THE EFFECT OF ANTRYCIDE ON PATTERNS OF RNA SYNTHESIS IN AMOEBA DISCOIDES

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SUMMARY

Antrycide is an aminoquinaldine whose inhibitory action on the growth of Trypanosoma and Crithidia is not fully understood at the cellular level. The growth of Amoeba discoides in concentrations of antrycide between 0.5 and 2 μg/ml was reduced considerably, while cells failed to divide in 4 μg/ml. The effects on growth rate were reversible at least up until 7 days in antrycide.

In order to assess the action of this synthetic drug on RNA synthesis in amoebae, the pattern of synthesis in normal cells was investigated using polyacrylamide gel electrophoresis. The profile of high molecular weight RNAs observed depended on the length of time in [3H]uridine, and was only fully developed after 66 h, when 5 peaks could be seen. The relative molecular weights of these peaks (I—V) were 2.45, 1.55, 1.13, 0.8 and 0.52 x 10^6 Daltons respectively. Those of 1.55 and 0.8 x 10^6 corresponded to ribosomal RNAs, the identity of the other peaks is unknown.

After growth in 2 μg/ml antrycide for 4 days, no high molecular weight RNA was found. Use of [14C]adenine/[3H]uridine showed that after 17 h in antrycide there was a loss of ribosomal RNA and increased levels of low molecular weight RNAs, due either to lack of synthesis or to degradation of newly synthesized material. Incorporation of [3H]leucine into hot acid-precipitable protein was inhibited in antrycide-treated cells by at least 50%. A possible explanation of the effect of antrycide on A. discoides was the inhibition of mRNA synthesis for ribosomal proteins, leading to degradation of newly synthesized rRNA. Reduced growth would continue on pre-existing ribosomes and previously synthesized long-lived mRNAs.

INTRODUCTION

The large free-living amoebae, Amoeba proteus and Amoeba discoides, require relatively high concentrations of actinomycin D to inhibit transcription rapidly and completely. Rao & Prescott (1970) showed that 1 mg/ml would inhibit the nuclear production of RNA within 20 min. These levels of actinomycin D contrast with 0.25 μg/ml for ribosomal RNA (rRNA) and 0.5 μg/ml for heterogeneous RNA (HnRNA) in L cells (Perry & Kelley, 1970) and 10 μg/ml for macromolecular RNA synthesis in Tetrahymena (Byfield, Lee & Bennett, 1970). The nature of this difference may reside in membrane permeability or in the organization of the amoeba genome. The need to use such high concentrations of antibiotic coupled with the problems of light instability and the adverse effects of this compound on mature RNA molecules (Harris, 1974) led us to look for an alternative inhibitor of RNA synthesis in amoebae.

While examining the responses of both strains of amoebae to a variety of compounds in a search for strain differences, we used antrycide, an active trypanocidal compound belonging to the aminoquinaldines. Antrycide inhibited division in amoebae at 4 μg/ml
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(Hawkins, 1973). Using the flagellate Crithidia oncopelti, Newton (1958) showed that the pattern of growth was changed from an exponential to a linear form in concentrations of antrycide ranging from 0.1 to 100 μg/ml. The effects could be reversed at any time by transferring the cells to a drug-free medium. After further extensive studies Newton (1966, 1973) concluded that antrycide reduced growth by inactivating cytoplasmic ribosomes rather than by inhibiting DNA-directed RNA synthesis. However, this hypothesis did not explain all the observed effects of antrycide, since RNA synthesis was inhibited when the organism was supplied with exogenous purines, for example [14C]adenine. Since antrycide appeared to affect amoebae at relatively low concentrations, we have investigated its effects on the pattern of RNA synthesis in Amoeba discoides using polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Living material

A. discoides, obtained originally from Taylor (Glasgow) were maintained in 'wheat grain' cultures (Lorch & Danielli, 1953) in Chalkley's medium at 17 ± 1 °C. Large numbers of cells required for the preparation of nuclear and cytoplasmic RNA were grown in mass cultures using a method modified from Griffin (1960). These cells were fed regularly on washed Tetrahymena pyriformis grown on sterile 1% proteose-peptone.

Growth rates were obtained by keeping the cells as 'singles' in solid watch glasses containing 2 ml Chalkley's medium and Colpidium sp. as food organisms (Hawkins & Willis, 1969) together with various concentrations of antrycide (I.C.I. Laboratories). The medium was renewed every 3 days.

Cells were labelled by the addition of radioactive precursors to the medium. Mass cultures were starved for 1 day before the addition of [5-3H]uridine (100 μCi/ml; sp. act. 28 Ci/mmol, Amersham) for various time periods. Small numbers of cells were taken from stock cultures, cleaned from food organisms and placed in solid watch glasses in Chalkley's medium containing either 50 μCi/ml [5-3H]uridine, [U-14C]adenine (sp. act. 225 mCi/mmol), [G-3H]hypoxanthine (sp. act. 1 mCi/mmol) or L-[4,5-3H]leucine (sp. act. 60 Ci/mmol) together with antrycide if required.

Preparation of RNA

Large numbers of amoebae were starved, bulked and lightly homogenized in 0.24 M sucrose-TKM buffer (0.05 M Tris-HCl, 0.025 M KCl, 0.005 M MgCl₂, pH 7.4) in the cold. Nuclear and ribosomal fractions were prepared by centrifugation at 2000 g for 10 min and 105 000 g for 2 h, respectively. RNA was obtained from these fractions by phenol/sodium dodecyl sulphate (SDS) extraction (Girard, 1967) 3 times at 4 °C. The purity of the RNA was assessed by O.D. 260:280 measurements.

The RNA from small numbers of amoebae (100-500) was prepared as follows: RNA from the post-mitochondrial supernatant of rat liver was extracted twice with phenol/SDS at 60 °C. The extract was cooled and the amoebae together with diethylpyrocarbonate (0.15 ml) added. A further phenol/SDS extraction was carried out at 4 °C.

Rat liver ribosomal RNA was prepared by phenol/SDS extraction at 60 °C and Escherichia coli ribosomal RNA was obtained from Miles Laboratories for use as 'markers'.

Polyacrylamide gel electrophoresis

Aqueous 2-9 % polyacrylamide gels were prepared using Cyanogum 41 (B.D.H.) and running buffer (40 mM Tris, 20 mM sodium acetate, 2 mM sodium EDTA, acetic acid being used to adjust the pH to 7.8 (Loening, 1971)) and were cast in cylindrical running tubes. Gels were pre-run for 1 h at 5 mA/gel at 4 °C and then samples containing RNA, 5 % (w/v) sucrose and
bromophenol blue were layered over the gels and electrophoresis continued at 5 mA/gel for variable time periods. Gels were removed from running tubes and either fixed in 1 M acetic acid and stained in methylene blue, or frozen on 'cardice' prior to slicing with a Mickle gel slicer (Mickle Engineering Co., Surrey). Gel slices were placed in scintillation vials, incubated in Nuclear Chicago Solubiliser (NCS) at 33 °C for 2 h, scintillation fluid added (PPO, 4 g; POPOP, 100 mg/l. toluene) and the radioactivity determined in a Beckman LS-230 liquid spectrometer.

**Amino acid incorporation**

Samples were acid-precipitated using an equal volume of hot (90 °C) 10 % trichloroacetic acid (TCA) for 15 min. Precipitates were filtered through Whatman GF/C filters and washed with a large volume of 5 % TCA, followed by ethanol/ether (3:1) and finally a small volume of ether. When dry, filters were placed in vials containing scintillation fluid and radioactivity determined in a Beckman LS-230 liquid spectrometer.

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**Fig. 1. Growth of *A. discoides* in antrycide.** ▲, 0.5 μg/ml antrycide in Chalkley’s medium; ■, 2 μg/ml antrycide in Chalkley’s medium; ●, controls in Chalkley’s medium.
RESULTS

Growth of amoebae in antrycide

Earlier experiments had shown that both *A. proteus* and *A. discoides* failed to divide in 4 μg/ml antrycide and no significant differences were noted between the 2 strains. Cells of *A. discoides* were taken at random from healthy 'wheat grain' cultures and were placed in concentrations of antrycide ranging between 0.1 and 2 μg/ml in Chalkley's medium containing food organisms. Cells were grown for 14 days, the medium being changed every 3 days. The results obtained for amoebae in 0.5 and 2 μg/ml antrycide are shown in Fig. 1. No reduction in growth rate occurred in 0.1 μg/ml, but in 0.5 μg/ml growth rate was reduced to 63% of that of control cells, while in 2 μg/ml
it was reduced to 40% of the controls. Reversibility was investigated by placing cells in 2 μg/ml antrycide for varying periods of time between 1 and 7 days. Even after 7 days in drug, cells placed into Chalkley's medium with food organisms resumed exponential growth.

**Patterns of RNA synthesis in A. discoides**

Before we could assess the effect of different levels of antrycide on the pattern of RNA synthesis in amoebae, it was necessary to establish the pattern seen in normal cells. Using polyacrylamide gel electrophoresis, no studies of this pattern had been published, so that we commenced by examining the distribution of [3H]uridine-labelled RNA extracted from nuclear and ribosomal fractions of large numbers (750,000) of amoebae. The radioactive precursor was added to Chalkley's medium in preference to feeding radioactive *Tetrahymena* so that we could be certain of obtaining labelled amoeba RNA, rather than risking contamination by partly degraded *Tetrahymena* RNA. After a preliminary starvation period, cells were labelled for 48 h; there were no free *Tetrahymena* in the medium, neither were there any living *Tetrahymena* in amoeba food vacuoles. Autoradiographs of these amoebae showed no incorporation in the food vacuoles.

After electrophoresis for 55 min on 2-9% aqueous polyacrylamide gels, a characteristic pattern of distribution of RNA was obtained. The ribosomal fraction (Fig. 2 A) clearly showed the 2 rRNAs together with some contaminating RNAs of lower molecular weight. The pattern of distribution of RNA in the nuclear fraction was more complex (Fig. 2 B) with 5 peaks in the first 2 cm of the gel, together with a considerable amount of low molecular weight RNA including transfer RNA (tRNA). Previously determined relative molecular weights for amoeba rRNAs (using both aqueous and formamide gel electrophoresis (Hawkins & Hughes, 1973)) of 1.55 and 0.8 x 10^6 Daltons respectively together with marker rRNAs from rat liver and E. coli enabled us to determine the relative molecular weights of the RNA in the 5 peaks (I–V). They were as follows (in Daltons x 10^6); I = 2.45; II = 1.55 (rRNA); III = 1.13; IV = 0.8 (rRNA); V = 0.52, corresponding to s values of approximately 35, 26, 23, 19 and 16. These RNAs did not disappear when the ribonuclease inhibitor diethylpyrocarbonate was added prior to phenol extraction, while other methods of extraction, e.g. pronase followed by hot (60 °C) phenol (Prescott, Stevens & Lauth, 1971) gave degraded RNA when examined on polyacrylamide gels. Prior treatment of samples with ribonuclease before phenol extraction eliminated over 95% of the radioactivity.

In order to examine the effects of antrycide, we needed more controlled growth conditions than those obtained in large mass cultures. Therefore we developed a method for the extraction of RNA from a small number of cells. The method finally adopted was to extract rat liver post-mitochondrial supernatant material twice with phenol/SDS at 60 °C, cool, add the required number of cells together with diethylpyrocarbonate and extract once using phenol/SDS at 4 °C. The rat liver RNA acted as a carrier for the relatively small amounts of amoeba RNA. Two hundred amoebae taken at random from ‘wheat grain’ cultures were adequate to give a consistent pattern of distribution of labelled RNA.
However the pattern of RNA seen varied with time in $[^{3}H]$uridine, and the patterns of distribution seen after 17, 48 and 66 h in radioactive precursor are shown in Fig. 3. Electrophoresis was carried out for 90 min in order to separate the 5 peaks seen in the top 2 cm of the gel (Fig. 2B), even though this meant loss of low molecular weight species. After 17 h in $[^{3}H]$uridine, only peaks III and V were prominent, although I and IV could be distinguished; 48 h were required to distinguish amoeba rRNAs II and IV, while 66 h in precursor were needed in order to give a pattern more normally seen in eukaryotes, with large to small rRNAs in a 1:2 to 1 ratio.

Fig. 3. Gel electrophoresis of RNA extracted from 200 A. discoides grown in $[^{3}H]$uridine for varying time periods. Electrophoresis on 2-9% polyacrylamide gels for 90 min. Peaks II and IV, rRNAs. A, B, C, cells grown in $[^{3}H]$uridine for 17, 48 and 66 h respectively.
Effects of antricyle on the pattern of RNA synthesis

Amoebae were placed in various concentrations of antricyle in Chalkley's medium plus food organisms for 24 h, washed free from food organisms in medium containing antricyle, and then grown for a further 66 h in antricyle and [3H]uridine (cells had been exposed to antricyle for 90 h). RNA was extracted from groups of 200 cells and the distribution of radioactivity examined on 2.9% polyacrylamide gels subjected to electrophoresis for 90 min. After 90 h growth in 2 μg/ml antricyle (Fig. 4c) no high molecular weight RNA could be detected on the gel, and a considerable amount of low molecular weight RNA was seen. This lack of high molecular weight RNA was also seen after 90 h in 1 μg/ml antricyle (Fig. 4b) while the pattern of RNA from cells grown in 0.25 μg/ml antricyle for 90 h also lacked peaks I, II and IV, only III and V being recognizable (Fig. 4a). After 90 h in antricyle cells appeared to be either unable to synthesize high molecular weight RNA including rRNA, or they might be capable of synthesis but breakdown was also occurring at an abnormally increased rate.

Fig. 4. Effect of antricyle on pattern of RNA synthesis in A. discoides. 200 cells grown in various concentrations of antricyle + food organisms for 24 h, washed free from food organisms, and grown for a further 66 h in antricyle + [3H]uridine; total time in drug, 90 h. Electrophoresis on 2.9% polyacrylamide gels for 90 min. A, B, C, 0.25, 1.0 and 2.0 μg/ml antricyle.
To investigate these possibilities we compared the profiles of cells labelled with \([^{14}\text{C}]\text{adenine}\) and then placed in \([^{3}\text{H}]\text{uridine} \pm \text{antricyde}\). Cells were cleaned from food organisms and grown in \([^{14}\text{C}]\text{adenine}\) to provide a ‘background’ pattern for 66 h. We had to use \([^{14}\text{C}]\text{adenine}\) rather than \([^{14}\text{C}]\text{uracil}\), since the latter gave very low levels of incorporation into RNA. The pattern of distribution of radioactivity in RNA when cells were grown in \([^{14}\text{C}]\text{adenine}\) could not be distinguished from that obtained using \([^{3}\text{H}]\text{uridine}\). Five hundred cells were rinsed briefly to free them from excess adenine and were placed in either 2 \(\mu\text{g/ml}\) antricyde and \([^{3}\text{H}]\text{uridine}\) or \([^{3}\text{H}]\text{uridine}\) only for further time periods.

The results obtained when cells were grown in \([^{3}\text{H}]\text{uridine}\) only for 17 h are shown

![Graph showing RNA synthesis pattern](image)

Fig. 5. Effect of growth for 17 h in 2 \(\mu\text{g/ml}\) antricyde on pattern of RNA synthesis in \(A.\text{discoides}\). Cells grown in \([^{14}\text{C}]\text{adenine}\) for 66 h, then grown in either \([^{3}\text{H}]\text{uridine}\) for 17 h or \([^{3}\text{H}]\text{uridine}\) plus 2 \(\mu\text{g/ml}\) antricyde for 17 h. Electrophoresis on 2-9% polyacrylamide gels for 90 min. A, 500 cells grown in \([^{14}\text{C}]\text{adenine}\) (66 h) followed by 17 h in \([^{3}\text{H}]\text{uridine}\). Notice clearly defined rRNAs (peaks II and IV). B, 500 cells grown in \([^{14}\text{C}]\text{adenine}\) (66 h) followed by 17 h in \([^{3}\text{H}]\text{uridine}\) plus 2 \(\mu\text{g/ml}\) antricyde. •, ○, \([^{14}\text{C}]\text{adenine}\) and \([^{3}\text{H}]\text{uridine}\), respectively, as precursors.
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in Fig. 5A. All 5 peaks were seen clearly after labelling in $[^{14}\text{C}]$adenine, the total time involved being 83 h, since radioactive precursors will continue to enter RNAs from the pool even though $[^{14}\text{C}]$adenine was no longer present in the external medium. The pattern of distribution of $[^3\text{H}]$uridine-containing RNA was typical of 17 h in this precursor, namely prominent peaks III and V, and also peak IV. The pattern of RNA obtained from cells grown for the last 17 h in $[^3\text{H}]$uridine together with 2 $\mu$g/ml antrycide (Fig. 5B), showed several differences. $[^3\text{H}]$uridine was incorporated into RNA peaks III and V, as in control cells, with peak IV (rRNA of the smaller subunit) being present perhaps as a shoulder to peak V and with increased levels of low molecular weight RNAs. However, the $[^{14}\text{C}]$adenine background showed a change in the ratio between the larger rRNA (peak II) and peak III, while the smaller subunit rRNA (peak IV) was not apparent. Longer time periods in $[^3\text{H}]$uridine were not successful because control cells began to show some evidence of changed synthesis, possibly due to the effects of starvation. It should be noticed that the actual levels of radioactivity for all types of RNA were considerably increased in the presence of antrycide, even though the same numbers of cells were used. Although considerable variation might be expected when using cells taken at random from cultures, higher levels of radioactivity were obtained consistently when antrycide was present in the medium.

These experiments indicated that at least 17 h after being placed in antrycide, some RNA of the types seen in control cells after that length of time in precursor were being produced. The difference in the $[^{14}\text{C}]$adenine background in antrycide, namely the loss of prominent rRNAs, suggested either the cessation of uptake of adenine into RNA, or the breakdown of newly synthesized RNA molecules. Maybe, like Crithidia, amoebae cannot use exogenous purines in the presence of antrycide. However, using $[^3\text{H}]$hypoxanthine in the presence of antrycide for 17 h gave a similar pattern of incorporation to that seen when cells were grown in $[^3\text{H}]$uridine and antrycide for the same time period, indicating use of exogenous purine into at least some RNAs. Thus antrycide might affect protein synthesis either directly or indirectly, leading to the breakdown of newly synthesized RNAs.

Effect of antrycide on protein synthesis

Some preliminary experiments were carried out to investigate the effects of antrycide on protein synthesis in amoebae, using $[^3\text{H}]$leucine as a general amino acid precursor. Batches of 200 cells were grown in 2 $\mu$g/ml antrycide and $[^3\text{H}]$leucine for varying time periods. They were rinsed in Chalkley's medium and the proteins precipitated with hot TCA prior to scintillation counting. Hot acid precipitation was used to eliminate the possibility of $[^3\text{H}]$leucyl-tRNA contaminating the filters. Although cells were taken from large cultures fed Tetrahymena daily, considerable variation was experienced between batches of cells taken on different days. However, the trend obtained was consistent throughout and Table 1 shows the results obtained from a typical experiment. Cells grown in antrycide for 4 h showed a 50% reduction in the radioactivity incorporated into hot-acid-precipitable protein when compared with control cells, while growth in antrycide for 18 h led to a 66% reduction. After longer times in drug, e.g. 40 h, this trend was reversed with an apparently higher incorporation in antrycide-
treated cells. Since this increased incorporation after long periods in drug had been noticed earlier, it could be that the presence of antrycide led to changes in membrane permeability or precursor pool usage. To test this apparent loss of inhibition of protein synthesis, cells were grown in antrycide only for 10 or 22 h before the addition of \[^{3}H\]leucine, and then incorporation into hot-acid-precipitable protein examined after a further 18 h. Now cells grown for 28 h in antrycide but with only 18 of those hours in precursor showed a reduction in acid-precipitable protein similar to those cells grown in drug plus precursor for 18 h. The effects of longer periods without food organisms on the level of incorporation can be seen in control cells (Table 1). The results obtained suggested a reduction in the level of protein synthesis in amoebae grown in antrycide, but the experiments do not differentiate between inhibition of translation or transcription.

### Table 1. The effects of antrycide on protein synthesis in Amoeba discoides

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### DISCUSSION

The major problem in assessing the effect of antrycide on RNA synthesis in \textit{A. discoides} lies in the absence of detailed information regarding patterns of normal RNA synthesis. Since the work of Craig & Goldstein (1969) and Prescott \textit{et al.} (1971) little has been published regarding patterns of synthesis of high molecular weight RNA, although recently Goldstein and his co-workers have been extensively investigating low molecular weight RNAs (Goldstein, 1976; Goldstein, Wise & Ko, 1977), which we have not considered in this study. Thus Prescott \textit{et al.} (1971) using phenol-extracted RNA and sucrose density gradient centrifugation could not detect any HnRNA in the amoeba nucleus. Goldstein (1973), again on the basis of sucrose density gradient analysis, records high molecular weight nuclear RNAs with \(s\) values of 39, 32–30, 19 and 16, with the \(s\) values of rRNAs as 32–30 and 19. No roles were proposed for the 39 and 16 \(s\) RNAs, other than to conclude from pulse-chase experiments that the 39 \(s\) molecules were not pre-ribosomal RNAs. Using both aqueous and formamide gel electrophoresis we established relative molecular weights for amoeba ribosomal RNAs of \(1.55\) and \(0.8 \times 10^{6}\) Daltons, corresponding to sedimentation values of approximately 26 and 19 \(s\) (Hawkins & Hughes, 1973). These were similar to the values obtained by Stevens & Pachler (1972) of 26 and 19 \(s\) (1.52 and \(0.89 \times 10^{6}\) Daltons) for the smaller amoeba \textit{Acanthamoeba castellani}. 

\[ A/C = \frac{\text{Antrycide}}{\text{Controls}} \]
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Using our values for amoeba rRNAs we have assigned relative molecular weights to the nuclear RNAs (peaks I–V) seen on aqueous gels of 2.45, 1.55, 1.13, 0.8 and 0.52 × 10^6 Daltons, corresponding to sedimentation values of 35, 26, 23, 19 and 16 s respectively. Except for the rRNAs we have no indication as to the roles of the other RNAs. These species do not disappear when the ribonuclease inhibitor diethylpyrocarbonate is added to the phenol extraction, and can be identified in RNA prepared by a modification of the method of Walsh & Fulton (1973) used for *Naegleria*, an amoeba-flagellate with high levels of nuclease activity. We attempted to identify the RNAs by use of [Me-3H]methionine since in eukaryotes 80% of the ribose becomes methylated at the 2'-o position at the level of the 45-s ribosomal precursor (Greenberg & Penman, 1966). However, we could detect no methylated nuclear RNA on our acrylamide gels, even after the precursor was microinjected into recipient amoebae. It could be that the methylated precursors are short-lived and are not detected using our long labelling times, or they may be present in very small amounts. The problem is that short periods in precursor yield very little detectable RNA when using phenol extraction, and we are attempting other methods of extraction to use with small cell numbers.

Since amoebae are not grown axenically it might be suggested that the 23 and 16 s nuclear RNAs are of bacterial origin. To investigate this possibility we extracted RNA from cells injected with [3H]uridine and then grown on unlabelled medium. All 5 peaks including the 23 and 16 s RNAs were present on polyacrylamide gels. However, some strains of amoebae are known to possess DNA-containing ‘bodies’ (Wolstenholme, 1966; Hawkins & Willis, 1969) in their cytoplasm, and thus these RNAs might be due to internal contaminants. Examination of our strain of *A. proteus* (TIP) which does not contain these bodies (Wolstenholme, 1966) still showed 23 and 16 s RNA. Furthermore, our strain of *A. discoides* is now free as judged by fluorescence microscopy and growth in dimidium bromide (Hawkins & Willis, 1969) and electron microscopy (G. Morgan, personal communication). In addition, cells were labelled by growth in [3H]uridine for 3 days and then their nuclei transferred into unlabelled amoebae and the transfers frozen immediately. RNA was extracted from batches of 200 transfers and examined on polyacrylamide gels. Although the amount of radioactive material was low, the 23 and 16 s peaks were present. Other evidence, such as the ratio of labelling of these peaks after 66 h, 1:1:3 rather than the converse, and the persistence of these peaks in the presence of certain inhibitors leads us to propose that there are at least 5 classes of high molecular weight nuclear RNA in amoebae.

*Crithidia oncopelti*, a trypanosomatid flagellate normally found in the gut of insects and cultured in defined media, has been used extensively as a model in the study of the activity of antrycide on blood-stream forms of pathogenic trypanosomes (Newton, 1966, 1973). These studies have suggested that the rate of growth of *Crithidia* is reduced by inactivation of cytoplasmic ribosomes rather than by direct action on DNA and RNA synthesis. However, detailed investigations comparing the incorporation of exogenous purines ([14C]adenine) with purines synthesized de novo ([14C]-glycine) into RNA showed a surprising difference in sensitivity to antrycide, the former being very sensitive. The inhibition of RNA synthesis occurred between formation
of acid-soluble nucleotides and synthesis of acid-precipitable nucleic acid. Attempts to examine the effects of drug on cell-free RNA synthesis using RNA polymerase from *E. coli* were difficult to interpret since, like neomycin, antrycide causes DNA-primer precipitation in reaction mixtures (Waring, 1965).

When amoebae were grown in levels of antrycide above 0.5 μg/ml, the growth rate was reduced with increasing antrycide concentration. As in *Crithidia*, the effects of the drug were reversible. Trypanosomes grown in mice treated with antrycide contain fluorescent granules, thought to be aggregates of drug and ribosomes (Ormerod & Shaw, 1963). Amoebae grown in antrycide were examined using fluorescent microscopy for the presence of ribosomal aggregates, but none were found. Electron micrographs of amoebae after 24 and 48 h in antrycide showed no abnormal 'clumping' of ribosomes. In contrast, *Tetrahymena* placed in antrycide rapidly accumulate fluorescent granules in their cytoplasm.

RNA extracted from amoebae grown in various levels of antrycide for 4 days and examined on polyacrylamide gels showed a concentration-linked loss of high molecular weight species, peaks II and IV, rRNAs appearing the most sensitive. Several possibilities can be proposed to account for these results: (a) RNA was not synthesized and cells survived at a reduced growth rate on pre-existing ribosomes and long-lived mRNAs; (b) antrycide prevented the utilization of radioactive precursor so that lack of synthetic activity was apparent rather than real; (c) in the presence of antrycide breakdown of newly synthesized RNA was occurring. Since cells grown in antrycide showed some incorporation into RNA molecules, the radioactive precursor appeared to be able to enter the cells and was utilized. Cells grown in [*14C]*adenine and then placed in antrycide plus [*3H]*uridine for 17 h showed some evidence of peaks I, III and V, but little rRNA. However, this might be expected to some extent since 17 h in [*3H]*uridine was not adequate to show rRNA in any clearly defined peaks. More interesting was the appearance of the [*14C]*adenine profile. In contrast to control cells where the rRNAs were very obvious, only the 26-s rRNA(II) was clear, but apparently in a different quantitative relationship to other RNAs. Since this was an exogenous purine, cells could perhaps no longer continue to use these pools in the presence of antrycide, but 17-h experiments using [*3H]*hypoxanthine did not support this proposal.

In all the experiments using antrycide an increased amount of low molecular weight RNA was seen, suggesting the possibility of breakdown of newly synthesized RNA. According to Apirion (1973) all classes of RNA are potentially degradable unless protected from endo- or exonuclease attack. In *Saccharomyces cerevisiae* processing of ribosomal precursor RNA depends on a continuous supply of ribosomal proteins, and unprocessed RNA is degraded (Warner & Gorenstein, 1977). We investigated the effects of antrycide on protein synthesis in amoebae by comparison of the levels of incorporation into hot-acid-precipitable protein using known numbers of cells. Even after 4 h in antrycide the level of incorporation had decreased by 50%. Further experiments are required to determine whether this inhibition is at the level of translation or transcription. In *Crithidia fasciculata*, results from cell-free experiments indicated the inhibition of the leucyl-tRNA synthetase reaction as well as protein synthesis (Kahan, Zahalsky & Hutner, 1968). Since cells continue to grow in drug at a reduced
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rate and this can be reversed readily, and amoebae are known to possess many long-lived mRNAs (Cameron & Hawkins, 1976), inhibition may be at the level of transcription. Warner & Gorenstein (1977) have shown that in yeast 35 coordinately regulated ribosomal proteins are synthesized on poly (A)-containing mRNAs with short lifetimes of between 8 and 15 min. When these are no longer transcribed, pre-ribosomal RNA, although transcribed, is degraded.

One explanation of the effects of antrycide on A. discoides is the reversible inhibition of transcription of mRNA for ribosomal protein(s) and possibly for other proteins required in the processing and protection of RNA molecules. Cells could survive on pre-existing ribosomes and pre-existing mRNAs. At higher concentrations of antrycide (4 µg/ml), cell division may be inhibited by the lack of RNAs synthesized in normal cells shortly before this event (Rao & Prescott, 1970). Much further experimentation is required, especially with regard to the production and processing of nuclear RNA molecules in this lower eukaryote.

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REFERENCES


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