INHIBITION OF THE ATTACHMENT OF MESSENGER RIBONUCLEIC ACID TO RIBOSOMES*

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In viral infections, messenger RNA (mRNA) copied from the virus genome attaches to the polyribosome complex of the host cell and directs the synthesis of viral proteins.[1-3] Inhibition of attachment represents one of several mechanisms by which virus replication might be prevented without interfering with the metabolism of the host cell. Since attachment of mRNA to ribosomes might be inhibited by small molecules, a number of organic compounds were examined for this activity. Several triphenylmethane dyes, including aurintricarboxylate (ATA), were found to be active in cell-free systems and appear to be novel inhibitors of mRNA translation. The binding of bacteriophage f2 RNA to *E. coli* ribosomes was used as a model system to study these interactions.

Materials and Methods.—Materials: The ammonium salt of aurintricarboxylic acid[4] was synthesized by the method of Smith *et al.*[5] and was isolated by repeated dissolution in acetone and precipitation with benzene. This material was further purified by paper chromatography in a solvent system composed of ethyl acetate-pyridine-water (55:25:20). Adenosine 5'-triphosphate (ATP) and guanosine 5'-triphosphate (GTP) were purchased from Pabst Biochemicals, *E. coli* B transfer RNA (tRNA) from Nutritional Biochemicals, pancreatic deoxyribonuclease from Worthington Biochemical Corporation, N^5-formyltetrahydrofolic acid from Lederle Laboratories, bovine serum albumin from Mann Research, and pyruvate kinase from Boehringer und Soehne. C^{14}-lysine and P^{32}-phosphoric acid were products of New England Nuclear Corporation; formylmethionyl-$tRNA_F$, labeled in the methione residue, was prepared as described by Salas *et al.*[6] Strains of *E. coli* Q13 were obtained from Dr. Jerard Hurwitz. *E. coli* MRE 600 was donated by Dr. Maxine Singer.

Phage RNA: Bacteriophage f2 was grown and purified as described by Webster *et al.*,[7] with *E. coli* Q13 as host cell. P^{32}-labeled bacteriophage f2 was prepared according to Takanami *et al.*[8] RNA was extracted from purified bacteriophage by the procedure of Shimura *et al.*,[9] and was lyophilized and stored at $-20°$. P^{32}-Q_β RNA, prepared as described by Weissmann and Feix,[10] was provided by Dr. Albert Wahba.

E. coli extracts and ribosomes: Extracts of *E. coli* MRE 600 were prepared by the procedure of Nirenberg and Matthaei.[11] Frozen cells were ground with alumina and extracted with a buffer composed of 0.01 *M* tris(hydroxymethyl)aminomethane-HCl (Tris HCl), pH 7.8, 0.014 *M* $MgCl_2$, 0.05 *M* NH_4Cl, and 0.006 *M* 2-mercaptoethanol (buffer A). Deoxyribonuclease (DNA), 2 μg/ml, was added, the extract was centrifuged twice at $30{,}000 \times g$, and the top $^4/_5$ of the supernatant (S-30) was removed. The S-30 extract was incubated for 45 min at 37° with the components required for amino acid incorporation and then was dialyzed overnight against buffer A (preincubated S-30).

Ribosomes were isolated from preincubated S-30 extracts by centrifugation at $105{,}000 \times g$ for 2.5 hr. The upper $^4/_5$ of the supernatant solution (S-100) was dialyzed overnight against buffer A; the lower $^1/_5$ was discarded, and the ribosomal pellet was rinsed and resuspended in buffer A. The isolation of ribosomes purified by *O*-(diethylaminoethyl) cellulose (DEAE-cellulose) chromatography and the isolation of initiation factors used in the experiment shown in Figure 2 have been described by Iwasaki *et al.*[12]

Amino acid incorporation: Peptide synthesis was measured as described by Nathans

et al.[13] The standard incubation mixture contained 0.05 *M* Tris HCl, pH 7.8; 0.05 *M* ammonium chloride; 0.003 *M* ATP; 0.0002 *M* GTP; 0.008 *M* magnesium acetate; 0.01 *M* phosphoenolpyruvate; 4×10^{-5} *M* C^{14}-lysine, 100 mc/mmole; 0.006 *M* 2-mercaptoethanol; 1.0 mg/ml tRNA; 30 μg/ml pyruvate kinase; 30 μg/ml N^5-formyltetrahydrofolate; 0.5 mg/ml f2 RNA; and preincubated S-30 extract equal to $^1/_{10}$ of the total volume. Except where otherwise indicated, the components of the reaction were mixed at 0°, the inhibitor being added after the S-30 extract and before the addition of f2 RNA. After incubation at 37° for 30 min, the reaction was terminated by the addition of an equal volume of 10% trichloroacetic acid (TCA). C^{12}-lysine (100 μg) and bovine serum albumin (200 μg) were added; the reaction mixture was heated at 95° for 15 min and chilled in an ice bath for 15 min. Precipitates were collected on Millipore filters and washed three times with 5% TCA, and the radioactivity was determined in a low-background counter or liquid scintillation spectrometer as previously described.[14]

Results.—Effects of ATA on the binding of mRNA to ribosomes: The effect of ATA on the attachment of P^{32}-f2 RNA to ribosomes in a preincubated S-30 extract is shown in Figure 1. Under these conditions, f2 RNA, which has a sedimentation coefficient of 27*S*, binds to 70*S* particles and to the more rapidly sedimenting ribosomes (Fig. 1*a*). This binding is prevented by the addition of ATA (Fig. 1*b*). The sedimentation coefficient of single ribosomes increases from 70*S* to 76*S* in the presence of ATA, as determined in separate experiments by the band centrifugation technique of Vinograd and Bruner.[15] The binding of H^3-polyuridylic acid to 70*S* ribosomes or to 30*S* subunits can likewise be inhibited by ATA.[16]

Effects of ATA on the mRNA-ribosome-tRNA complex: Purified ribosomes, initiation factors, P^{32}-Q_β RNA, and C^{14}-formylmethionyl-$tRNA_F$ were used to

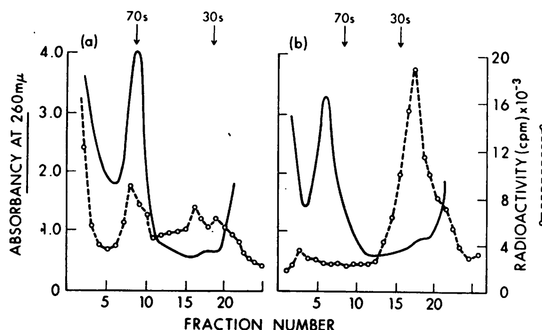

FIG. 1.—Effect of ATA on the attachment of f2 RNA to ribosomes. (*a*) 0.4 ml of preincubated S-30 extract and 50 μg of P^{32}-f2 RNA, spec. act. 2×10^6 cpm/mg, were added to the components of the standard reaction mixture in a final volume of 1.0 ml, as described in *Materials and Methods*. (b) An identical reaction mixture containing 5×10^{-5} *M* ATA.

Reactions were incubated at 37° for 5 min, chilled, and layered over 5–20% sucrose gradients prepared in 0.01 *M* Tris-HCl, pH 7.8, 0.01 *M* Mg acetate, and 0.05 *M* NH_4Cl. Gradients were centrifuged for 2 hr at 30,000 rpm in the no. 30 rotor of a Spinco ultracentrifuge. To determine radioactivity, 1-ml fractions were collected from the bottom of the tube and added to scintillation vials. Absorbancy at 260 mμ was determined during collection of the gradient by means of a flow cell attached to a Gilford spectrophotometer. The sedimentation constants shown in the figure are approximate.

study the effects of ATA on the formation and stability of the initiation complex. This experiment was kindly performed for us by M. J. Miller and A. J. Wahba, who used a recently published procedure.[12] Q_β RNA and formylmethionyl-$tRNA_F$ bind to the 70*S* ribosomes in a complex that is dependent on the addition

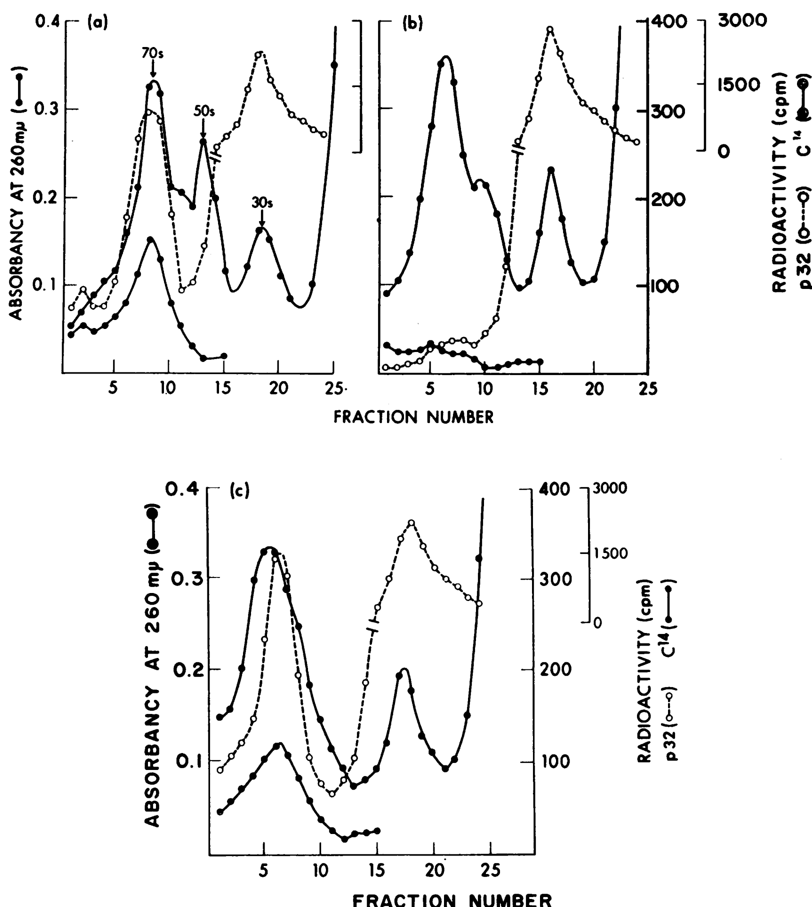

FIG. 2.—Effect of ATA on the formation of the initiation complex. (*a*) The complete reaction mixture was incubated at 37° for 15 min without ATA. (*b*) Ribosomes were preincubated with 1×10^{-4} *M* ATA for 5 min at 0° before the addition of the factors and mRNA, and were incubated at 37° for 15 min. (*c*) The complete reaction mixture was incubated at 37° for 15 min and then cooled to 5°. After 1×10^{-4} *M* ATA had been added, the reaction was allowed to proceed for 5 min at 0°.

All reaction mixtures contained in a volume of 0.125 ml: Tris-HCl buffer, pH 7.8, 50 mM; NH_4Cl, 50 mM; magnesium acetate, 6 mM; 2-mercaptoethanol, 6 mM; GTP, 0.08 mM; tRNA, 125 μg; purified *E. coli* MRE 600 ribosomes, 3.9 A_{260} units; P^{32}-Q_β RNA, 1.0 A_{260} unit (20,000 cpm); formyl-C^{14}-methionyl-$tRNA_F$, 33 μμmoles (7000 cpm); initiation factors F_1 (34 μg), F_2 (9 μg), and F_3 (10 μg). After incubation, 0.1-ml aliquots were layered on 5 ml of a 5–20% linear sucrose gradient in a solution containing 50 mM Tris-HCl buffer, pH 7.8, 60 mM NH_4Cl, and 6 mM magnesium acetate. The samples were centrifuged for 100 min at 39,000 rpm at 4° in the Spinco SW39 rotor. Eight drops of each fraction were analyzed for optical density at 260 mμ, and the remainder was collected for use in the determination of radioactivity.

of initiation factors and crude tRNA (Fig. 2*a*). If ATA is added prior to the addition of Q_β RNA, attachment of mRNA to the ribosomes is prevented (Fig. 2*b*). If Q_β RNA is incubated with ribosomes and formylmethionyl-tRNA prior to the addition of ATA, neither mRNA nor formylmethionyl-tRNA is displaced from the ternary complex (Fig. 2*c*). Q_β RNA does not appear to bind to that fraction of the ribosomes which sediment more rapidly in the presence of ATA. In addition to the increase in the sedimentation rate of single ribosomes, the number of 50*S* ribosomes diminishes in the presence of ATA.

Effects of ATA on the translation of mRNA: An inhibitor of mRNA binding would be expected to decrease the rate of protein synthesis. Some effects of ATA on f2 RNA-stimulated peptide synthesis under various conditions are shown in Figure 3. Peptide synthesis was inhibited by 50 per cent at a 3×10^{-6} *M* con-

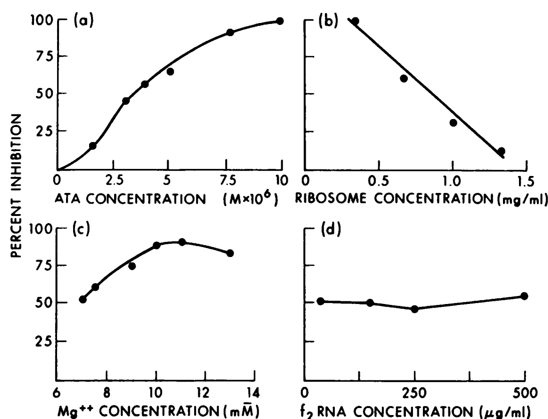

FIG. 3.—Effect of changing various parameters on the ATA inhibition of f2 RNA-stimulated peptide synthesis. (*a*) Amino acid incorporation was measured in a reaction volume of 0.2 ml, as described in *Materials and Methods*. ATA was present at the indicated concentrations. The uninhibited control reaction incorporated 6025 cpm in the presence of f2 RNA. All values were corrected for endogenous protein synthesis which, in the control reaction, was 68 cpm. (*b*) Conditions were as in (*a*), except that 0.1 ml of S-100 and varying concentrations of ribosomes were substituted for the S-30 extract, as indicated; ATA was present at a concentration of 9×10^{-6} *M*. (*c*) Conditions were as in (*a*), except that varying concentrations of Mg^{++} were present, as indicated; ATA was present at a concentration of 6×10^{-6} *M*. (*d*) Conditions were as in (*a*) except that varying concentrations of f2 RNA were present, as indicated; ATA was present at a concentration of 5×10^{-6}*M*.

centration of ATA (Fig. 3*a*). The inhibition observed at a given concentration of ATA was overcome by increasing the concentration of ribosomes (Fig. 3*b*), but not by increasing the concentration of f2 RNA (Fig. 3*d*). The degree of inhibition varied with the concentration of magnesium in the reaction mixture, being maximal at 10 mM (Fig. 3*c*).

The effect of ATA on peptide synthesis was less marked if the ternary complex of ribosomes, mRNA, and tRNA was first allowed to form. This is illustrated by the experiment shown in Table 1, in which ATA was added at various times after peptide synthesis had begun. The rate of synthesis in this experiment was

TABLE 1. *Effect of time of addition of ATA on inhibition of peptide synthesis.*

Time of addition (min)	Rate of incorporation (cpm/ml/min)	Inhibition (%)
0	0	100
10	210	73
15	230	70
25	290	61

Amino acid incorporation was measured in a reaction volume of 1.0 ml, as described in *Materials and Methods*. At the indicated intervals, 5×10^{-5} *M* ATA was added to the reaction mixture, and the subsequent rate of peptide synthesis was determined by removing 0.1-ml aliquots at 5-min intervals. The control reaction showed a linear rate of incorporation of 700 cpm/ml/min during the entire period of the experiment.

only partially inhibited by ATA, although the concentration of ATA was five times higher than that necessary to completely inhibit peptide synthesis when added prior to the addition of mRNA.

Inhibition by ATA was more pronounced when peptide synthesis was stimulated by the addition of exogenous mRNA than when endogenous protein synthesis was allowed to proceed. As shown in Figure 4, inhibition of peptide synthesis stimulated by the addition of f2 RNA to a preincubated S-30 extract was greater than the inhibition of endogenous protein synthesis. Complete inhibition of f2 RNA-directed peptide synthesis was induced by 10^{-5} *M* ATA, a concentration that allows endogenous protein synthesis to continue at 50 per cent of the control rate.

Discussion.—We conclude from these experiments that ATA prevents the attachment of f2 or Q_β mRNA to *E. coli* ribosomes, thereby inhibiting viral peptide synthesis. ATA does not compete with f2 RNA for the mRNA binding site on the ribosome and may inhibit allosterically.[16] The increased sedimentation rate of ATA-treated ribosomes may reflect a conformational change in the ribosome induced by such an allosteric modification.

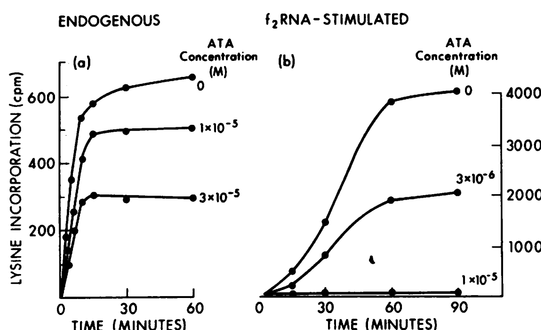

FIG. 4.—Effects of ATA on endogenous and f2 RNA-stimulated peptide synthesis. (*a*) Amino acid incorporation was measured in a reaction volume of 2.0 ml as described in *Materials and Methods*, except that f2 RNA was omitted and the preincubated S-30 was replaced with an extract that was not preincubated. ATA was present at the concentrations indicated in the figure. (*b*) Conditions were as in (*a*), except that the standard reaction mixture contained f2 RNA and preincubated S-30. To determine radioactivity, 0.2-ml aliquots were taken out at the indicated intervals.

The chemical basis for the binding of mRNA to ribosomes of animal and bacterial origin is poorly understood. The studies of Moore[17] suggest that an amino group of ribosomal RNA is involved in mRNA binding and that divalent cations are required only to neutralize the exposed phosphate groups. A different model, involving magnesium bridges between mRNA and ribosomal phosphates, has been proposed by Furano *et al.*[18] A protein factor required for the binding of viral RNA but not of synthetic polynucleotides has also been reported.[12, 19] It is unlikely that ATA interacts with this protein since H^3-ATA binds to ribosomes washed free of initiation factors and inhibits polyphenylalanine synthesis on such particles.[16]

The present studies were undertaken in the hope that inhibition of attachment of viral mRNA would offer a means of preventing virus replication without interfering with the metabolism of the host cell. Evidence is accumulating that such a mechanism accounts for the antiviral activity of interferon.[20-22] Treatment of vaccinia virus-infected L-cells with interferon prevents the attachment of viral mRNA to ribosomes.[20] Ribosomes of interferon-treated cells appear to be altered, possibly by the attachment of a protein.[21, 22] Although restricted in their ability to interact with viral mRNA, these ribosomes are still able to translate host-cell mRNA.[21] A similar selectivity was observed in our experiments. Attachment of viral mRNA to ribosomes was inhibited at concentrations of ATA that interfered minimally with the translation of preattached endogenous mRNA. The demonstrated resistance of the preformed ternary complex to inhibition by ATA may contribute to the greater sensitivity of virus-directed peptide synthesis. It has not yet been determined whether this selective inhibition is manifested *in vivo*.

The significance of our observations lies in the demonstration that a small organic molecule, structurally unrelated to nucleic acids, can inhibit the attachment of natural mRNA to ribosomes. It is firmly established that the replication of animal viruses may be inhibited by chemotherapeutic agents, some of which are clinically effective.[23] The chemical synthesis of compounds related to ATA in structure and sharing its mode of action could potentially lead to the design of novel antiviral agents.

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