Proceedings of the National Academy of Sciences
Vol. 68, No. 1, pp. 97–101, January 1971

Aurintricarboxylic Acid: Inhibitor of Initiation of Protein Synthesis

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Communicated by Alfred Gilman, September 8, 1970

ABSTRACT **Aurintricarboxylic acid prevents the attachment of bacteriophage messenger RNA to ribosomes. As a consequence, initiation of protein synthesis in cell-free extracts prepared from *Escherichia coli* or rabbit reticulocytes is inhibited at concentrations of dye that do not prevent chain extension. Its properties can be distinguished from other agents that inhibit protein synthesis, including sodium fluoride, cycloheximide, and pactamycin.**

Aurintricarboxylic acid (ATA) is a triphenylmethane dye that prevents attachment of bacteriophage mRNA to *Escherichia coli* ribosomes but does not dissociate complexes composed of ribosomes, mRNA, formymethionyl-tRNA, and initiation factors (1). The results reported in the present paper indicate that the primary inhibitory effect of ATA in cell-free extracts prepared from *E. coli* or rabbit reticulocytes is on the initiation of protein synthesis. Protein synthesis is inhibited at concentrations of dye that show little or no effect on the rate or extent of chain extension; the inhibition is accompanied by sequential release of ribosomes and completed peptides from the polyribosomes.

MATERIALS AND METHODS

The preparation and sources of ATA, bacteriophage f2 RNA, and the other materials used in these experiments are described elsewhere (1–3). As prepared, ATA is impure and contains significant quantities of isomeric and inactive materials. [^{14}C]hemoglobin, 4 μCi/mg, was prepared by incubating intact rabbit reticulocytes for 24 hr at 30°C in medium (3) containing [^{14}C]leucine. Preincubated, dialyzed S-30 extracts (1), containing 30–35 mg of ribosomes per ml, were centrifuged at 150,000 × *g* for 2 hr and the top half of the supernatant solution (S-150) removed. Reticulocyte lysates were prepared and the synthesis of globin was measured, as described by Maxwell and Rabinovitz (4). Density gradient centrifugation (5), acrylamide gel electrophoresis (6), and the determination of radioactivity in precipitates collected on Millipore membrane filters (3) or on crushed acrylamide gels (6) were performed according to published procedures.

RESULTS

Effects of ATA on initiation of protein synthesis in extracts of E. coli:

The inhibitory effects of ATA on f2 RNA-directed peptide synthesis in extracts of *E. coli* are shown in Fig. 1*A*. If the dye is added prior to the addition of f2 RNA, complete inhibition of peptide synthesis is observed. On the other hand, if the same concentration of ATA is added 10 or 15 min after initiating the reaction, the rate of protein synthesis remains unaffected for several minutes, then decreases rapidly. In contrast to this delayed inhibitory effect of ATA, chloramphenicol, an antibiotic that inhibits chain elongation (7), stops peptide synthesis immediately after addition to the reaction mixture (Fig. 1*B*).

Effects of ATA on the synthesis of phage coat and other proteins

The effects of ATA on the synthesis of coat and non-coat proteins are compared in the experiments shown in Fig. 2. Synthesis of coat protein is measured by the incorporation of valine (8); synthesis of non-coat proteins is measured by the incorporation of histidine [95% of which enters RNA synthetase (9)].

Incorporation of valine is linear during the first 20 min of the reaction and precedes that of histidine, indicating the polarity of the cell-free system (Fig. 2*A* and *B*). ATA rapidly inhibits valine incorporation if added during the first several minutes following the addition of mRNA to the reaction. If the dye is added 5 min after mRNA (Fig. 2*C*), the degree of inhibition becomes progressively less marked. The onset of inhibition is delayed if ATA is added at later times during the reaction (Fig. 1*A*).

In contrast to the effects of ATA on incorporation of valine, histidine incorporation is immediately inhibited if the dye is added 1.5 min or less after initiation (Fig. 2*D*). If ATA is added at 2 min, protein synthesis continues for 1 min before the onset of inhibition is observed; if ATA is added more than 2 min after mRNA, the delay increases to a maximum of approximately 5 min.

Effects of ATA and other antibiotics on ribosome–mRNA–peptide complexes

The nature of the polyribosome–peptide complexes formed in extracts of *E. coli* in the presence and absence of ATA are shown in Fig. 3. In the control experiment (Fig. 3*A*), nascent peptide appears on the polyribosome 5 min after protein synthesis is initiated by the addition of mRNA. After 10 min, completed peptides begin to be released from the polyribosome and appear at the top of the gradient (Fig. 3*B* and *C*). A small and variable amount of released peptide probably coat protein bound to f2 RNA (10) usually sedimented in the 30 S region of the gradient in such experiments.

ATA, added 5 min after f2 RNA, induces sequential release of peptides and monomeric ribosomes from polysomes, be-

Abbreviation: ATA, aurintricarboxylic acid.

* Career Investigator of the Health Research Council of the City of New York.

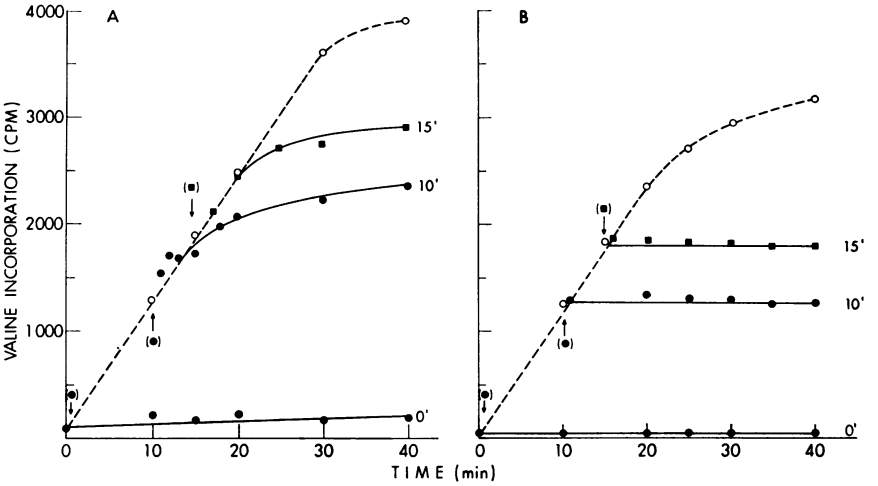


FIG. 1. Effects of ATA and chloramphenicol on phage RNA-directed polypeptide synthesis. (*A*) Four identical reaction mixtures, containing 50 mM Tris·HCl, pH 7.8; 50 mM NH_4Cl; 3 mM ATP; 0.2 mM GTP; 8 mM magnesium acetate; 10 mM phosphoenolpyruvate; a 50 μM (each) mixture of 19 [^{12}C]amino acids; 50 μM [^{14}C]valine, 100 Ci/mol; 6 mM 2-mercaptoethanol; 1.2 μg/ml *N*-formyltetrahydrofolate; 2.9 units/ml of pyruvate kinase; 1 mg/ml of f2 RNA; 1.2 mg/ml of stripped *E. coli* transfer RNA; and 10 μl each of S-150 and S-30 extracts, in a final volume of 0.2 ml, were incubated at 35°C. f2 RNA was added to initiate the reaction after all of the other components had been incubated for 2 min at 35°C.

ATA (final concentration 70 μM) was added to three of the reactions at the times indicated; the fourth reaction (O- - -O) served as the uninhibited control. In the reaction indicated as 0′, ATA was added just prior to f2 RNA.

Aliquots, 20 μl, were removed at the indicated times and radioactivity in hot Cl_3CCOOH-insoluble material was determined. Data are corrected for endogenous incorporation of valine which, after 15 min, was 100 cpm in the control and 92 cpm in the ATA-treated sample. (*B*) represents a separate experiment in which conditions were identical to (*A*) except that chloramphenicol (final concentration 0.6 mM) was substituted for ATA.

ginning with the larger complexes' (Fig. 3*D* and *E*). The peak of radioactivity corresponding to the coat protein–mRNA complex is also observed in the presence of the dye. The major radioactive product formed in the presence of ATA (and released from polysomes) coelectrophoresed with authentic phage coat protein on acrylamide gels.

The effects of ATA on ribosome-bound peptide, the peptide–f2 RNA complex, and free peptide are compared with those of puromycin, streptomycin, and chloramphenicol in Table 1. Puromycin induces the immediate release of nascent peptide from the ribosome, while streptomycin and chloramphenicol had no effect on this process during the first 9 min after they were added to the reaction. In other experiments (not shown), uniform breakdown of polyribosome structure, with concomitant release of peptide, was observed after 20 min of further incubation (29-min total) with streptomycin but not with chloramphenicol. Peptide–f2 RNA complexes were observed only in the uninhibited control and in the reaction containing ATA.

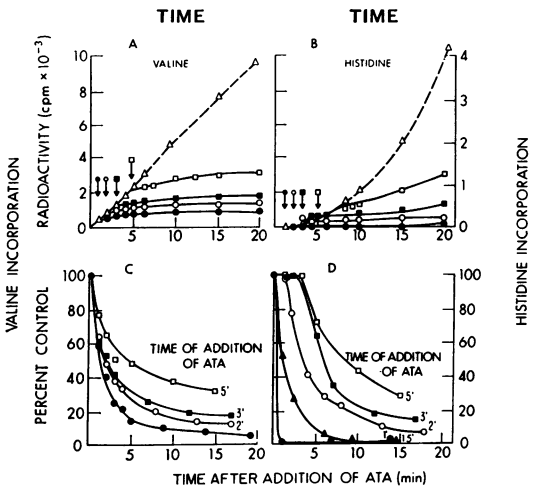
Effects of ATA on the initiation of globin synthesis

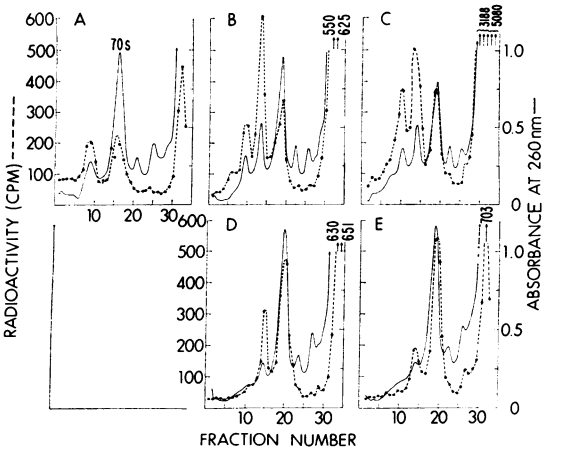
Crude reticulocyte lysates, capable of initiating new globin chains in the presence of hemin (11), were used to study the effects of ATA on protein synthesis in extracts prepared from a mammalian cell. As shown in Fig. 4, there was a delay in the onset of inhibition after the addition of either ATA or

TABLE 1. *Effect of ATA and other antibiotics on the synthesis of coat protein*

Additions	Ribosome-bound nascent peptide (min)			Peptide–f2 RNA complex (min)			Free peptide (min)		
	0	5	9	0	5	9	0	5	9
None	8,292	11,919	16,535	36	148	741	2,311	6,935	10,701
Puromycin	...	281	...	..	20	...	...	8,325	...
ATA	...	8,922	7,787	..	120	251	...	2,952	3,955
Streptomycin	...	8,124	7,943	..	30	35	...	2,600	2,714
Chloramphenicol	...	...	8,493	..	...	40	...	...	2,579

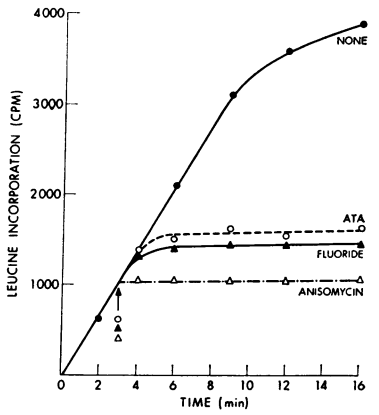
The experiment was performed as described in Fig. 3, with certain minor modifications. Antibiotics were added after 8 min of incubation (0-time) at the following concentrations: ATA, 80 μM; puromycin, 500 μM; streptomycin, 70 μM; and chloramphenicol, 600 μM. Reaction volumes were 0.7 ml; 150-μl aliquots were layered on gradients. Radioactivity incorporated into each fraction was determined by integrating the appropriate areas of the gradient: free peptide was assumed to be in the top four fractions, the peptide–f2 RNA complex was estimated from the peak of radioactivity at 30 S, and ribosome-bound nascent peptide included the radioactivity that was bound to the 70S, dimeric, and trimeric ribosomes.

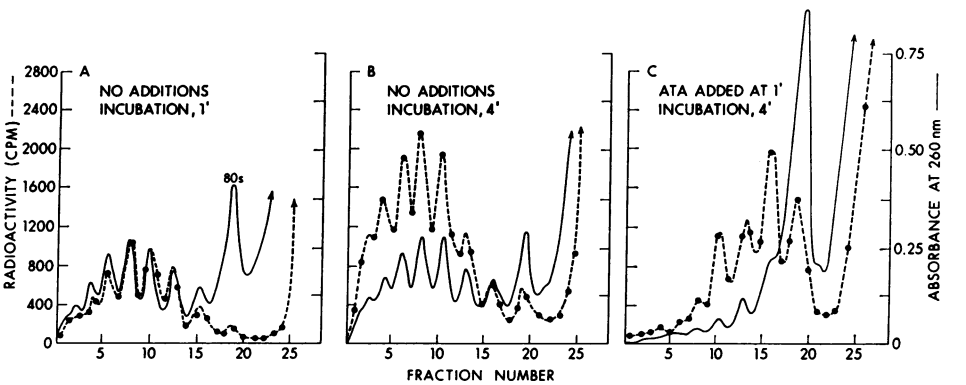




(*Left*) FIG. 2. Effects of ATA on incorporation of [3H]histidine and [^{14}C]valine into phage proteins. Six reaction mixtures were prepared, as described in Fig. 1, as well as six additional mixtures, in which [^{3}H]histidine, 3.3 Ci/mmol, was substituted for [^{14}C]valine. The final volume was 1.0 ml. Reactions were initiated by the addition of f2 RNA; ATA (final concentration 70 μM) was added at the times indicated by the arrows and at 1.5 min. Samples without inhibitor served as controls. Aliquots, 100 μl, were removed at the indicated times, and the radioactivity in the hot Cl_3CCOOH-insoluble fractions determined. All values are corrected for endogenous incorporation of histidine or valine. Panels *C* and *D* are the same data as in *A* and *B*, respectively, expressed relative to the uninhibited control, and shown at various time intervals after addition of the inhibitor. Data for the experiment in which ATA was added at 1.5 min was included only in Panel *D*.

(*Right*) FIG. 3. Sucrose density gradient analysis of ribosomes and nascent peptides formed at various times in the presence and absence of ATA. Two identical reaction mixtures, with the composition described in Fig. 1, were incubated for 2 min at 35°C in a final volume of 0.5 ml. 5 min after the addition of f2 RNA, a 90-μl aliquot was removed from the uninhibited control reaction and chilled; ATA (final concentration 70 μM) was added to the other reaction. Aliquots, 15 μl, were removed from both samples after 5 and 10 min of further incubation for analysis of hot Cl_3CCOOH-insoluble radioactivity. Aliquots, 90 μl, removed at the same intervals, were chilled and layered onto 12 ml of a 5–20% sucrose gradient, prepared in a buffer composed of 10 mM Tris·HCl (pH 7.8)–10 mM magnesium acetate–50 mM NH_4Cl. Gradients were centrifuged for 100 min at 41,000 rpm at 4°C in a Beckman SW-41 rotor. Absorbancy at 260 nm was determined during collection of the gradient, and fractions of 0.4 ml were collected from the bottom of the tube for determination of hot Cl_3CCOOH-insoluble radioactivity. *A*, no additions, incubation 5′; *B*, no additions, incubation 10′; *C*, no additions, incubation 15′; *D*, ATA added at 5′, incubation 10′; *E*, ATA added at 5′, incubation 15′.





(*Left*) FIG. 4. Effect of ATA on globin synthesis in reticulocyte lysates. Four reaction mixtures, containing 10 mM Tris·HCl, pH 7.4; 75 mM KCl; 1 mM ATP; 0.2 mM GTP; 15 mM creatine phosphate; 2 mM $MgCl_2$; 170 μM [^{14}C]leucine, 250 Ci/mol; 6 mM 2-mercaptoethanol; a mixture of 19 amino acids (12); 0.9 mg/ml of creatine phosphokinase, and 0.1 ml of lysate were incubated at 33°C for the times indicated in a final volume of 0.5 ml. ATA, 0.1 mM; NaF, 25 mM; or anisomycin 0.25 mM, were present where indicated. Aliquots, 50 μl, were removed from the reactions at the indicated times for determination of radioactivity.

(*Right*) FIG. 5. Sucrose density gradient analysis of reticulocyte polyribosomes and nascent globin peptides formed in the presence and absence of ATA. Three reaction mixtures, with the composition described in Fig. 4, were prepared. Each contained 0.14 ml lysate in a final volume of 0.25 ml. All reactions were incubated for 1 min at 35°C then treated as follows: in reaction *A*, the incubation was stopped after 1 min; in reaction *B*, the incubation was continued for 3 min longer; in reaction *C*, ATA (final concentration 0.2 mM) was added and the incubation continued 3 min longer. Reactions were stopped by diluting to 1.0 ml with cold buffer. Samples were layered onto 36 ml of a 10–25% sucrose gradient prepared in a buffer composed of 10 mM Tris·HCl (pH 7.4)–10 mM KCl–1.5 mM $MgCl_2$, then centrifuged in the sucrose density gradient for 2.5 hr at 25,000 rpm (4°C) in an SW-27 rotor; fractions of 1.2-ml were collected.

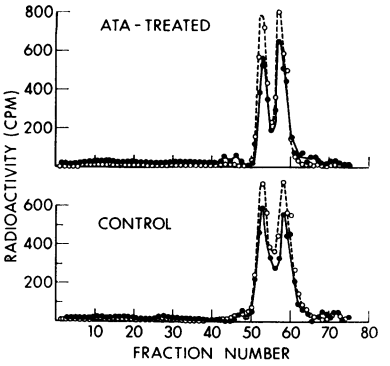


FIG. 6. Comparison by acrylamide gel electrophoresis of the reaction products formed in the presence or absence of ATA. Two reaction mixtures were incubated exactly as described for *B* and *C* in Fig. 5, except that [^{3}H]leucine, 20 Ci/mmol, was used in place of [^{14}C]leucine. The reactions were chilled and centrifuged for 2 hr at 100,000 × *g*. The supernatant solutions, containing globin peptides, were dialyzed for 24 hr against three changes of distilled water. 19 μg of globin peptides were mixed with 12 μg of authentic [^{14}C]globin and subjected to electrophoresis for 15 hr at 4.5 V/cm on 20-cm acrylamide gels prepared in phosphate buffer containing 0.1% sodium dodecyl sulfate. Sectioned gels were crushed automatically, suspended in 5 ml of a solution containing 5 g of 2,5-diphenyloxazole and 100 g of napthalene dissolved in 1 liter of dioxane (6), and their radioactivity was determined. ●——●, [^{14}C]globin; O- - -O, [^{3}H]peptides. Migration is from the anode, which is on the left side of the figure.

NaF to the reaction. By contrast, anisomycin, an inhibitor of chain elongation in animal cells (3, 13, 14), inhibits globin synthesis immediately after addition to the reaction.

The effect of ATA on the synthesis of globin peptides is also demonstrated by the experiment shown in Fig. 5. Fig. 5*A* shows the presence of nascent peptides bound to polyribosomes 1 min after the addition of [^{14}C]leucine. Only 30% of the radioactivity that was recovered from these ribosomes after digestion with pancreatic RNase, had the same electrophoretic mobility as globin, indicating that 70% of the peptides were incomplete at this stage of the reaction. Incubation of the uninhibited control for 3 min longer (4-min total; Fig. 5*B*) resulted in a net increase in ribosomes involved in protein synthesis, ribosome-bound peptides, and completed globin chains. When ATA was added after the initial 1 min incubation, and the incubation was continued for an additional 3 min, 80 S ribosomes were sequentially released, initially from the larger polyribosome complexes (Fig. 5*C*). There is an increase in the amount and specific activity of the peptides that remain bound to the ribosome; this increase is accounted for by the addition of amino acid residues to existing peptide chains. At lower concentrations of ATA (10^{-4} M), breakdown of polyribosomes and peptide release was complete in 3 min, indicating that the higher concentrations of dye affect chain elongation as well as initiation. Centrifugation of extracts for extended peroids of time revealed that the number of ribosomal subunits had increased 2- to 3-fold in the presence of ATA.

Peptides released from the ribosomes during incubation with ATA were indistinguishable from those isolated from untreated controls, as tested by acrylamide gel electrophoresis (Fig. 6). The two radioactive peptides separated by gel electrophoresis under these conditions have tentatively been identified as α and β chains of globin (T. Hunt, personal communication).

DISCUSSION

We have previously reported (1) that ATA inhibits the attachment of mRNA to the ribosome. Other effects of ATA include a delay in the onset of inhibition of protein synthesis, the systematic release of ribosomes and peptides from polyribosomes—initially affecting the larger complexes, and an accumulation of ribosomal subunits. Peptides formed in the presence of ATA and subsequently released from the polyribosome appear to be complete, as shown in the present study and, previously, by Webster and Zinder (15). Taken together, these observations distinguish the effects of ATA from those of other reported inhibitors of the initiation of protein synthesis such as pactamycin (16), cycloheximide (17, 18), and sodium fluoride (17, 19).

The effects of ATA on hemoglobin synthesis in reticulocyte lysates resemble those observed in extracts of *E. coli*. Experiments with purified transfer factors and washed reticulocyte ribosomes have shown that ATA has no direct effect on translocation or peptide bond formation (20). Initiation of protein synthesis on certain plant ribosomes, with tobacco mosaic virus as a source of mRNA, is also inhibited by ATA (21). It appears that ATA affects eukaryotes as well as prokaryotes, suggesting that chain initiation in both types of cells involves a cyclic process of release and reattachment of ribosomes to mRNA.

ATA immediately inhibits incorporation of histidine if the dye is added 1.5 min or less after the addition of mRNA to the reaction. The onset of the inhibitory effect is delayed if ATA is added 2 min or longer after chain initiation has begun. Since we found that the average rate of reading of the coat protein cistron was 25–30 amino acids per molecule of RNA per minute, in good agreement with the results of others (15), translational polarity (22, 23) appears to be lost at about the addition of the 40th amino acid of the f2 coat protein. This position lies between the end of the codon for the polar amber mutant, sus 3 (the 6th amino acid) (15) and that of nonpolar amber mutant amB11 (the 50th amino acid) (24). Apparently, ribosomes do not bind to the internal cistron coding for RNA polymerase until translation has progressed to about the 40th amino acid (see ref. 25 and 31).

The reported effects of ATA as a specific inhibitor of the binding of mRNA to bacterial ribosomes (1), and, thereby, of initiation, have been confirmed by Webster and Zinder (15) and by Wilhelm and Haselkorn (26). Other reports suggest that ATA may also inhibit the transfer factor, T_s (27), the charging of tRNA (28), ribosome-dependent GTP hydrolysis (28), and ribonuclease V activities (28, 29). These effects do not occur during translation of natural mRNA, since chain elongation is unaffected by inhibitory concentrations of the dye, as shown in the present and other (1, 15) studies. ATA binds strongly to various proteins, including bovine serum albumin (30), and induces conformational changes in certain ribosomes (1). Binding of the dye to protein factors or to ribosomes, thereby compromising their function, may account for the observed effects on isolated preparations. Ribosomes are not susceptible to the effects of ATA when complexed to natural mRNA (1, 20), and enzymes and transfer factors may be similarly protected when engaged in protein synthesis.

We conclude that ATA selectively inhibits the initiation of protein synthesis in cell-free extracts prepared from *E. coli* and from rabbit reticulocytes at concentrations of dye that do not affect chain extension. The unique properties of ATA should prove useful in studying molecular events involved in the initiation of protein synthesis in bacteria and eukaryotes.

We thank Dr. Irving H. Goldberg for allowing some of these experiments to be performed in his laboratory and Dr. Tim Hunt for showing us his procedure for the acrylamide gel electrophoresis of globin.

Supported by grants from the USPHS (CA-10666) and the American Cancer Society. Number 3 in a series of papers on the inhibition of attachment of mRNA to ribosomes; the preceding paper is ref. 2. This paper is communication 215 from the Joan and Lester Avnet Institute of Molecular Biology.

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