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AURINTRIBOXYLIC ACID AS BOTH A STIMULATOR AND AN INHIBITOR OF CELL-FREE PROTEIN SYNTHESIS BY RAT HEART POLYRIBOSOMES

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SUMMARY. Aurintricarboxylic acid is shown to be a powerful stimulator at lower concentrations and a strong inhibitor at higher concentrations of protein synthesis in a heart ribosomes brain supernatant cell free system. The stimulation appears to arise from the prevention of low affinity abortive complex formation.

INTRODUCTION

Aurintricarboxylic acid (ATA) is a substituted phenolic dye antibiotic, which has been reported to be a specific inhibitor of protein synthesis. It was shown by Lebleu et al [1] to inhibit the binding of labelled globin mRNP to reticulocyte 40s subunits, and the dose-response characteristics were reported more fully by Lodish et al [2]. These workers showed that at a sufficiently high concentration, elongation as well as initiation of protein synthesis in reticulocyte lysates was inhibited. Dettman and Stanley [3] have reported that ATA also inhibited ternary complex formation by a system using purified components, a result confirmed by other groups [4,5] although Hunter et al [6] could find no effect of ATA on ternary complex formation in the wheat-germ system. This pattern of inhibitory effects is further complicated by reports of a stimulatory effect of ATA on protein synthesis directed by artificial message in an ascites cell-free system [7] and on

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Abbreviation: ATA Aurintricarboxylic Acid

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endogenous protein synthesis by a cell-free system from liver [8]. We report here that while ATA acts as an inhibitor of ternary complex and initiation complex formation in a preparation of cardiac polyribosomes incubated in brain high speed supernatent, at lower concentrations the dye stimulates amino acid incorporation by this system by up to 50%. The stimulatory, though not the inhibitory, effect can be abolished by high salt washing the polyribosomes.

MATERIALS AND METHODS

Aluminon (tri-ammonium aurine tricarboxylate: ATA) was obtained from BDH Chemicals, Poole, UK. L-[^{35}S] methionine (~ 600 Ci/mmol) and L-[4,5-^{3}H] leucine (~ 50 Ci/mmol) were provided by the Radiochemical Centre, Amersham, UK.

PREPARATION OF SUBCELLULAR FRACTIONS

Polyribosomes from perfused rat heart were prepared as described in Mackie et al [9]. High-speed supernatant from rabbit brain was prepared by the method of Metafora et al [10].

Assays Total protein synthesis was assayed by measuring the incorporation of [^{3}H] leucine into TCA-insoluble material as previously described [11].

Ternary complex formation in brain high-speed supernatant was assayed by trapping of complexes on Millipore filters after incubation for 30 min at 37°C in the presence of 80 mM KCl, 2 mM $MgCl_2$, 30 mM Tris HCl (pH 7.4), 1 mM APT, 0.6 mM CTP, 0.5 mM GTP, 10 mM creatine phosphate, 160 µg/ml creatine phosphokinase and approx. 60 µCi/ml [^{35}S]-methionine. Samples (20 µl) from incubations were diluted into 2 ml ice-cold buffer A (80 mM KCl, 50 mM Tris-HCl, pH 7.4, 5 mM methionine) before passage through Millipore filters. Each filter was washed three times with 2 ml cold buffer A before treatment with formic acid and counting. Initiation assays used the same incubation medium as that for incorporation assays except that the tritiated leucine was replaced by 40 µM unlabelled leucine and the unlabelled methionine by [^{35}S]-methionine (4 µCi/100 µl; 780-1250 Ci/mmole). Incubations were for 5 min at 37°C in the presence of 10^{-3}M emetine and stopped by addition of 2 volumes ice-cold 250 mM sucrose/100 mM KCl/5 mM $MgCl_2$/1 mM dithiothreitol/20 mM Tris-HCl buffer pH 7.4. The mixture was cooled on ice and layered on to 12.5 ml 15-68% exponential sucrose gradient in 100 mM KCl/2 mM $MgCl_2$/20 mM Hepes pH 7.4 and centrifuged at 4° for 15 h at 38,000 rpm (Beckman SW40 Ti, 181,000 g av.). The gradients were scanned [12] and collected as 0.4 ml fractions each of which was diluted with 5 ml buffer containing 5 mM unlabelled methionine, filtered through a 0.45 µm Millipore filter presoaked in the same medium and washed three times with 5 ml buffer. The radioactivity was assayed by liquid scintillation counting at an efficiency of about 80% in a Beckman 355 spectrometer using external quench correction.

RESULTS AND DISCUSSION

The effect of ATA on initiation in a heart ribosome-brain sap cell-free system

Figure 1 shows that 5 x 10^{-4} M ATA inhibited the methionine radio-activity associated with both the 80s and the 40s regions of the gradient

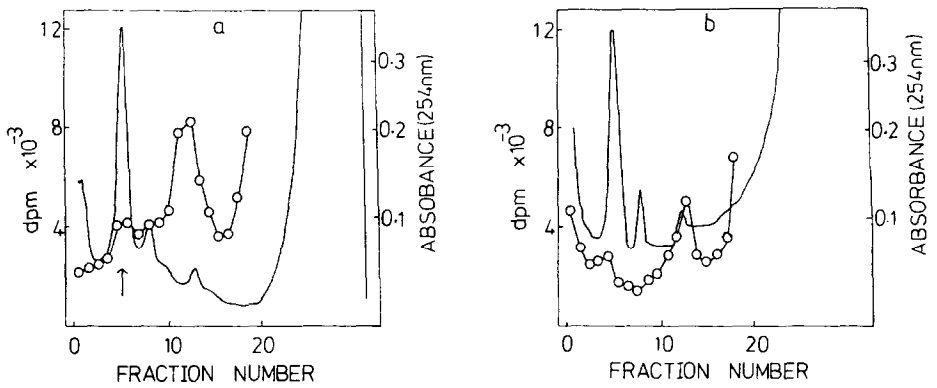

Figure 1: Initiation complex formation by cardiac ribosomes in the presence of brain high-speed supernatant and 10^{-3} M emetine (a) alone (b) in the presence of 5 x 10^{-4} M ATA.
Continuous line shows A_{254}, O———O [^{35}S]-Methionine bound to Millipore filters in each gradient fraction. Arrow shows position of 80s peak. Sedimentation was from right to left.

when a rat heart ribosome preparation supported by a soluble extract of rabbit brain was incubated in the presence of 10^{-3} M emetine. In addition the peak around 40s was shifted towards the lighter end of the gradient presumably indicating a reduction in 40s complex binding to mRNA in the presence of ATA [1]. The marked reduction in 40s radioactivity may well be the result of reduced ternary complex formation which was inhibited in brain high-speed supernatant at concentrations greater than 2 x 10^{-5} M ATA (figure 2). The exact equivalence between these two assays is hard to assess since the effective free concentration of ATA was presumably greater in the ternary complex assay owing to the absence of ribosomes and associated proteins [see 8].

The effect of ATA on amino acid incorporation

Figure 3 shows the effect of a range of ATA concentrations on the incorporation of ^{3}H -leucine into protein by cardiac polyribosomes in the presence of brain high-speed supernatant. The minimum effective concentration for inhibition of protein synthesis was 2 x 10^{-4} M. This is higher than that reported [2] for amino-acid incorporation by reticulocyte lysates (8 x 10^{-5} M). Leader [8] has shown that the effective concentration of ATA depends on the protein concentration and this could well explain the discrepancy. At higher concentrations (> 8 x 10^{-4} M) complete inhibition of

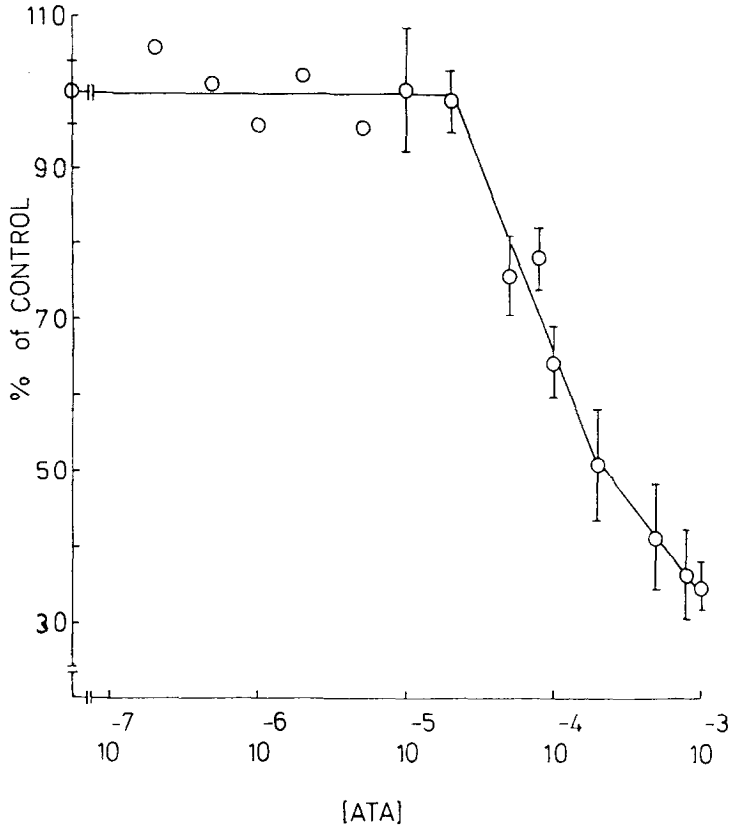

Figure 2: Formation of ternary complexes by brain post-ribosomal supernatant as a function of ATA concentration. Incubation was for 30 min. Results are expressed as percent of the value in the absence of ATA and points show the mean ± SEM at least six separate extimates. Points without error bars show the means of duplicate estimates.

protein synthesis is seen, indicating that ATA is effectively inhibiting both the initiation and elongation processes while between 10^{-5} and 10^{-4} M, the range of concentrations used by other workers to inhibit initiation specifically, ATA was observed to stimulate protein synthesis significantly ($p < .005$).

It is not clear whether this represents a stimulation of initiation, of elongation or of both. Mathews [7] using an ascites cell-free system found that although ATA was a powerful inhibitor of incorporation directed by poly U, it was ineffective up to 10^{-4} M against, and even stimulatory of, incorporation on poly U containing the initiation codons AUG and GUG. This suggests ATA is especially effective against non-specific subunit messenger interactions which could stimulate elongation by clearing subunit complexes which are clogging

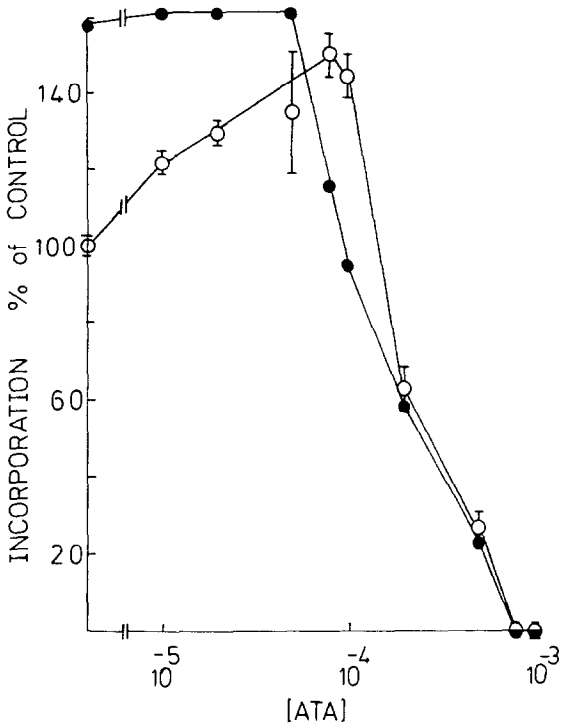

Figure 3: Incorporation of [^{3}H]-leucine into TCA-insoluble material by cardiac ribosomes in the presence of brain post-ribosomal supernatant as a function of ATA concentration. Incubation was for 60 min. Results are expressed as percent of incorporation in the absence of incorporation in the absence of ATA.

O———O cardiac ribosomes; points show the mean ± SEM of at least four separate estimates; all points are significantly different from control ($P < 0.005$).

●———● cardiac ribosomes prepared in the presence of high salt; points show the mean of duplicate estimates as a percent of the ribosomes prepared in the absence of added NH_4Cl.

polysome message; or it could stimulate Met $tRNA_f$ initiation complex formation by releasing initiation factors or subunits from such abortive complex formation. Leader [8] has reported one experiment in which 10^{-7} - 10^{-5} M ATA stimulated endogenous protein synthesis by a cell-free system from rat liver. Since he also reported that his soluble support medium was deficient in initiation factors, he interpreted the effect of ATA as a stimulation of elongation although the presence of polyribosome-bound factors cannot be excluded.

The effect of ATA on the time-course of incorporation

The time-course of [^{14}C]-leucine incorporation into TCA-insoluble material was examined at a stimulatory (10^{-4} M) and mildly inhibitory

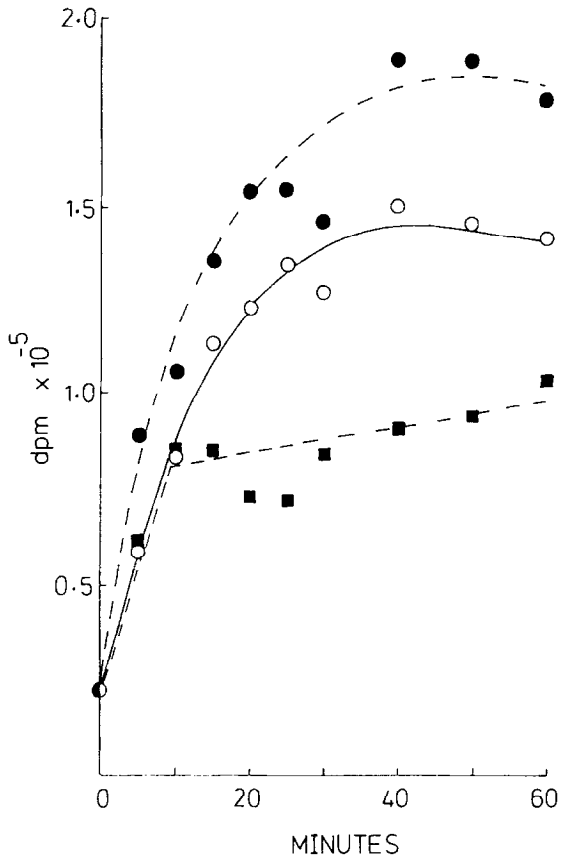

Figure 4. Time-course of incorporation of [^{3}H]-leucine into TCA-insoluble material by cardiac ribosomes in the presence of brain post-ribosomal supernatant O———O alone, ●— —● + 10^{-4} ATA, ■- - - -■ + 2 x 10^{-4} m ATA.

(2 x 10^{-4} M) concentration of ATA. Figure 4 gives an example of the results consistently obtained. ATA at 10^{-4} M stimulated incorporation at all time-points which is the result to be expected from either increased initiation or elongation. At 2 x 10^{-4} M, ATA had no significant effect for the first 10 minutes of incubation but caused an almost complete cessation of incorporation thereafter. This could well represent inhibition of initiation alone although the proportion of incorporation dependent on initiation (35%) is substantially greater than the 10-20% suggested by other studies with this cell-free system using edeine as an inhibitor of initiation (C.M. Mackie, unpublished). The virtually unchanged initial rate may be the net resultant of inhibited initiation and slightly stimulated elongation. The

use of strongly inhibitory concentrations of ATA resulted in the inhibition of incorporation at all time-points (data not shown).

The effect of high salt washing the polysomes on ATA stimulation

The possibility that ATA might be selectively removing inhibitory material associated with the polysomes was examined by using a high salt preparation of ribosomes which others have found effective in removing polysome bound factors [13-15]. This was done by adding NH_4Cl to one half of the post-mitochondrial supernatant to give a final concentration of 250 mM [16]. These high salt conditions reduced the protein content of the ribosomes preparation by around 25% and increased their incorporating ability by about 50% (figure 3; [16]). Not only were these high salt prepared ribosomes as active as the maximally ATA stimulated ones but ATA addition produced no appreciable further stimulation (figure 3). This finding suggests that the high salt washing removed the same inhibitory components as did ATA at stimulatory concentrations. Others [17,18] have reported that one action of ATA is to release proteins from 40s subunits.

The sensitivity of the high salt ribosomes to the inhibitory action of ATA is first seen at concentrations above 5×10^{-5} M which agrees very well with that (8×10^{-5} M) reported for reticulocyte lysates [2] and compares well with the concentration at which ATA begins to inhibit ternary complex formation (figure 2). As assessed by increased sensitivity to edeine [16] and by binding of [^{35}S]-methionine to 40s and 80s components (D.B. Buxton, unpublished findings) initiation as well as elongation is increased in these NH_4Cl prepared ribosomes.

COMMENT

ATA appears to disrupt non-specific RNA-protein interactions at lower concentrations that it does the (presumably) more tightly-bound selective catalytic associations. Hence although it may still be useful in examining these latter reactions provided its effective concentration is established for a given preparation, it could prove more beneficial both in assays and

in polysome preparations in preventing the lower affinity associations which may be, at least partly, responsible for the poor performance of cell-free protein synthesising systems from complex eukaryotic cells.

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