Role of Purine Biosynthetic Intermediates in Response to Folate Stress in *Escherichia coli*

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Received 26 July 1990/Accepted 24 September 1990

Folic acid plays a central role in anabolic metabolism by supplying single-carbon units at varied levels of oxidation for both nucleotide and amino acid biosyntheses. It has been proposed that 5-amino-4-imidazole carboxamide riboside 5'-triphosphate (ZTP), an intermediate in de novo purine biosynthesis, serves as a signal of cellular folate stress and mediates a physiologically beneficial response to folate stress in *Salmonella typhimurium* (B. R. Bochner, and B. N. Ames, Cell 29:929-937, 1982). We examined the physiological response of *Escherichia coli* to folate stress induced by the drugs psicofuranine, trimethoprim, and sodium sulfathiazole or by *p*-aminobenzoic acid (pABA) starvation. Analysis of nucleotide pools showed that psicofuranine or trimethoprim treatment of a prototrophic strain or growth of a pABA auxotroph on limiting pABA induced the production of the nucleotide ZTP, as previously observed in *S. typhimurium* by Bochner and Ames. Accumulation of ZTP and its precursor 5-amino-4-imidazole carboxamide riboside 5'-monophosphate (ZMP) did not correlate well with folate stress in *E. coli*, as measured by determination of the folate/protein ratios of extracts of treated cells. Treatment of cells with psicofuranine caused a marked accumulation of 5-amino-4-imidazole carboxamide ribonucleotides (Z-ribonucleotides) but a statistically insignificant drop in the folate/protein ratio of cell extracts. Sodium sulfathiazole treatment at a drug concentration that led to a threefold drop in the growth rate and in the folate/protein ratio of treated cells led to little accumulation of Z-ribonucleotides in *E. coli* A *purF* *his*⁺ strain which produces ZTP and ZMP when treated with trimethoprim was constructed. In this strain, histidine represses the synthesis of both ZMP and ZTP. Treatment of cells of this strain with trimethoprim resulted in a decrease in the folate/protein ratio of cell extracts, but a blockade of Z-ribonucleotide accumulation did not affect the extent of folate depletion seen in treated cells and had only a small effect on the resistance of this strain to growth inhibition by trimethoprim. The patterns of protein expression induced by treatment of this strain with trimethoprim or psicofuranine were examined by two-dimensional electrophoretic resolution of the total cellular proteins. No differences in protein expression were seen when the treatments were performed in media containing or lacking histidine. These studies failed to provide evidence in *E. coli* for a folate stress regulon controlled by ZTP.

Reduced forms of folic acid play a central role in *Escherichia coli* anabolic metabolism by supplying single-carbon units at varied levels of oxidation. Both amino acid and nucleotide biosynthetic pathways rely upon this cofactor at several steps for the introduction of methyl, methylene, and formyl groups. A blockade in the supply of these single-carbon units causes an interruption of these pathways. Bochner and Ames (6) proposed a provocative hypothesis for the response of *Salmonella typhimurium* to conditions that lead to reduced levels of 10-formyltetrahydrofolate. Under these conditions, an intermediate in purine biosynthesis, 5-amino-4-imidazole carboxamide riboside 5'-monophosphate (ZMP), accumulates (Fig. 1). 5-Amino-4-imidazole carboxamide riboside 5'-triphosphate (ZTP), the triphosphate analog of ZMP, also accumulates. In mammalian cells, phosphoribosylpyrophosphate (PRPP) synthetase catalyzes the conversion of ZMP to ZTP (28, 29) as shown in equation 1:

Bochner and Ames proposed that ZTP is an alarmone signalling 10-formyltetrahydrofolate deficiency and that it mediates a physiologically beneficial response to folate stress. They constructed a *purF* *his*⁺ strain of *S. typhimurium* in which ZMP and ZTP production could be blocked by

repression of the histidine biosynthetic operon by exogenous histidine and demonstrated that when histidine was present this strain was hypersensitive to treatment with drugs thought to induce folate stress. For these studies, Bochner and Ames used a variety of treatments to induce folate stress. These treatments included growth of a *p*-aminobenzoic acid (pABA) auxotroph on limiting pABA or treatment of prototrophic strains with sulfathiazole, trimethoprim, methotrexate, or psicofuranine. The treatments were not directly shown to decrease the intracellular folate pool sizes or alter the distribution of folate derivatives in the cell; the folate stress was inferred from the observation of ZTP accumulation. Furthermore, no direct evidence was provided that the addition of histidine to the medium repressed the synthesis of ZTP in the *purF* *his*⁺ strain, nor was it shown that the addition of histidine to the medium exacerbated the folate stress induced by drugs in this strain. Therefore, it was not clear whether the effect of histidine on the sensitivity of the test strain to sulfa drugs or to trimethoprim or methotrexate was attributable to an exacerbation of folate stress in the absence of ZTP.

In the studies reported in this paper, the responses of *E. coli* K-12 strains to treatments that lead to ZMP and ZTP accumulation were examined. The inhibitors used in this research were psicofuranine (6-amino-9-D-psicofuranosylpurine), sodium sulfathiazole (monosodium 4-amino-*N*-2-thiazolylbenzene-sulfonamide), and trimethoprim [2, 4-diamino-5-(3, 4, 5-trimethoxybenzyl)pyrimidine]. Psicofuranine inhibits

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FIG. 1. Interconnections between purine, histidine, and folate pathways. Abbreviations: PRA, phosphoribosylamine; GAR, phosphoribosyl glycinamide; f-GAR, phosphoribosyl *N*'-formylglycinamide; f-GAM, phosphoribosyl *N*'-formylglycinamide; AIR, phosphoribosyl aminoimidazole; c-AIR, phosphoribosyl aminoimidazole-4-carboxylate; PRuf-ZMP, *N*-(5'-phosphoribosyl)formimino-5-amino-1-(5'-phosphoribosyl)-4-imidazole carboxamide; H₂Ptd-CH₂OPP, (6-hydroxymethylpyrophosphoryl)dihydropteridine; H₂Pte, dihydropterolate; H₂PteGlu, dihydrofolate; H₄PteGlu, tetrahydrofolate; CH₂-H₄PteGlu, 5,10-methylenetetrahydrofolate; CH=H₄PteGlu, 5,10-methenyltetrahydrofolate; HCO-H₄PteGlu_n, 10-formyltetrahydrofolate with *n* glutamyl residues. s-AMP, succinyl-AMP. Gene names are shown in italics. Inhibitor targets are indicated by solid squares. This figure was adapted from one of Bochner and Ames (6) and is reproduced with permission (copyright held by Cell Press).

XMP amidotransferase (31), blocking the conversion of XMP to GMP and depleting the cellular pool of GTP, which is the substrate for the first committed step of folate biosynthesis. Sodium sulfathiazole acts as a competitive inhibitor of pterate formation, displacing pABA (9, 39). Trimethoprim prevents the conversion of dihydrofolate to its active tetrahydrofolate form by inhibiting dihydrofolate reductase (14). We also used growth of a pABA auxotroph on limiting pABA to induce ZMP and ZTP accumulation. The relevant metabolic pathways and points of inhibition are summarized in Fig. 1. Three areas of investigation were emphasized: we determined the effects of treatments on nucleotide pools by using the methods for nucleotide separation by thin-layer chromatography developed by Bochner and Ames (7); we used two-dimensional gel electrophoresis to examine changes in the patterns of protein synthesis induced by

treatments; and we examined the effects of treatments on the growth and survival of *E. coli*. Bochner and Ames (6) suggested that psicofuranine might induce the accumulation of ZMP and ZTP because treatments with this drug dramatically alter cellular levels of GTP, which is the precursor for the biosynthesis of the pteridine ring of folic acid. If this interpretation is correct, then three of the treatments used to induce ZMP and ZTP accumulation—pABA restriction and treatment with psicofuranine or sulfathiazole—should result in a diminution of the total folate pool size. We measured the total intracellular folate pools in a *purF his*⁺ strain of *E. coli* grown in the presence or absence of histidine in media containing trimethoprim and psicofuranine and also measured total folate pools in a pABA auxotroph grown on limiting pABA.

Using this approach, we hoped to establish whether the

TABLE 1. Bacterial and bacteriophage strains used in this work^a

Strain	Description of genotype	Source or reference
AB3295	F ⁻ <i>pabB3 hisG4 rfbD1</i> <i>rpsL704 xyl-5 mtl-1 ilvC7</i> <i>argE3(Oc) thi-1</i>	B. Bachmann (15)
AT2092	F ⁻ <i>argG his pheA purF</i> <i>lysS⁺ Str^r</i>	F. Neidhardt (11)
ATCC 7469	<i>L. casei</i>	American Type Culture Collection (18)
CE2093	F ⁻ <i>argG pheA purF lysS⁺</i> <i>Str^r</i>	This work
W3110	F ⁻ λ^- IN(<i>rrnD-rrnE</i>)	F. Neidhardt (13)
P1 vir	Bacteriophage P1, lytic	G. Weinstock (34)
TA2795	<i>S. typhimurium</i> LT2 <i>his⁺</i> <i>purF</i>	B. Ames (6)

^a Strains were *E. coli* K-12, unless noted otherwise. Oc, Ochre mutation.

effects of these stresses in *E. coli* mirrored those seen in *S. typhimurium* (6) and whether there was a correlation between ZTP synthesis and the induction of folate stress. Bochner and Ames (6) made use of a *purF his⁺* strain of *S. typhimurium* to test whether ZTP production enhanced growth under conditions thought to induce folate stress. We used a similar *E. coli* strain to examine the proposed role of ZTP as an alarmone in the response to folate stress.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The bacterial and phage strains used in these studies are described in Table 1. Strain CE2093 was constructed by P1 transduction of strain AT2092 with W3110 as the donor strain and selection for histidine prototrophy.

Media and growth conditions. Bacteria were grown aerobically at 37°C in a New Brunswick model G76D rotary-action water bath at 200 rpm. Growth was monitored spectrophotometrically at 420 nm (optical density at 420 nm [OD₄₂₀]). Cells were grown either in LB medium (20) or in defined MOPS minimal medium with 0.4% glucose as the carbon source (21). Disk sensitivity assays were conducted with nutrient broth or Vogel-Bonner minimal medium (37) as previously described (2). Amino acid, nucleoside, or vitamin supplements were added as required by each strain. All growth media were supplemented with 10 μ M thiamine. Growth medium for strain CE2093 required supplementation with arginine (200 μ M), phenylalanine (200 μ M), and inosine (500 μ M). Histidine was used at a concentration of 50 μ M. Media for cell viability studies also included methionine (100 μ M) and glycine (400 μ M). Streptomycin was added to Str^r strains to a final concentration of 25 μ g/ml. Working cultures were maintained on plates with the above-described media combined with 1.5% agar. Long-term cultures were kept at -80°C in 15% glycerol.

Nucleotide assays. Cellular nucleotides were labeled, separated, and quantitated as previously described (7, 8), with slight modifications. In each case, the treated culture was compared with an identical culture that was not subjected to stress. For treatments with psicofuranine or trimethoprim, cultures were sampled just prior to drug addition and 15 to 60 min after drug addition. Drug was added to a culture in exponential growth in medium containing 100 μ Ci of H₃³²PO₄ per ml. Growth was monitored spectrophotometrically at 420 nm in an unlabeled control culture. For treatments with pABA limitation or with sodium sulfathiazole, the medium contained the limiting supplement or drug at the

time of introduction of the cells. Cultures were harvested from 30 to 240 min after the initiation of growth by inoculation. Nucleotides were separated by thin-layer chromatography on cellulose MN300 polyethyleneimine plates (Brinkmann). The sample volume applied to each plate was normalized to the control at time zero by use of culture optical density measurements and the formula shown in equation 2:

$$\text{Volume of extract spotted at time } t = (\text{OD}_{420} \text{ at } t_0 / \text{OD}_{420} \text{ at } t) \times \text{volume of control spotted at time } t_0 \quad 2$$

First-dimension separation based on charge was achieved with solvent Ta or Tb (7). Second-dimension separation based on the structure of the nucleotide base was achieved with solvent Sb (7). Plates were exposed to autoradiographic film with Valca II High Plus screens for 16 to 24 h. Nucleotide spot locations were traced to the plates from the autoradiograms, and the spots were cut out and counted in scintillation fluid with a Beckman model LS 7500 scintillation counter. ATP concentrations at time zero were assumed to be 3 mM (7), and the concentrations of other nucleotides in the culture were calculated on the basis of the counts found in the ATP spot from the time-zero sample following subtraction of the background.

Disk sensitivity assays. Sensitivity to various antifolate drugs was tested by the method of Alper and Ames (2). Drug-impregnated disks were purchased from Organon Teknika or prepared in our laboratory. Disks impregnated with trimethoprim (5 μ g), trimethoprim-sulfamethoxazole (1.25 μ g/23.75 μ g), or triple sulfa (300 μ g; sulfamerazine-sulfadiazine-sulfamethazine, 1:1:1) were purchased. We prepared our own disks impregnated with sulfathiazole (10 μ g), sulfacetamide (100 μ g), or sulfaguanidine (500 μ g).

Growth rate inhibition studies. Growth rate studies were conducted with strain CE2093 to observe the effect of histidine supplementation on growth inhibition by trimethoprim. The concentration of inhibitor at which the growth rate was one-half that in the uninhibited culture (I₅₀) was determined by measuring growth rates over a range of drug concentrations in the presence or absence of a 50 μ M histidine supplement to test whether the repression of 5-amino-4-imidazole carboxamide ribonucleotide (Z-ribonucleotide) biosynthesis altered the I₅₀ of either drug. Cultures were inoculated to a starting OD₄₂₀ of 0.050 in flasks containing 10 to 10,000 ng of trimethoprim per ml (34.5 nM to 34.5 μ M) and no histidine or 50 μ M histidine, and growth rates were monitored spectrophotometrically for 300 min. The fractional inhibition of growth rate, *i*, was plotted against log inhibitor concentration [I] (25), and the inhibitor concentration at *i* = 0.5 was determined.

Cell viability studies. The effect of trimethoprim on the viability of strain CE2093 was tested under bactericidal conditions. Overnight cultures were grown in the appropriate medium with or without histidine. Cells were inoculated into cultures at an OD₄₂₀ of 0.050 and grown to an OD₄₂₀ of approximately 0.300. Subsequently, second inoculations were made into medium containing methionine (100 μ M), glycine (400 μ M), inosine (500 μ M), and trimethoprim (5 μ g/ml; 17.2 μ M) and with or without histidine (50 μ M) to a starting OD₄₂₀ of 0.050. The viability of the cultures was measured by the protocol of VanBogelen et al. (36). Samples were removed at intervals, and serial dilutions were plated on LB agar. Viable colonies were counted after incubation overnight at 37°C.

Folate quantitation. Total folate pools were assayed in

FIG. 2. Nucleotide pools of strain CE2093 before and after treatment with trimethoprim. Cultures were grown in glucose MOPS minimal medium supplemented with arginine, phenylalanine, inosine, and thiamine. Cultures were steady-state labeled with $H_3^{32}PO_4$. When cultures were in exponential growth ($OD_{420} \sim 0.3$), a control sample was removed and trimethoprim was added to a final concentration of 300 ng/ml. Treated cultures were sampled 60 min after drug addition. Nucleotides were separated by two-dimensional thin-layer chromatography (see the text) and detected by autoradiography.

Lactobacillus casei, which cannot synthesize folate de novo (16). Folate levels were measured with an *L. casei* microbiological assay (strain ATCC 7469). The protocol has been described by Wilson and Horne (38). γ -Glutamyl hydrolase was prepared as previously described and stored in 0.5-ml aliquots at $-80^\circ C$. Protein in cell extracts was determined by the bicinchoninic acid assay method (33) with an assay kit obtained from Pierce. Extracts were precipitated with trichloroacetic acid in the presence of sodium deoxycholate (27) to prevent reaction with the reducing agents ascorbic acid and β -mercaptoethanol.

Extract preparation for folate quantitation. Strains W3110, AB3295, and CE2093 were grown in glucose MOPS minimal medium with appropriate supplements. Separate cultures were grown for each time point. After drug treatment, 15 ml of each culture was transferred to sterile conical tubes and centrifuged for 25 min ($1,000 \times g$ at $4^\circ C$). Cell pellets were washed twice in 1 ml of cold 0.9% NaCl solution. Cell pellets were stored at $-80^\circ C$ or processed directly. Pellets were resuspended in 1 ml of cold 50 mM phosphate buffer (pH 6.1) containing 0.15% ascorbate. This suspension was sonicated with a Branson sonicator (three 15-s pulses at setting 1.75; ~ 25 W) on ice. Cell debris was pelleted in a 15-min Microfuge spin at $4^\circ C$. The supernatant extract was transferred to a clean 1.5-ml Microfuge tube. A 500- μ l aliquot of extract was transferred to a second tube for treatment with γ -glutamyl hydrolase. The remaining extract was stored at $-80^\circ C$ for protein assay. Extracts were stored under N_2 at $-80^\circ C$ (38).

Radioactive labeling of protein and resolution on two-

dimensional gels for autoradiography. Cells were pulse-chase labeled with [^{35}S]methionine (1.25 mCi/mmol; 100 μ Ci/ml). At the appropriate time points, 2 ml of cell culture was labeled for 5 min in a 20-ml sterile vial and chased for 3 min after the addition of 167 μ l of 0.2 M methionine. Extracts were prepared and resolved by two-dimensional polyacrylamide gel electrophoresis as described by O'Farrell et al. and with the modifications of Pederson et al. (23, 24, 26). Gels were fixed and stained with Coomassie blue. All gels were subsequently dried and exposed to Kodak XAR autoradiographic film for 2 to 16 days at $-80^\circ C$.

RESULTS

Nucleotide studies. We have investigated the effect of treatments that have been shown to cause ZTP synthesis in *S. typhimurium* (6) on nucleotide pool sizes in *E. coli*. Autoradiograms of the nucleotide pools of strain CE2093 (*purF his⁺*) after resolution by two-dimensional thin-layer chromatography are shown in Fig. 2. For these studies, the strain was grown in medium lacking histidine but supplemented with inosine. Within 60 min after the addition of trimethoprim at 300 ng/ml, the doubling time of strain CE2093 had increased from 58 to 122 min. Table 2 shows changes in the nucleotide pool sizes of strain CE2093 induced by treatment with trimethoprim in the presence or in the absence of histidine. ZMP levels were elevated from 0.14 to 0.79 mM after 60 min of drug treatment, and ZTP levels increased from 0.05 to 0.49 mM. The results obtained are very similar to those reported by Bochner and Ames (6) for

TABLE 2. Strain CE2093 nucleotide pools after trimethoprim treatment^a

Nucleotide	Concn (mM) with:			
	No histidine		50 μ M histidine	
	0 min	60 min	0 min	60 min
ATP	3.0	3.46	3.0	3.49
GTP	1.62	1.39	1.48	1.39
ppGpp	0.04	0.41	0.04	0.51
pppGpp	0.06	0.08	0.07	0.15
ZTP	0.05	0.49	0.02	0.02
ZMP	0.14	0.79	0.08	0.08

^a Changes in nucleotide pools were monitored for strain CE2093 before and after treatment with 300 ng of trimethoprim per ml and while growing in the presence or absence of 50 mM histidine. The radioactivity associated with each spot detected on the autoradiograms was determined by tracing the spot locations from the autoradiograms to the plates, cutting out the spots on the plates, and quantitating by scintillation counting. ATP concentrations before treatment were assumed to be 3 mM (7), and the concentrations of other nucleotides in the culture were calculated on the basis of the counts found in the ATP spot at time zero following subtraction of the background.

the treatment of cells of *S. typhimurium* LT2 with trimethoprim. Depletion of ATP and GTP pools did not occur on treatment of strain CE2093 with trimethoprim. Control studies with prototrophic strain W3110 showed that ATP and GTP pools were significantly depleted on treatment of this

strain with trimethoprim in medium lacking inosine but that supplementation of the medium with inosine prevented the depletion of the ATP and GTP pools (data not shown). These observations are consistent with ZMP and ZTP accumulation resulting from a blockade in de novo purine biosynthesis at the step at which AICAR (5-amino-4-imidazole carboxamide ribonucleotide 5'-monophosphate or ZMP) is converted to formylAICAR. Table 2 also shows that ZMP and ZTP were not detected at significant levels when strain CE2093 was grown in medium supplemented with histidine, even after the addition of trimethoprim. Such results are consistent with the observed repression of histidine biosynthesis in the presence of exogenous histidine (6, 19).

Psicofuranine treatment of strains CE2093 (data not shown) and W3110 (Table 3) also led to a significant accumulation of ZMP and ZTP, to levels of 0.4 and 0.3 to 0.4 mM, respectively. XMP, which is the substrate for the XMP amidotransferase reaction that is inhibited by psicofuranine, also accumulated on psicofuranine treatment. GTP concentrations dropped drastically, from 1.7 to 0.28 mM over the 60-min treatment course, and supplementation of the medium with inosine did not prevent the observed drop in GTP pool sizes. Again, our results for the treatment of *E. coli* strains are completely consistent with those reported for the treatment of *S. typhimurium* strains by Bochner and Ames (6).

Treatment of strain W3110 with sufficient sodium sulfathi-

TABLE 3. Changes in folate and protein contents on treatment of *E. coli* K-12 strains with drugs thought to induce folate stress^a

Strain	Drug supplement	Time (min)	No. of samples	OD ₄₂₀	Folate (ng/ μ l)	Protein (μ g/ μ l)	Folate (ng)/protein (μ g)	ZTP (mM)	ZMP (mM)	Doubling time (min)
W3110	Psicofuranine (500 μ g/ml)	0	3	0.350 $\pm$ 0.013	0.116 $\pm$ 0.012	0.130 $\pm$ 0.005	0.892 $\pm$ 0.010	0.060	0.150	72
		60	3	0.455 $\pm$ 0.005	0.228 $\pm$ 0.011	0.284 $\pm$ 0.019	0.803 $\pm$ 0.066	0.310	0.440	174
	Trimethoprim (300 ng/ml)	0	4	0.371 $\pm$ 0.073	0.202 $\pm$ 0.079	0.228 $\pm$ 0.063	0.887 $\pm$ 0.424	0.030	0.060	70
		15	2	0.350 $\pm$ 0.028	0.097 $\pm$ 0.001	0.204 $\pm$ 0.021	0.475 $\pm$ 0.071	0.080	0.170	
		30	2	0.352 $\pm$ 0.003	0.101 $\pm$ 0.007	0.189 $\pm$ 0.032	0.534 $\pm$ 0.098	0.130	0.220	
		60	4	0.395 $\pm$ 0.071	0.104 $\pm$ 0.030	0.190 $\pm$ 0.056	0.547 $\pm$ 0.226	0.260	0.350	195
	Trimethoprim (300 ng/ml) + 500 μ M inosine	0	3	0.305 $\pm$ 0.005	0.151 $\pm$ 0.012	0.174 $\pm$ 0.004	0.868 $\pm$ 0.072	0.043	0.041	85
		60	2	0.390 $\pm$ 0.000	0.070 $\pm$ 0.021	0.133 $\pm$ 0.023	0.526 $\pm$ 0.182	0.137	0.250	195
	None Sulfathiazole (500 μ g/ml)	210	4	0.334 $\pm$ 0.012	0.081 $\pm$ 0.005	0.146 $\pm$ 0.028	0.554 $\pm$ 0.112	0.029	0.048	60
		150 ^b	1	0.150				0.047	0.165	240
		360	4	0.181 $\pm$ 0.017	0.011 $\pm$ 0.003	0.061 $\pm$ 0.013	0.180 $\pm$ 0.063			190
AB3295	10 μ M pABA	210	1	0.400				0.044	0.166	57
		240	3	0.677 $\pm$ 0.018	0.188 $\pm$ 0.020	0.408 $\pm$ 0.004	0.461 $\pm$ 0.050			48
	3 nM pABA	210 ^c	1	0.240				0.115	0.299	153
		300	4	0.220 $\pm$ 0.007	0.016 $\pm$ 0.003	0.090 $\pm$ 0.015	0.178 $\pm$ 0.044	0.115	0.394	92
CE2093	Trimethoprim (300 ng/ml) + no histidine	0	3	0.295 $\pm$ 0.000	0.102 $\pm$ 0.019	0.126 $\pm$ 0.014	0.810 $\pm$ 0.050	0.050	0.140	58
		60	3	0.447 $\pm$ 0.011	0.101 $\pm$ 0.008	0.202 $\pm$ 0.018	0.500 $\pm$ 0.060	0.490	0.790	122
	Trimethoprim (300 ng/ml) + 50 μ M histidine	0	4	0.314 $\pm$ 0.053	0.104 $\pm$ 0.040	0.151 $\pm$ 0.042	0.689 $\pm$ 0.327	0.020	0.080	45
		60	4	0.445 $\pm$ 0.098	0.079 $\pm$ 0.035	0.185 $\pm$ 0.057	0.427 $\pm$ 0.230	0.020	0.080	205

^a Samples were measured with the *L. casei* microbiological assay for total folate and the bichinchoninic acid assay for protein. The averages $\pm$ standard deviations are reported. Propagation of error in the calculations was performed by the method of Bevington (5). The concentrations of ZTP and ZMP were determined in separate experiments with different cultures. Folate quantitation during treatment with sodium sulfathiazole was performed 1 year after the other folate quantitation experiments with a different culture of *L. casei*. The differences in the baseline folate levels observed in this experiment may be attributable to the use of a different culture of the assay organism.

^b Experiments that are not shown established that both ZTP and ZMP concentrations and the doubling time of the sulfathiazole-treated culture reached stable values within 120 min of treatment.

^c Experiments that are not shown established that both ZTP and ZMP concentrations and the doubling time of the pABA-limited culture reached stable values within 180 min after shifting of the culture to the lower pABA concentration.

azole (500 $\mu\text{g/ml}$) to increase the doubling time from 70 to 240 min led to a minimal induction of Z-ribonucleotides, and after 150 min of treatment, ZMP and ZTP had reached maximum concentrations of 0.165 and 0.047 mM, respectively (Table 3). In contrast, Bochner and Ames (6) reported significant elevations of ZMP and ZTP pools in *S. typhimurium* strains treated with sulfathiazole.

The growth of strain AB3295 (*pabB*) in medium containing limiting pABA resulted in a moderate accumulation of ZMP and ZTP, to maximum levels of 0.4 and 0.12 mM, respectively (Table 3). These results are in agreement with similar observations made by Bochner and Ames (6) with an *S. typhimurium* strain that was a pABA auxotroph.

Folate quantitation. The results of measurements of the folate and protein contents in extracts of strains of *E. coli* K-12 subjected to treatment with trimethoprim, sulfathiazole, or psicofuranine and in extracts of cells of strain AB3295 (*pabB*) grown in medium containing limiting pABA are shown in Table 3. We have also presented measurements of the folate/protein ratio in cell extracts because of the possibility of the loss of cells from the pellet during the washing procedures conducted prior to the microbiological assay of the extracts and the resultant greater variability in the measured folate content per cell. However, measurements of folate normalized to the A_{420} of the cell culture when harvested follow the same trends as those of the folate/protein ratio in cell extracts during the course of drug treatments. Extracts of cells of strain AB3295 showed a marked decrease in folate/protein ratios when cells were grown on limiting pABA. The ratio of total folate to protein was reduced in extracts from the pABA-limited cultures to 32% of that measured in cultures with a full pABA supplement. The reduced levels of intracellular folate were directly correlated with growth rates. Cultures supplemented with 10 μM pABA grew with a doubling time of 57 min, while cultures grown in medium containing only 3 nM pABA took 153 min to double. Thus, the growth of a pABA auxotroph in medium containing limiting pABA appears limited by the rate of folate biosynthesis, and the accumulation of ZMP and ZTP that occurs on pABA limitation may reasonably be attributed to the restricted availability of formyltetrahydrofolate for de novo purine biosynthesis.

Cultures of strain W3110 supplemented with 500 μg of sodium sulfathiazole per ml grew with a doubling time of 190 min, while control cultures in the absence of drug had a doubling time of 60 min. This 3.2-fold reduction in the growth rate on treatment with sodium sulfathiazole was paralleled by a 3.1-fold reduction in the intracellular folate/protein ratio. Given the marked effect of sodium sulfathiazole treatment on the folate content of cells, it is surprising that little induction of Z-ribonucleotides was associated with this treatment.

Psicofuranine treatment of strain W3110 produced only a 10% decrease in the folate/protein ratios of these extracts, even though ZTP levels increased 4-fold under this stress condition and the growth rate decreased ~ 2.4 -fold. These results suggest that the accumulation of ZMP and ZTP in cells treated with psicofuranine is unlikely to result from a failure to synthesize adequate amounts of folic acid.

Trimethoprim treatments of strains W3110 and CE2093 caused decreases in the folate/protein ratios but they were less than twofold over the 60-min treatment period, when maximal ZMP accumulation was observed. Trimethoprim reduced the growth rates of these strains ca. threefold. Histidine did not affect the decrease in folate levels in strain CE2093. Folate/protein ratios were reduced 38% from their

time-zero levels both in cultures containing no histidine and in cultures containing 50 μM histidine. Small differences were observed in the initial folate levels between the two cultures; the histidine-supplemented culture exhibited lower folate levels over the time course. Treatment of strain W3110 with trimethoprim induced a similar 38% decrease in folate/protein ratios over 60 min. Concern that inosine might be alleviating some of the effect of trimethoprim on CE2093 folate pools prompted us to measure folate/protein ratios in extracts of strain W3110 grown in the presence of a 500 μM inosine supplement. In these samples, the folate/protein ratios fell 39% from the time-zero values, indicating that inosine provided no significant protection against the depletion of intracellular folate. Thus, on treatment of *E. coli* strains with trimethoprim, accumulation of ZMP and ZTP was temporally associated with modest decreases in the folate/protein ratios in the cells, but the abolition of the accumulation of ZMP and ZTP by repression of histidine biosynthesis in a *purF* strain did not have an observable effect on the decrease in the folate/protein ratios on trimethoprim treatment.

Disk sensitivity assays. Disk sensitivity studies were conducted to determine whether *E. coli* exhibited increased sensitivity to antifolate inhibitors when Z-ribonucleotide synthesis was repressed, as has been previously observed in *S. typhimurium* (6). The results of parallel studies on the sensitivity of *purF* *his*⁺ strains of *E. coli* and *S. typhimurium* are shown in Table 4. Sensitivity to the same panel of drugs, including those used originally by Bochner and Ames (6), was measured in media containing or lacking histidine. With one exception, *E. coli* CE2093 failed to demonstrate increased sensitivity to sulfanilamides and/or trimethoprim when Z-ribonucleotide synthesis was repressed. There was a statistically significant difference ($P = 0.04$) in the diameter of the inner zone of inhibition when trimethoprim was used as the inhibitor, but no difference in sensitivity was seen with the combination of trimethoprim and sulfamethoxazole. In contrast, *S. typhimurium* TA2795 exhibited significantly increased sensitivity to the same drugs when Z-ribonucleotide synthesis was repressed by exogenous histidine. The difference in the response of the two kinds of enterobacteria to sulfanilamide drugs such as sodium sulfathiazole may relate to our observation that sodium sulfathiazole treatment led to minimal accumulation of ZMP and ZTP in *E. coli*, whereas this same drug led to marked accumulation of Z-ribonucleotides in *S. typhimurium* (6). Thus, the repression of Z-ribonucleotide synthesis would not be expected to affect significantly the response of *E. coli* to sodium sulfathiazole. However, trimethoprim treatment led to a significant accumulation of ZMP and ZTP in both *E. coli* and *S. typhimurium*, but the addition of histidine to the medium produced much more dramatic increases in drug sensitivity in *S. typhimurium* than in *E. coli*. These results are completely consistent with the observation that the repression of Z-ribonucleotide biosynthesis did not affect the decrease in cellular folate/protein ratios that occurred when cells of *E. coli* CE2093 were treated with trimethoprim.

Growth rate inhibition and determination of I_{50} . The growth rate of strain CE2093 was measured in the presence of various levels of trimethoprim in media with and without added histidine to test whether the induction of Z-ribonucleotides affected the level of drug required to reduce the growth rate by one-half. The I_{50} was determined with equation 3:

$$\log[I] = \log[I_{50}] + \log[(g_t - g_0)/g_0] \quad 3$$

TABLE 4. Zones of inhibition caused by antifolate drugs

Organism	Drug (μg)	Double-ringed zone diam (mm) around a 6-mm paper disk ^a				<i>P</i> value ^b	
		Without histidine		With histidine			
		Inner	Outer	Inner	Outer	Inner	Outer
<i>E. coli</i> CE2093	Trimethoprim-sulfamethoxazole (25)	34 ± 1 (c)	38 ± 1 (t)	33.3 ± 0.6 (c)	37 ± 1 (t)	0.26	0.11
	Trimethoprim (5)	24 ± 1 (c)	34.3 ± 1.2 (t)	26.3 ± 0.6 (c)	33.3 ± 0.6 (t)	0.04	0.21
	Triple sulfa (300)	34.7 ± 0.6 (st)	40.3 ± 1.2 (t)	35 ± 0 (st)	40 ± 1 (t)	0.21	0.37
	Sodium sulfathiazole (10)	26.7 ± 0.6 (st)	32 ± 0 (t)	26.7 ± 0.6 (st)	32.7 ± 1.2 (t)	Identical	0.21
	Sulfacetamide (100)	28 ± 0 (st)	33.3 ± 0.6 (t)	28 ± 0 (st)	33.3 ± 0.6 (t)	Identical	Identical
	Sulfaguanidine (500)	26.7 ± 0.6 (st)	32 ± 0 (t)	26.7 ± 0.6 (st)	32 ± 0 (t)	Identical	Identical
<i>S. typhimurium</i> TA2795	Trimethoprim-sulfamethoxazole (25)	24.5 ± 2.1 (c)	31.2 ± 1.7 (st)	29.7 ± 1.0 (c)	34 ± 1.5 (st)	0.004	0.04
	Trimethoprim (5)	21 ± 1 (c)	28.3 ± 0.6 (st)	22 ± 0 (c)	32 ± 0 (st)	0.11	0.004
	Triple sulfa (300)	20 ± 0 (c)	33 ± 0 (st)	29.3 ± 0.6 (c)	39.7 ± 0.6 (st)	0.000065	0.001
	Sodium sulfathiazole (10)	13.7 ± 0.5 (t)	26.2 ± 1.3 (vt)	19 ± 1.3 (c)	28.7 ± 3.0 (vt)	0.00005	0.07
	Sulfacetamide (100)	14.3 ± 1.7 (t)	25.5 ± 1.2 (vt)	19.7 ± 0.8 (c)	29.3 ± 3.4 (t)	0.001	0.04
	Sulfaguanidine (500)	13.7 ± 1.5 (t)	24.2 ± 1.6 (vt)	14.3 ± 2.2 (c)	28.2 ± 3.5 (vt)	0.05	0.01

^a Letters in parentheses denote the extent of cell growth in the zone as clear (c), slightly turbid (st), turbid (t), or very turbid (vt). All values are averages $\pm$ standard deviations.

^b Calculation of *P* values for comparison of values obtained without or with histidine was performed with Student's two-tailed *t* test.

where g_0 is the uninhibited doubling time and g_I is the doubling time with inhibitor concentration [I]. Growth rates were measured at concentrations of trimethoprim ranging from 10 ng/ml to 10 μ g/ml. The best estimates of the I_{50} were obtained from the linear portion of the curve described by equation 3, from approximately an 0.7 to an 0.3 fraction of uninhibited growth or 30 to 1,000 ng of trimethoprim per ml. Values from the extremes varied greatly and were discarded. The calculated I_{50} values are summarized in Table 5. The presence of histidine in the medium caused a small but significant decrease in the I_{50} for trimethoprim, indicating increased sensitivity to the drug under conditions in which Z-ribonucleotide synthesis was repressed. With psicofuranine, inclusion of histidine in the medium did not lead to a change in the I_{50} for the drug.

Cell viability. After conducting studies on the inhibitory levels of the various drugs with disk sensitivity assays and liquid culture growth studies, we wished to evaluate the actual viability of the cells in the presence and absence of ZTP production. Trimethoprim is bacteriostatic under normal culture conditions. A source of purine bases (e.g., inosine, xanthine, and hypoxanthine), methionine, and glycine are required for continued growth (3, 4). Trimethoprim then becomes bactericidal (4). Using such a medium, we compared the viability of CE2093 after trimethoprim treatment in the presence of histidine, when the cells are incapable of ZMP or ZTP synthesis, or in the absence of histidine,

when ZMP and ZTP syntheses are uninhibited (Table 6). The histidine-supplemented cultures exhibited an earlier onset of cell death; when compared with the percent viability in the unsupplemented culture, the percent viability in medium containing trimethoprim was reduced by exogenous histidine. The observed differences were small, and the difference in survival was less than a factor of three at all times tested.

Alterations in protein synthesis. Trimethoprim treatment of strain CE2093 caused marked changes in the observed patterns of protein expression. A total of 18 proteins were induced during trimethoprim treatment of either strain CE2093 (Fig. 3) or strain W3110 (data not shown). Since equal counts from sonicates of washed cells were applied to the gels of treated and control cells, changes in these patterns reflect relative levels rather than absolute levels of proteins. In fact, trimethoprim treatment for 60 min led to a 22% reduction in the protein/OD₄₂₀ ratio, as expected for cells growing more slowly and exhibiting a marked stringent response, as judged by the elevation of ppGpp in the nucleotide assay. Comparison of samples from strain

TABLE 5. I_{50} values for drug treatment of strain CE2093 in liquid medium with or without histidine supplementation

Drug	Supplement	I_{50} (μ g/ml) ^a	<i>P</i> value
Trimethoprim	None	0.334 $\pm$ 0.058	0.0088
	50 μ M histidine	0.206 $\pm$ 0.039	
Psicofuranine	None	156 $\pm$ 25	Identical
	50 μ M histidine	156 $\pm$ 41	

^a Values are the averages of at least three determinations, and the standard deviations are shown. *P* values were calculated for the significance of the differences in I_{50} values for cells treated with drug in the presence or absence of histidine with Student's two-tailed *t* test.

TABLE 6. Survival of cells of *E. coli* CE2093 during trimethoprim treatment in liquid medium containing methionine, glycine, and inosine

Time after drug addition (h)	% Viable colonies ^a	
	Without histidine	With 50 μ M histidine
0	100 $\pm$ 0	100 $\pm$ 0
1	108 $\pm$ 20	107 $\pm$ 12
2	90 $\pm$ 5	70 $\pm$ 5
3	47 $\pm$ 7	25 $\pm$ 3
4	19 $\pm$ 3	7 $\pm$ 2
5	7 $\pm$ 1	4 $\pm$ 0.6
6	3 $\pm$ 0.6	1 $\pm$ 0.06

^a Cells were grown to the log phase and diluted into medium containing 5 mg of trimethoprim per ml and the proper supplements (methionine, 100 mM; glycine, 400 mM; inosine, 500 mM; histidine, 50 mM). Samples were removed at intervals, diluted, and plated on LB agar. Viable colonies were counted after 18 h of growth at 37°C. Values are the averages of at least three determinations of viability, and associated standard deviations are shown.

FIG. 3. Synthesis of individual proteins in strain CE2093 before and after treatment with trimethoprim. (A) No histidine, 0-min control. (B) No histidine, 60 min after drug addition. (C) 50 μ M histidine, 0-min control. (D) 50 μ M histidine, 60 min after drug addition. Boxed protein were induced in strain CE2093 or W3110 after trimethoprim treatment. The arrowhead indicates IMP dehydrogenase, which is repressed by trimethoprim treatment.

CE2093 grown in the presence and in the absence of histidine showed only one significant difference in the patterns of protein induction in response to trimethoprim treatment. Protein 7 (coordinates on the gene-protein index [22], 34 $\times$ 92), which corresponds to the *glyA* gene product, serine hydroxymethyltransferase, appeared to be induced more in cells exposed to drug in the absence of histidine than in cells treated in the presence of 50 μ M histidine. One protein that was not detectably induced by short-term trimethoprim treatment was dihydrofolate reductase (96 $\times$ 46). Trimethoprim treatment is a standard method of causing the overproduction of dihydrofolate reductase (12, 32) by selection for resistance to the drug. However, dihydrofolate reductase overproduction was not seen during transient exposure to trimethoprim.

We also examined the changes in the patterns of protein

expression when strain CE2093 was treated with psicofuranine in medium with or without histidine supplementation (data not shown). Psicofuranine also induced marked changes in the patterns of protein expression, but again the same changes were seen whether histidine was present in or absent from the medium. When changes in the patterns of protein expression following trimethoprim and psicofuranine drug treatments were compared in strain CE2093 at 15 and 60 min, four common proteins, proteins 8 (35 $\times$ 29), 10 (39 $\times$ 102), 15 (58 $\times$ 34), and 18 (56 $\times$ 35), were found. These four proteins were also induced by drug treatments of prototrophic strain W3110. A summary of the changes in protein expression induced by treatments of strain CE2093 with trimethoprim or psicofuranine, with the coordinates of each protein on the gene-protein index, is available on request.

DISCUSSION

Bochner and Ames (6) first reported that treatment of *S. typhimurium* strains with psicofuranine, trimethoprim, and sulfa drugs or growth of a pABA auxotroph in medium containing limiting pABA leads to the accumulation of ZMP and ZTP. We have shown that strains of *E. coli* respond in a similar manner to these treatments, except that treatment of *E. coli* with sodium sulfathiazole does not lead to the substantial accumulation of Z-ribonucleotides. Accumulation of Z-ribonucleotides would be expected to occur whenever the conversion of ZMP to formyl-ZMP becomes the rate-limiting step in purine biosynthesis, either because of a deficiency of 10-formyltetrahydrofolate or because of inhibition of the enzyme catalyzing this reaction. 10-Formyltetrahydrofolate is also used for the formylation of glycineamide ribonucleotide, an earlier intermediate in purine biosynthesis, but this reaction appears to be less sensitive to folate stress. Treatment of *E. coli* with sulfanilamide drugs results in the excretion of 5-amino-4-imidazole carboxamide, indicating that folate deficiency leads to a blockade of later steps in purine biosynthesis (30, 35). Investigation of the sources of single-carbon units introduced into the purine ring has established that serine and glycine contribute 100% of the C-2 carbons of the purine ring (the reaction catalyzed by AICAR transformylase) but only 50% of the C-8 carbons (the reaction catalyzed by phosphoribosyl glycineamide transformylase). Formate is utilized extensively for the C-8 carbon, but there is no 10-formyltetrahydrofolate synthetase activity in *E. coli*. Thus, formate must enter purine biosynthesis by a mechanism independent of phosphoribosyl glycineamide transformylase (10).

Our studies with strains of *E. coli* indicate that Z-ribonucleotide accumulation does not correlate well with the induction of folate stress, as measured by decreases in the folate/protein or folate/OD₄₂₀ ratios of cell extracts. Sodium sulfathiazole treatment of *E. coli*, which is known to inhibit dihydrofolate biosynthesis (9) and which leads to a marked depletion of the intracellular folate levels, does not lead to a substantial accumulation of ZMP or ZTP. In contrast, the correlation between the reduction in the growth rates associated with treatment and the reduction in the intracellular folate levels is excellent. pABA limitation of a pABA auxotroph leads to an ~70% reduction in the folate pool sizes and to a moderate accumulation of Z-ribonucleotides. Treatment with psicofuranine leads to a statistically insignificant folate stress (~10% decrease) but to the accumulation of high levels of Z-ribonucleotides. These observations belie a simple correlation between decreased levels of formyltetrahydrofolate and ZTP and ZMP accumulation. We have not directly measured the formyltetrahydrofolate pools in psicofuranine-treated cells, so we cannot eliminate the possibility that Z-ribonucleotide accumulation results from an effect of psicofuranine on the distribution of folates in cells rather than by an inhibition of folate biosynthesis. At present, such measurements are not technically feasible. However, an alternate possibility should be considered, namely, that psicofuranine is responsible for primary or secondary inhibition of the AICAR transformylase reaction rather than for depletion of the formyltetrahydrofolate pools. The lack of correlation between the folate stress induced by sodium sulfathiazole treatment and the induction of Z-ribonucleotides is particularly puzzling. Sodium sulfathiazole does induce marked stringency, as indicated by an elevation in the levels of ppGpp to 0.5 to 0.7 mM during treatment, and it is possible that this treatment affects histidine biosynthe-

sis. Alternatively, secondary effects of sodium sulfathiazole treatment may include inhibition of PRPP synthetase, the enzyme responsible for the conversion of ZMP to ZTP, or decreases in PRPP levels. *E. coli* and *S. typhimurium* clearly differ in their response to sodium sulfathiazole, both in the extent of induction of ZTP in response to treatment and in their tolerance of this drug (see below).

Trimethoprim treatment leads to a moderate folate stress (~38% decrease in the folate/protein ratios of cell extracts) and to a marked accumulation of ZTP and ZMP. In strain CE2093, in which Z-ribonucleotide accumulation can be blocked in medium containing histidine, no greater depletion of folate stores is seen during trimethoprim treatment than in cells treated under conditions in which Z-ribonucleotide accumulation occurs. In this case, the degree of folate stress may not be simply measured by the depletion of total intracellular folate stores. In contrast to treatment with sulfa drugs or pABA limitation, which affect the biosynthesis of folic acid, trimethoprim treatment would be expected to affect the distribution of the existing forms of folic acid in cells. By inhibiting dihydrofolate reductase, trimethoprim treatment effects the accumulation of dihydrofolate at the expense of cellular pools of tetrahydrofolate derivatives, including formyltetrahydrofolate. Furthermore, at least in mammalian cells, there is a direct inhibition of AICAR transformylase by dihydrofolate (1).

There is evidence that conditions that lead to the repression of Z-ribonucleotide biosynthesis in *E. coli* increase the sensitivity of cells to the action of trimethoprim, although only by a factor of two to three. These effects were observed in disk sensitivity assays on solid media, on growth in liquid medium, and in cell viability studies. However, we found no evidence that the sensitivity of cells of *E. coli* to psicofuranine or sulfanilamide was affected by the repression of Z-ribonucleotide biosynthesis. In contrast, *S. typhimurium* cells show increased sensitivity to sodium sulfathiazole under conditions in which Z-ribonucleotide synthesis is repressed, and this effect correlates with the higher levels of Z-ribonucleotide induction seen in *S. typhimurium* cells during treatment with this drug. These differences provide support for the idea that Z-ribonucleotide induction may be associated with an amelioration of the effects of drug treatment under those conditions in which ZTP is synthesized. At least in *E. coli*, there is no evidence that the response to Z-ribonucleotide induction prevents the depletion of intracellular folate pools.

The killing action of trimethoprim in supplemented medium results from continued cell growth and division in the presence of the inosine, methionine, and glycine supplements but a failure to produce thymidylate because of a lack of methylenetetrahydrofolate. The lack of thymidylate for DNA synthesis leads to cell death (3, 4). The effect of histidine on viability in this medium needs to be interpreted with caution. Histidine biosynthesis consumes up to 15% of the intracellular PRPP pools (17) and a significant portion of the ATP pool. Exogenous histidine spares these pools, making them available for nucleotide and nucleic acid syntheses. Greater rates of nucleic acid synthesis resulting from the provision of exogenous histidine might increase the demand for thymidylate, advancing the point at which the lack of this nucleotide becomes limiting and therefore lethal to cells.

We have not observed any major effect of Z-ribonucleotide accumulation on the changes in the patterns of protein expression induced by either trimethoprim or psicofuranine. The only minor effect is a slight decrease in the induction of

serine hydroxymethyltransferase by trimethoprim treatment when histidine is present in the medium. Thus, we have been unable to obtain convincing evidence for a direct effect of ZTP and/or ZMP on the patterns of protein synthesis in drug-treated cells. If ZTP were functioning as an alarmone, as proposed, one might expect to see a regulon of genes responsive to the accumulation of this alarmone. It is of course possible that the proteins formed by transcription of the genes of such a regulon are all present at such low levels in cells that they are not detectable with our protocols. For instance, the enzymes involved in folate biosynthesis would probably be hard to detect in autoradiograms of two-dimensional gels of the total proteins of *E. coli*. However, our failure to see an increased synthesis of folic acid under conditions in which Z-ribonucleotides accumulate in folate-stressed cells also argues that the genes of the folate biosynthetic pathway are not members of a ZTP-induced regulon.

In summary, in our studies of *E. coli* many of the observations originally made with *S. typhimurium* by Bochner and Ames (6) have been confirmed. Treatments of both *S. typhimurium* and *E. coli* strains by exposure to trimethoprim or psicofuranine or starvation of a pABA auxotroph for pABA result in the accumulation of Z-ribonucleotides. We have also confirmed their observation that an *S. typhimurium purF* strain exhibits hypersensitivity to trimethoprim and/or sulfa drugs in disk sensitivity assays when Z-ribonucleotide production is repressed by histidine. However, our further studies with strains of *E. coli* cast some doubt on their hypothesis that ZTP serves as an alarmone and mediates a physiologically beneficial response to folate stress, at least in *E. coli*. We have not been able to obtain evidence for a stress regulon under the control of ZTP in *E. coli*, and we have observed only a weak correlation between the induction of Z-ribonucleotides and resistance to agents that induce folate stress in *E. coli*. Our results do not preclude the possibility that ZTP plays a role as an alarmone in *S. typhimurium*; indeed, one interpretation of the disk sensitivity studies conducted by both Bochner and Ames (6) and us is that ZTP induction does protect *S. typhimurium* strains against folate stress induced by trimethoprim and sulfa drugs.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant GM 24908 from the National Institutes of Health.

We thank Frederick Neidhardt and Robert Bender for helpful discussions during the course of this work.

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