body weight minus tumor weight), TW (tumor weight), FTS (final tumor size), spleen weight, ovarian weight, uterine weight, adrenal weight, thymus weight, and pituitary weight. The results of the test are shown in Tables II and III.

Acknowledgment. This investigation was supported in part by a Public Health Service Research Grant (AM-05016) from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service, and by funds from the Academic Senate, University of California, San Francisco, Calif. The testing for antitumor activity, performed without charge at Mason Research Institute, Worcester, Mass., is acknowledged with gratitude.

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In Vivo Inhibitors of *Escherichia coli* Phenylalanyl-tRNA Synthetase

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N-Benzyl-D-amphetamine is a potent in vitro and in vivo inhibitor of phenylalanyl-tRNA synthetase of *Escherichia coli*. The concentration of this inhibitor necessary for the in vivo inhibition is approximately 100-fold greater than that necessary for inhibition of the purified enzyme. Treatment of *rel*$^+$ strains of *E. coli* with the inhibitor results in a decreased percentage of $tRNA^{Phe}$ which is charged, guanosine tetraphosphate formation, cessation of RNA synthesis, and growth arrest. Evidence is presented which demonstrates that the primary and perhaps sole mode of action of *N*-benzyl-D-amphetamine is inhibition of phenylalanyl-tRNA synthetase.

In addition to the direct role of aminoacyl-tRNA's in protein biosynthesis, the charged/uncharged tRNA ratios function as regulators of growth and gene expression in prokaryotes and probably in eukaryotes.[1-3] Specific inhibitors of the aminoacyl-tRNA synthetases would be most useful tools for studying and manipulating the varied effects which might accompany depletion of intracellular charged tRNAs and should show profound effects on cell growth. While numerous inhibitors of cell-free preparations of these enzymes have been developed, relatively few have been convincingly demonstrated to be effective in vivo. The most studied of such inhibitors have been L-histidinol and *O*-methylthreonine, competitive inhibitors of His- and Ile-tRNA synthetases, respectively; while it is not clear that inhibition of the synthetases is the sole in vivo action of these analogues, it has been established that they do decrease the intracellular levels of charged $tRNA^{His}$ and $tRNA^{Ile}$.[3,4]

Recently, we have found that analogues of *N*-benzyl-2-phenylethylamine are extremely potent inhibitors of the Phe-tRNA synthetase from *E. coli*.[5] In this report, we describe experiments which demonstrate that this class of inhibitors is effective in vivo; one analogue, *N*-benzyl-D-amphetamine, has been studied in detail and demonstrated to specifically inhibit in vivo charging of $tRNA^{Phe}$ and produce the manifestations of the stringent response in *rel*$^+$ strains of *E. coli*.

Experimental Section

Carrier-free [^{32}P]phosphoric acid, [5-^{3}H]uracil (1.43 Ci/mmol), L-[^{3}H]phenylalanine (9.1 Ci/mmol), L-[^{3}H]isoleucine (105 Ci/mmol), and L-[^{3}H]valine (2.14 Ci/mmol) were products of New England Nuclear Corp. Polyethyleniminecellulose thin-layer sheets were obtained from Brinkmann Instruments and casamino acid was a product of Difco. The *N*-benzyl-2-phenylethylamine derivatives assayed for bacterial growth inhibition have been previously described.[5] Partially purified preparations of Phe-, Ile- and Val-tRNA synthetases were obtained from *E. coli* B (General Biochemicals) by published procedures[6-8] and were approximately 50% pure with respect to published values of the maximal rate of ATP–PP_i exchange.

Escherichia coli B/r, donated by N. Lee, is prototrophic and phenotypically stringent. Growth inhibition and reversal experiments with *E. coli* B/r were conducted at 37 °C in a basal salts medium containing 0.2% glucose, 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.1% $(NH_4)_2SO_4$. The medium used for all other experiments with *E. coli* B/r was the Tris–glucose minimal medium of Gallant and Suskind,[9] with or without

0022-2623/79/1822-1260$01.00/0

Table I. Inhibition of Phenylalanyl-tRNA Synthetase and Growth of *E. coli* B/r by Analogues of *N*-Benzyl-2-Phenylethylamine

inhibitor	K_i, μM[a]	IC_{50}, μM	$C(IC_{50}/K_i)$	% reversal by 2.5 mM L-Phe
N-benzyl-D-amphetamine	0.14	15	107	100
N-benzyl-2-phenylethylamine	0.67	77	115	65
N-benzyl-D-phenylalaninol	0.77	82	106	69
N-benzyl-L-amphetamine	5.6	590	105	77
2-phenylethylamine	93	10900	117	

[a] Data from ref 5.

0.2% casamino acids (as specified). *E. coli* NP2 and NP29[10] were obtained from F. C. Neidhardt. Both strains are phenotypically stringent, and NP29, derived from NP2, possesses a temperature-sensitive Val-tRNA synthetase which shows normal activity at 30 °C and essentially no activity at 42 °C.[10] Cultures of NP2 and NP29 were grown in Tris–glucose minimal medium supplemented with 0.2% casamino acids. Bacterial cultures were grown in a reciprocating shaker bath at 37 °C (unless otherwise specified), and growth rates were monitored turbidometrically at 600 nm. Addition of inhibitor or temperature shifts to 42 °C were performed at specified times during logarithmic growth of B/r, NP2, and NP29.

All radioactive samples were counted in a Nuclear-Chicago Isocap-300 scintillation counter under toluent-base cocktail containing 2.6% NCS (Amersham Searle), 0.4% Omnifluor (Nuclear Chicago), and 0.43% water.

Growth Inhibition of *E. coli* B/r. Aliquots (0.3 mL) of an exponentially growing culture of *E. coli* B/r ($A_{600} \simeq 0.3$) in the basal salts minimal medium were diluted with minimal medium (1.7 mL) containing various levels of the inhibitor to be tested; controls did not contain inhibitor. The subcultures were incubated until the controls reached an A_{600} reading of ca. 0.4 to 0.45 (ca. three generations). The extent of growth in each tube was determined turbidometrically at 600 nm, and the percent growth inhibition was calculated with respect to control turbidity. IC_{50} values (inhibitor concentration required to produce 50% growth inhibition) were obtained from plots of percent growth inhibition with respect to inhibitor concentration. In studies of reversal of inhibition by amino acids, the exponentially growing B/r culture (0.3 mL, $A_{600} \simeq 0.3$) was diluted with minimal medium containing the specified amino acid and the inhibitor at its IC_{50} concentration. Inhibitor was omitted from controls and the extent of growth inhibition was determined as described above. For each inhibitor, the fraction of reversal by added amino acid was calculated as the difference in percent growth inhibition in the absence and presence of added amino acid divided by the percent growth inhibition in the absence of added amino acid.

In Vivo Determinations of Charged tRNA. For each determination, two bottles containing 100 mL of prewarmed (37 °C) Tris–glucose minimal media,[9] with or without 0.2% casaminoacids (as specified in Table II), were innoculated with 2 mL of *E. coli* B/r subculture ($A_{600} \simeq 0.3$) and incubated at 37 °C until A_{600} was 0.4 to 0.45. At this time, a prewarmed (37 °C) solution of *N*-benzyl-D-amphetamine was added to one culture and the other was kept as a control. After incubation for 30 min, cultures were rapidly cooled by the addition of ice and NaN_3[11] and collected by centrifugation. Disruption of cells and analysis of the percentage of charged tRNA were performed as described by Atherly and Suchanek[12] with the following modifications: (i) cells were immediately disrupted rather than washed with TCA, (ii) tRNA was collected by ethanol precipitation prior to periodate treatment or stripping, (iii) 1 mM periodate was used instead of 50 mM, (iv) aminoacyl-tRNA was stripped by treatment with 1.8 M Tris-HCl (pH 8.0) for 45 min, and (v) purified preparations of aminoacyl-tRNA synthetases were used.

The procedure used for *E. coli* NP2 and NP29 was essentially the same, except cells were grown in the Tris–glucose minimal media containing 0.2% casamino acids, and temperature of incubations was 30 °C. When 30 to 42 °C temperature shifts were performed, or inhibitor added, the control culture was kept at 30 °C.

Determination of ppGpp Formation. The amounts of [^{32}P]ppGpp produced in cultures of B/r, NP2, and NP29 under various growth conditions were assayed essentially by the procedure of Cashel.[13] Exponentially growing cultures in the Tris–glucose minimal medium (3.1 mL) were labeled by the addition of carrier-free [^{32}P]phosphoric acid (final specific activity ca. 0.1–0.4 Ci/mmol, ca. 2–8 × 10^5 cpm/5 μL) at least 30 min before sampling to allow equilibration of the label with the intracellular phosphate pools.[14] At appropriate times, 25-μL aliquots of culture were added to 25 μL of ice-cold 2 M formic acid to disrupt cells. After 30 min at 0 °C, 5 μL of the extracts was chromatographed in one dimension on PEI-cellulose using 1.5 M KH_2PO_4 (pH 3.4).[13] Labeled ppGpp was localized by autoradiography and quantitated by counting the appropriate cutouts under the NCS scintillation fluid.

RNA Synthesis. [5-^{3}H]Uracil (75 Ci/mol) was added to exponentially growing cultures of *E. coli* B/r, and [^{3}H]RNA was assayed by reported methods.[15] At various times, 25-μL aliquots of labeled culture were diluted into 1 mL of ice-cold trichloroacetic acid. After 5 min at 0 °C, samples were filtered through Whatman GF/C filters and washed with four 4-mL portions of ice-cold trichloroacetic acid and then with ice-cold 95% ethanol. Filters were dried and counted under NCS scintillation fluid.

Results

Table I compares K_i values[5] of five inhibitors of *E. coli* B Phe-tRNA synthetase with the concentrations required for 50% inhibition of growth (IC_{50}) of *E. coli* B/r. The K_i and IC_{50} values span nearly three orders of magnitude and are linearly related by the equation $IC_{50} = CK_i$, where $C = 109 \pm 6$ (SD); the value C may be taken to represent composite factors which influence the in vivo effectiveness of the analogues (e.g., transport, endogenous Phe, etc.). Growth inhibition by the most potent inhibitor, *N*-benzyl-D-amphetamine, at its IC_{50} concentration was completely reversed by 2.5 mM L-Phe, whereas Val, Ser, Tyr, Ile or Pro had no effect on inhibition. Under similar conditions, the inhibition produced by other analogues examined was reversed 65 to 77% by 2.5 mM L-Phe; the incomplete reversal suggests that a small part of the growth inhibition by the less potent Phe-tRNA synthetase inhibitors involves other unidentified sites of action.

E. coli NP26 possesses a temperature-sensitive Val-tRNA synthetase.[10] In accord with reported results, when NP29 is shifted from its permissive to nonpermissive temperature there is a specific decrease in the percentage of Val-tRNA, increases in charging of Phe- and Ile-tRNA, and production of ppGpp (Table II); a similar temperature shift of the parent NP2 strain shows none of these effects. At its permissive temperature, treatment of NP29 with *N*-benzyl-D-amphetamine results in a decrease of charged $tRNA^{Phe}$, increases in the percentage of charged $tRNA^{Val}$ and $tRNA^{Ile}$, and production of ppGpp.

The composite experiments shown in Figure 1 illustrate some of the effects which occur when an exponentially growing culture of *E. coli* B/r is treated with 92 μM *N*-benzyl-D-amphetamine. Characteristic of the stringent response, there is a rapid appearance of ppGpp, followed by its gradual decline to steady-state levels. Concurrently, there is a cessation of growth and RNA synthesis. As shown in Table II, increasing concentrations of *N*-benzyl-D-amphetamine results in a parallel decrease in the charging levels of $tRNA^{Phe}$ but not $tRNA^{Ile}$ or $tRNA^{Val}$.

Table II. tRNA Charging and ppGpp Formation in *E. coli* Treated with *N*-Benzyl-D-amphetamine or Subjected to Temperature Shifts

strain	*N*-benzyl D-amphetamine concn, μM	temp, °C	% charged tRNA[a] Phe	Val	Ile	ppGpp[b]
NP29[c]	0	30	70	30	40	
	0	42	90	5	65	+
	230	30	5	95	45	+
NP2[c]	0	30	100	100	90	−
	0	42	100	100	*e*	−
B/r[d]	0	37	60	85	40	−
	4.6	37	45	*e*	40	+
	9.2	37	35	*e*	40	+
	23	37	20	*e*	45	+
	230	37	10	100	45	+
B/r[c]	0	37	90	100	50	−
	230	37	15	100	55	+

[a] tRNA charging levels and ppGpp determinations were made 30 min after addition of inhibitor or temperature shift. [b] Detection of ppGpp is denoted by a plus sign; a minus sign indicates that ppGpp could not be detected. [c] The Tris-glucose minimal medium described in ref 9 supplemented with 0.2% casamino acids was used. [d] The casamino acid supplement was omitted from the media used in these experiments. [e] Determinations were not made.

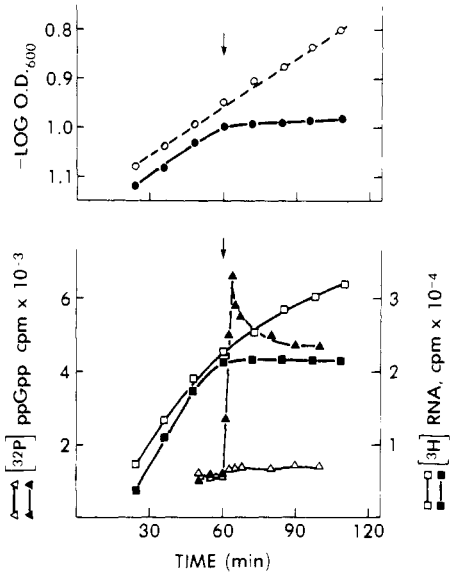

Figure 1. Stimulation of the stringent response in *E. coli* B/r by 92 μM *N*-benzyl-D-amphetamine. The inhibitor was added to logarithmically growing cultures at the indicated time (arrow), and measurements of cell density (●), ppGpp formation (▲), and RNA synthesis (■) were made as described under the Experimental Section. Similar measurements were made on cultures not containing inhibitor (○, △, □). RNA and ppGpp measurements refer to counts per minute in 2.5 μL of culture.

Reduction of the in vivo level of charged $tRNA^{Phe}$ from 60 to 45% is sufficient to activate the stringent factor to produce ppGpp, and further reductions in charged tRNA are all accompanied by ppGpp formation. The concentration of the inhibitor necessary to reduce charging of $tRNA^{Phe}$ by 50% is 13 μM, nearly identical with the concentration required for 50% growth inhibition. As with the effect upon charging of $tRNA^{Phe}$, the initial rate of ppGpp formation is also related to the concentration of *N*-benzyl-D-amphetamine in the media (Figure 2); here, half maximal rate was observed with ca. 70 μM inhibitor.

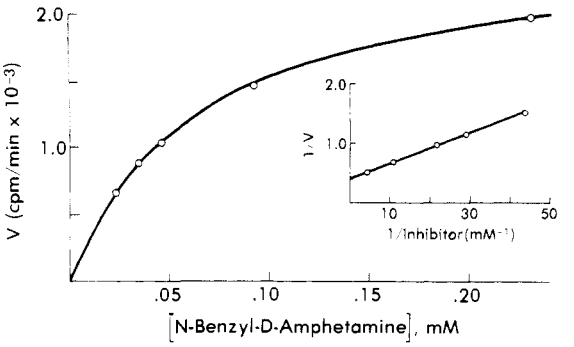

Figure 2. Initial rate of ppGpp formation in *E. coli* B/r as a function of concentration of *N*-benzyl-D-amphetamine; inset: double-reciprocal plot. Procedures were as described under the Experimental Section. Four to five 25-μL aliquots were removed from cultures at ca. 1-min intervals after addition of the inhibitor, rapidly quenched, and analyzed for ppGpp. Initial rates were determined as the linear increase in ppGpp with respect to time; ppGpp measurements refer to counts per minute contained in 2.5-μL of culture.

Discussion

Investigations of the substrate binding sites of *E. coli* Phe-tRNA synthetases[5,16,17] have resulted in the finding that analogues of *N*-benzyl-2-phenylethylamine are potent inhibitors, competitive with respect to Phe, of this enzyme.[5] The efficacy of these analogues appears to be a manifestation of binding to a unique region of this bacterial enzyme, since Phe-tRNA synthetase from rat liver is not inhibited by these analogues,[18] and similar structural modifications of other amino acids do not provide effective inhibitors of their cognate synthetases. Because of their high affinity for Phe-tRNA synthetase and remote structural resemblance to the substrate, it appeared reasonable to believe that other enzymes which utilize L-phenylalanine as substrate would not be affected at concentrations required to effectively inhibit Phe-tRNA synthetase, and such analogues might exhibit a high degree of in vivo selectivity for this enzyme. The results described in this paper clearly illustrate that *N*-benzyl-D-amphetamine, the most potent of this class of inhibitors, arrests the growth of *rel*$^+$ strains of *E. coli* by inhibition of Phe-tRNA synthetase and the consequent regulatory events.

There is an excellent correlation between the K_i values of five analogues of *N*-benzyl-2-phenylethylamine and their IC_{50} values for growth inhibition of *E. coli* B/r, although their potencies span three orders of magnitude, $IC_{50}/K_i = 109 \pm 6$ (SD). Inhibition of growth by all of the inhibitors was reversed to a large degree by 2.5 mM exogenous Phe. Indeed, growth inhibition by *N*-benzyl-D-amphetamine was completely reversed by Phe but not several other amino acids tested. The small deviation in the ratio IC_{50}/K_i, together with the reversal of inhibition by exogenous Phe, strongly suggest that bacterial growth inhibition by these compounds resulted from inhibition of Phe-tRNA synthetase.

Direct evidence that the target for *N*-benzyl-D-amphetamine was Phe-tRNA synthetase was obtained by demonstrating that this inhibitor reduced the intracellular levels of charged $tRNA^{Phe}$ in both NP29 and B/r strains of *E. coli*. Moreover, the charged/uncharged $tRNA^{Phe}$ in B/r was reduced in parallel to the amount of inhibitor present in the culture medium. Interestingly, the concentration of *N*-benzyl-D-amphetamine required to reduce the charging level of $tRNA^{Phe}$ by 50% is identical with that required to inhibit growth by 50%. The inhibitor did not decrease charging levels of $tRNA^{Val}$ or $tRNA^{Ile}$ but rather

resulted in slight increases. This is an expected consequence of idling of protein synthesis, since intracellular aminoacyl-tRNAs are not being consumed and increased charging of unaffected tRNAs should occur. In addition, we have recently observed that for 12 tRNAs examined only the charging level of $tRNA^{Phe}$ is significantly reduced by treatment of *E. coli* with 230 μM inhibitor (unpublished results).

The advantage of using *rel*$^+$ strains of *E. coli* for this study is that a well-defined series of biochemical events (the stringent response) is triggered when the charging level of one or more tRNAs is decreased, which ultimately leads to arrest of growth.[1] Basically, the presence of uncharged tRNA on the A site of ribosomes confers enzymatic activity to the ribosomal-bound stringent factor which results in the formation of ppGpp. The presence of high concentrations of ppGpp controls a series of intracellular events, which includes, among others, idling of protein synthesis, cessation of stable RNA synthesis, and growth arrest. While the stringent response is usually effected by amino acid starvation or temperature-sensitive aminoacyl-tRNA synthetase mutants, correlation of manifestations of this response with decreases in charging levels of $tRNA^{Phe}$ induced by *N*-benzyl-D-amphetamine provide a direct link between inhibition of Phe-tRNA synthetase and growth arrest caused by this inhibitor.

When *E. coli* NP29 is shifted to a temperature nonpermissive for Val-tRNA synthetase activity, there is the expected decrease in intracellular charged $tRNA^{Val}$, production of ppGpp, and growth arrest. At its permissive temperature, treatment of NP29 with *N*-benzyl-D-amphetamine results in an analogous response, except the charged $tRNA^{Phe}$ is depleted and triggers the stringent response. Treatment of *E. coli* B/r with the Phe-tRNA synthetase inhibitor results in specific depletion of charged $tRNA^{Phe}$, ppGpp production, cessation of RNA synthesis, and growth arrest. Moreover, the extent of depletion of charged $tRNA^{Phe}$, the rate of ppGpp production, and growth arrest are all related to the concentration of inhibitor present in the culture medium. As previously noted, 50% of charging of $tRNA^{Phe}$ and 50% inhibition cell growth are both achieved with ca. 15 μM inhibitor. A concentration of inhibitor (4.6 μM) which reduces charging of $tRNA^{Phe}$ by as little as 15% was sufficient to produce ppGpp, as were all higher concentrations. In addition, the rate of ppGpp synthesis increases as the concentration of *N*-benzyl-D-amphetamine in the medium is increased; half-maximal rate of ppGpp production is achieved with 70 μM inhibitor. Here it should be noted that the higher concentration of *N*-benzyl-D-amphetamine required for maximal rate of ppGpp synthesis than for growth inhibition is not a discrepancy. Rate measurements were made during the first few minutes after addition of the inhibitor and reflect complex phenomena including cell permeation, the rate at which charged $tRNA^{Phe}$ is depleted, and the steps intermediate to ppGpp production which are induced by uncharged tRNA. The concentration of ppGpp reaches its peak within ca. 5 min and is then degraded to a steady-state level. In the previously described experiments demonstrating a correspondence of uncharged $tRNA^{Phe}$ and the presence of ppGpp, measurements were made 30 min after addition of the inhibitor; at this time, the charging level of tRNAs and ppGpp concentrations had presumably reached a steady state, and the pleiotropic effects of ppGpp were completely manifested.

In summary, analogues of *N*-benzyl-2-phenylethylamine are potent in vivo inhibitors of bacterial Phe-tRNA synthetase. While it is not possible to ascertain whether these analogues bind to other intracellular proteins, it is clear that their primary effect on growth control is a direct result of inhibition of this enzyme. One of these analogues, *N*-benzyl-D-amphetamine, is the most potent inhibitor of Phe-tRNA synthetase yet reported and should be a useful biochemical tool for studies aimed at further understanding the regulatory effects of tRNA in bacteria.

Acknowledgment. This work was supported by USPHS Grant CA 14266 from the National Cancer Institute. D.V.S. is the recipient of an NIH Career Development Award.

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