Aurintricarboxylic Acid, a Preferential Inhibitor of Initiation of Protein Synthesis

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The effect of aurintricarboxylic acid (ATA) was tested on various aspects of protein synthesis directed by the natural messenger ribonucleic acid (RNA) isolated from R17 RNA bacteriophage. The effects of various levels of ATA (up to 1,000 μM) were tested on overall protein synthesis as well as on binding of messenger RNA and met-transfer RNA to ribosomes and on the addition of the 50S ribosome to the 30S ribosome initiation complex. All of the reactions tested could be inhibited by ATA, and none of the tested steps was found to be uniquely sensitive to it. However, the total initiation steps were more sensitive to this chemical than the elongation steps; thus, under appropriate conditions this chemical can preferentially inhibit initiation while elongation of the polypeptide chain is not appreciably affected.

The trimethylpentane dye aurintricarboxylate (ATA) has been used as a novel inhibitor of protein synthesis in vitro (4, 6, 10, 12). It has been proposed that this compound functions by specifically inhibiting the attachment of messenger ribonucleic acid (mRNA) to the ribosome (4).

Our studies on the effect of ATA on polyphenylalanine synthesis directed by polyuridylic acid (Siegelman and Apirion, J. Bacteriol., in press), as well as the studies by Weissbach and Brot (11), suggested that ATA can interfere with other steps in protein synthesis besides binding of messenger. The available experiments (4, 10, 12) demonstrated inhibition of the initiation complex by ATA when a natural messenger was used, but did not show which of the steps involved in the complex formation is affected. Therefore, we studied the effects of ATA on protein synthesis directed by the natural mRNA, isolated from R17 bacteriophage, in Escherichia coli cell-free extracts.

Overall protein synthesis as well as specific steps in the initiation process were studied. We found that all steps tested were almost equally sensitive to ATA inhibition. However, we found concentrations of ATA that completely inhibited initiation of protein synthesis without appreciably affecting elongation. Therefore, we suggest that none of the initiation steps is uniquely sensitive to ATA, but it is rather the total sum of the initiation steps that are more sensitive to ATA inhibition than the total elongation steps. Thus, this drug (ATA) is still a unique inhibitor of protein synthesis.

MATERIALS AND METHODS

Radioisotopes. l-14C-methionine (51 mCi/m mole) was purchased from Schwarz BioResearch Inc., 35S-methionine (910 mCi/m mole) was purchased from Amersham-Searle, 32P-phosphate (5 mCi/ml) was purchased from Schwarz BioResearch Inc., and 3H-R-17 RNA was a gift from M. Kuwano of this laboratory.

Chemicals. ATA was purchased from Aldrich Chemical Co., polyadenylic-uridylic-guanylic acid (poly AUG; a 1:1:1 random copolymer) was a Miles Laboratory product, and calcium leucovorin (folinic acid) was purchased from Lederle Laboratories. Other chemicals were of reagent grade, when available.

Growth of cells and preparations of subcellular fractions. Strain D10 (met rns ) (3) was used. It was grown in Casamino broth medium (1) in a New Brunswick Scientific Ferracell Fermentor and was harvested at an absorbancy of about 1 at 560 nm. Preparation of crude extracts (S-30) and fractionation of extracts into ribosomes and supernatant enzymes (S-100) were carried out by modifications of published techniques (8). Cell-free extracts were prepared by alumina grinding of frozen cells and by extracting them with 1.5 ml of buffer [0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6), 0.01 M magnesium acetate, 0.05 M potassium chloride, and 0.006 M β-mercaptoethanol] per g (wet weight) of cells.

Ribosomes were sedimented at 150,000 x g in a Ti50 Spinco rotor for 90 min. The upper three-fourths of the tube contents was removed and recentrifuged at 150,000 x g for 150 min. The upper two-thirds of the contents of this tube was removed, dialyzed against buffer containing 0.01 M Tris-hydrochloride (pH 7.6), 0.01 M magnesium acetate, 0.05 M potassium chloride, and 0.006 M β-mercaptoethanol, and stored at −80 C for use as supernatant enzymes. The initial pellet of ribosomes was drained, resuspended in 0.01 M Tris-hydrochloride (pH 7.6) and 0.01 M magnesium acetate, and sedimented again at 150,000 x g for 90 min. The resulting pellet was resuspended in the same buffer at 20 mg/ml and stored in small amounts at −80 C. The 30S
ribosomes were a gift from L. Gorelick of this laboratory. They were prepared by zonal centrifugation in an IEC B15 rotor (International Equipment Co.) in a B 60 ultracentrifuge.

Preparation of phage RNA and in vitro protein synthesis. Growth of RNA bacteriophage R17 on host AB301, purification of viral RNA, and incorporation of amino acids directed by the viral RNA in cell-free extracts depleted of endogenous mRNAs were carried out as recommended by Capechi (2). To deplete endogenous mRNA, S-30 was incubated for 30 min at 37 C, in a mixture containing, per ml: 150 μg of adenosine triphosphate (ATP), 10 μg of guanosine triphosphate (GTP), 40 nmoles of 14C-amino acids, 15 μmole of magnesium acetate, 55 μmole Tris-hydrochloride (pH 7.6), 10 μmoles of reduced glutathione (GSH), 36 μmoles of ammonium acetate, 4 μg of pyruvate kinase (EC 2.7.1.40), and 5.4 μmoles of 2-phosphoenolpyruvate (PEP). The protein synthesis mixture contained, per ml: 500 μlitters of preincubated S-30 mixture, 150 μg of ATP, 100 μg of GTP, 5.4 μmoles of PEP, 50 μmoles of Tris-hydrochloride (pH 7.6), 10 μmoles of GSH, 800 μg of transfer RNA (tRNA), 5 μCi of 14C-L-alanine, 44 μmoles of ammonium acetate, and 300 μg of R17 RNA. Incubation times and volumes are indicated in Results. The reactions were terminated by the addition of 3 ml of 5% trichloroacetic acid, and the radioactivity in material insoluble in hot trichloroacetic acid (20 min at 90 C) was determined with a Nuclear-Chicago low-background counter with a 25% efficiency for 14C.

R17 RNA binding to 30S ribosomes. Reaction mixtures contained, in 50 μliters: 0.9 A260 units of 30S ribosomes, purified by zonal centrifugation; 20.5 μmoles of 3H-R17 RNA (800 counts/min); 0.3 μmoles of magnesium acetate; 2.5 μmoles of ammonium acetate; 1.25 μmoles of Tris-hydrochloride (pH 7.6); and 0.25 μmole of GTP. The mixtures were incubated for 25 min at 25 C. Ribosomes were added last, preceded by the addition of the appropriate concentration of ATA. The reactions were terminated by putting them on ice and the addition of 3 ml of cold buffer used in the experiments. Samples were filtered through HA (0.45 μm) filters (Millipore Corp.). The filters were washed three times with 5-ml portions of cold buffer, dried, and counted (scintillation counting (5)).

Preparation of fmet-tRNA. A modification of the technique of Marker (7) was used. Labeled methionine (either 14C or 35S as indicated in the text and legends) was incubated for 15 min at 37 C in a 1-ml volume containing: Tris-hydrochloride (pH 7.6), 100 μmoles; magnesium acetate, 14 μmoles; potassium chloride, 10 μmoles; GSH, 7.5 μmoles; ATP, 5 μmoles; PEP, 9 μmoles; 19 other amino acids (no methionine), 0.05 μmole; pyruvate kinase, 6 μg; tRNA (commercial), 16 mg; calcium leucovorin, 180 μg; S-100, 400 μliters; and either 14C-methionine (5 μCi) or 35S-methionine (50 μCi). The reaction was terminated by chilling to 0 C, and the mixture was passed through several HA Millipore filters. The filtrate was used directly in the various binding reactions. The filtrate was incapable of catalyzing peptide synthesis with R17 RNA as the messenger, either by itself or with the addition of ribosomes. Charging of met-tRNA was routinely between 30 and 35%, assuming methionyl-tRNA was one-twentieth of the total tRNA. Under these conditions (leucovorin included), the labeled product was 75% resistant to enzymatic discharge as assessed from the pyrophosphate exchange reaction (7).

Binding of fmet-tRNA. In a modification of the method of Nirenberg and Leder (9), 100-μlter reaction mixtures were incubated for 25 min at 25 C. They contained 8.1 A260 units of D10 ribosomes; 30 μg of poly(AU); 20 μlitters of fmet-tRNA filtrate, prepared as indicated above; 0.5 μmole of GTP; 5 μmoles of ammonium chloride; 2.5 μmoles of Tris-hydrochloride (pH 7.6); and magnesium acetate and ATA as indicated. The reactions were terminated by chilling to 0 C and dilution with 5 ml of cold buffer containing concentrations of Tris-hydrochloride (pH 7.6), magnesium acetate, and ammonium chloride identical to those in the incubation mixtures. They were filtered on HA Millipore filters. The filters were washed three times with 5-ml portions of cold buffer, dried, and counted.

RESULTS

In vitro protein synthesis, directed by R17 bacteriophage RNA, was sensitive to ATA (Fig. 1). Inhibition was observed at 10 μM concentrations of ATA, and inhibition was almost complete at 40 μM ATA. In a second experiment (Table 1), ATA was added at the start and during the course (10 min) of the incubation (10 to 300 μM tested). The results show that the inhibition by ATA was greater when it was added at the beginning of the reaction. This was observed for ATA concentrations ranging from 10 to 80 μM. This indicates a preferential inhibition of initiation at this range of concentrations.

In a subsequent experiment, a preferential inhibition of initiation by ATA, as opposed to elongation, was clearly demonstrated (Fig. 2). It can
be seen from this experiment that, at 30 C, 70 μM ATA completely inhibited polypeptide synthesis when added at zero time, whereas it did not markedly affect polypeptide synthesis for as long as 4 min when added during the course of the reaction (5 min after the incubation started), and initiation took place. (It should be emphasized that variation in the extent of inhibition by ATA was noticed in different extracts within the range of 50 μM concentrations.) To find out whether a step uniquely sensitive to ATA exists in the initiation process, several steps in initiation were examined. In the first experiment in this series, the effect of ATA on the poly AUG-stimulated binding of f-^{14}C-met-tRNA to ribosomes was studied (Fig. 3). Binding assays were carried out at 6 and 15 mM Mg^{2+}. Binding at both Mg^{2+} concentrations was sensitive to ATA, with maximal inhibition occurring at approximately 200 μM ATA. (ATA in the concentrations used does not affect the binding of ribosomes to the filters; unpublished observations.) Since in this experiment ATA could affect the

<table>
<thead>
<tr>
<th>ATA (μM)</th>
<th>ATA at zero time</th>
<th>ATA at 10 min</th>
</tr>
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<tr>
<td></td>
<td>Counts/min</td>
<td>Per cent</td>
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<tr>
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<td>100</td>
</tr>
<tr>
<td>10</td>
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<td>340</td>
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</tr>
<tr>
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<td>60</td>
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</tr>
<tr>
<td>300</td>
<td>21</td>
<td>1.5</td>
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</tbody>
</table>

* R17 RNA-directed protein synthesis was carried out as described in Materials and Methods. The indicated concentrations of ATA were added to incubation (37 C) either at zero time or 10 min after the incubation started. Samples (50 μl) were removed at 0, 10, 20, and 30 min of incubation. Values for 20 min of synthetic activity (30 min – 10 min) are presented. Values for incubations without R17 RNA but with the proper concentration of ATA added at the appropriate times have been subtracted (e.g., no ATA, 30-min incubation was 306 counts/min). In this experiment, incorporation was approximately linear during the first 0.5 hr. When ATA was added at time zero, the extent of inhibition was similar throughout the length of the reaction.

**FIG. 2.** Preferential inhibition of initiation by ATA. S-30, reaction mixtures, and preincubation were as described in Materials and Methods. Samples (50 μl) were removed from an incubation mixture at 30 C, at the indicated times, precipitated with 5% trichloroacetic acid, and analyzed as indicated in Materials and Methods. ATA was added to a concentration of 70 μM at zero time or 5 min after the start of the incubation. Equivalent incubation mixtures without R17 RNA and without ATA or with ATA added to a concentration of 70 μM at either zero time or at 5 min were tested and their values were subtracted (e.g., no ATA, after 12 min incubation, 237 counts/min).

**FIG. 3.** Binding of fmet-tRNA to ribosomes. Experiments were conducted as described in Materials and Methods. Reaction mixtures contained 20 μl of f-^{14}C-met-tRNA (prepared as described in Materials and Methods), and either 0.6 μmole (6 mM final concentration) or 1.5 μmole (15 mM final concentration) of magnesium acetate, as indicated in the figure by 6 mM and 15 mM, respectively. The results given are the net stimulated binding due to the poly AUG. Values for incubations without poly AUG, but with the appropriate concentration of ATA have been subtracted (no ATA; 0.6 μmole of Mg^{2+}, 6.2 pmoles; 1.5 μmole of Mg^{2+}, 2.0 pmoles); 100% activity was 1.00 pmoles and 2.65 pmoles bound, respectively.
reaction by inhibiting messenger binding to ribosomes or fmet-tRNA binding to the messenger-ribosome complex, these two reactions were dissected. In Fig. 4, the inhibition of \(^{3}H\)-R17 RNA binding to purified 30S ribosomes is presented. The inhibition obtained (maximum at 350 \(\mu M\)) is similar to that obtained with the poly AUG-stimulated binding of f-\(^{14}C\)-met (Fig. 3).

In another experiment, the inhibition by ATA of the binding of fmet-tRNA to the messenger-ribosome complex was tested. The poly AUG was bound to ribosomes by incubation for 10 min at 25 C; then the appropriate concentrations of ATA, f-\(^{3}C\)-met-tRNA, and GTP were added, and the incubation was continued for an additional 15 min at 25 C. The results (Fig. 5) indicate that the binding of fmet-tRNA to the messenger-ribosome complex is also sensitive to ATA and only slightly less sensitive than the binding of messenger to the 30S ribosome.

The effect of ATA on the addition of the 50S ribosome to the 30S initiation complex was also examined (Table 2). This reaction was inhibited about 55% at a 100 \(\mu M\) concentration of ATA.

**DISCUSSION**

ATA has been used as a novel inhibitor of initiation (4, 6, 10, 12) of protein synthesis, and it has been proposed that this compound functions by specifically inhibiting the attachment of mRNA to the ribosome (4).

The results presented here, all of which are in agreement with previous results (4, 10, 12), demonstrate that under proper conditions ATA is a preferential inhibitor of initiation. However, elongation can be inhibited as well (see Fig. 2). Inhibition of elongation is further supported by the facts that ATA inhibits polyuridylic acid-directed protein synthesis and ribosome-dependent guanosine triphosphatase (Siegelman and Api- rion, J. Bacteriol., in press), as well as the function of the Ts elongation factor (11).

The concentrations necessary to inhibit the G-specific, ribosome-dependent guanosine triphosphatase, R17 RNA binding, and fmet-tRNA binding are similar and are higher than the concentrations necessary to inhibit in vitro protein synthesis.

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**Fig. 4.** \(^{3}H\)-R17 RNA binding to 30S ribosomes. Experiments were carried out as described in Materials and Methods; 100% activity equals 351 counts/min bound after subtraction of background binding; 18 counts/min (value of an incubation without ribosomes). Values for incubations containing ATA were corrected for quenching, determined in a series of incubations in which ATA was added after incubation.

**Fig. 5.** f-\(^{3}C\)-met-tRNA binding to the messenger-30S complex. D10 ribosomes were incubated for 10 min at 25 C in 50-\(\mu l\) volumes containing 30 \(\mu g\) of poly AUG, 2.1 \(\mu moles\) of Tris-hydrochloride (pH 7.6), 0.425 \(\mu moles\) of magnesium acetate, and 2.5 \(\mu moles\) of ammonium chloride followed by the addition of the indicated concentrations of ATA, 0.5 \(\mu moles\) of GTP and 20 \(\mu l\) of f-\(^{3}C\)-met-tRNA (prepared as indicated in Materials and Methods), and incubation was continued for 15 min at 25 C in a 100-\(\mu l\) volume. The reactions were terminated by chilling to 0 C and dilution with 5 ml of buffer (0.042 M Tris-hydrochloride, pH 7.6; 0.0085 M magnesium acetate; 0.05 M ammonium chloride). The reaction mixtures were filtered through HA Millipore filters, washed three times with 5-\(ml\) volumes of buffer, dried, and counted. The results presented are for poly AUG-stimulated binding; values for incubations without poly AUG, but with the appropriate concentration of ATA, have been subtracted (no ATA, 1,428 counts/min; 100% activity equals 854 counts/min.

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**Values**

- **Km**;
- **40**
- **60**
- **80**
- **100**
- **200**
- **600**
- **1000**

**GTP and 20 \(\mu l\) of f-\(^{3}C\)-met-tRNA (prepared as indicated in Materials and Methods), and incubation was continued for 15 min at 25 C in a 100-\(\mu l\) volume. The reactions were terminated by chilling to 0 C and dilution with 5 ml of buffer (0.042 M Tris-hydrochloride, pH 7.6; 0.0085 M magnesium acetate; 0.05 M ammonium chloride). The reaction mixtures were filtered through HA Millipore filters, washed three times with 5-\(ml\) volumes of buffer, dried, and counted. The results presented are for poly AUG-stimulated binding; values for incubations without poly AUG, but with the appropriate concentration of ATA, have been subtracted (no ATA, 1,428 counts/min; 100% activity equals 854 counts/min.**

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**REFERENCES**

2. The effect of ATA on the addition of the 50S ribosome to the 30S initiation complex was also examined (Table 2). This reaction was inhibited about 55% at a 100 \(\mu M\) concentration of ATA.

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The concentrations necessary to inhibit the G-specific, ribosome-dependent guanosine triphosphatase, R17 RNA binding, and fmet-tRNA binding are similar and are higher than the concentrations necessary to inhibit in vitro protein synthesis.
TABLE 2. Inhibition of binding of fmet-tRNA and the 
SOS ribosomal subunit to the initiatio

complex

<table>
<thead>
<tr>
<th>ATA (nM)</th>
<th>Counts/min</th>
<th>Ribosomes (μmol)</th>
<th>50S/30S</th>
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<tr>
<td>0</td>
<td>3,170</td>
<td>0.932</td>
<td>1.09</td>
</tr>
<tr>
<td>20</td>
<td>2,280</td>
<td>0.926</td>
<td>0.91</td>
</tr>
<tr>
<td>100</td>
<td>1,690</td>
<td>0.771</td>
<td>0.45</td>
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a In experiment 1, 200-μlitter volumes were incubated 
for 25 min at 25 C. They contained 21.5 units of A, ribosomes, 30 μliters of f,met-tRNA (prepared as 
described in Materials and Methods), 9,600 counts/min of [3H]-R17 RNA, 1 μmole of GTP, 8.4 μmoles of Tris-
hydrochloride (pH 7.6), 1.7 μmoles of magnesium acetate, and the indicated concentrations of ATA. The in-
cubations were stopped by chilling to 0 C and were layered on 5 to 20% sucrose gradients (42 mM Tris-hydro-
chloride, pH 7.6; 8.5 mM magnesium acetate; and 50 mM ammonium acetate). The gradients were 
centrifuged for 135 hr at 35,000 rev/min in a Spincos SW50 rotor. One-drop fractions were collected from the 
bottom of the tube, and the determination of either absorbance at 260 nm or radioactivity, by precipitation 
with 5% trichloroacetic acid, filtration on DA (0.65-
μm) Millipore filters, and scintillation counting, was 
performed on every other drop. The values presented in 
the first two columns are taken from the region of the 
gradient where R17 RNA was bound.

Expt 1 (°P3S met-tRNA) | Expt 2°
<table>
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<tbody>
<tr>
<td>3.170</td>
<td>0.932</td>
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<td>2.280</td>
<td>0.926</td>
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<tr>
<td>1.690</td>
<td>0.771</td>
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</table>

b Experiment 2 was performed under identical condi-
tions, with the exception that °P-labeled ribosomes 
(45,000 counts/min, 25 A₂₆₀) were used. The regions of 
the gradient that contained messenger were pooled, unla
teled ribosomes (100 A₂₆₀) were added, and the mixture 
was dialyzed against 0.01 mM Tris-hydrochloride, 
PH 7.6, 0.0001 mM magnesium acetate. The ribosomes 
were reanalyzed in 5 to 20% sucrose gradients (0.01 mM Tris-hydrochloride pH 7.6; 0.0001 mM magnesium acetate), centrifuged for 11.5 hr at 21,000 rev/min in the 
SB110 rotor in an IEC B-60 ultracentrifuge. The molar 
ratios of 50S to 30S were calculated from the °P counts 
recovered, assuming that 2.2 counts per min of 50S to 1 
count/min of 30S is equal to a 1:1 molar ratio. This is 
based on the following molecular weights: 23S ribo-
somal RNA, 1.1 x 10²; 16S ribosomal RNA, 0.5 x 
10². Since, under the conditions studied, binding of 30S 
itself to R17 RNA is undetected in a sucrose gradient, 
the deficiency in 50S particles probably reflects a spe-
cific inhibition of the addition of 50S ribosome subunits 
to the messenger-fmet-tRNA-30S ribosome complex.

synthesis directed by natural mRNA (see Fig. 1- 
5). Therefore, we assume that the preferential 
inhibition of initiation by ATA is the result of the 
sum of the inhibitions of the various steps of initia-
tion, and therefore perhaps the total initiation 
process is more complex, involving more steps 
than the elongation process. It may be that some 
as yet unexamined step in initiation is sensitive to 
ATA to a greater extent than those already 
tested. However, even if such were the case, it is 
still an unavoidable conclusion that ATA is an 
inhibitor of many of the reactions of protein syn-
thesis.

Our conclusions can be summarized as follows: 
(i) ATA can inhibit many steps in protein syn-
thesis; (ii) none of the steps tested is uniquely 
sensitive to ATA; (iii) ATA inhibits the total initia-
tion process of a protein chain to a greater extent 
than the elongation process; (iv) by use of proper 
concentrations of ATA, the initiation process can 
be inhibited completely while the elongation 
process is almost unaffected.

We do not know at present the mechanism by 
which ATA exerts its effects, i.e., whether it 
binds mainly to RNA or to protein, and through 
which chemical groups. However, there are cer-
tain indications that its effects, although of wide 
range, are nonetheless quite specific and probably 
cannot be attributed, for instance, to chelating of 
Mg²⁺ ions. This is indicated by the fact that 
increasing Mg²⁺ concentrations do not alleviate 
inhibition by ATA [see Fig. 3 and Siegelman and 
Apirion, (J. Bacteriol., in press)]. (In this last-
mentioned experiment, no reversal of inhibition 
of polyuridylic acid-directed protein synthesis 
was observed when the Mg²⁺ concentration was 
raised.)

It is interesting to compare a chemical like 
ATA to naturally occurring antibiotics that inter-
fere with protein synthesis. Whereas the anti-
biotics apparently block unique steps in the reac-
tion and therefore can be adapted to by the organ-
ism by single-step mutations, ATA is interfering 
with more than one step of the reaction and an 
organism would not be able to adapt to it by a 
single-step mutation [some of our studies (unpublished observation) with E. coli strains 
made permeable to ATA indicate that this is in-
deed very likely]. Perhaps antibiotics that neces-
sitate adaptation by more than a few mutations 
upset the ecological balance to such an extent that 
their perpetuation in nature is selected against.

ACKNOWLEDGMENTS

We thank M. Kuwano for a gift of R17 RNA and L. Goretic 
for a gift of 30S ribosomes.

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grant HD01956, National Science Foundation grant GB-17888, 
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