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Biochemical Bases for the Antimetabolite Action of L-Serine Hydroxamate

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The amino acid analogue L-serine hydroxamate, which is bacteriostatic for *Escherichia coli*, has been shown to inhibit protein synthesis. The antimetabolite is a competitive inhibitor of seryl-transfer ribonucleic acid (tRNA) synthetase with a K_i value of 30 μM. Mutants resistant to L-serine hydroxamate have been selected, and three were shown to have seryl-tRNA synthetases with increased K_i values. One mutant contains a 3-phosphoglycerate dehydrogenase which is insensitive to inhibition by L-serine.

In the preceding paper (20), we identified L-serine hydroxamate as a potentially useful serine analogue for the study of the control of serine metabolism (20). The suitability of this analogue depends on the observation that it rapidly inhibits growth of *Escherichia coli* and that its inhibitory action is specifically reversed by low levels of exogenous serine. It is expected, therefore, that a mutant which has the capacity to increase its intracellular pool of serine would have lower sensitivity to the action of the antimetabolite. Serine biosynthesis is subject to end-product inhibition (18, 21), a mechanism for increasing the pool size would be to lose this control. An alternative possibility would be to enhance the activity of the biosynthetic enzymes by lowering their K_m values, increasing their V_{max} values, or elevating their concentration in the cell by derepressing enzyme synthesis. Resistance to the analogue could also arise from an alteration in the enzyme system(s) which are inhibited by serine hydroxamate. This type of mutation would be expected to occur frequently, not necessarily be associated with serine biosynthesis, and often take the form of a reduced affinity of the sensitive enzyme(s) for the analogue.

There are several ways an amino acid analogue can function as an antimetabolite (for review, see references 14 and 15), and there is precedent for an amino acid hydroxamate acting as a feedback inhibitor as well as producing repression (11).

To study the regulation of serine synthesis and to determine the mechanism by which L-serine hydroxamate inhibits growth, we have investigated the effects of the analogue on cellular processes and correlated the ability of mutant organisms to resist the analogue with alterations in the properties of their enzymes. The results of the experiments reported in this paper identify the primary site of serine hydroxamate action as the inhibition of the seryl tRNA synthetase and substantiate the proposal that some mutants resistant to its action would be altered in the regulation of serine synthesis.

MATERIALS AND METHODS

Bacteria and selection of mutants. *E. coli* K-12 (λ) was the laboratory stock culture. It lacks auxotrophic markers and is identified as carrying the prophage (λ) by its nonpermissive behavior towards T4 RII bacteriophage. This strain plates T4r$^+$ with the same efficiency as *E. coli* strain B. This differential phage sensitivity was used to check the parentage of the mutants isolated. The conditions for growing bacteria and the methods for measuring growth were described in the previous paper (20).

The procedure of Adelberg et al. was used for obtaining mutants (1). Bacteria in mid-log phase were filtered, suspended in tris(hydroxymethyl)aminomethane (Tris)-maleate buffer (*p*H 6.0), and treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanadine (100 μg/ml) for 30 min at 37 C with gentle shaking. This treatment killed 99.5% of the cells. The cells were filtered, washed, and suspended in Brain Heart Infusion medium. Incubation in this medium was continued for 3 hr at 37 C with aeration. The cells were again filtered and washed with M9 medium; a portion was spread on gradient plates which contained DL-serine hydroxamate-hydrochloride at a concentration range from 0 to 1 mg/ml. Discrete colonies that appeared at the high concentration of hyxroxamate were picked and subsequently purified by three subcultures on different levels of serine hydroxamate. Eight clones showing high levels of resistance to Ser-Hdx were selected for subse-

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quent studies. The bacterial strains used in this study are listed in Table 1.

Chemical compounds and procedures. Adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), DL-serine hydroxamate-hydrochloride, L-leucine hydroxamate, L-lysine hydroxamate, DL-threonine hydroxamate, glycine hydroxamate were purchased from Sigma Chemical Co. (St. Louis, Mo.). The following compounds were purchased from Schwarz Research (Orangeburg, N.J.): *E. coli* transfer ribonucleic acid (tRNA), ^{14}C-L-serine (stan-star grade, 50 μCi/μmole), and ^{14}C-glycine (stan-star grade, 50 μCi/μmole). ^{14}C-phosphoglycerate (PGA), dithiothreitol (DTT), and unlabeled amino acids were purchased from Calbiochem (Los Angeles, Calif.) ^{3}H-uracil, ^{14}C-L-threonine, and ^{14}C-L-leucine were purchased from New England Nuclear (Boston, Mass.). L-serine hydroxamate-hydrochloride was synthesized as described in the accompanying paper (20), and chloramphenicol (CM) was a gift from Parke Davis Co.

Protein was determined by the method of Lowry et al. (10). The uptake of ^{14}C-labeled amino acids and ^{3}H-uracil into acid-precipitable material was measured as described by Smith and Pizer (16). The experiments were performed in the same manner as the growth studies described in the previous paper (20). After dilution from a Klett reading of 80 to 40, the culture was divided into growth tubes; ^{3}H-uracil (10 μCi/μmole) and ^{14}C-leucine (1.5 μCi/μmole) at final concentrations of 0.2 and 0.1 mM were added followed by other compounds as required. Samples were removed to filter paper discs at the desired times, and Klett readings were recorded. Radioactivity was counted either in a gas flow counter or in a Packard scintillation spectrophotometer, which had an efficiency of 45% for ^{14}C-labeled compounds.

3-Phosphoglycerate dehydrogenase. PGA dehydrogenase was assayed in crude extracts by measuring the conversion of radioactive PGA into serine phosphate (12). Overnight cultures grown in minimal media with 2 mg of glucose/ml were collected by centrifugation, washed with cold medium, suspended in 10 mM Tris-hydrochloride buffer (*p*H 7.5) containing 5 mM DTT (5 ml/g, wet weight, of cells), and disrupted by sonic oscillation for 1.5 min in a sonic oscillatior (Measuring & Scientific Equipment, Ltd.). After removal of debris by centrifugation, the cell extract was diluted with buffer until serine phosphate synthesis was directly proportional to the amount of extract added. The components of the assay, incubation conditions, and column fractionation were the same as previously reported (12). One unit of enzyme activity is 1 μmole of serine phosphate formed in 30 min at 37 C.

Amino acyl-tRNA synthetase. Amino acyl-tRNA synthetase was assayed by measuring the conversion of ^{14}C-labeled amino acids into an acid-precipitable form as described by Katze and Konigsberg (8). The activating enzymes were partially purified by the following procedure. Cells grown in minimal media with 2 mg of glucose per ml overnight were collected by centrifugation, washed with minimal medium, and disrupted by grinding with alumina. The disrupted cells were extracted with 20 mM Tris-hydrochloride buffer (*p*H 7.5) containing 10 mM $MgCl_2$, 1 mM DTT, and 0.5 mM ethylenediaminetetraacetic acid (EDTA). After removal of debris by centrifugation at 12,000 $\times$ *g* for 10 min, the supernatant was subjected to ultracentrifugation at 105,000 $\times$ *g* for 2 hr. Streptomycin sulfate (0.3 volume, 5% weight by volume) was added to precipitate the nucleic acids. After 30 min, the precipitate was removed by centrifugation, and 2 volumes of saturated ammonium sulfate was added to the supernatant to precipitate the protein. After 30 min, the protein was collected by centrifugation, dissolved in 20 mM Tris-hydrochloride buffer (*p*H 7.5) containing 10 mM $MgCl_2$, 1 mM DTT, and 0.5 mM EDTA. The protein was dialyzed against two changes of this buffer overnight, and the dialyzed preparation was stored frozen at −20 C.

The assay mixture contained in a final volume of 0.3 ml: 100 mM Tris-hydrochloride buffer (*p*H 7.4), 10 mM $MgCl_2$, 10 mM KCl, 2 mM ATP, 1 mM DTT, 300 μg of

TABLE 1. *Bacteria used for this study*

Strains	Origin	Characteristics
K-12 (λ)	S. S. Cohen	Nonpermissive for T4rII
K-12 ser/gly	S. Simmonds	Requires serine or glycine for growth, lacks PGA dehydrogenase
K-12 (λ) met⁻	J. McKitrick	2-Amino-purine mutagenesis of K-12 (λ) requires methionine for growth
Hfr-C	J. S. Gots	Met⁻, *rel*
A-111g	Nitrosoguanadine mutagenesis of K-12 (λ)	Completely resistant to 100 μg of L-serine hydroxamate per ml
C-111d	Nitrosoguanadine mutagenesis of K-12 (λ)	Completely resistant to 100 μg of L-serine hydroxamate per ml
D-111a	Nitrosoguanadine mutagenesis of K-12 (λ)	Completely resistant to 100 μg of L-serine hydroxamate per ml
C-211g	Nitrosoguanadine mutagenesis of K-12 (λ)	Partially resistant to 100 μg of L-serine hydroxamate per ml
A-211d	Nitrosoguanadine mutagenesis of K-12 (λ)	Inhibition initiated at 60 min
B-111e	Nitrosoguanadine mutagenesis of K-12 (λ)	Inhibition initiated at 30 min
B-211e	Nitrosoguanadine mutagenesis of K-12 (λ)	Inhibition initiated at 30 min
D-221d	Nitrosoguanadine mutagenesis of K-12 (λ)	Inhibition initiated at 60 min

E. coli B tRNA, 50 μM ^{14}C-L-serine (25 μCi/μmole), and enzyme. The reaction was initiated with substrate, and incubation was at 37 C. At 5 and 10 min after the start of the reaction, 50-μliter portions were removed to filter paper discs, and the discs were immediately immersed in cold 10% trichloroacetic acid. After one washing with cold 10% trichloroacetic acid, two washings with cold 5% trichloroacetic acid, and one washing with acetone, the radioactivity on the discs was determined by use of a scintillation spectrophotometer. Radioactive glycine, threonine, and leucine were substituted for serine where the activating enzymes for these amino acids were to be assayed. The attachment of 1 nmole of amino acid to tRNA during a 10-min incubation constitutes a unit of enzyme activity. For the kinetic studies, the enzyme was diluted so that activity was proportional to the amount of protein present.

RESULTS

Effect of serine hydroxamate on the growth of mutant strains. The growth of eight mutant strains selected for their resistance to serine hydroxamate was tested in the presence of 1.28 mM DL-serine hydroxamate-hydrochloride. The growth response fit three patterns (Fig. 1). The first group was not inhibited (A-111g, C-111d, and D-111a). The second group (C-211g) was partially resistant, and the third group (A-211d, B111e, B-211e, and D-211d) was resistant for periods of time between 30 and 60 min and then became partially resistant. The generation times of some of the mutants are presented in Table 2. At higher levels of serine hydroxamate, the growth of resistant strains D-111a and A-111g was inhibited. Strain D-111a had generation times of 150 and 350 min when 1.28 and 2.55 mM of L-serine hydroxamate was in the medium.

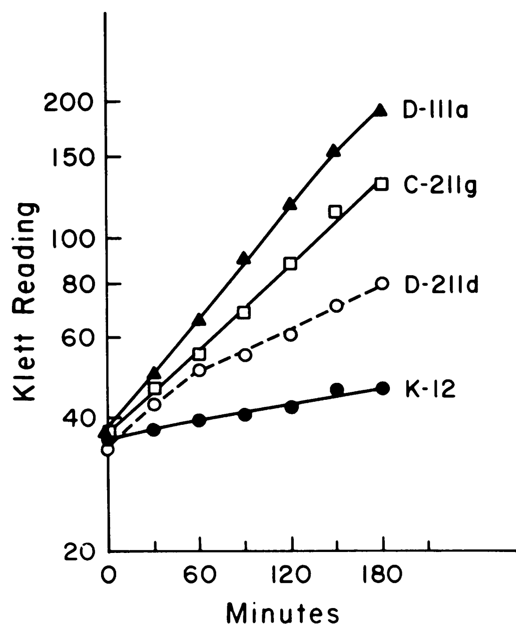

FIG. 1. *Growth of mutants in the presence of L-serine hydroxamate. The growth of K-12 and serine-hydroxamate-resistant mutant strains was determined as described in the preceding paper (20). All cultures contained DL-serine hydroxamate-hydrochloride at 1.28 mM.*

Growth of strain K-12 ser$^-$, which lacks PGA dehydrognease and consequently has a nutritional requirement for serine or glycine, was used to test the possibility that the analogue was acting as a false feedback inhibitor shutting off the supply of serine. The experiment was possible because glycine failed to reverse the serine hydroxamate inhibition (20). Figure 2 shows that a strain using exogenous glycine for the production of serine, and not possessing the feedback-sensitive enzyme, was still inhibited by serine hydroxamate in a manner indistinguishable from the wild-type organism. This result indicates that the analogue does not act on PGA dehydrogenase to cause inhibition of growth. Moreover, the capacity of exogenous glycine to give rise to serine was below the level necessary to reverse serine hydroxamate inhibition, even when the cells were utilizing this pathway for serine biosynthesis.

Effect of serine hydroxamate on macromolecular synthesis. By using ^{14}C-leucine to measure protein synthesis and ^{3}H-uracil to measure nucleic acid synthesis, it was possible to follow simultaneously the effect of L-serine hydroxamate on the two major classes of macromolecules found in the cell. Because RNA accumulation is connected to the ability to make protein, the effect of the antibiotic CM was tested in conjunction with serine hydroxamate. CM allows RNA synthesis to occur under conditions of amino acid starvation (9). In the presence of either serine hydroxamate, CM, or a combination of these two compounds, protein synthesis was completely inhibited (Fig. 3A). The addition of serine hydroxamate alone also inhibited the accu-

TABLE 2. *Mass doubling times of E. coli K-12 and serine hydroxamate-resistant mutants*[a]

L-Serine hydroxamate (μM)	Bacterial strain				
	K-12	A-111g	C-111d	C-211g	D-111a
0	70	70	100	70	70
64	195				
128	282				
191	400				
320	∞				
640	∞	70	100	100	70

[a] The mass doubling times were calculated from plots of Klett readings against time. The time for the Klett reading to increase from 50 to 100 is shown.

mulation of uracil in nucleic acids (Fig. 3B). In contrast to the situation with protein synthesis, addition of CM or CM plus serine hydroxamate leads to an appreciable amount of uracil incorporation into nucleic acid. The *E. coli* K-12 strain used in these experiments was stringent (17) since it failed to accumulate RNA in the absence of an amino acid requirement (Table 3). CM had the effect of allowing RNA synthesis either in the absence of amino acid requirement or the presence of serine hydroxamate. These results suggested that the effect of serine hydroxamate on RNA synthesis was a secondary one related to the fact that protein synthesis was inhibited by the serine analogue. This possibility was tested directly by measuring the effect of serine hydroxamate on the accumulation of uracil into nucleic acids in a relaxed strain (Hfr-C; reference 17). In this strain, the inhibition of protein synthesis by serine hydroxamate was somewhat slower in appearing and less complete. The effect on uracil incorporation shows that RNA synthesis occurred in the presence of L-serine hydroxamate when protein synthesis was blocked.

The synthesis of nucleic acids in the presence of serine hydroxamate (Table 3) indicates that the analogue does not limit the supply of serine. Serine is the source of glycine, which is required for purine biosynthesis; had the supply of serine been reduced RNA synthesis and protein synthesis should have stopped. The results in Table 3 indicate that serine hydroxamate must be acting directly on the synthesis of protein.

Inhibition of L-seryl-tRNA synthetase. Since the serine analogue appeared to be acting directly on protein synthesis, its effect on the seryl-tRNA synthetase was determined. The activity of seryl-tRNA synthetase was reduced in the presence of L-serine hydroxamate (Fig. 4A). When the enzyme activity was measured as a function of L-serine concentration alone and in the presence of serine hydroxamate, the analogue acted as a competitive inhibitor towards the amino acid (Fig. 4B). The K_m for L-serine obtained from these studies was 50 μM and the K_i for L-serine hydroxamate was 30 μM. The enzyme apparently has a higher affinity for the analogue than it does for its natural substrate. To test the specificity of the inhibition, seryl-tRNA synthetase activity was measured in the presence of several amino acid hydroxamates. The results (Table 4) show that 10 mM DL-threonine hydroxamate produced 50% inhibition. This concentration of DL-threonine-hydroxamate is approximately 10 times the concentration that failed to effect growth of *E. coli* K-12 (20).

The specificity of inhibition by L-serine hydroxamate was also tested. Table 5 demonstrates

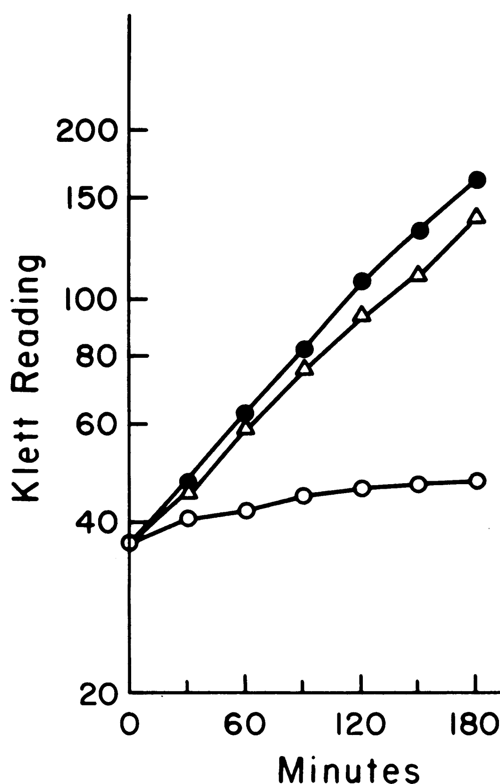

FIG. 2. *Inhibition of the growth of E. coli K-12 Ser⁻ by serine hydroxamate. The experiment was performed as described in the accompanying paper. The nutritional requirement of the organism was satisfied by 400 μg of glycine per ml. The* DL-*serine hydroxamate concentrations were: none,* ●; *128 μM,* Δ; *and 1.28 mM,* O.

that, although leucine hydroxamate, threonine hydroxamate, and glycine hydroxamate inhibit their acyl-tRNA synthetase to an appreciable extent, L-serine hydroxamate at concentrations that would completely inhibit the seryl-tRNA synthetase had no effect on the leucine-activating enzyme, relatively little effect on the threonine activating enzyme, but inhibited the glycyl-activating enzyme approximately 60%. The effect of the glycyl-activating enzyme may be of interest, considering that glycine hydroxamate was not appreciably better as an inhibitor than the serine analogue. It is apparent that the inhibition of seryl-tRNA synthetase by the L-serine hydroxamate is sufficiently sensitive and specific to account for the bacteriostatic action of this compound. The significance of the inhibition of acyl-tRNA synthetases by other amino acid hydroxamates in relation to earlier growth studies is evaluated in the discussion section.

Mutant studies. To obtain direct evidence that the inhibition of seryl-tRNA synthetase by serine

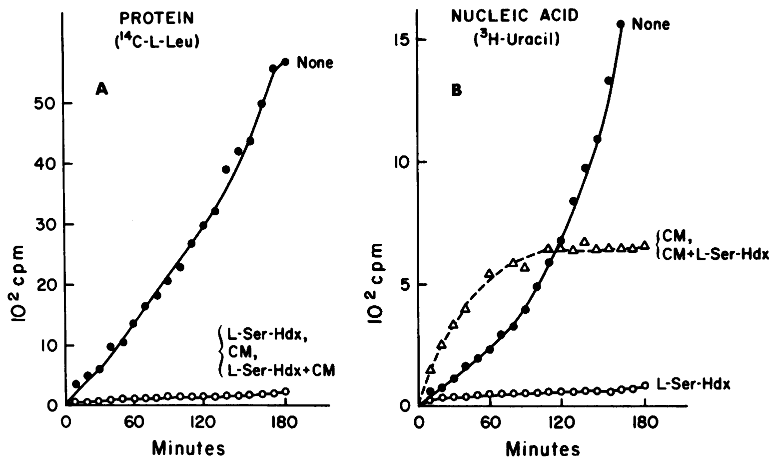

FIG. 3. *Effect of serine hydroxamate on protein and nucleic acid synthesis in E. coli K-12. After a Klett reading of 80 was reached, the culture was diluted with an equal volume of medium and divided into growth tubes. The isotopically labeled compounds and the desired antibiotics were then added. The additions are shown on the appropriate curves.* L*-serine hydroxamate and CM were present at a final concentration of 100 μg/ml. The ^{14}C-leucine incorporation in the presence of* L*-serine hydroxamate, CM, or both compounds was the same and is shown by a single curve* (○). *The ^{3}H-uracil incorporation in the presence of CM or CM + serine hydroxamate was the same and is shown by a single curve* (Δ).

TABLE 3. *Effect of* L*-serine hydroxamate (Ser-Hdx) on uracil and leucine incorporation into acid-precipitable material*

Strain	Compound added (counts/min)						
	None (A)	L-Ser-Hdx[a] (B)	CM[b]	L-Ser-Hdx[a] + CM	Per cent of relative incorporation (B/A × 100)[c]	-MET	-MET + CM
^{3}H-uracil							
K-12	680	80	650	650	12		
K-12 Met$^-$	1,780	216	1,080	1,106	12	112	870
Hfr-C[3]	2,600	1,150	600	600	58		
^{14}C-leucine							
K-12	2,950	120	20	30	4		
K-12 Met$^-$	7,020	128	60	36	2	340	154
Hfr-C	4,150	300	0	0	8		

[a] L-Ser-Hdx-hydrochloride concentration was 0.64 mM (100 μg/ml).
[b] CM (chloramphenicol) concentration was 100 μg/ml.
[c] Isotope incorporation is presented for the samples removed from the cultures 120 min after initiation of the experiment. The incorporation kinetics were similar for strains K-12 (λ)Met$^-$ and Hfr-C to those found for strain K-12 (λ) (Fig. 3). In cultures of K-12 Met$^-$ and Hfr-C, methionine was present at 40 μg/ml unless otherwise indicated.

hydroxamate was responsible for the bacteriastatic properties of this compound, mutant strains which had been selected for their ability to grow in the presence of the analogue were studied. Assuming that the point of L-serine hydroxamate inhibition was the activating enzyme, there would be two ways to generate a resistant strain. One way would be modification of the activating enzyme itself; another would be to increase the intracellular level of L-serine. Described in the introduction are the characteristics in which the activating enzyme could be altered and the mechanisms for increasing the supply of serine.

The possibility that the resistance of the eight mutants which grew in the presence of serine hydroxamate was due to an increased supply of serine was tested by analyzing the capacity for serine phosphate formation in crude extracts. It was observed (Table 6) that the enzyme levels did not differ from those found in the wild-type organism, and no evidence was obtained for derepression of enzyme synthesis. Whether the enzymes were altered in their sensitivity to feedback control was tested by measuring serine

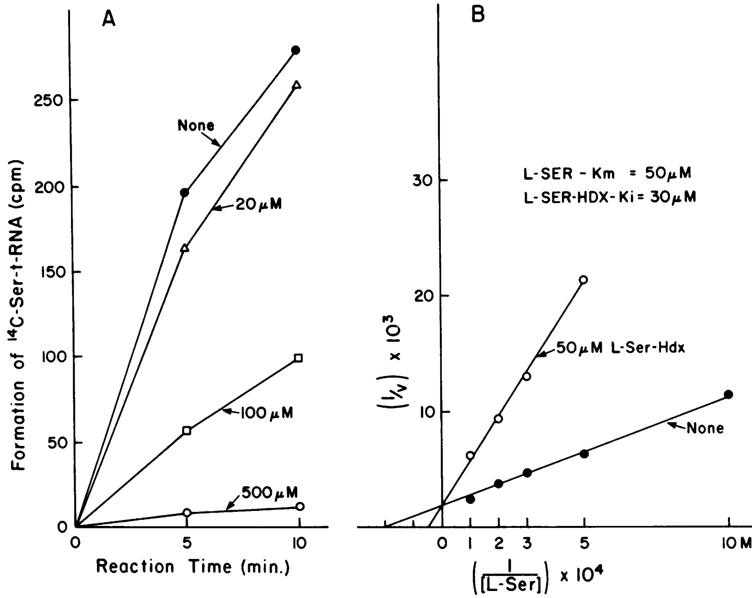

FIG. 4. *Effect of serine hydroxamate on L-seryl-tRNA synthetase. (A) The assay was performed with 0.1 unit of enzyme activity in the incubations, a serine concentration of 50 μM,* and the *L-serine hydroxamate concentrations indicated. (B) The standard assay was used with 0.16 units of enzyme, and the serine concentration varied as shown. The concentration of L-serine is in moles per liter, and the Ser-Hdx concentration was 50 μM* (O). *The radioactivity (counts per minute) present in the sample removed at 5 min was used as the measure of velocity, and the reciprical of counts/min is plotted on the 1/v axis. In both experiments, serine had a specific activity of 25 μCi/μmole.*

TABLE 4. *Effect of amino acid hydroxamates on L-seryl-tRNA synthetase*[a]

Amino acid hydroxamates (5 mM)	Activity (counts/min)	Inhibition (%)
None	676	0
L-Leucine hydroxamate	658	3
DL-Threonine hydroxamate	328	50
Glycine hydroxamate	640	5
L-Lysine hydroxamate	744	0
L-Serine hydroxamate	0	100

[a] Standard assay was performed with 0.09 units of enzyme, ¹⁴C-L-serine (50 μCi/μmole), and the L-amino acid hydroxamates at 5 mM. The concentration of DL-threonine-hydroxamate was 10 mM. The activity is shown for the 10-min time point.

phosphate production in the presence of 40 μM L-serine. One strain (C-111d) failed to show any inhibition, whereas the wild-type organism and remaining seven mutants were inhibited by approximately 70% (Table 6). A detailed study of the feedback inhibition of this mutant strain (Fig. 5) showed that the enzyme in this mutant was highly resistant to serine inhibition. Mixing experiments and experiments in which the serine was preincubated with the mutant extract unequivocally showed that the failure to observe inhibition was *not* due to destruction of L-serine. We

TABLE 5. *Effect of amino acid hydroxamates on amino acyl-t-RNA synthetases*[a]

Substrates	Amino acid hydroxamates	Activity (counts/min)	Inhibition (%)
¹⁴C-L-leucine (40 μCi/μmole)	None	127	0
	L-Leucine hydroxamate	59	54
	L-Serine hydroxamate	127	0
¹⁴C-L-threonine (50 μCi/μmole)	None	877	0
	DL-Threonine hydroxamate	41	95
	L-Serine hydroxamate	685	21
¹⁴C-glycine (50 μCi/μmole)	None	50	0
	Glycine hydroxamate	16	78
	L-Serine hydroxamate	20	60

[a] The standard assay was performed with the activity shown for the 10-min time point. The substrates were at 50 μM, and the L-amino acid hydroxamates were at 5 mM. The DL-threonine-hydroxamate was at 10 mM. The amount of enzyme added was equivalent to 0.09 units of seryl-tRNA synthetase.

conclude that in strain C-111d the mutation alters the PGA dehydrogenase, making the enzyme less sensitive to inhibition by serine, and infer that, as a consequence of this alteration the pool

of serine in the cell increases to the level where serine hydroxamate no longer acts as an effective inhibitor of seryl-tRNA synthetase.

An analysis of the amino acyl-tRNA synthetase activity in three other mutants was then undertaken. One of these mutants (A-111g) exhibited a four-fold increase in the level of seryl-tRNA synthetase (Table 7). One of the mutants, (C-211g), contained 15% of the activity of the wild-type, the third (D-111a) slightly higher activity than the wild-type organism. The strain A-111g which had the greater activity for serine activation had approximately wild-type levels of threonyl- and leucyl-activating enzymes (Table 7). The lowered level of enzyme activity detected in strain C-211g suggested that the structure of this enzyme had been altered. This was verified by

TABLE 6. *Serine-phosphate formation inhibition by L-serine*[a]

Bacterial strain	Serine-phosphate formation (units/mg of protein)	Relative activity (%)	Inhibition by 40 μM L-serine (%)
K-12	1.32	100	65
A-111g	1.60	122	80
A-211d	1.55	120	76
B-111e	1.86	141	77
B-211e	1.97	149	76
C-111d	1.65	125	0
C-211g	1.01	83	76
D-111a	2.07	157	77
D-221d	1.34	102	72

[a] The specific activity of the substrate (phosphoglycerate) was 20,000 counts per min per μmole.

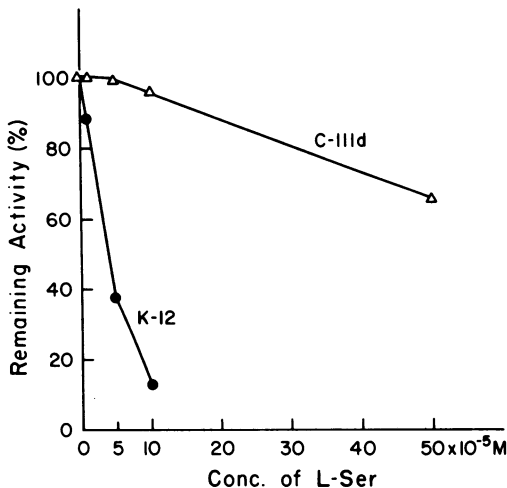

FIG. 5. *Inhibition of serine-P formation by L-serine. Standard assay conditions were used with the final concentration of L-serine in the assay indicated.*

measuring the temperature stability of this enzyme. The data obtained by using the procedure of Sundharadas et al. (19) showed that the mutant enzyme was 50% inactivated by 5 min of treatment at 57 C, whereas the wild-type organism was considerably more stable (Fig. 6). The instability of the activating enzyme from C-211g did not affect bacterial growth at 42 C. The tRNA synthetase from mutant strains A-111g and D-111g showed the same temperature stability as the enzyme from K-12. A detailed study of the kinetic properties of the activating enzymes from the mutant strains was undertaken. It was observed (Fig. 7) that the wild-type and mutant enzymes had approximately the same K_m values for serine and that, in all cases, serine hydroxamate acted as a competitive inhibitor. K_i values were obtained for the mutant strains that were three to five times higher than those found with the parental type K-12. These results are summarized in Table 8.

DISCUSSION

The primary observation described in this paper is that L-serine hydroxamate inhibits the activity of seryl-tRNA synthetase. The inhibition is competitive with L-serine. Since the hydroxamate group eliminates activation of the analogue, the inhibition presumably results from exclusion of the amino acid substrate from the enzyme. The in vivo consequences of reduced seryl-tRNA synthetase activity are inhibition of protein synthesis and growth. In strains that exert stringent control over RNA synthesis, there is, together with the inhibition of protein synthesis, a marked reduction in the accumulation of uracil into RNA. It appears that, even in the presence of an adequate supply of serine, RNA synthesis does not proceed normally if the attachment of the amino acid to the seryl-tRNA is inhibited. Serine behaves like other amino acids

TABLE 7. *Comparison of aminoacyl-tRNA synthetase activities*[a]

Bacterial strains	Aminoacyl-tRNA synthetase activities (units/mg of protein)		
	L-Serine	L-Threonine	L-Leucine
K-12	11.6	1.5	11.3
A-111g	51.3	1.6	6.9
C-211g	1.8		
D-111a	16.6		

[a] Standard assay conditions were used with L-serine present at 25 μM (specific activity, 50 μCi/μmole), and the enzyme was diluted to give proportionately with added protein. The activity of the sample taken at 5 min was used to calculate the specific activity shown.

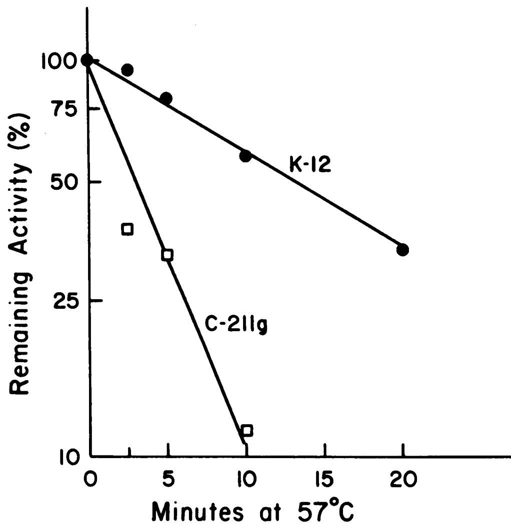

FIG. 6. *Thermal inactivation of seryl-tRNA synthetase. The procedure of Sundharadas et al. (19) for determining the rate of heat inactivation was modified by the substituting DTT for β-mercaptoethanol. After removal of enzyme samples to chilled tubes, the enzyme was diluted 10 times with reaction buffer; tRNA, ATP, and ¹⁴C-serine were then added to initiate the assay. The amount of enzyme added was adjusted to give the same amount of activity before heating.*

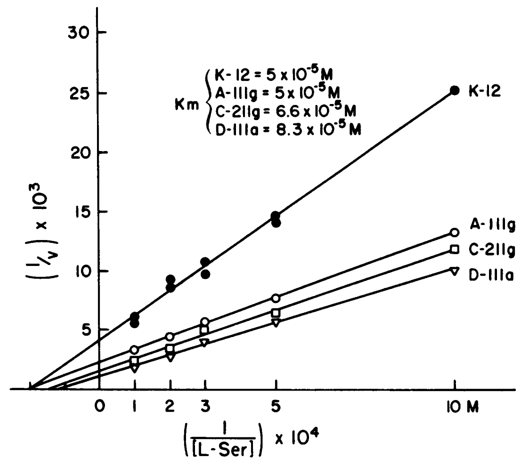

FIG. 7. *Kinetics of L-seryl-tRNA synthetase. The standard assay was employed with between 0.05 to 0.2 units of enzyme purified from the mutant strains. The serine (25 μCi/μmole) concentration was varied as shown, and the reciprocal of the molarities was multiplied by 10⁴ plotted on the 1/s axis. The velocity of the reaction was measured by determining the radioactivity in the sample taken at 5 min. The reciprocals of the number of counts per minute in these samples were multiplied by 10³ and have been plotted on the 1/v axis.*

TABLE 8. *Summary of the properties of L-seryl-tRNA synthetases from mutant strains*

Bacterial strains	Growth inhibition by 1.28 mM DL-serine hydroxamate	Relative L-seryl-tRNA synthetase activity (%)	Thermal inactivation at 57 C relative activity (%) remaining after		Kinetic parameters (μM)		Characteristics of mutant seryl-tRNA synthetase
			5 min	10 min	K_m[a] for L-serine	K_i for L-serine hydroxamate	
K-12	Complete inhibition	100	70–90	48–67	50	30	
A-111g	Complete resistance	450	91	53	50	167	Excess enzyme, K_i alteration
C-211g	Partial resistance	15	33	13	66	85	Heat sensitive, K_i alteration
D-111a	Complete resistance	140	78	47	83	167	K_i alteration

[a] Reported K_m, 7×10^{-5} M (8).

(e.g., valine and glycine) in this respect (4, 5). Because serine hydroxamate is a competitive inhibitor, its effectiveness in stopping growth depends on the concentration of serine hydroxamate and serine in the cell and the relative affinities of these two compounds for the activating enzyme. The second parameter can be expressed as the ratio of K_i (Ser-Hdx) to K_m (Ser). This ratio is approximately 1; therefore, the sensitivity of the bacteria to low concentrations of analogue must be due to low intracellular concentrations of serine.

Evidence that the bacteriostatic action of serine hydroxamate is due to the inhibition of the activating enzyme and that the in vitro measurements of enzyme activity are pertinent to the conditions in the cell comes from an analysis of the properties of the mutants resistant to the analogue. Three of the resistant mutants have activating enzymes with increased K_i (Ser-Hdx)

values and, in keeping with the competitive character of the inhibition, higher levels of serine hydroxamate are required to inhibit their growth. In addition to this kinetic evidence that mutations to resistance are accompanied by changes in seryl-tRNA synthetase, one resistant strain possesses an enzyme with an altered structure which leads to increased lability at 57 C. These data confirm that the activating enzyme is the point of inhibition and that the K_i (Ser-HDX) to K_m (Ser) ratio is critical in establishing the level of sensitivity.

The second factor which should effect sensitivity is the intracellular concentration of serine. The normal endogenous serine concentration appears to be maintained at a low level. Addition of serine to the medium relieves the inhibition of growth by increasing the intracellular concentration. A mutation which relieves end-product inhibition confers resistance to serine hydroxamate, presumably by allowing expansion of the endogenous pool. It appears that serine production is tightly regulated by the end-product inhibition of PGA dehydrogenase. The addition of exogenous glycine did not overcome serine hydroxamate inhibition; we conclude that, although the conversion of glycine to serine was adequate for growth, it did not lead to an elevated concentration of serine in the cell. The possibility that the conversion of serine to glycine is regulated warrants further investigation.

The properties of the mutants support our ideas on the mechanism of action of the analogue. By making some assumptions we can further test the relationship between the inhibition of seryl-tRNA synthetase and the inhibition of growth. If we equate the rate of growth (reciprocal of generation times given in Table 2) in the absence of serine hydroxamate with V_{max} ($1/V_{max}$, time in minutes for the Klett reading to go from 50 to 100 in the uninhibited culture) of the activating enzyme and the rate of growth in the presence of different concentrations of serine hydroxamate to v, the inhibited level of enzyme activity, we can attempt to fit the equation

$$[(1/v)/(1/V_{max})] - 1 = \{K_m - (K_m[I]/K_i\}1/[S] \quad (1)$$

to the growth data obtained in the presence of different levels of serine hydroxamate. Equation 1 is a convenient rearrangement of the standard Michaelis equation for a competitive system (2): $v = V_{max}[S]/\{K_m(1 + [I]/K_i) + [S]\}$. Equation 1 describes the competitive inhibition of an enzyme reaction (2); to use it, we make the additional assumption that $[I]$, the inhibitor concentration in the cell, is equal to the concentration of serine hydroxamate added to the culture medium. This is probably justified since we have demonstrated the passive entry of serine hydroxamate into the cell (20). If our assumptions are valid and the reduction in growth is solely due to a drop in the rate of charging seryl-tRNA, we predict that the serine concentration in the cell $[S]$ should remain relatively constant at the different levels of growth inhibition. We could then equate the computed value of $[S]$ to the in vivo serine concentration maintained by end-product inhibition. Substituting the numerical values for growth rates (Table 2) and the average values for K_m (ser) and K_i (Ser-Hdx) (Table 7) into equation 1, the intracellular concentration of serine was computed to be 88, 87, and 79 μM for cells of strain K-12 growing in the presence of 64, 128, and 192 μM L-serine hydroxamate, respectively. The relative constancy of the calculated serine concentrations support the validity of our assumptions and strongly suggests (i) that the pool of serine is not expandable by endogenous synthesis, (ii) that the inhibition of seryl-tRNA synthetase reduces growth to a level, v, (iii) that the uninhibited rate of growth represents V_{max} of seryl-tRNA synthetase.

Points i and iii above require further amplification. If the serine pool was expandable by endogenous synthesis, it should increase when the activating enzyme is inhibited and, in the case of a competitive inhibitor, thereby overcome the inhibition. More than a transient inhibition of growth by serine hydroxamate depends, therefore, on the constancy of the intracellular serine concentration. The constant serine concentration could result from limitations on substrate supply for the biosynthetic pathway, e.g., PGA, NAD or glutamate, or end-product inhibition of PGA dehydrogenase. The second explanation appears likely because the calculated intracellular serine pool of approximately 90 μM would markedly inhibit the PGA dehydrogenase (Fig. 5). Also, substrate limitations cannot be restricting serine synthesis, since a mutant without this feedback control increases the serine pool, as judged by its resistance to the antibiotic. It appears that serine synthesis is proceeding in the cell at a greatly inhibited rate. Derepression of the biosynthetic enzymes did not occur during growth in the presence of the analogue, and resistant mutants with elevated enzyme levels were not found.

By equating the velocity of the seryl-tRNA synthetase with the rate of growth, we assume that the activity of this enzyme is the pacemaker for bacterial growth. This assumption is plausible when the culture is growing in the presence of the antibiotic (i.e., the determination of v), but is less likely in the absence of serine hydroxamate.

We have tested the consequences of inaccuracy in the growth rate measurements by substituting alternative values for v and V_{max} into equation 1. Recalculations of $[S]$ with changes in v and V_{max} of ± 10 min have shown that $[S]$ remained relatively constant if the same V_{max} value was used for a series of cultures and that this analysis is not sensitive to small alterations in growth rate.

In view of these calculations and the fact that an intracellular serine concentration of 90 μM would not saturate the activating enzyme with a K_m of 50 μM, we conclude that we are not using the true V_{max} value in equation 1 and seryl-tRNA synthetase alone does not limit normal growth. However, it does appear that, unlike the PGA dehydrogenase, the seryl-charging enzyme is not regulated by a feedback control and is functioning in the cell at the maximum rate allowed by the availability of its substrate. Direct measurements of $[S]$ are being undertaken to determine whether the computed value of 90 μM is in agreement with experimentally determined pools.

When growth data and the K_i(Ser-Hdx) obtained for strain C-111d was put into equation 1, the calculated serine concentrations were 380 and 202 μM for growth in the presence of 1.25 and 2.5 mM serine hydroxamate. The variation and the magnitude of the $[S]$ values suggest that at high serine hydroxamate concentrations equation 1 does not hold and the slow growth is not solely due to a reduction in seryl-tRNA synthetase activity.

The inhibition of the leucyl-, threonyl- and glycyl-tRNA synthetases by their corresponding amino acid hydroxamates shows that the inhibition of the activating enzymes is not restricted to serine hydroxamate and raises the question why these analogues fail to inhibit bacterial growth. The concentration of analogues, 5 mM, that gave marked inhibition of the activating enzymes was approximately 10 times higher than that used in the growth studies; although growth might have been inhibited by higher concentrations, the large quantitative difference in the effectiveness of the amino acid hydroxamates is noteworthy. From the few preliminary measurements of the inhibition of the activating enzymes, it appears that the K_i values for the other analogues are higher than the K_i for serine hydroxamate. The differences in K_i could account to a large extent for the absence of an observed effect on growth. In addition, the extent of enzyme inhibition at a given analogue concentration depends on the K_m for the amino acid and its concentration in the cell. For the amino acid hydroxamate to be a potent antimetabolite, the pool size of the amino acid must be regulated to avoid an increase when the hydroxamate inhibits the charging of the tRNA. The serine concentration is maintained at a low level by end-product inhibition. Other amino acids might be maintained at a higher concentration if their biosynthetic pathways lack this type of control or had a less sensitive feedback enzyme. Derepression of enzymes that are repressed by amino acyl tRNA would also effect the pool size. Different species of bacteria might vary considerably in the intracellular concentrations of the amino acids and the mechanisms of regulating these concentrations. The susceptibility to the bacteriostatic action of amino acid hydroxamates might provide a simple method for finding similarities in regulatory processes and identifying differences among bacterial species.

The inhibition of the activating enzyme by the amino acid hydroxamates should be considered when using hydroxamate formation to assay enzyme activity. The degree to which inhibition occurs will vary from amino acid to amino acid and possibly account for the observation by Hoagland, Keller, and Zamecnic (7) and by Hirsh and Lippman (6) that there is a lack of correspondence when some amino acyl-tRNA synthetases are assayed by hydroxamate formation, pyrophosphate exchange, and amino acyl-tRNA formation. Although the concentration of amino acid hydroxamate produced would be initially low, its synthesis would occur in the active site, the ideal location for it to act as an inhibitor.

In conclusion, there is the possibility that amino acid hydroxamates could be a useful class of chemotherapeutic agents affective against viral and bacterial infections or malignant conditions. Whether these compounds are effective in a particular situation will depend on the same factors that distinguish serine hydroxamate from other hydroxamates in inhibiting the growth of *E. coli*. Cultured human cells do not possess tight feedback controls on the synthesis of the nonessential amino acids (3). In the case of serine, there is a report that blood cells, unlike other tissues, require exogenous serine (13). These types of differences from organism to organism or tissue to tissue might provide a basis for selective hydroxamate toxicity. An experimental program based on the considerations presented in this paper appears warranted.

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