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Effects of Aurintricarboxylic Acid on Ribosomes and the Biosynthesis of Globin in Rabbit Reticulocytes

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SUMMARY

The triphenylmethane dye, aurintricarboxylic acid (ATA), inhibits the synthesis of globin in reticulocyte lysates. The principal effect of low concentrations of ATA (0.01–0.1 mM) is on initiation of protein synthesis. This conclusion is based on the following observations: (a) a delay of 1–2 min occurs before inhibition of globin synthesis by ATA is observed; (b) complete breakdown of polyribosomes is induced by ATA; (c) peptide chains, previously identified as completed α and β chains of globin, are completed and released from the ribosome in the presence of the dye; and (d) an increase in the number of ribosomal subunits is observed in the presence of the dye.

ATA inhibits binding of polyuridylic and polycytidylic acids to reticulocyte ribosomes. This observation is in accordance with the view that a primary action of the dye is the inhibition of attachment of mRNA to ribosomes. Translocation, peptide bond formation, and rate of release of nascent globin peptides are unaffected by 0.1 mM concentrations of ATA. The activity of guanosine triphosphatase is inhibited, but only when the enzyme is assayed as an isolated preparation. If ATA is incubated with guanosine triphosphatase in the presence of all components required for amino acid polymerization, hydrolysis of GTP is not affected.

High concentrations of ATA (1 mM) prevent chain elongation. Under these conditions, breakdown of polyribosomes is incomplete and single ribosomes unfold, forming discrete particles sedimenting at 54–56 S. Such unfolding can be prevented by increasing the concentration of magnesium ions. ATA also induces a decrease in the sedimentation rate of polyribosomes, single ribosomes, and the smaller ribosomal subunit.

[^{3}H]ATA binds to polyribosomes and 80 S and 40 S particles, but not to the 60 S subunit. Taken together, our results suggest that the dye binds to the 40 S ribosomal subunit, preventing the subsequent attachment of mRNA. As a consequence of this action, ATA inhibits initiation of globin synthesis in reticulocyte lysates.

INTRODUCTION

Previous communications from this laboratory (1, 2) have shown that the triphenylmethane dye, aurintricarboxylic acid, prevents attachment of natural and synthetic

These investigations were supported in part by National Institutes of Health Grants CA-10666 and GM-00065 and by American Cancer Society Grant T-418. This is Publication 243 from the Joan and Lester Avnet Institute of Molecular Biology and is the fourth in a series of papers on the inhibition of attachment of mRNA to ribosomes; the preceding paper is ref. 2. A preliminary report of this work was presented before the American Society of Biological Chemists [*Fed. Proc.* **29**, 537 (1970)].

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mRNA to *Escherichia coli* ribosomes and, as a consequence, inhibits initiation of protein synthesis. In the present paper, we show that ATA[2] has similar effects on the synthesis of globin in extracts prepared from reticulocyte ribosomes. Our results indicate that elongation of nascent globin chains is relatively unaffected by ATA at concentrations of dye that inhibit initiation of new peptides. Reticulocyte ribosomes and subunits treated with low concentrations of ATA are unable to bind synthetic polynucleotides and, at higher concentrations, unfold reversibly to form discrete particles with lower sedimentation coefficients. The effects of ATA can be distinguished from those of cycloheximide, sodium fluoride, pactamycin, and other agents that are believed to inhibit initiation of protein synthesis in animal cells.

EXPERIMENTAL PROCEDURE

Preparation of rabbit reticulocytes. Reticulocytosis was induced in New Zealand rabbits by a slight modification of the protocol originally described by Borsook *et al.* (3). Acetophenylhydrazide (15 mg/kg) was injected subcutaneously on the first day, 10 mg/kg were injected on each of the next 3 days, and blood was obtained by cardiac puncture on the sixth day. Under these conditions, 95% of the erythrocytes obtained were present in the form of reticulocytes. Plasma was separated from packed reticulocytes by centrifugation at 1500 × *g* for 15 min at 4°; the cells were suspended in a solution composed of 0.13 M NaCl, 5 mM KCl, and 7.5 mM $MgCl_2$, and washed twice with the same solution.

Isolation of ribosomes. Crude ribosomes were prepared from reticulocytes by a modification (4) of the procedures described by Allen and Schweet (5) and by Grayzel, Horchner, and London (6). Ribosomes were further purified according to the method of Felicetti and Lipmann (7) as follows. Crude ribosomes (20 mg), suspended in 10 ml of a buffer (0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4, and 2 mM $MgCl_2$) were layered onto 10 ml of a solution composed of 1 M sucrose, 0.05 M Tris-HCl (pH 7.4), and 2 mM $MgCl_2$ and centrifuged at 78,480 × *g* for 10 hr at 4° in a Spinco No. 30 rotor. The ribosomal pellets were resuspended in a buffer composed of 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.4), 10 mM $MgCl_2$, and 0.5 M NH_4Cl, at a concentration of approximately 1 mg/ml, stirred for 20 min at 4°, and centrifuged at 30,000 rpm for 3 hr. This process was repeated twice, and the washed ribosomes were finally resuspended in a buffer composed of 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.4), and 2 mM $MgCl_2$, at a concentration of approximately 10 mg/ml, and stored in liquid nitrogen. Ribosomes prepared in this manner are free from TF-I and TF-II activity.

Partial purification of transfer factors. Crude reticulocyte lysates were prepared and a 40–70% ammonium sulfate fraction was obtained, as described by Allen and Schweet (5). Partial resolution of TF-I and TF-II from this fraction was achieved by a slight modification of the procedure described by McKeehan and Hardesty (8). Sucrose was added to the 40–70% ammonium sulfate fraction at a final concentration of 10% (w/v). The suspension was clarified by centrifugation at 12,000 × *g* for 10 min, and 20 ml of the supernatant solution, containing 1 g of protein, were applied to a 15 × 5 cm column of Sephadex 4B. This column had previously been washed for 24 hr with a buffer composed of 10 mM Tris-HCl (pH 7.5), 6 mM 2-mercaptoethanol, and 0.2 mM EDTA. Enzymes were eluted with the buffer used for washing the column, at a flow rate of 40 ml/hr. Fractions eluted between 560 and 680 ml of buffer contained TF-I activity, and fractions containing primarily TF-II activity were eluted between 830 and 940 ml. The eluate containing TF-I activity was concentrated by ultrafiltration to approximately 10 mg/ml of protein and dialyzed twice for 18 hr against 2 liters of 0.02 M Tris-HCl, pH 7.5. This enzyme could be frozen and stored at −40° without loss of activity. The eluate containing TF-II activity was concentrated in a similar manner, dialyzed for 18 hr against a buffer containing 20 mM Tris-HCl (pH 7.4), 0.2 mM

[2] The abbreviations used are: ATA, aurintricarboxylic acid; TF-I, aminoacyl transfer ribonucleic acid-binding enzyme; TF-II, aminoacyltransferase II.

EDTA, and 4 mM glutathione, and stored at −90° in liquid nitrogen.

Measurement of globin biosynthesis by reticulocyte lysates. Lysates were prepared by adding an equal volume of 1 mM $MgCl_2$ to a packed suspension of reticulocytes and shaking gently for 2 min. Cell membranes and debris were removed by centrifugation for 10 min at 10,000 rpm in a Sorvall SS-34 rotor; the crude lysate was divided into small aliquots and stored in liquid nitrogen.

Biosynthesis of globin was measured, as described by Maxwell and Rabinovitz (9), by modification of the method originally described by Zucker and Schulman (10) and by Adamson, Herbert, and Godchaux (11). The standard reaction mixture contained 10 mM Tris-HCl (pH 7.4), 75 mM KCl, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 2 mM magnesium chloride, 2 μM [^{14}C] leucine (315 Ci/mole), 6 mM 2-mercaptoethanol, a 0.1 mM mixture of 19 amino acids (4), 0.9 mg/ml of creatine phosphokinase, 60 μM hemin, and 0.10–0.13 ml of lysate in a final volume of 0.5 ml. Following incubation at 33°, aliquots (50 μl) were removed from the reaction mixture and added to 3 ml of 5% trichloroacetic acid. The solution was heated for 15 min at 95° and then chilled in an ice bath. Precipitates were collected on Millipore membrane filters and washed three times with cold 5% trichloracetic acid, and the radioactivity was determined as described below.

Density gradient centrifugation. Density gradient centrifugation was performed according to the procedure of Britten and Roberts (12). Samples of 1 ml were layered over 36 ml of 10–25% (w/v) sucrose gradients prepared in "reticulocyte standard buffer" (13), composed of 0.01 M Tris-HCl (pH 7.4), 0.01 M KCl, and 1.5 mM $MgCl_2$. All gradients were centrifuged at 4° for various lengths of time at 25,000 rpm (average force, 80,900 × *g*) in a Spinco SW 27 swinging bucket rotor, and the absorbance at 260 nm was determined during collection of the gradient by means of a flow cell (light path-0.5 cm) attached to a Gilford spectrophotometer. Fractions of 1.2 ml were collected from the bottom of the tube for determination of radioactivity.

Determination of radioactivity. Membrane filters containing ^{14}C were dried, and their radioactivity was determined in a low-background counter (counting efficiency, 20%) or by liquid scintillation counting in a toluene solution containing 0.5% 2,5-diphenyloxazole and 0.003% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene efficiency, 80%). Filters containing tritium were treated with 0.6 ml of NH_4OH for 15 min and dissolved in Bray's solution (14) (efficiency, 20%). Aqueous samples containing tritium were counted by adding 10 ml of Bray's solution (efficiencies, 10–60%).

Preparation of aurintricarboxylic acid. None of the published procedures for the synthesis of ATA (15–19) includes a satisfactory chemical characterization of the reaction product. The experiments reported in this paper were performed with a single lot of aluminon, purchased from Aldrich Chemical Company.[3] This lot (No. 761145) was equally active, on a weight basis, with material used in this laboratory for earlier studies (1, 2, 20). The average molecular weight of aluminon is assumed to be 475.

[^{3}H]ATA was prepared as described by Welcher (18). Sodium nitrite, 0.4 g, was dissolved in 4.4 ml of cold concentrated sulfuric acid, and 1.2 g of salicylic acid were slowly added, with vigorous stirring, over a period of 10 min. Then 10 μl (0.12 mg) of [^{3}H]formaldehyde (specific activity, 125 Ci/mole) were added, in an ice-water bath, to 200 μl of the sulfuric acid–sodium nitrite

[3] The sodium salt of aurintricarboxylic acid is marketed under the name chrome violet (C.I. 43810), and the ammonium salt is known as aluminon, a reagent used in analysis of aluminum (18). Preparations of chrome violet and aluminon obtained from Aldrich, Baker, Fisher Scientific, Eastman Kodak, Sandoz, K & K Laboratories, Geigy, and several other commerical sources proved to be grossly impure and vary widely in their ability to inhibit protein synthesis. Certain samples had little inhibitory activity at concentrations as high as 100 μg/ml. The sample used for the experiments reported in this paper inhibited globin synthesis by 50% at a concentration of 3 μg/ml. Preparations of aluminon have also been reported to vary in their suitability as analytical reagents for the detection of aluminum (19).

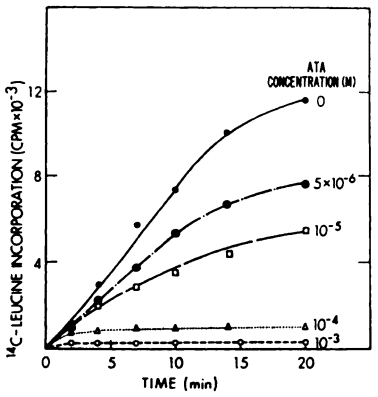

FIG. 1. *Effect of various concentrations of ATA on synthesis of globin*

Synthesis of globin in reticulocyte lysates was measured in the presence of the indicated concentrations of ATA, using the standard reaction mixture (0.5 ml) described under EXPERIMENTAL PROCEDURE. [^{14}C]-Leucine incorporation represents hot trichloroacetic acid-insoluble radioactivity found in a 50-μl aliquot.

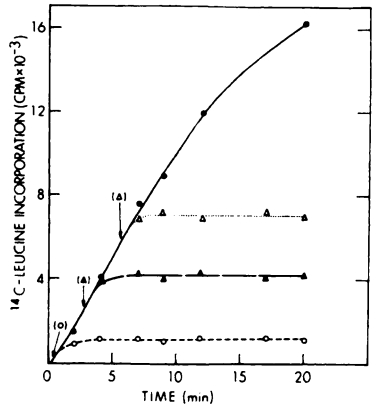

FIG. 2. *Effect of ATA, added at various times, on synthesis of globin*

ATA, at a final concentration of 0.1 mM, was added to the standard reaction mixture at three different times, as indicated by the arrows. Synthesis of globin was measured as described under EXPERIMENTAL PROCEDURE. [^{14}C]-Leucine incorporation represents hot trichloracetic acid-insoluble radioactivity found in a 50-μl aliquot.

solution. The reaction mixture was vigorously mixed for several minutes and allowed to stand at 4° overnight. The resulting red precipitate was collected by centrifugation, washed three times with 0.5 M HCl and twice with water, and dried under vacuum over P_2O_5. The final yield was 25 mg of crude ATA; specific activity, 3.5 μCi/μmole.[4] This material displayed numerous components when subjected to paper chromatography in a solvent system composed of ethyl acetate–pyridine–water (55:25:20), identical with those found in the sample of aluminon obtained from Aldrich Chemical Company. The synthetic sample of [^{3}H]ATA inhibits globin synthesis by 50% at a concentration of 3 μg/ml.

To determine whether inhibition of protein synthesis by aluminon could be due to the presence of a contaminating product of the synthetic procedure, Dr. A. Focella, Chemical Research Department, Hoffman–La Roche, Inc., prepared a chemically pure sample of aurintricarboxylic acid. The procedure differs from those reported (19) in that the intermediate, methylenedisalicylate, was purified prior to the condensation reaction.

Dimethyl 5,5-methylenedisalicylate (compound I) (21) was saponified with 10% sodium hydroxide at 90° for 30 min and acidified. The resulting amorphous precipitate was dissolved in ether and precipitated again with petroleum ether. The product, methylenedisalicylic acid, melted at 265–267°.[5] Infrared, ultraviolet, and NMR spectra were compatible with the proposed structure, analysis for carbon and hydrogen agreed with ±0.4% of the theoretical values, and two carboxyl groups could be titrated with sodium hydroxide.

Methylenedisalicylic acid was condensed with salicylic acid in the presence of a mixture of sodium nitrite and concentrated sulfuric acid, according to the procedure of Smith *et al.* (19). The reaction product was dissolved in the minimum volume of acetone, filtered free of insoluble material, and diluted with 3 volumes of benzene. The precipitate formed was removed by filtration, and the filtrate was evaporated under reduced pres-

[4] Tritium is presumably incorporated into several positions in the aromatic ring by this procedure. Since the product is impure, binding studies using [^{3}H]-ATA are reported as counts per minute bound.

[5] Melting points for this compound have been reported to be 238° (22) and 247° (19).

sure to 50% of its original volume. The resulting brick-red, amorphous solid was vacuum-dried at 56° for 4 hr to yield pure aurintricarboxylic acid (II). This product exhibited a single component (R_f 0.57), as detected by thin-layer chromatography on silica gel using dimethylformamide–methanol–acetic acid (10:8:3) as a solvent system. Analysis for carbon and hydrogen agreed within ±0.4% of the theoretical values. Ultraviolet spectra of this material in 2-propanol displayed peaks at 232 (ϵ = 32,500), 280 (ϵ = 10,800), 303 (ϵ = 13,250), 428 (ϵ = 2,500), and 550 nm (ϵ = 6,100). The NMR spectrum was compatible with the structure assigned. Gradual decomposition of compound II was noted even after storage in the dark under vacuum.

Further identification of compound II was obtained by catalytic reduction to tris(3-carboxy-4-hydroxyphenyl)methane (III). A solution of compound II, prepared in 50% ethanol, was subjected to catalytic hydrogenation on carbon in the presence of 10% palladium at 1000 psi at room temperature. The catalyst was filtered, the colorless filtrate was evaporated, and the residue was crystallized from acetic acid (m.p. 298°). A single component (R_f 0.9) was detected by thin-layer chromatography on silica gel using a solvent system composed of ether–acetic acid–methanol (120:60:18:1). Infrared, ultraviolet, and NMR spectra were compatible with the assigned structure.

Compounds II and III inhibited globin synthesis by 50% at concentrations of 15 and 40 μg/ml, respectively, when assayed in the standard reaction mixture described above. Compound I had no inhibitory activity when tested at a concentration of 100 μg/ml.

Materials. Fusidic acid was obtained through the courtesy of Dr. W. O. Godfredsen of Leo Pharmaceutical Products and pactamycin was a gift from Dr. I. H. Goldberg. Anisomycin was obtained from Pfizer Laboratories; cycloheximide, from Nutritional Biochemicals Corporation; creatine phosphate and creatine phosphokinase, from Boehringer/Manneheim Corporation; and [^{3}H]polyuridylic acid (78.1 Ci/mole of phosphorus), [^{3}H]polycytidylic acid (67.3 Ci/mole of phosphorus), polyuridylic acid, and polycytidylic acid, from Miles Laboratories, Inc. [^{14}C]Leucine (315 Ci/mole), [^{14}C]L-phenylalanine (194 Ci/mole), [^{14}C]L-phenyl-

TABLE 1

Effects of ATA, cycloheximide, pactamycin, and sodium fluoride on binding of synthetic polynucleotides to ribosomes

Each reaction mixture contained $MgCl_2$, 0.008 M; KCl, 0.06 M; Tris-HCl, pH 7.5, 0.025 M; ammonium chloride-washed ribosomes, 33 μg; and [^{3}H]poly U, 0.5 μg (36,070 cpm), or [^{3}H]poly C, 1.7 μg (155,424 cpm), in a volume of 0.25 ml. Inhibitors were added to the reaction mixture after the ribosomes and before the addition of [^{3}H]poly U or [^{3}H]poly C. Samples were incubated at 36° for 10 min, filtered through a Millipore membrane filter, and washed seven times with a buffer containing the same ionic composition as that used in the reaction mixture. Filters used in this experiment were first treated by immersion in 0.5 M NaOH at 23° for 30 min, then rinsed with distilled water, and stored in 0.1 M Tris-HCl, pH 7.4. Filters thus treated retain ribosomes, but not poly U (23) or poly C. In the absence of ribosomes, 543 or 349 cpm of poly U and poly C, respectively, were bound to the filter.

Inhibitor added	Inhibitor concentration	[^{3}H]Poly U bound	Inhibition	[^{3}H]Poly C bound	Inhibition
	M	*cpm*	%	*cpm*	%
None		12,479		23,556	
ATA	1×10^{-4}	1,130	91	5,573	76
	1×10^{-5}	1,574	87	14,246	40
	1×10^{-6}	10,171	19	22,227	6
	3×10^{-7}	11,362	9	23,045	2
Cycloheximide	1×10^{-3}	13,515	0	20,655	12
Pactamycin	1×10^{-4}	11,391	9		
Sodium fluoride	1×10^{-2}	12,667	0		

alanyl-tRNA (1.91 μCi/mg), and [^{14}C]L-leucyl-tRNA (0.355 μCi/mg) were products of New England Nuclear Corporation. The sources of acetophenylhydrazide, nucleotides, salts, buffers, scintillators, and other reagents used in these experiments were described previously (4).

RESULTS

Effects of ATA on rate of synthesis of globin. As shown in Fig. 1, ATA inhibits the synthesis of globin in crude reticulocyte lysates. At 1 mM ATA, inhibition occurs without delay; at 0.1 mM and lower concentrations of dye, there is a relative lag before the inhibitory effect is observed. As shown in Fig. 2, this delay does not depend on the time of addition of the inhibitor. The following experiments were designed to determine which of the established individual steps in globin synthesis are specifically affected by ATA.

Effects of ATA on binding of synthetic polynucleotides to ribosomes. ATA prevents attachment of polyuridylic acid and polycytidylic acid to washed reticulocyte ribosomes, as determined by measuring retention of the polynucleotide-ribosome complex on membrane filters (Table 1). Inhibition of polynucleotide attachment by ATA is inversely proportional to the number of ribosomes in the reaction at ATA to ribosome

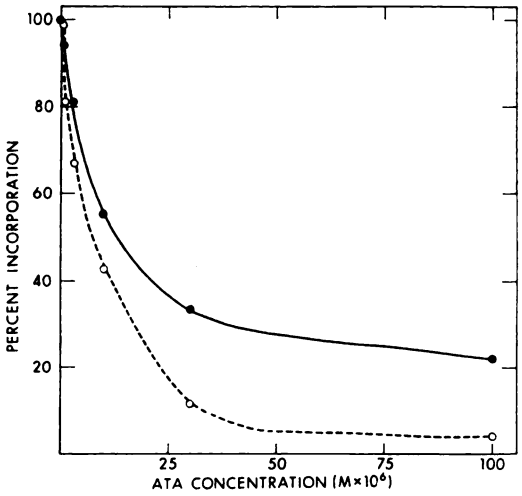

FIG. 3. *Effect of ATA on synthesis of polyphenylalanine*

The reaction mixture (0.125 ml) contained 0.025 M Tris-HCl (pH 7.4), 0.06 M KCl, 0.008 M $MgCl_2$, 0.016 M dithiothreitol, 0.008 M GTP, 200 μg/ml of poly U, 224 μg/ml of *E. coli* [^{14}C]-phenylalanyl-tRNA (4312 cpm), 100 μg of washed ribosomes, 20 μg of TF-I, and 20 μg of TF-II. The final components added to the reaction were ribosomes, poly U, and [^{14}C]-phenylalanyl-tRNA, in that order. ATA was added before poly U (O- - -O) or after the addition of [^{14}C]-phenylalanyl-tRNA (●- - -●). Reaction mixtures were incubated for 10 min at 37°, and reactions were stopped by the addition of 5% trichloracetic acid. Hot acid-insoluble radioactivity was determined as described under EXPERIMENTAL PROCEDURE for measuring the synthesis of globin. In the uninhibited control, 2442 cpm were incorporated.

TABLE 2

Effect of ATA on translocation and peptide bond formation

The first incubation mixture (0.25 ml), containing 0.06 M Tris-HCl (pH 7.4), 0.07 M KCl, 0.004 M $MgCl_2$, 0.01 M dithiothreitol, 0.2 mM GTP, and 500 μg of ammonium chloride-washed ribosomes, was incubated at 37° for 15 min, after which the reaction was stopped by chilling at 0°. [^{14}C] Leucyl-tRNA, 15 μg (9000 cpm); TF-I, 40 μg; NaF, 8 μmoles, and the indicated antibiotic were added. Sufficient Tris, KCl, $MgCl_2$, GTP, and dithiothreitol were also added to maintain their original concentration in the reaction mixture, the final volume being 0.5 ml. This second reaction mixture was incubated at 37° for 20 min, and incorporation of [^{14}C]leucine into hot trichloracetic acid-insoluble peptide was measured as described under EXPERIMENTAL PROCEDURE. TF-II (40 μg) was added before the first or second incubation as indicated below.

Inhibitor added	Inhibitor concentration	Inhibitor added after translocation[a]		Inhibitor added before translocation[b]	
		Leucine incorporation	Inhibition	Leucine incorporation	Inhibition
	mM	*cpm*	%	*cpm*	%
None		940		942	
ATA	0.1	1669	0	1379	0
Cycloheximide	1.0	1062	0	513	45
Anisomycin	0.1	110	88	73	92

[a] TF-II was added before the first incubation.

[b] TF-II was added before the second incubation.

TABLE 3

Effect of ATA on activity of GTPase

All reaction mixtures contained, in a final volume of 0.125 ml, 0.05 M Tris-HCl (pH 7.4), 0.08 M KCl, 8 mM $MgCl_2$, 8 mM dithiothreitol, and 4 μM [^{32}P]GTP (168 Ci/mole). Ribosomes, 200 μg; TF-I, 50 μg; TF-II, 70 μg; polyuridylic acid, 25 μg; and phenylalanyl-tRNA, 30 μg, were present as indicated below. Reaction mixtures were incubated at 37°, and reactions were terminated by addition of 2.5 ml of 0.02 M silicotungstic acid dissolved in 0.02 M H_2SO_4. [^{32}P]Phosphate, liberated by hydrolysis during the first 10 min of incubation, was determined as the phosphomolybdate complex following extraction into 2.5 ml of a 1:1 mixture of isobutyl alcohol–benzene (w/v). An aliquot of the organic phase was evaporated to dryness, and the radioactivity was determined. ATA and fusidic acid were present at final concentrations of 0.1 and 1 mM, respectively.

Components present	Inhibitor added	[^{32}P] Phosphate released	Inhibition
		cpm	%
Ribosomes	None	781	
	ATA	159	80
	Fusidic acid	367	53
TF-II	None	3,302	
	ATA	718	79
	Fusidic acid	1,954	41
Ribosomes, TF-I, poly U, and phenylalanyl-tRNA[a]	None	3,665	
	ATA	2,733	25
	Fusidic acid	3,500	4
Ribosomes and TF-II	None	4,805	
	ATA	3,026	37
	Fusidic acid	3,177	34
Ribosomes, TF-I, TF-II, poly U, and phenylalanyl-tRNA[b]	None	13,163	
	ATA	11,753	11
	Fusidic acid	7,382	44

[a] Components required for binding of aminoacyl-tRNA.

[b] Components required for amino acid polymerization.

molar ratios of less than 220.[6] Cycloheximide, sodium fluoride, and pactamycin, agents that also inhibit initiation of protein synthesis, had no effect on binding of polyuridylic acid to ribosomes in this assay.

Effects of ATA on synthesis of polyphenylalanine and on binding of phenylalanyl-tRNA to ribosomes. Polyphenylalanine synthesis on washed reticulocyte ribosomes was measured using polyuridylic acid as mRNA (Fig. 3). The effect of ATA on polyphenylalanine synthesis is most pronounced when the dye is added prior to the addition of poly U. If the ternary complex of phenylalanyl-tRNA, poly U, and ribosomes is allowed to form before adding ATA, the effect on the reaction is less; however, significant inhibition still occurs.

The effect of ATA on the TF-I-catalyzed binding of phenylalanyl-tRNA to ribosomes was similar to that on polyphenylalanine synthesis. Binding was measured as described by Felicetti and Lipmann (7), and the product was shown to contain less than 1% diphenylalanine and triphenylalanine. ATA inhibits enzymatic binding of phenylalanyl-tRNA to ribosomes by 50% at a concentration of 10 μM if the dye is added to

[6] Calculations of the molar ratio of ATA to ribosomes are based on approximate molecular weights of 475 and 4×10^6, assigned to ATA and reticulocyte ribosomes, respectively. A 1 mg/ml suspension of ribosomes was assumed to have an optical density of 11.2 at 260 nm (24).

the reaction mixture before the addition of polyuridylic acid.

Effects of ATA on translocation and peptide bond formation. Elongation of peptide chains occurs after formation of the initiation complex. Two of the enzymatic reactions required for chain elongation involve translocation of peptidyl-tRNA and formation of the peptide bond. The method of McKeehan and Hardesty (25) was used to determine the effect of inhibitors on these reactions. In this assay, translocation of nascent globin peptide is allowed to take place during the first incubation in the presence of TF-II. Labeled aminoacyl-tRNA, TF-I, and sodium fluoride are then added, and peptide bond formation is permitted to occur during a second incubation. By adding the inhibitor before or after translocation, the assay can distinguish inhibitors of translocation and peptide bond formation. The data shown in Table 2 indicate that ATA has no inhibitory effect on either process when tested at concentrations as high as 0.1 mM. Cycloheximide, tested under conditions that inhibit translocation (25, 26), and anisomycin, an inhibitor of peptide bond formation (27, 28), served as controls in this experiment.

Effects of ATA on activity of guanosine triphosphatase. The effect of ATA on hydrolysis of GTP was assayed under various conditions, as described by Felicetti and Lipmann (7). GTP hydrolysis, associated with TF-II, is inhibited significantly by the dye; however, the degree of inhibition is altered by the presence of additional components (Table 3). Inhibition of GTPase is greatest when the enzymatic activity is determined on the ribosome or TF-II alone; it is least when assayed in the presence of all components required for protein synthesis. The concentration of free ATA in those experiments containing ribosomes is calculated, from data shown in Fig. 10, to be reduced by 40%.

The assay used for these experiments measures the activity of all enzymes that

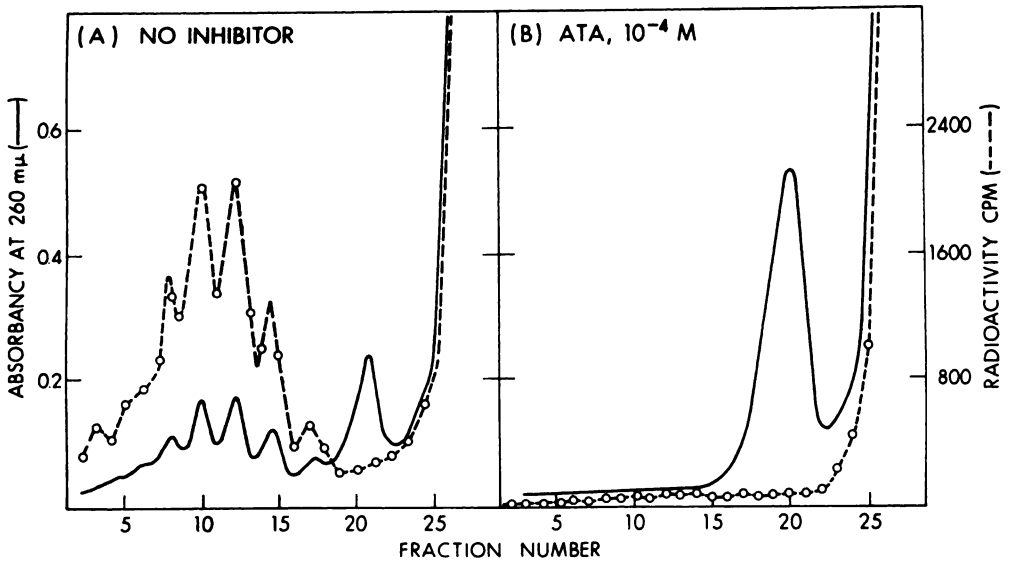

FIG. 4. *Effect of ATA on release of nascent peptides from polyribosomes*

Two reaction mixtures, prepared as described for measuring the synthesis of globin, were incubated for 1 min at 33°; ATA was added to one of these at a final concentration of 0.1 mM, and both were incubated 3 min longer. Reactions were terminated by chilling and diluting the mixtures to 1.0 ml with reticulocyte standard buffer, after which they were layered onto sucrose density gradients. Gradients were centrifuged for 2.5 hr, and fractions were collected as described under EXPERIMENTAL PROCEDURE. Trichloracetic acid was added to each fraction at a final concentration of 5% and heated at 90° for 15 min, and the radioactivity was determined as described under EXPERIMENTAL PROCEDURE.

hydrolyze GTP, including those that are specific for GTP. Nevertheless, fusidic acid, a compound that inhibits the specific GTPase associated with TF-II (7, 29), prevents hydrolysis of GTP under the conditions of this experiment.

Effects of ATA on chain completion and release of nascent peptides from ribosomes.

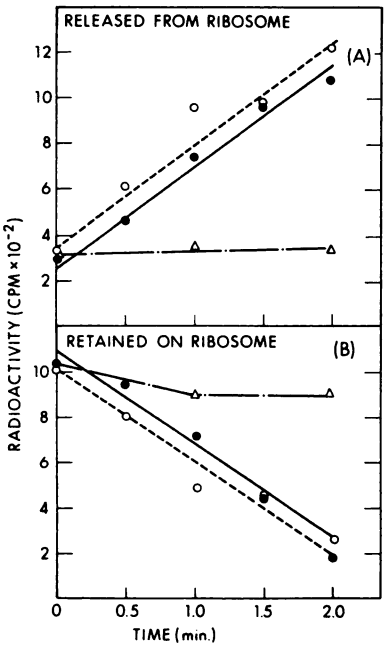

FIG. 5. *Effect of ATA on the completion and release of nascent peptides from labeled ribosomes*

Reaction mixtures, prepared as described for measuring the syntheis of globin, were incubated for 1 min at 33°, and the reactions were terminated by chilling and adding 20 μmoles of [^{12}C]-leucine. ATA or cycloheximide was added at a final concentration of 0.1 or 0.5 mM, respectively, and sufficient amounts of the other reactants were added to maintain their original concentration in the diluted solution. These reaction mixtures were incubated further at 33° and terminated at the times indicated by diluting them to 10 ml with a cold solution composed of 7.5 mM $MgCl_2$, 0.13 M NaCl, and 5 mM KCl. The diluted reaction was centrifuged for 3 hr at 30,000 rpm (average force, 78,480 × *g*) in a Spinco No. 30 rotor. Hot trichloracetic acid-insoluble material in the resulting supernatant solution is indicated as radioactivity "released from ribosome," and that remaining in the ribosomal pellet, as radioactivity "retained on ribosome." ●——●, uninhibited control; ○- - -○, ATA; △-·-·△, cycloheximide.

ATA effects complete dissociation of polyribosomes, releasing radioactive peptides from the ribosome in less than 3 min, when tested at a concentration of 0.1 mM (Fig. 4). During this time, there is an increase in the number of polyribosomes in the uninhibited control reaction (2). Peptides released from the ribosome in the presence of ATA have previously been identified as completed chains of globin (2).

The over-all rate of chain completion and release of nascent peptides from previously labeled reticulocyte ribosomes was determined by experiments in which ribosomes containing nascent globin chains were labeled for 1 min by addition of [^{14}C]leucine. Further initiation was prevented by adding ATA and an excess of unlabeled amino acids. The effects of ATA on release of completed globin chains from the ribosome were measured (Fig. 5A), and the residual peptide bound to the ribosome was simultaneously determined (Fig. 5B). The rate of peptide release was essentially the same in the ATA-treated sample as in the untreated control. Cycloheximide, which inhibits chain elongation and peptide release at the concentration used in this experiment, served as a control. The slight decrease in peptide bound to the ribosomes observed in the presence of cycloheximide is not considered to be significant.

Effects of ATA on polyribosomes, ribosomes and ribosomal subunits. Incubation of reticulocyte lysates in the presence of low concentrations of ATA results in the dissociation of polyribosomes into single ribosomes and ribosomal subunits (Fig. 6B and C). During the same period of time, polyribosomes remain intact in the uninhibited control reaction (Fig. 6A). At ATA concentrations of 0.5 mM or higher, dissociation of polyribosomes is incomplete (Fig. 6D–F). Under these conditions, there is a decrease in the number of single ribosomes, a concomitant increase in particles sedimenting in the 54–56 S region of the gradient, and a small but significant decrease in the rate of sedimentation of single ribosomes and polyribosomes.

The effects of ATA on ribosomal subunits can be seen more clearly when centrifugation of the sucrose gradients is continued for

longer periods of time (Fig. 7). At concentrations of dye that induce complete dissociation of polyribosomes, the number of larger and smaller subunits increases by 2–3-fold (Fig. 7C). Single ribosomes and the smaller subunit sediment more slowly as the concentration of ATA is increased (Fig. 7B and C); the monosomes appear to unfold

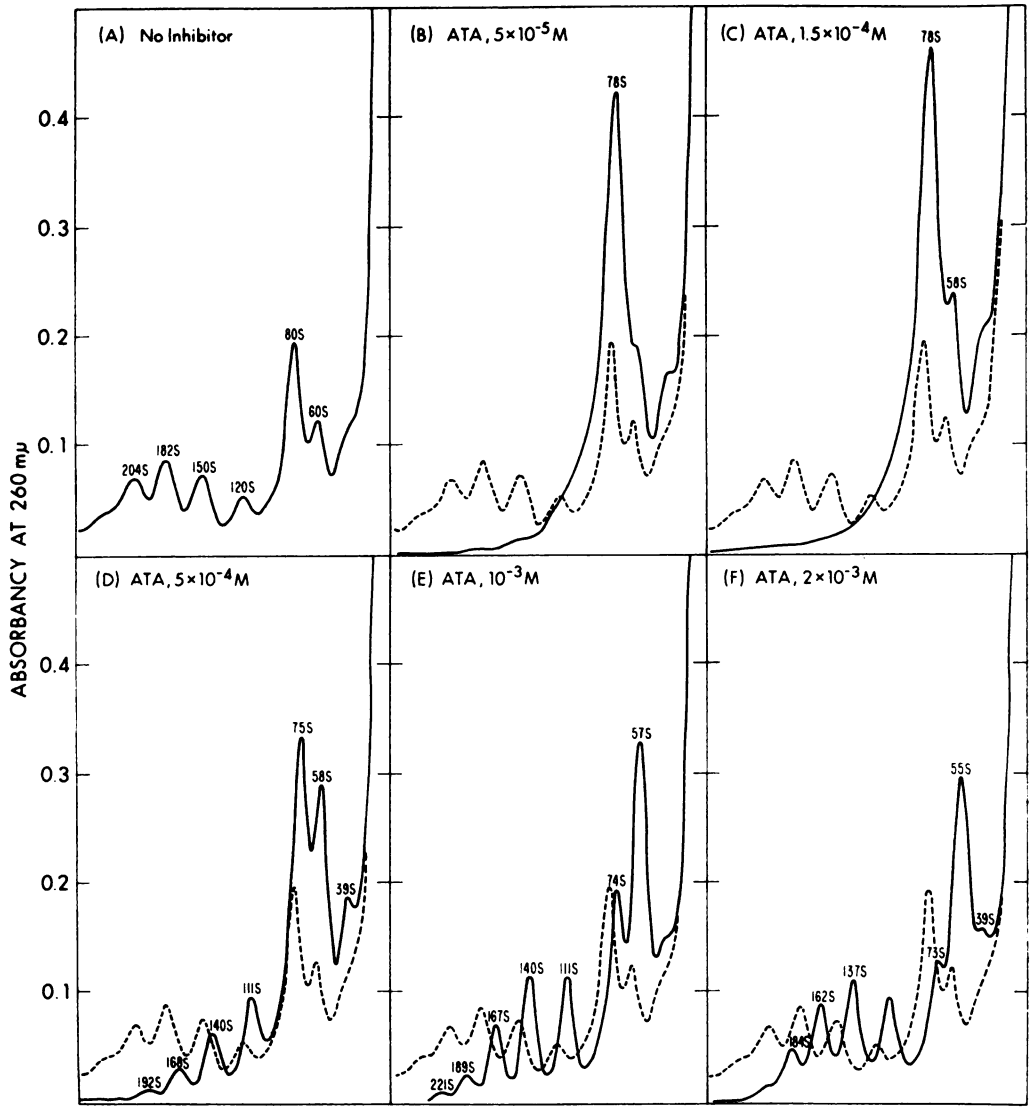

FIG. 6. *Effect of ATA on polyribosomes in reticulocyte lysates*

Reaction mixtures (0.24 ml), containing ATA at the final concentrations indicated in the figure, were prepared as described for measuring the synthesis of globin. These solutions were incubated for 4 min at 37°, the reactions were terminated by chilling and diluting to 1.0 ml with reticulocyte standard buffer, and the solutions were layered onto sucrose density gradients. Gradients were centrifuged for 2.5 hr as described under EXPERIMENTAL PROCEDURE. The dashed lines (B–F) represent the optical density pattern of the uninhibited control, taken from panel A. Sedimentation coefficients of 80, 120, 150, 182, and 204 S for monosomes, disomes, trisomes, tetrasomes, and pentasomes, respectively, were adopted from values given by Hoerz and McCarty (30). A linear relation exists between these assignments and the position of the ribosomes in the sucrose gradient. This relationship was used to estimate the sedimentation coefficients observed in the presence of ATA.

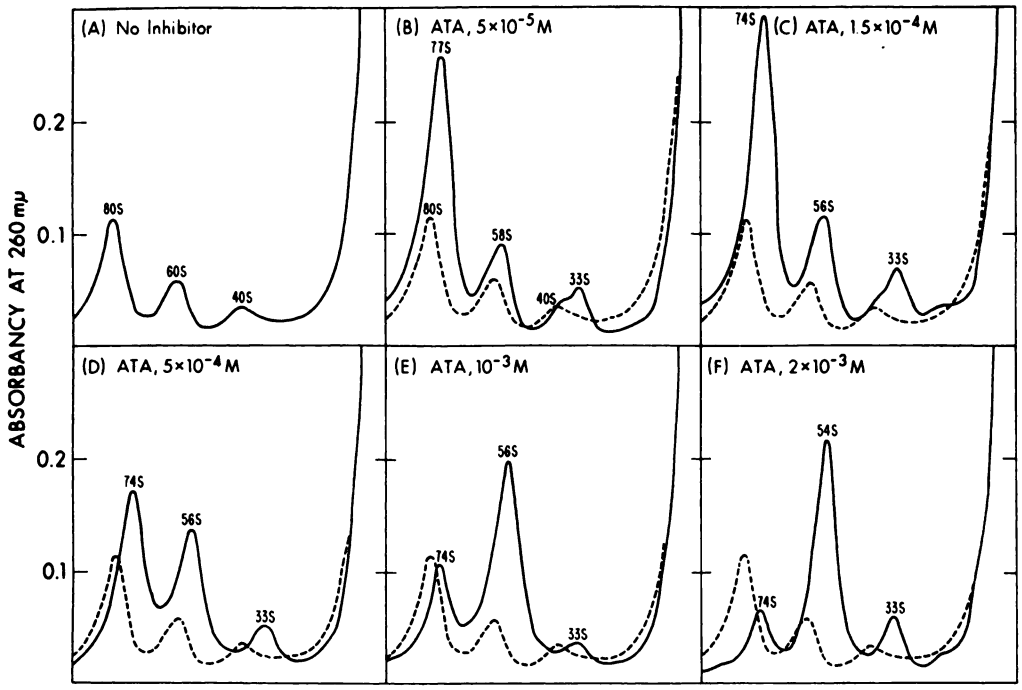

FIG. 7. *Effect of ATA on ribosomes and ribosomal subunits in reticulocyte lysates*

This experiment was performed as described in the legend to Fig. 6, except that the sucrose density gradients were centrifuged for 7.8 hr. The dashed lines (B–F) represents the optical density pattern observed in the uninhibited control (panel A). Sedimentation coefficients of 80, 60, and 40 S were arbitrarily assigned to the monosome and its subunits. Approximate sedimentation coefficients of ATA-treated particles were derived by interpolation, as described in the legend to Fig. 6.

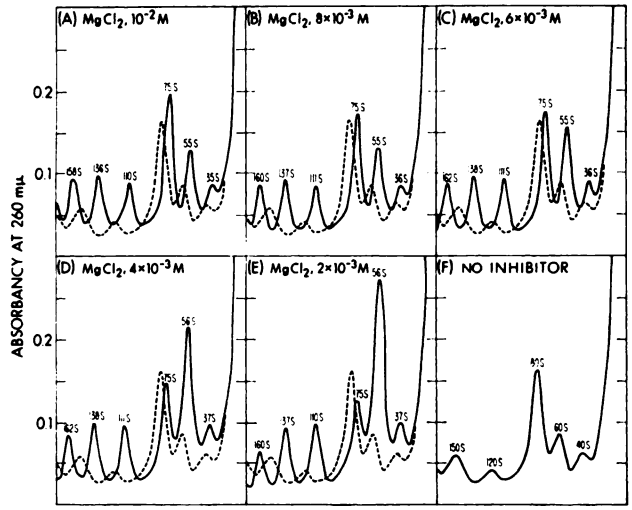

FIG. 8. *Effect of ATA on polyribosomes at various concentrations of magnesium chloride*

The standard reaction mixture (0.24 ml) for the synthesis of globin was prepared as described under EXPERIMENTAL PROCEDURE. The final concentration of magnesium ions in each solution was adjusted to that indicated in the figure. In the experiments shown in panels A–E, ATA was present in the reaction mixture at a final concentration of 2 mM. Solutions were incubated for 4 min at 37°, chilled, diluted to 1 ml with reticulocyte standard buffer, and subjected to density gradient centrifugation for 2.5 hr. The dashed lines (A–E) represent the optical density pattern observed in the uninhibited control (panel F). Approximate sedimentation coefficients were assigned as described in the legend to Fig. 6.

(Fig. 7D–F) as the concentrations of ATA and $MgCl_2$ approach 2 mM.

RNA was extracted from particles sedimenting in the 54–56 S region of the gradient by the addition of sodium dodecyl sulfate and EDTA, and then was analyzed by density gradient centrifugation. From the proportion of 18 S RNA present, it was calculated that 65% of these particles consist of unfolded 80 S ribosomes, the remainder being 60 S subunits.

Unfolding of 80 S ribosomes could not be duplicated by adding EDTA or pyrophosphate to the reaction mixture and to the sucrose gradient. At concentrations of 2 mM, these chelating agents merely served to dissociate polyribosomes into subunits. The ATA-induced conversion of monosomes to 54–56 S particles could be prevented by increasing the concentration of magnesium ions in the reaction prior to the addition of the dye (Fig. 8). The decreased sedimenta-

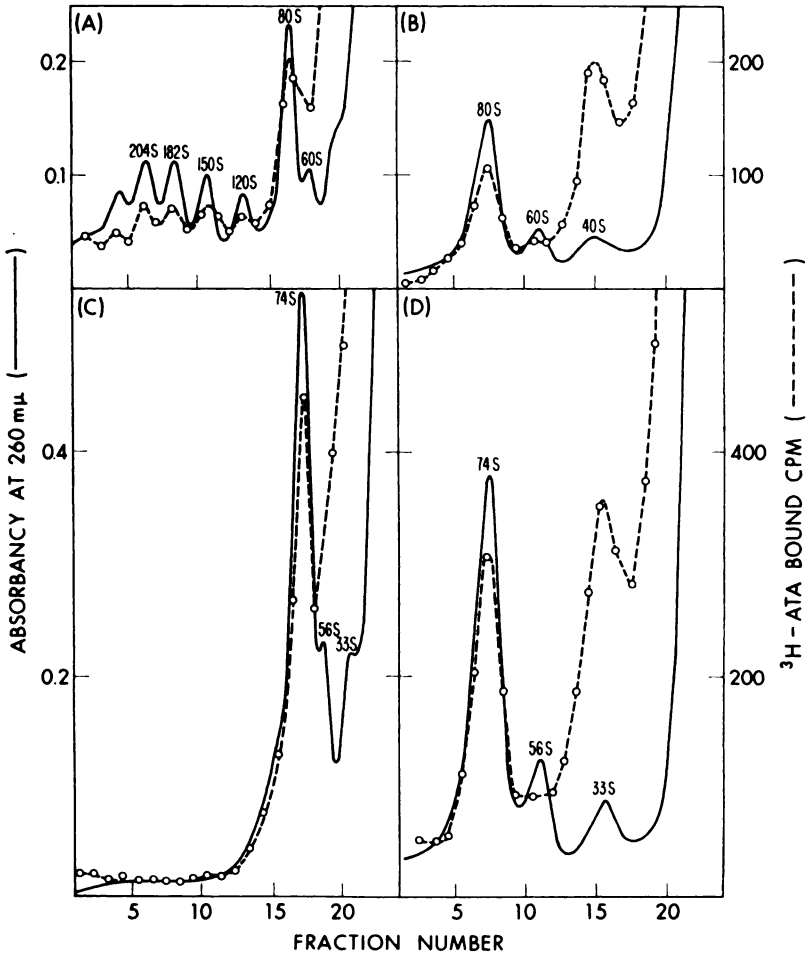

FIG. 9. *Binding of* [^{3}H]*ATA to polyribosomes and ribosomal subunits*

[^{3}H]ATA (25,300 cpm), at a final concentration of 0.1 mM, was added to the standard reaction mixture described under EXPERIMENTAL PROCEDURE. The final volume was 0.24 ml. Mixtures for the reactions shown in panels A and B were chilled immediately, while those for panels C and D were incubated at 35° for 4 min. All four samples were diluted to 1.0 ml by addition of reticulocyte standard buffer, then subjected to sucrose density gradient centrifugation. Gradients shown in panels A and C were centrifuged for 2.5 hr, and those shown in panels B and D, for 7.8 hr. Fractions (1.4 ml) were collected directly into scintillation vials, and the radioactivity was determined as described under EXPERIMENTAL PROCEDURE.

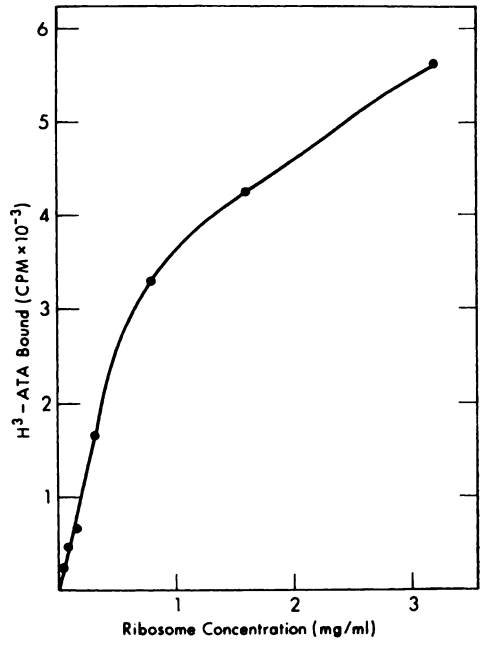

FIG. 10. *Binding of [³H]ATA to ribosomes*

The reaction mixture (0.125 ml) contained 25 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 60 mM KCl, varied concentrations of washed ribosomes, and 80 μM [³H]-ATA (11,093 cpm). Following incubation for 20 min at 0°, ribosomes were collected on Millipore filters, using minimal vacuum, and rapidly washed seven times with 3 ml of buffer composed of 25 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, and 60 mM KCl. The radioactivity retained on the filters was determined as described under EXPERIMENTAL PROCEDURE.

tion of polysomes and subunits was not prevented by the highest concentration of magnesium ions tested.[7]

Binding of [³H]ATA to ribosomes and subunits. If [³H]ATA is added to a crude reticulocyte lysate at 0°, the dye binds to polysomes, monosomes, and 40 S subunits (Fig. 9A and B). If the lysate is incubated for 4 min at 35°, polyribosomes dissociate and [³H]ATA associates with 80 S monomers and 40 S subunits (Fig. 9C and D). In a parallel experiment, the lysate used for the studies shown in Fig. 9C and D was centrifuged for 9.0 hr to separate [³H]ATA bound to the 40 S subunit from [³H]ATA remaining at the top of the gradient (not shown). The specific activity of the [³H]ATA–40 S particle was approximately twice that calculated for the [³H]ATA–80 S complex.

Binding of [³H]ATA to ribosomes was also determined by measuring retention of the complex on Millipore membrane filters. When the molar ratio of dye to ribosome is high, binding is proportional to the concentration of ribosomes (Fig. 10). Saturation with ATA begins to occur at a concentration of ribosomes (1.8 mg/ml) at which the molar ratio of dye to ribosomes is approximately 680.

The membrane filter technique was used to examine the effects of various parameters on the binding reaction. Binding of ATA to ribosomes is complete in less than 20 sec and shows no dependence on temperature between 0° and 35°. Magnesium, calcium, and manganese (not shown) are optimally effective in stimulating the binding of ATA at a concentration of 6 mM, while monovalent cations are required at concentrations greater than 0.1 M (Fig. 11). Spermine and spermidine (not shown in figure), at concentrations of 6 mM, were equally effective in promoting the binding of ATA to the ribosome.

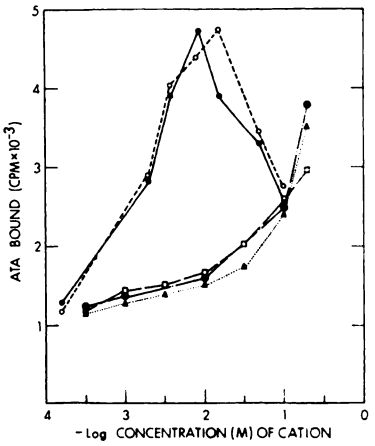

FIG. 11. *Effect of various cations on binding of [³H]-ATA to ribosomes*

Binding of [³H]-ATA was determined as described in the legend to Fig. 10, except that the divalent and monovalent cations indicated in the figure were substituted for magnesium chloride in the reaction mixture and in the buffer used for washing the ribosomes. ●——●, $CaCl_2$; ○---○, $MgCl_2$; ◉—·—◉, NaCl; ▲····▲, NH_4Cl; □— —□, KCl.

[7] The purified preparation of ATA (compound II) did not cause unfolding of single ribosomes at a concentration of 1 mM.

TABLE 4

Effect of concentration of poly U on binding of [^3H]ATA to ribosomes

Binding of [^{3}H]ATA to ammonium chloride-washed ribosomes was determined as described in the legend to Fig. 10. All reaction mixtures contained 200 μg of washed ribosomes. [^{3}H]ATA was added in experiments A, B, and C at final concentrations of 80 μM (11,097 cpm), 20 μM (2,774 cpm), and 10 μM (1,387 cpm), respectively.

Poly U	[^{3}H]ATA bound		
	A	B	C
mg/ml	*cpm*	*cpm*	*cpm*
0	3583	1256	675
0.2		1179	594
0.4	3575	1086	593
0.8	3352	1158	563
1.2	3124	1079	590

No significant difference in the amount of ATA bound to ribosomes was detected over a pH range of 6–9. Binding cannot be overcome by adding 1.2 mg/ml of polyuridylic acid to the reaction mixture if the concentration of ATA is 10 μM (Table 4). At 80 μM concentrations of ATA, addition of the same amount of polyuridylic acid displaces 13% of the bound ATA.

DISCUSSION

Effect of ATA on initiation of protein synthesis. We have previously shown that ATA affects chain initiation in extracts of *E. coli*, using bacteriophage f2 RNA as a source of mRNA (1, 2, 20). In related studies, Webster and Zinder (31) reached similar conclusions. On the basis of results reported in the present paper and previously (2), it seems probable that the dye exerts similar effects on protein synthesis in reticulocyte lysates. Evidence supporting this view includes the following observations. (a) Following addition of ATA to a lysate, a delay of 1–2 min occurs before inhibition of globin synthesis is observed. (b) During this period of time, peptide chains, previously identified as completed α and β chains of globin (2), are completed and released from the ribosome. (c) ATA induces sequential breakdown of polyribosomes, beginning with the larger complexes (2). (d) A 2–3-fold increase in the number of ribosomal subunits is observed in the presence of the dye. Additional evidence is derived from the recent report of Lodish *et al.* (32), in which ATA was shown to inhibit incorporation of formylmethionine into globin from yeast formylmethionyl-$tRNA_f^{Met}$. In these experiments, formylmethionine residues are introduced at the amino terminal of α and β chains (33) but are not subsequently removed.

Initiation of protein synthesis comprises initial binding of mRNA to the smaller ribosomal subunit, subsequent addition of the larger subunit, and binding of aminoacyl-tRNA to form the ternary complex (34). The effect of ATA on the reactions involved in initiation was examined using synthetic polynucleotides as a source of mRNA. Binding of poly U and poly C to the ribosome was strongly inhibited by the dye. As expected, poly U-directed enzymatic binding of phenylalanyl-tRNA and synthesis of polyphenylalanine were also affected; however, inhibition of polyphenylalanine synthesis was diminished if ATA was added after polyuridylic acid had been allowed to bind to the ribosome. This inhibitory effect of ATA on attachment of synthetic mRNA to ribosomes is supported by recent reports that the dye prevents binding of 40 S ribosomal subunits to "natural" globin mRNA (35, 36).

Effects of ATA on chain elongation. The individual reactions involved in chain elongation, i.e., binding of aminoacyl-tRNA, peptide bond formation and translocation, and the rate of completion and release of globin peptide chains do not appear to be affected by low concentrations of ATA. Inhibitory effects of the dye were detected on GTPase activity associated with ribosomes and TF-II. Under the conditions used in our experiments, it cannot be determined whether this inhibition represents effects on specific GTPase(s) involved in protein synthesis. In any event, GTPase is protected from the effects of ATA, since inhibition of enzymatic activity by the dye was greatly decreased in the presence of other components required for amino acid polymerization. Furthermore, any significant inhibition of specific GTP hydrolysis in crude reticulocyte lysates by ATA would have diminished

the over-all rate of chain elongation. High concentrations of ATA partially inhibit chain elongation, but only at concentrations of the dye that induce conformational changes in the ribosome.

Conformational changes in ribosomes induced by ATA. In reticulocyte lysates, selective effects of ATA on initiation of protein synthesis are obtained if the ATA to ribosome molar ratio is 330 or less (ribosomes, 1.2 mg/ml; ATA, 0.1 mM or less). Under these conditions, polyribosomes dissociate to 80 S monomers, and 40 S and 60 S subunits accumulate. At higher concentrations of dye (e.g., 1 mM), dissociation of polyribosomes is incomplete, suggesting that ATA, or a minor constituent of the crude dye, affects chain elongation. At these higher concentrations, the sedimentation coefficient of all classes of ribosomes decreases, the effect on monosomes being particularly marked. The latter partially unfold at ATA to ribosome molar ratios greater than 330, becoming discrete particles sedimenting at 54–56 S (35). The 40 S subunit displays similar behavior, sedimenting progressively slower as the concentration of dye is increased and, in turn, becoming a discrete particle with a sedimentation coefficient of 33 S. Reversibility of the unfolding effect on single ribosomes argues against the interpretation that the lower sedimentation coefficient reflects loss of ribosomal protein or RNA. In the case of the 40 S subunit, this possibility cannot be excluded, since the lower sedimentation coefficient is not reversed by increasing the concentration of magnesium ions.

ATA is a weak chelator of divalent metals (37), but its effects on reticulocyte ribosomes do not resemble those observed with other chelating agents. Addition of EDTA dissociates polyribosomes into ribosomal subunits, 9 S RNA (mRNA), 5 S RNA, and tRNA (38). Pyrophosphate, at concentrations sufficient to chelate all the available magnesium in the solution, dissociates ribosomes into subunits (39). The effects of ATA on ribosomes are clearly different from those of EDTA and pyrophosphate. Furthermore, the concentrations of dye required for inhibition of initiation (0.1–0.01 mM) are much lower than the concentration of magnesium ions (2 mM) present in the solution. Sequestration of divalent cations, therefore, cannot account for the inhibitory effects of ATA on initiation of protein synthesis. However, the unfolding of ribosomes and subunits observed at high concentrations of ATA is reversed by increasing the concentration of magnesium ions in the solution.

Binding of ATA to reticulocyte ribosomes. Certain requirements for binding of ATA to the ribosome are reported in the present paper. Divalent cations are required for the binding reaction, and much higher concentrations of monovalent cations are required to obtain the same effect. Spermine and spermidine are as effective as magnesium ions in stimulating binding, and it appears that the fundamental requirement for the binding reaction is neutralization of the negative charge on the ribosomal phosphate groups. Similar dependence on charge neutralization is observed when synthetic polynucleotides are bound to rRNA (40).

The binding of ATA to ribosomes is rapid and independent of temperature and pH over the range tested. The dye can be only partially displaced from the ribosome by addition of excess mRNA. A primary binding site for the dye appears to lie on the 40 S subunit, since ATA binds to polyribosomes, 80 S ribosomes, and 40 S subunits, but not to 60 S ribosomes. The binding site for mRNA is also found on the smaller subunit (34, 39).

Comparison with other inhibitors of initiation of protein synthesis. Biopolymers, such as polyadenylic acid (41, 42), dextran sulfate (43), and viral RNA (44), bear certain functional similarities to ATA in their effects on protein synthesis. This is not surprising, since these polyanions may also compete with mRNA for binding sites on the ribosome. The effects of ATA can be differentiated from those of other low molecular weight compounds, such as cycloheximide, pactamycin, and sodium fluoride, agents that have been reported to inhibit initiation of protein synthesis in reticulocyte extracts and other eukaryotes. Cycloheximide (45) acts primarily as an inhibitor of translocation in cell-free extracts derived from reticu-

locytes (25, 46) or rat liver (26), but may also inhibit initiation (46, 47) and chain termination and release (48, 49). Cycloheximide induces prolonged "freezing" of the polyribosome-peptide complex, inhibiting protein synthesis almost immediately after addition to the reaction. In contrast, ribosomes systematically disengage from polyribosomes in the presence of ATA, and a significant delay occurs before the inhibitory effect of the dye on protein synthesis becomes evident.

Pactamycin, at low concentrations, inhibits initiation of protein synthesis in reticulocyte lysates (32, 50–52) and extracts of *E. coli* (53). In contrast to ATA, pactamycin does not inhibit binding of polyuridylic acid to *E. coli* ribosomes; instead, it appears to alter the structure of the initiation complex so as to lead to its destabilization and dissociation (53).

The mode of action of ATA also differs from that of sodium fluoride (2, 35, 36, present paper). Treatment of rabbit reticulocytes with sodium fluoride produces intermediate complexes composed primarily of mRNA and 40 S subunits (30). Sodium fluoride does not prevent attachment of globin mRNA to the smaller subunit (30, 35, 36). These results were interpreted as showing that fluoride inhibits attachment of 60 S ribosomal subunits to the initiation complex formed between mRNA and the smaller subunit. Such complexes do not accumulate in lysates treated with ATA; instead, there is an increase in the number of 40 S and 60 S subunits. Finally, we have shown that ATA inhibits the binding of polyuridylic acid to ribosomes, while sodium fluoride has no effect on this process.

Active component of ATA. Although ATA is used as a standard analytical reagent for the detection of aluminum (17–19), it has never been prepared in a homogeneous form. Previously reported biochemical studies employing ATA (e.g., 1, 2, 20, 31,32, 35, 36, 44) have used either commerical or partially purified preparations. Since all commerical preparations seem to contain several components,[3] purified ATA was synthesized to determine which biochemical effects could be unequivocally assigned to the triphenylmethane structure proposed for this compound. Unfortunately, the purified dye proved unstable, even when protected from the light, and the lengthy procedure required for its chemical synthesis precluded its use for all of the studies reported in this paper.

The basic actions of the crude dye, i.e., inhibition of attachment of mRNA to ribosomes, were demonstrated using pure ATA, but unfolding of ribosomes was not observed with the latter material. This gross conformational change may underlie the observed effects on chain elongation induced by high concentrations of ATA, and it is possible that such effects are caused by minor constituents of the crude dyestuff.

Conclusion. We conclude from our experiments that ATA binds to the smaller subunit, preventing subsequent attachment of mRNA. As a result, initiation of globin synthesis is inhibited in cell-free extracts prepared from rabbit reticulocytes. Inhibition of initiation is achieved at concentrations of dye that do not affect chain extension; however, high concentrations of dye exert an unfolding effect on the ribosome and inhibit chain elongation. The unique properties of ATA should prove useful in studying molecular events involved in the initiation of protein synthesis.

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REFERENCES

1. A. P. Grollman and M. L. Stewart, *Proc. Nat. Acad. Sci. U. S. A.* **61**, 719 (1968).
2. M. L. Stewart, A. P. Grollman and M. T. Huang, *Proc. Nat. Acad. Sci. U. S. A.* **68**, 97 (1971).
3. H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley and P. H. Lowy, *J. Biol. Chem.* **196**, 669 (1952).
4. A. P. Grollman, *J. Biol. Chem.* **242**, 3226 (1967).
5. E. H. Allen and R. S. Schweet, *J. Biol. Chem.* **237**, 760 (1968).
6. A. I. Grayzel, P. Horchner and I. M. London, *Proc. Nat. Acad. Sci. U. S. A.* **55**, 650 (1966).

7. L. Felicetti and F. Lipmann, *Arch. Biochem. Biophys.* **125,** 548 (1968).
8. W. McKeehan and B. Hardesty, *J. Biol. Chem.* **244,** 4330 (1969).
9. C. R. Maxwell and M. Rabinovitz, *Biochem. Biophys. Res. Commun.* **35,** 79 (1969).
10. W. V. Zucker and H. M. Schulman, *Biochim. Biophys. Acta* **138,** 400 (1967).
11. S. D. Adamson, E. Herbert and W. Godchaux, III, *Arch. Biochem. Biophys.* **125,** 671 (1968).
12. R. J. Britten and R. B. Roberts, *Science* **131,** 32 (1960).
13. J. R. Warner, P. M. Knopf and A. Rich, *Proc. Nat. Acad. Sci. U. S. A.* **49,** 122 (1963).
14. G. A. Bray, *Anal. Biochem.* **1,** 279 (1960).
15. G. B. Heisig and W. M. Lauer, *Org. Syn.* **9,** 8 (1929).
16. D. A. Holaday, *J. Amer. Chem. Soc.* **62,** 989 (1940).
17. J. A. Scherrer and W. H. Smith, *J. Res. Nat. Bur. Stand.* **21,** 113, Research Paper 1118 (1938).
18. "Organic Analytical Reagents," F. J. Welcher, Editor Vol. II, p. 94. Van Nostrand, New York, 1947.
19. W. H. Smith, E. E. Sager and I. J. Siewers, *Anal. Chem.* **21,** 1334 (1949).
20. A. P. Grollman, in *Antimicrob. Agents Chemother.* 36 (1969).
21. M. Nakanishi and T. Kuriyama, *Chem. Abstr.* **58,** 7881a (1963).
22. E. Clemmensen and A. H. C. Heitman, *J. Amer. Chem. Soc.* **33,** 733 (1911).
23. M. Smolarsky and M. Tal, *Biochim. Biophys. Acta* **199,** 447 (1970).
24. P. O. P. Ts'o and J. Vinograd, *Biochim. Biophys. Acta* **49,** 113 (1961).
25. W. McKeehan and B. Hardesty, *Biochem. Biophys. Res. Commun.* **36,** 625 (1969).
26. B. S. Baliga, A. W. Pronczuk and H. N. Munro, *J. Biol. Chem.* **244,** 4480 (1969).
27. D. Vazquez, E. Battaner, R. Neth, G. Heller and R. E. Monro, *Cold Spring Harbor Symp. Quant. Biol.* **34,** 369 (1969).
28. M.-T. Huang and A. P. Grollman, *Fed. Proc.* **29,** 609 (1970).
29. M. Malkin and F. Lipmann, *Science* **164,** 71 (1969).
30. W. Hoerz and K. S. McCarty, *Proc. Nat. Acad. Sci. U. S. A.* **63,** 1206 (1969).
31. R. E. Webster and N. D. Zinder, *J. Mol. Biol.* **42,** 425 (1969).
32. H. F. Lodish, D. Housman and M. Jacobsen, *Biochemistry* **10,** 2348 (1971).
33. D. Housman, M. Jacobs-Lorena, U. L. Rajbhondary and H. Lodish, *Nature* **227,** 913 (1970).
34. P. Lengyel and D. Soll, *Bacteriol. Rev.* **33,** 264 (1969).
35. B. Lebleu, G. Marbaix, J. Wérenne, A. Burny and G. Huez, *Biochem. Biophys. Res. Commun.* **40,** 731 (1970).
36. W. Hoerz and K. S. McCarty, *Biochim. Biophys. Acta* **228,** 526 (1971).
37. A. K. Mukherji and A. K. Dey, *Z. Anal. Chem.* **152,** 424 (1956).
38. G. Huez, A. Burny, G. Marbaix and B. Lebleu, *Biochim. Biophys. Acta* **145,** 629 (1967).
39. J. W. Holder and J. B. Lingrel, *Biochim. Biophys. Acta* **204,** 210 (1970).
40. P. B. Moore and K. Asano, *J. Mol. Biol.* **18,** 21 (1966).
41. B. Hardesty, J. J. Hutton, R. Arlinghaus and R. Schweet, *Proc. Nat. Acad. Sci. U. S. A.* **50,** 1078 (1963).
42. B. Hardesty, R. Miller and R. Schweet, *Proc. Nat. Acad. Sci. U. S. A.* **50,** 924 (1963).
43. A. Korner, *in* "Inhibitors, Tools in Cell Research" (T. Bucher and H. Sies, eds.), p. 132. Springer, New York, 1969.
44. M. B. Mathews and A. Korner, *Eur. J. Biochem.* **17,** 339 (1970).
45. H. D. Sisler and M. R. Siegel, *in* "Antibiotics Mechanism of Action" (D. Gottlieb and P. D. Shaw, eds.), Vol. II, p. 283. Springer, New York, 1969.
46. T. G. Obrig, W. J. Culp, W. L. McKeehan and Hardesty, B. *J. Biol. Chem.* **246,** 174 (1971).
47. S.-Y. Lin, R. D. Mosteller and B. Hardesty, *J. Mol. Biol.* **21,** 51 (1966).
48. W. Godchaux, III, S. D. Adamson and E. Herbert, *J. Mol. Biol.* **27,** 57 (1967).
49. S. Rajalakshmi, H. Liang, D. S. R. Sarma, R. Kisilevsky and E. Farber, *Biochem. Biophys. Res. Commun.* **42,** 259 (1971).
50. B. Colombo, L. Felicetti and C. Baglioni, *Biochim. Biophys. Acta* **119,** 109 (1966).
51. J. S. Macdonald and I. H. Goldberg, *Biochem. Biophys. Res. Commun.* **41,** 1 (1970).
52. M. L. Stewart-Blair, I. S. Yanowitz and I. H. Goldberg, *Biochemistry* **10,** 4198 (1971).
53. L. B. Cohen, A. E. Herner and I. H. Goldberg, *Biochemistry* **8,** 1312 (1969).