determine whether the drug was actually assimilated by *L. arabinosus* or whether it exerted its action merely by binding with the surface of the cell.

Washed cells of *L. arabinosus* were permitted to take up *p*-aminobenzoylglutamic acid in the absence of pyrimethamine. Then the cells were washed and added to the usual folinic acid-synthesis system containing pyrimethamine, but lacking *p*-aminobenzoylglutamic acid. It was observed that 7 μg of pyrimethamine per ml produced a 58 per cent inhibition of the conversion of the intracellular *p*-aminobenzoylglutamic acid to folinic acid. This was the same concentration of drug required to inhibit by 50 per cent the conversion of extracellular *p*-aminobenzoylglutamic acid to folinic acid (see Table 1). Evidently, then, pyrimethamine does actually enter the cell and

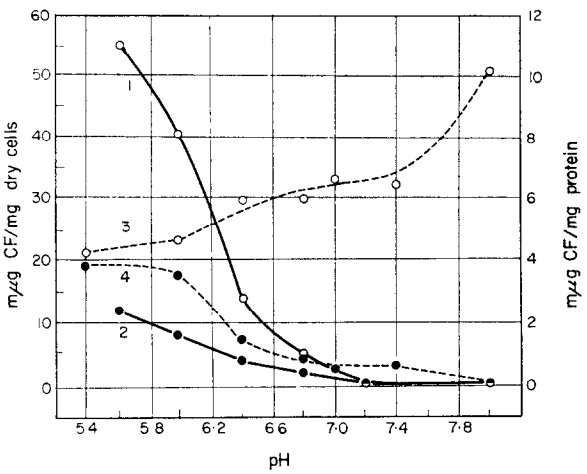

FIG. 4. Effect of pH upon the inhibition of folinic acid synthesis produced by pyrimethamine in *L. arabinosus*: a comparison of whole cells and cell-free extracts. *Extracts*: Curve no. 1 depicts the control set; curve no. 2 depicts the set containing 40 mμg of pyrimethamine per ml. With the exception of the inclusion of glucose (0·006 M) in this experiment, the same reaction system as that described in Table 1 for the *L. arabinosus* extract was employed here. *Whole cells*: Curve no. 3 depicts the control set; curve no. 4 depicts the set containing 7 μg of pyrimethamine per ml. The components of the reaction systems were identical with those described in Table 1 for the *L. arabinosus* washed-cell preparations. "CF" means folinic acid.

inhibit the conversion of endogenous *p*-aminobenzoylglutamic acid to folinic acid. Therefore, the equivalent uptake of pyrimethamine-2-^{14}C by living and by dead cells of *L. arabinosus* appears to be similar to the situation in which dead micro-organisms, e.g. yeast cells,[39] have been observed to absorb quantitative amounts of dyes such as methylene blue.

DISCUSSION

There is a tremendous range in the concentrations of amethopterin and pyrimethamine required to produce inhibition of the growth of the three species of bacteria examined in this study. However, the basic site of action of these drugs seems to be the same in all cases, viz. the conversion of folic acid to folinic acid. Assuming that the reaction studied in the extract experiments is the natural one carried out by the cell, then the profound differences in sensitivity to the drugs seem to lie in species differences in permeability to the drugs and not in differences in the sensitivity of their intracellular enzymes.

The uptake characteristics of *L. arabinosus* are unusual. It can assimilate amethopterin but not equivalent concentrations of folic or folinic acids. The reason for this

seems to be that the transport of amethopterin, but not that of the folic acids, is facilitated by a system which normally takes up thiamin. This transport system works best in the presence of an energy source (glucose) and would, by definition, be termed an active transport system.[40] Since thiamin interferes with the uptake of aminopterin, but not of pyrimethamine, only the transport of aminopterin (and presumably amethopterin) would seem to be mediated by this system. This transport system seems to have a high degree of specificity, since the uptake of folic acid, which differs from aminopterin only in the substitution of a hydroxyl group for an amino group, is not influenced by thiamin. Perhaps the presence of a 4-amino group on the pteridine ring is necessary for the molecule to fit the site on the cell surface where thiamin and aminopterin are absorbed. Pyrimethamine seems to enter the cell via a passive transport system, since glucose inhibits its uptake (Table 3) and cellular viability is unnecessary. Its uptake is influenced principally by its degree of ionization (see below).

According to general theory,[41] the state of ionization of an ionizable drug should influence its uptake and also its subsequent effect within the cell. The drug must be relatively undissociated in order to enter the cell. Within the cell, the ionized form should be the more active. The results of the experiments dealing with the effect of pH on the action of pyrimethamine on intact cells as compared with cell-free extracts fit this theory. Pyrimethamine is absorbed best and is most inhibitory toward the intact cells at pH values near its pK_a (7·2*); it is most effective against the extracts at the lower pH values, at which it is more dissociated.

The anti-folic acid drugs seem to occupy a unique position among the antimetabolites. They are extremely potent drugs and their action on the conversion of folic acid to folinic acid is non-competitive,[31] making their reversal with folic acid extremely difficult. Furthermore, the reaction catalyzing the reduction of folic acid is sensitive to many drugs which are not very similar structurally to folic acid. However, there is yet another quality of the folic acid system which makes it of value from the standpoint of selective chemotherapy. The capacity to take up folic acid and its derivatives evidently is present only in certain cells. When this transport system is lacking, it usually requires a concentration of these metabolites which is at least 1000-fold higher than the physiological concentration before they can be forced into the cell. Since the anti-folic acid drugs such as amethopterin or aminopterin generally enter via the folic acid transport system,[4] only those cells which can assimilate the vitamin will be affected by the drugs.

Several different interrelationships between the cellular transport of a metabolite and its corresponding antimetabolite seem to exist in nature. One case is that in which both are assimilated with facility. This is illustrated by the uptake of folic acid, folinic acid and their analogs by *S. faecalis* and *L. casei*.[4] A second situation is one in which only the antimetabolite is assimilated, as with the folic acid analogs in *B. subtilis*.[21, 37, 42] Another conceivable situation is one in which only the metabolite is taken up. An example of this is unknown. Finally, there is the situation in which neither the metabolite nor the antimetabolite can be assimilated. This apparently applies to *E. coli*, which cannot take up folic or folinic acids[4, 12, 15] and is inhibited only by extremely high concentrations of the anti-folic acid drugs.[11, 23–27]

As more and more data are gathered concerning the complement of intracellular enzymes possessed by various species, it is becoming increasingly apparent that there

* Unpublished observation of Bases and Hitchings.

is a far greater degree of enzymatic similarity among species than there is dissimilarity. That is, the intracellular enzymic differences are quantitative rather than qualitative. On the other hand, it is possible to observe what might be called for practical purposes a qualitative difference in the permease constitution of various bacterial species. This means that practical chemotherapy may be able to exploit these differences.

REFERENCES

1. V. M. MCGLOHON, B. H. PETERSON and O. D. BIRD, *Canad. J. Microbiol.* **3**, 569 (1957).
2. V. MCGLOHON and O. D. BIRD, *Biochem. Pharmacol.* **2**, 299 (1959).
3. D. HENDLIN, L. K. KODITSCHEK and M. H. SOARS, *J. Bact.* **65**, 466 (1953).
4. R. C. WOOD and G. H. HITCHINGS, *J. Biol. Chem.* **234**, 2381 (1959).
5. R. C. WOOD and G. H. HITCHINGS, *J. Bact.* **79**, 524 (1960).
6. R. D. WILLIAMS, *J. Biol. Chem.* **156**, 85 (1944).
7. J. O. LAMPEN, R. R. ROEPKE and M. J. JONES, *J. Biol. Chem.* **164**, 789 (1946).
8. B. D. DAVIS, *J. Bact.* **62**, 221 (1951).
9. J. LASCELLES and D. D. WOODS, *Brit. J. Exp. Path.* **33**, 288 (1952).
10. S. C. AGARWALA and W. H. PETERSON, *Arch. Biochem.* **27**, 304 (1950).
11. C. DAVISON, H. G. MANDEL, E. A. BROWN and P. K. SMITH, *Proc. Soc. Exp. Biol., N.Y.* **91**, 160 (1956).
12. F. WEYGAND, A. WACKER, A. TREBST and O. P. SWOBODA, *Z. Naturf.* **11b**, 689 (1956).
13. H. E. SAUBERLICH and C. A. BAUMANN, *J. Biol. Chem.* **176**, 165 (1948).
14. C. A. NICHOL, *Nature, Lond.* **183**, 550 (1959).
15. H. R. ALIMCHANDANI and A. SREENIVASAN, *J. Bact.* **73**, 538; **74**, 171, 175 (1957).
16. H. F. HAVAS and A. P. MCGEADY, *J. Bact.* **66**, 531 (1953).
17. R. TSCHESCHE, F. KORTE and L. REICHLE, *Z. Naturf.* **10b**, 346 (1955).
18. J. O. LAMPEN and M. J. JONES, *J. Biol. Chem.* **166**, 435 (1946).
19. A. WACKER and F. WEYGAND, *Congr. intern. biochim.*, Resumes communs, 3 *Congr., Brussels* 91 (1955).
20. L. W. HEDGECOCK, *J. Bact.* **72**, 839 (1956).
21. M. E. LOEBECK, *J. Bact.* **79**, 384 (1960).
22. M. E. SWENDSEID and J. F. NYC, *J. Bact.* **75**, 654 (1958).
23. M. WEBB and W. J. NICKERSON, *J. Bact.* **71**, 140 (1956).
24. A. L. FRANKLIN, E. L. R. STOKSTAD, C. E. HOFFMANN, M. BELT and T. H. JUKES, *J. Amer. Chem. Soc.* **71**, 3549 (1949).
25. R. H. NIMMO-SMITH and D. J. BROWN, *J. Gen. Microbiol.* **9**, 536 (1953).
26. W. J. NICKERSON and M. WEBB, *J. Bact.* **71**, 129 (1956).
27. D. W. WOOLLEY and A. PRINGLE, *J. Biol. Chem.* **174**, 327 (1948).
28. M. WEBB, *Biochem. J.* **70**, 472 (1958).
29. T. SHIOTA, *Arch. Biochem. Biophys.* **80**, 155 (1959).
30. E. BRESNICK, S. SINGER and G. H. HITCHINGS, *Biochim. Biophys. Acta* **37**, 251 (1960).
31. R. C. WOOD and G. H. HITCHINGS, *J. Biol. Chem.* **234**, 2377 (1959).
32. *Clinical Manual for Klett–Summerson Photoelectric Colorimeter*, Klett Manufacturing Company, New York.
33. V. M. DOCTOR and J. R. COUCH, *J. Biol. Chem.* **200**, 223 (1953).
34. J. O. LAMPEN and M. J. JONES, *J. Biol. Chem.* **170**, 133 (1947).
35. B. W. KOFT, M. G. SEVAG and E. STEERS, *J. Biol. Chem.* **185**, 9 (1950).
36. R. GUTHRIE, M. HILLMAN and E. HYATT, *Bact. Proc.* 122 (1956).
37. M. J. PINE, *Bact. Proc.* 108 (1959).
38. M. J. PINE and R. GUTHRIE, *J. Bact.* **78**, 545 (1959).
39. W. BORZANI and M. L. R. VAIRO, *J. Bact.* **76**, 251 (1958).
40. E. F. GALE, *J. Gen. Microbiol.* **1**, 53 (1947).
41. A. ALBERT, *Selective Toxicity*. John Wiley, New York (1951).
42. M. J. PINE, *J. Bact.* **79**, 827 (1960).
43. M. J. PINE, *J. Bact.* **79**, 835 (1960).