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Effects of 5-Azacytidine on Nucleolar RNA and the Preribosomal Particles in Novikoff Hepatoma Cells†

John W. Weiss‡ and Henry C. Pitot*

ABSTRACT: Examination of nucleolar RNA from cultured Novikoff hepatoma cells treated for 3 hr with 5×10^{-4} M 5-azacytidine shows that significant amounts of analog-substituted 45S RNA are processed to the 32S RNA species, but 28S RNA formation is completely inhibited. Under these conditions of analog treatment 37% of the cytidine residues in the 45S RNA is replaced by 5-azacytidine. During coelectrophoresis of nucleolar RNA from 5-azacytidine-treated and control cells, the analog-substituted 45S RNA and 32S RNA display reduced mobilities compared to the control 45S RNA and 32S RNA. Coelectrophoresis of analog-substituted and control RNA after formaldehyde denaturation shows no differences in electrophoretic mobility between the two RNA samples, suggesting that 5-azacytidine incorporation may alter the secondary structure of the 45S RNA and the 32S RNA. 5-Azacytidine at 5×10^{-4} M severely inhibits protein synthesis in Novikoff cells by 3 hr. After this length of treatment, however, CsCl buoyant density analysis reveals no difference in density of either the 80S or 55S preribosomal ribonucleoprotein particles when compared to normal particles. Also 5-azacytidine treatment does not appear to cause major changes in the polyacrylamide gel electrophoresis patterns of the proteins in the 80S and 55S preribosomal particles. These results together with previous findings suggest that 5-azacytidine's inhibition of rRNA processing is possibly related to its alteration of the structure of the ribosomal precursor RNAs and is not a consequence of a general inhibition of ribosomal protein formation.

In mammalian cells rRNA is formed by a unique maturation process that occurs within the nucleolus. The mature 28S and 18S RNAs are formed from a large initial precursor, the 45S RNA. The 45S RNA becomes methylated and undergoes a sequence of specific cleavages forming various intermediate RNA species and finally the mature rRNAs (Weinberg *et al.,* 1967; Weinberg and Penman, 1970; Attardi and Amaldi, 1970; Burdon, 1971). rRNA processing does not occur with free RNA molecules. Studies have indicated that the ribosomal precursor RNAs exist as ribonucleoprotein complexes (Liau and Perry, 1969; Warner and Soeiro, 1967). Proteins become attached to the 45S RNA during or immediately after its synthesis. Warner and Soeiro (1967) were able to isolate discreet nucleolar RNP[1] particles designated as 80 S and 55 S based on their sedimentation properties and found these particles to contain 45S and 32S RNA, respectively.

At present little is known about the cleavage enzymes involved in processing of ribosomal precursor RNA or how the cell regulates the maturation process. Several factors are known to be necessary for normal processing. Proper methylation is important. During maturation the methylated portions of the 45S RNA are conserved (Wagner *et al.,* 1967). Processing is inhibited if the ribosomal precursor RNAs are undermethylated (Vaughan *et al.,* 1967). In addition to methylation, studies have shown a close correlation between rRNA processing and protein synthesis. Cycloheximide decreases 45S RNA synthesis and inhibits its processing in HeLa cells (Willems *et al.,* 1969). Puromycin also alters rRNA maturation (Soeiro *et al.,* 1968). HeLa cells starved for the essential amino acid, L-valine, display reduced rRNA processing (Maden *et al.,* 1969). When HeLa cells were incubated in hypertonic media which causes a gradual inhibition of protein synthesis the first effect on ribosome synthesis observed was reduced processing of the 45S RNA (Pederson and Kumar, 1971).

In our laboratory we have been interested in the effects of base analogs on the processing of rRNA. Numerous analogs of both purine and pyrimidine bases have been found to inhibit rRNA maturation (Perry, 1965; Tavitian *et al.,* 1968; Wilkinson *et al.,* 1971; Wilkinson and Pitot, 1972; Weiss and Pitot, 1974a,b). Evidence suggests that the analogs must be incorporated into the ribosomal precursor RNAs in order to exert their inhibitory effect (Tavitian *et*

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‡ Trainee in Biochemical Pathology of the National Institutes General Medical Sciences (GM-00130).

[1] Abbreviations used are: RNP, ribonucleoprotein; SDS, sodium dodecyl sulfate; 5-azaCR, 5-azacytidine; RSB buffer, 0.01 M NaCl-0.0015 M $MgCl_2$-0.01 M Tris-HCl (pH 7.4); NEB, 0.01 M NaCl-0.01 M EDTA-0.01 M Tris-HCl (pH 7.4).

al., 1968; Wilkinson and Tlsty, 1974). It has been proposed that incorporation of the analogs alters the structure of the ribosomal precursor RNAs and does not allow normal processing. Analog-substitued tRNA has been found to possess changes in secondary structure in studies with 5-fluorouracil (Lowrie and Bergquist, 1968) and 8-azaguanine (Levin and Litt, 1965). Exposure to 5-fluorouracil causes the formation of abnormal ribosomal particles in *Escherichia coli* and the RNA moieties of these particles possessed altered structure as indicated by reduced hyperchromicity and T_m values (Andoh and Chargaff, 1965).

It is possible that the base analogs exert their inhibition by affecting some other aspect important in the maturation process. While methylation does not appear to be affected as indicated in studies with toyocamycin (Tavitian *et al.*, 1968), 5-fluorouridine (Wilkinson and Pitot, 1972), and 5-azacytidine (Weiss and Pitot, in press), it is possible that some of the base analogs exert their effect by inhibiting protein synthesis or more specifically ribosomal protein synthesis. 5-Azacytidine (Doskocil *et al.*, 1967; Reichman and Penman, 1973), tubercidin (Acs *et al.*, 1964), and 8-azaguanine (Brockman, 1963) are effective inhibitors of protein synthesis.

In this paper, the inhibition of rRNA maturation caused by 5-azacytidine is studied in some detail. Both the amount and mobility of the ribosomal precursor RNAs synthesized in the presence of 5-azacytidine are evaluated by acrylamide–agarose gel electrophoresis. The possible importance of inhibition of protein synthesis by 5-azacytidine as the mechanism by which the analog inhibits rRNA processing is investigated by examining the proteins in nucleoli and in the preribosomal RNP complexes.

Materials and Methods

Materials

RNase-free density grade sucrose and optical grade CsCl were purchased from Schwarz/Mann. Biological grade glutaraldehyde, 50% w/w, was purchased from Fischer Scientific Co. Reagent grade formaldehyde, 37% w/w, was obtained from the Mallinckrodt Chemical Co.; DNase, electrophoretically purified, was obtained from Worthington Biochemical Corp. 5-Azacytidine was purchased from Calbiochem. 5-Aza[4-^{14}C]cytidine (50 Ci/mol) and [2,6-^{14}C]uridine (101 Ci/mol) were purchased from Nuclear Dynamics Inc. [5-^{3}H]Uridine (28 Ci/mmol), L-[U-^{14}C]leucine (280 Ci/mol), L-[4,5,-^{3}H]leucine (67.6 Ci/mmol), and [G-^{3}H]amino acid mixture (1 mCi/ml) were obtained from New England Nuclear; [8-^{3}H]guanosine (16 Ci/mmol) was purchased from Amersham/Searle. [8-^{14}C]Guanosine (34.1 Ci/mol) was purchased from Schwarz/Mann.

Methods

Cell Culture and Analog Treatment. Experiments were performed with Novikoff hepatoma cells growing in suspension culture as previously described (Wilkinson and Pitot, 1972; Weiss and Pitot, 1974a). Cells were used when growing in mid-log phase at 4–6 × 10^5 cells/ml. For the various studies, cells were resuspended in fresh media at 1–4 × 10^6 cells/ml and allowed to equilibrate 1 hr before using. Exposure to 5-azacytidine was done by adding the analog to the culture media from concentrated stock solutions in 0.9% saline which were prepared just before use. All analog studies were done at a final concentration of 5 × 10^{-4} M 5-azacytidine, in keeping with earlier work (Weiss and Pitot, 1974a).

Preparation of Nucleoli. Nucleoli were prepared by a modification of the method described by Penman (1966). Cells harvested by centrifugation (500 g, 3 min, 0°) were washed twice in ice-cold 0.9% saline and then resuspended in RSB buffer (0.01 M NaCl–0.0015 M $MgCl_2$–0.01 M Tris-HCl (pH 7.4)) at 1–2 × 10^8 cells/ml. The cells were allowed to swell for 15–20 min at 0°, and then were ruptured by homogenization in a Potter-Elvejhem homogenizer (0.004–0.006 in. clearance) with 30 up and down strokes. The homogenate was adjusted to 2% Triton X-100 and 0.4% sodium deoxycholate and vortexed for 30 sec. The nuclei were then collected by centrifugation (500*g*, 3 min) and washed in RSB buffer containing 2% Triton X-100 and 0.4% sodium deoxycholate. The pellet of nuclei was washed a second time in RSB buffer alone. The final nuclear pellet was then suspended in a solution of 0.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris-HCl (pH 7.2) at 1 × 10^8 nuclei/ml. To the resulting viscous solution DNase was added to a final concentration of 50 μg/ml. The suspension was warmed to room temperature and mixed by pipetting for several minutes until the viscosity was reduced and all visible clumps disappeared. The suspension was then chilled to 0° and centrifuged (1000*g*, 15 min). The resulting pellet of nucleoli was used for RNA extraction or preparation of preribosomal RNP particles.

Preparation of Preribosomal RNP Particles. Nucleolar ribonucleoprotein particles were isolated by the method of Warner and Soeiro (1967). Nucleolar pellets from 2 × 10^8 cells were suspended in 2 ml of NEB (0.01 M NaCl–0.01 M EDTA–0.01 M Tris-HCl (pH 7.4)) containing 0.01 M dithiothreitol and incubated at 37° for 7 min with occasional mixing. The samples were then vortexed for 15 sec, chilled to 0°, and centrifuged (1000*g*, 15 min). The resulting supernatants containing the extracted RNP particles were then analyzed on 30 ml of 15–30% w/w sucrose gradients prepared in NEB buffer. Absorbance at 260 nm was monitored by passing the gradients through a flow-cell in a Gilford 2400 spectrophotometer, and fractions of 1 ml or less were collected. Fractions containing the 80S and the 55S preribosomal particles were pooled and used for buoyant density and protein gel electrophoresis studies. In accordance with earlier work (Warner and Soeiro, 1967; Craig and Perry, 1970) electrophoretic analysis of the RNA from fractions containing the 80S particles yielded predominantly 45S RNA; those containing the 55S particles yielded predominantly 32S RNA (gels not shown).

RNA Extraction. Nucleolar RNA was extracted by the SDS–phenol method described previously (Wilkinson and Pitot, 1972; Weiss and Pitot, 1974a). The extracted RNA was precipitated with 2 volumes of 95% ethanol containing 2% potassium acetate at −20° overnight. After washing twice in 75% ethanol containing 1% potassium acetate, the RNA samples were dissolved in distilled water at 30–40 A_{260} units/ml.

Polyacrylamide-Agarose Gel Electrophoresis of RNA. Electrophoretic analysis of nucleolar RNA employed the method described earlier (Wilkinson *et al.*, 1971; Weiss and Pitot, 1974a) in which 2.1% acrylamide-0.6% agarose gels were used. RNA samples of 0.3–0.75 total A_{260} unit were analyzed per gel. Electrophoresis was done at 6 mA/gel for 3–5 hr as indicated in the figure legends. Electrophoresis beyond 3 hr resulted in the 18S RNA migrating off the end of the gel. Absorbance at 260 nm was monitored in a Gilford 2400 spectrophotometer equipped with a linear trans-

Table I: Degree of 5-Azacytidine Incorporation into 45S RNA.

Exposure Time (hr) to 5-Aza-cytidine	$\frac{^{14}C\ dpm}{^{3}H\ dpm}$	GTP Specific Activity[b] (Ci/mol)	% Cytidine Replaced
1	$\frac{224 \pm 11}{1136 \pm 46}$	0.37 ± 0.04	28.3 ± 2.1
3	$\frac{828 \pm 17}{3166 \pm 40}$	0.37 ± 0.03	37.2 ± 0.7

[a] To 4×10^8 Novikoff cells in 100 ml of media [^{3}H]guanosine (5×10^{-4} M, 1 Ci/mol) was added, 15 min later 5-aza[^{14}C]cytidine (5×10^{-4} M, 0.26 Ci/mol) was introduced. 1 and 3 hr after analog addition cells were harvested and nucleolar RNA was prepared. RNA samples, 0.5 A_{260} unit, were electrophoresed on 2.1% acrylamide–0.6% agarose gels for 3 hr. The 45S peaks located from A_{260} profiles were cut out and assayed for radioactivity. From the results the micromoles of 5-azacytidine and GMP present in the 45S samples were calculated using the known specific activity of 5-aza[^{14}C]cytidine and the calculated specific activity of GTP. With these values 5-azacytidine incorporation expressed at % cytidine replaced was determined (% = μmol of 5-azacytidine/μmol of GMP × 100). Values represent averages of five determinations. [b] Specific activity of GTP was determined as described under Methods. Both 1- and 3-hr values represent averages of seven separate determinations.

port. Gels were frozen for sectioning. They were routinely sliced into 2-mm sections except in several experiments as noted where 1-mm sections were taken.

Formaldehyde Denaturation of RNA and Electrophoresis in the Presence of Formaldehyde. RNA denaturation by formaldehyde and the gel electrophoresis method reported by Boedtker (1971) were modified and used to evaluate the electrophoretic properties of nucleolar RNA, free of differences in secondary structure. RNA samples were denatured with 1.1 M formaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) by heating to 63° for 15 min. This treatment effectively denatures RNA (Boedtker, 1971). After heating, the samples were chilled on ice and immediately electrophoresed.

To assure full denaturation was maintained during electrophoresis, gels were prepared in the presence of formaldehyde. The electrophoresis buffer used contained 1.1 M formaldehyde–3 mM EDTA–0.03 M sodium phosphate, (pH 7.3). Initially 2.1% acrylamide-0.6% agarose gels were prepared. The presence of formaldehyde, however, was found to stiffen the texture of the gels as noted elsewhere (Bernhardt and Darnell, 1969). While the RNA mobilities on these gels were quite similar to those seen in the system described by Boedtker (1971), the migration of the large RNA species was not great enough to allow adequate resolution of minor mobility differences even after prolonged electrophoresis. Therefore 1.5% acrylamide-0.6% agarose gels were prepared. In these gels the denatured nucleolar RNA species had mobilities adequate for our studies.

The gels were preelectrophoresed for 2 hr at 6 mA/gel prior to use. Then the denatured RNA samples were applied and coelectrophoresed for 5 hr at 6 mA/gel at room temperature. After electrophoresis the gels were scanned for absorbance at 260 nm, then frozen and sliced into 1-mm sections for radioactivity determination.

Protein SDS–Polyacrylamide Gel Electrophoresis. Protein electrophoresis was carried out by the SDS–acrylamide method (Shapiro *et al.*, 1967); 7.5% acrylamide gels were prepared in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS. Gel dimensions were 12 cm × 0.6 cm. Protein samples were prepared as follows: Sucrose gradient fractions containing the 80S and 55S RNP particles were precipitated with Cl_3CCOOH (5% final concentration). After 1 hr at 0°, the fractions were centrifuged (1000*g*, 30 min, 4°) and the resulting pellets were washed twice with ice-cold 100% ethanol and then air dried. The pellets were next dissolved in sample buffer (0.01 M sodium phosphate (pH 7.2), 0.5 M urea, 0.5% SDS, 1.0% mercaptoethanol, and 10% glycerol) by heating to 65° for 15 min. For samples of nucleolar proteins, nucleoli prepared as described above were further purified by resuspending in NEB buffer and centrifuging (1500*g*, 30 min) through NEB containing 30% sucrose. The resulting nucleolar pellets were then dissolved in the sample buffer with heating.

Samples were applied to the gels with Bromophenol Blue added as the tracking dye. After the samples had entered the gels at 4 mA/gel, the current was increased to 8 mA/gel and electrophoresis was performed for 10 hr at room temperature. The gels were stained with 0.05% Coomassie Blue in methanol–acetic acid–water (5:1:5) for 5 hr and then destained in 10% methanol. The gels were subsequently fractionated into 1-mm crushed sections with a mechanical gel fractionator (Gilson Medical Electronics) for radioactivity measurements.

Cesium Chloride Buoyant Density Analysis. Buoyant density studies were carried out by the method reported by Baltimore and Huang (1968). Fractions from preparative sucrose gradients containing the 80S and 55S RNP particles were fixed with 6% glutaraldehyde neutralized with sodium bicarbonate just prior to use. Performed 5-ml cesium chloride gradients were prepared in RSB buffer containing 0.5% Brij-35. After fixation the samples were immediately layered onto the chilled gradients and centrifuged in a Spinco SW41 rotor for 24 hr at 30,000 rpm (4°). The bottoms of the tubes were then punctured and 10-drop fractions were collected. Density was determined by refractometry; 500 μg of bovine serum albumin was added to each fraction as carrier and the fractions were precipitated with 10% Cl_3CCOOH. After 1 hr at 0° the samples were transferred onto fiberglass filter discs by successive rinses with 10% Cl_3CCOOH, 5% Cl_3CCOOH, and finally 70% ethanol. After drying, the filters were placed in toluene–2,5-diphenyloxazole scintillation fluid and their radioactivity was determined.

Determination of GTP Specific Activity. To calculate the amount of GMP present in samples of 45S RNA it was necessary to know the specific activity of the precursor pool of GTP. Determinations were made on cell cultures handled as described in Table I. Novikoff cells, 4×10^8 cells in 100 ml of media, were incubated with [^{3}H]guanosine (5×10^{-4} M, 1 Ci/mol). After 15 min 5×10^{-4} M 5-azacytidine was added; 1 and 3 hr after analog addition, 2×10^8 cells were harvested and their acid-soluble fractions were obtained by three 2-ml extractions with 0.5 N perchloric acid at 0°. The three combined extracts were neutralized with potassium hydroxide. After standing for 30 min the potassium perchlorate precipitate was removed by centrifugation. The supernatants were then evaporated to dryness and resuspended in 1-ml volumes of distilled water.

GTP was isolated from the acid-soluble sample using

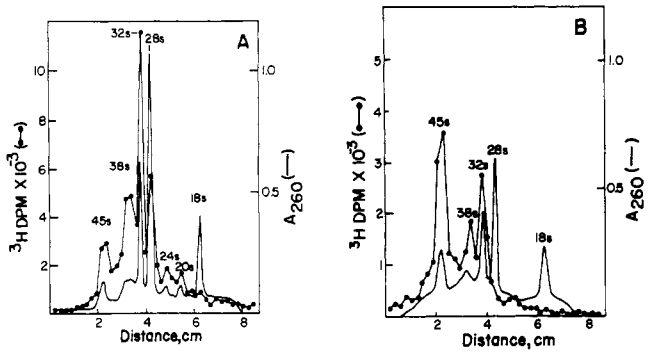


FIGURE 1: Electrophoresis of nucleolar RNA from control and 5-azacytidine treated Novikoff cells. Two cultures of Novikoff cells, each containing 1.5×10^8 cells in 300 ml of media, were prepared. One culture was incubated with 5×10^{-4} M 5-azacytidine for 3 hr. During the last 2 hr of incubation [^{3}H]uridine (0.5 μCi/ml, 1×10^{-5} M) was added to both cultures. Cells were harvested and nucleolar RNA was prepared. RNA samples were electrophoresed for 3 hr on 2.1% acrylamide-0.6% agarose gels. Radioactivity (dpm) and absorbance (A_{260}) are plotted *vs.* distance of migration (cm). (A) Control nucleolar RNA, 0.5 A_{260} unit; (B) 5-azacytidine-treated nucleolar RNA, 0.3 A_{260} unit.

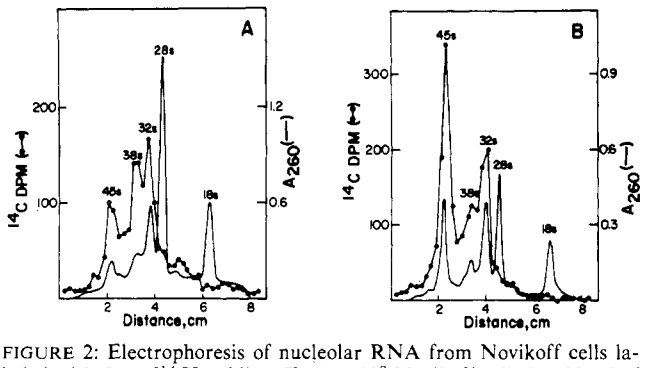


FIGURE 2: Electrophoresis of nucleolar RNA from Novikoff cells labeled with 5-aza[^{14}C]cytidine. To 4×10^8 Novikoff cells in 100 ml of media 5-aza[^{14}C]cytidine (0.13 μCi/ml, 5×10^{-4} M) was added. After 1 and 3 hr of incubation in the presence of the analog, cells were harvested and nucleolar RNA prepared. RNA samples were electrophoresed for 3 hr on 2.1% acrylamide-0.6% agarose gels. (A) 1-hr nucleolar RNA sample, 0.75 A_{260} unit; (B) 3-hr nucleolar RNA sample, 0.5 A_{260} unit.

thin-layer chromatography essentially as described by Randerath and Randerath (1967). Chromatography was performed on polyethyleneimine-impregnated cellulose plates (Brinkman). GTP standards were run in parallel with the unknown samples. The chromatograms were first developed with methanol–water (1:1) to remove salts and organic material. Then stepwise development without intermediate drying was performed: first with 0.4 M LiCl to 2 cm beyond the origin; then 1 M LiCl to 4 cm; and finally with 1.6 M LiCl to 17 cm beyond the origin. The plates were dried and then rinsed in absolute methanol to remove the LiCl. The location of GTP was found by examining the chromatograms under uv light. The GTP regions were cut out, and GTP was eluted with a solution of 0.7 M $MgCl_2$–0.02 M Tris-HCl (pH 7.4) for 1 hr at room temperature. The eluents were cleared of cellulose particles by centrifugation. Absorbance at 253 nm, λ_{max} for GTP (Hurlbert, 1957), and the radioactivity of the eluents were measured. From these values the specific activity of GTP was calculated.

Liquid Scintillation Counting. Gel fractions containing ^{14}C and ^{3}H radioactivity were hydrolyzed in 0.5 ml of 0.5 N NaOH at 37° overnight. The fractions were neutralized with HCl; 10 ml of Scintisol (Isolab) was added to each sample and its radioactivity was measured. Gels containing only ^{3}H radioactivity were incubated in 0.5 ml of 30% H_2O_2 at 75° overnight. Scintisol was then added and their radioactivity was determined. Counting was done in a Nuclear Chicago liquid scintillation counter with attached computer, programmed to calculate dpm values.

Results

Effect of 5-Azacytidine on Nucleolar RNA Processing. Previous work from this laboratory indicated that treatment of cultured Novikoff cells with 5-azacytidine caused an inhibition of rRNA processing (Weiss and Pitot, 1974a). Analysis of total cell RNA by gel electrophoresis showed complete inhibition of 28S and 18S RNA formation while some processing of the 45S RNA to the 32S intermediate continued. Sucrose gradient analysis of nucleolar RNA from HeLa cells treated with 5-azacytidine showed greatly reduced processing of the 45S RNA to the 32S RNA, suggesting that most of the 45S component formed in the presence of the analog was degraded (Reichman *et al.*, 1973). To appreciate more fully the extent of rRNA processing in the presence of 5-azacytidine, nucleolar RNA was examined by acrylamide-agarose gel electrophoresis.

Figure 1A shows the electrophoretic analysis of an RNA sample extracted from the nucleoli of Novikoff cells growing in log phase which were labeled for 2 hr with [^{3}H]uridine. The absorbance and radioactivity profiles indicate all the major RNA species in the rRNA maturation sequence. In addition two small peaks, 24S and 20S, are observed. While the 24 S is not thought be a normal intermediate in the processing sequence, the 20 S is considered to be the immediate precursor to the 18S RNA (Weinberg *et al.*, 1967; Weinberg and Penman, 1970).

Figure 1B shows the electrophoretic pattern of nucleolar RNA isolated from Novikoff cells treated with 5×10^{-4} M 5-azacytidine for 3 hr. The absorbance profile shows a relative increase in the 45S and 32S RNA precursors. [^{3}H]Uridine labeling carried out in the same manner as in Figure 1A shows a well-labeled 45S peak. A fair amount of labeled 45 S has been processed to the 32S RNA; however, no label appears in either the 28S or 18S peak. While a small peak of radioactivity is seen in the 24 S, no labeling of the 20S RNA is observed. In comparing the 5-azacytidine sample with the control it must be pointed out as noted in the figure legend that different sample sizes were analyzed. When this is taken into account labeling of the 45S RNA in the 5-azacytidine sample is nearly twice that seen in a control sample of equal size while 32S RNA labeling is slightly less than ½ that in a comparable control sample. From these and subsequent results to be shown it can be appreciated that a significant amount of 32S RNA is formed in the presence of 5-azacytidine.

Incorporation of 5-Aza[^{14}C]cytidine into Ribosomal Precursor RNA. Experiments with 5-aza[^{14}C]cytidine were performed to establish that the analog was incorporated into the ribosomal precursor RNAs. Figure 2 shows the electrophoretic profiles of nucleolar RNA isolated from Novikoff cells exposed to 5×10^{-4} M 5-aza[^{14}C]cytidine. After 1 hr of exposure (Figure 2A) label is present in the 45S RNA and has progressed into the 32S RNA. A small peak of radioactivity is also in the 24S RNA, but no label has progressed into the 28S or 18S RNAs. After 3 hr of exposure (Figure 2B), a relative increase in the quantity of precursor RNAs in the nucleolar sample is apparent from the absorbance profile. The radioactivity pattern also shows

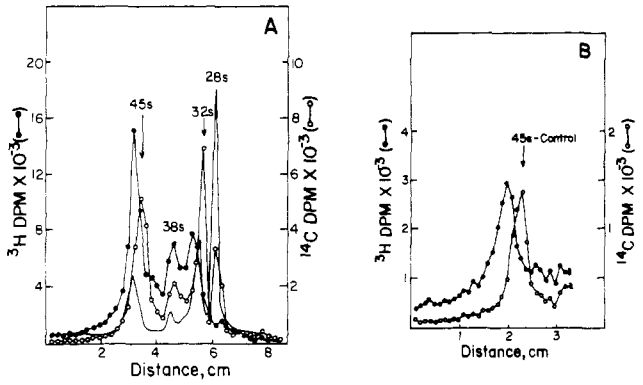


FIGURE 3: Coelectrophoresis of nucleolar RNA from 5-azacytidine-treated and control Novikoff cells. Two identical cultures of 1.5×10^8 Novikoff cells in 100 ml of media were prepared. One culture was treated with 5×10^{-4} M 5-azacytidine for 3 hr. [^{3}H]Guanosine (0.5 μCi/ml, 1×10^{-5} M) was present during the last 2 hr of incubation. The other culture incubated with 5×10^{-4} M cytidine (control) for 3 hr, [^{14}C]Guanosine (0.25 μCi/ml, 1×10^{-5} M) was added during the last 2 hr of incubation. Nucleolar RNA was prepared after the 3-hr incubation. Equivalent analog and control RNA samples, 0.25 A_{260} unit each, were mixed and coelectrophoresed on a 2.1% acrylamide-0.6% agarose gel for 4 hr as follows: (A) 5-Azacytidine-treated, ^{3}H-labeled (●); control, ^{14}C-labeled (○). Radioactivity was determined from 2-mm gel sections. Arrows point to positions of RNA peaks in control sample. Absorbance at 260 nm (——); full scale equals 1.2 A_{260} units. (B) 5-Azacytidine treated (●); control (○). Radioactivity was determined on 1-mm gel sections. Upper one-third of the gel is shown.

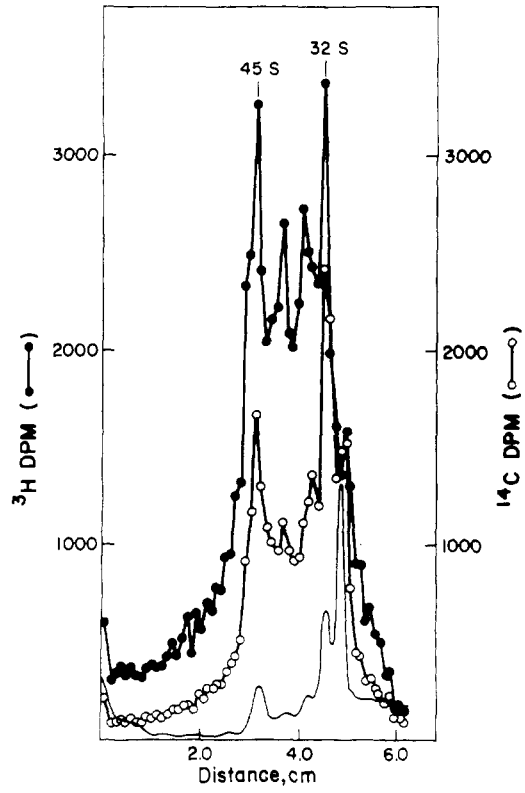


FIGURE 4: Coelectrophoresis of formaldehyde denatured nucleolar RNA samples from 5-azacytidine-treated and control Novikoff cells. Nucleolar RNA samples were prepared as described in the legend to Figure 3. Samples of ^{3}H-labeled 5-azacytidine RNA and ^{14}C-labeled control RNA, 0.25 A_{260} unit each, were separately denatured by heating at 63° with 1.1 M formaldehyde for 15 min. The samples were cooled, mixed, and coelectrophoresed for 5 hr on a 1.5% acrylamide-0.6% agarose gel in the presence of 1.1 M formaldehyde. The gel was sectioned into 1-mm slices and counted. 5-Azacytidine treated (●); control (○). Absorbance at 260 nm (——); full scale equals 1.5 A_{260} units.

increased labeling of the 45S and 32S RNA. Still, after this length of time no label has entered into the 28S or 18S peaks. Comparison of Figures 1B and 2B shows that the inhibition pattern seen when a labeled nucleoside precursor is used in the presence of unlabeled 5-azacytidine truly reflects the inhibition of processing of analog-substituted precursors.

The increased labeling of the 45S and 32S RNA after 3 hr as compared with 1 hr in Figure 2 could reflect a greater frequency of analog incorporation in addition to increases in the quantities of the precursors. To determine this, the degree of analog incorporation into the 45S RNA was measured. 5-Azacytidine incorporation into the 45S RNA, expressed as per cent cytidine replaced, was estimated by double labeling with 5-aza[^{14}C]cytidine and [^{3}H]guanosine making use of the reported values for the base composition of the 45S RNA. The nucleotide composition of the the 45S RNA from Novikoff hepatoma cells has been found to contain essentially equal amounts of GMP (33.5%) and CMP (33.1%) (Nakamura *et al.*, 1967; Busch and Smetana, 1970). Thus the micromoles of GMP present in a given 45S sample can be equated to the micromoles of CMP that should normally be present. Per cent cytidine replacement can then simply be computed by the ratio of micromoles of 5-azacytidine to micromoles of GMP present in 45S RNA samples.

Table I shows the incorporation data. To calculate the micromoles of GMP present it was necessary to know the specific activity of the GTP pool, the immediate precursor utilized for RNA synthesis, since the exogenously supplied guanosine would be diluted with guanosine synthesized *de novo*. GTP specific activity was determined as described under Materials and Methods, and as can be seen in Table I the values were substantially lower than the specific activity of the added guanosine (1 Ci/mol). After 1 hr of exposure, about 28% of the cytidine residues in the 45S RNA is replaced by 5-azacytidine; after 3 hr of exposure, approximately 37% of cytidine is replaced. Thus analog incorporation is increasing with time.

Alteration of the Ribosomal Precursor RNAs by 5-Azacytidine. The inhibition of rRNA maturation by base analogs has been postulated to be a result of changes in the secondary structure of the analog-substituted ribosomal precursor RNAs. Examination of total cell RNA from Novikoff hepatoma cells treated with 5×10^{-4} M 5-azacytidine for 3 hr revealed that the mobilities of the 45S and 32S precursor RNAs were reduced during gel electrophoresis (Weiss and Pitot, in press). Because this was direct evidence for abnormal ribosomal precursor RNA formation the phenomenon was studied in more detail. To substantiate that this finding truly represented differences in the ribosomal precursors and not spurious changes in nonribosomal RNA which possibly occurred during treatment, the effect was reexamined with nucleolar RNA samples from cells treated in the same manner.

Figure 3 shows the coelectrophoresis of nucleolar RNA from 5-azacytidine treated and control cells. Equivalent samples of ^{3}H-labeled nucleolar RNA from 5-azacytidine-treated cells and ^{14}C-labeled nucleolar RNA from control cells were mixed and electrophoresed together for 4 hr. In Figure 3A the 32S RNA in the 5-azacytidine sample displays a definitely slower mobility than the control 32S peak. The analog-containing 45S RNA also appears to have a slower mobility than the control 45S but the difference is

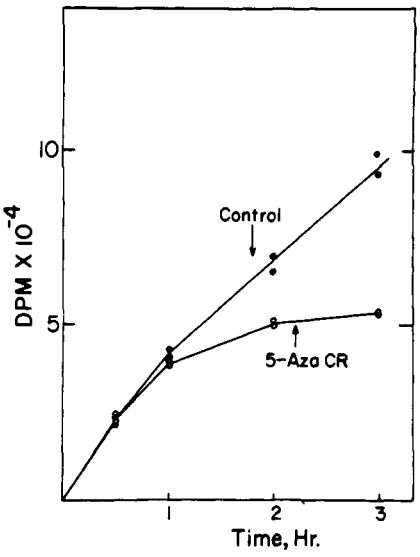


FIGURE 5: Incorporation of ^{3}H-labeled amino acids into acid-insoluble material of Novikoff cells. Novikoff cells were resuspended in fresh media and divided into two 25-ml cultures at 1×10^6 cells/ml; 5×10^{-4} M 5-azacytidine was added to one culture and 5×10^{-4} M cytidine (control) was added to the other. A mixture of ^{3}H-labeled amino acids was added simultaneously to give 0.2 μCi/ml final concentration. Equal cell aliquots of 10^6 cells were collected in duplicate at subsequent time points. Acid-insoluble material was collected with ice-cold 10% Cl_3CCOOH and washed twice with ice-cold 5% Cl_3CCOOH. The resulting pellets were incubated at 37° in 0.2 N NaOH for 1 hr. Aliquots of the supernatants were counted. Radioactivity is expressed as dpm/10^6 cells.

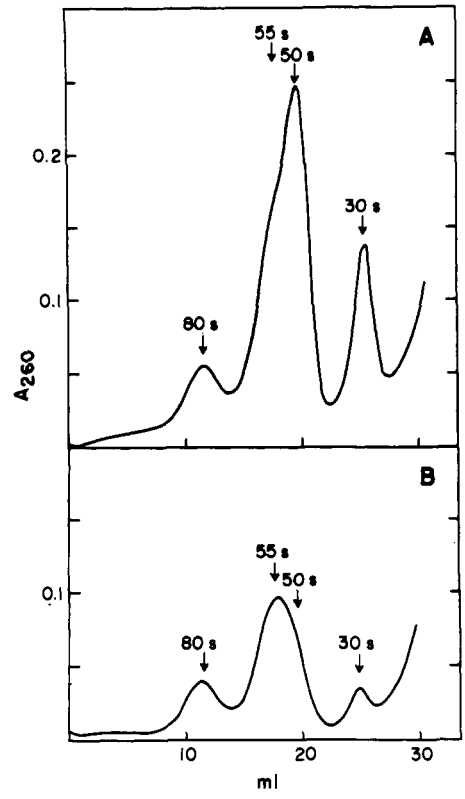


FIGURE 6: Sucrose gradient preparation of nucleolar RNP particles. Two cultures of 2×10^8 Novikoff cells in 100 ml of media were treated with 5×10^{-4} M 5-azacytidine or 5×10^{-4} M cytidine (control) for 3 hr. Nucleolar RNP particles were then prepared as described under Methods and analyzed on 30 ml of 15–30% (w/w) sucrose gradients in a Spinco SW27 rotor. Centrifugation was at 22,000 rpm for 16 hr at 4°. The gradients were eluted through a flow-cell and absorbance at 260 nm was monitored. Sedimentation coefficients of the preribosomal particles and ribosomal subunits were assigned in conformity with the original designations of Warner and Soeiro (1967). (A) Control; (B) 5-azacytidine treated.

not as pronounced. No difference in the 38S region is observed.

It was considered important to confirm that the analog-containing 45S RNA definitely had a slower mobility compared to the normal 45S RNA because this would indicate the altered precursors did not arise from aberrant processing since the 45S RNA is a direct product of transcription. Faulty processing could logically be argued to result in the formation of an abnormal 32S RNA. To improve detection of subtle differences in the 45S RNA, a gel containing the coelectrophoresed analog and control samples was sliced into 1-mm gel sections rather than 2 mm which is the slice thickness routinely taken. A more distinct difference between analog and control 45S RNA with 1-mm sectioning is apparent in Figure 3B. Hence there is no question that the analog containing 45S RNA has a slower mobility than the control 45S RNA.

Conformation Independent Gel Electrophoresis. Work by Boedtker (1971) utilizing formaldehyde to destroy the secondary structure of RNA allowed her to determine RNA molecular weights by acrylamide gel electrophoresis, free of conformational differences between the RNA species. A slightly modified version of this technique as described under Materials and Methods was used to study 5-azacytidine's effect on the ribosomal precursor RNAs. Samples of nucleolar RNA from control and 5-azacytidine-treated cells were separately denatured by heating to 63° in 1.1 M formaldehyde for 15 min. The denatured samples were then mixed and coelectrophoresed for 5 hr on a 1.5% acrylamide-0.6% agarose gel in the presence of 1.1 M formaldehyde. After electrophoresis the gel was sectioned into 1-mm slices for greater resolution. Under these electrophoretic conditions both the 5-azacytidine and control 45S peaks are coincident, showing no migration differences (Figure 4). Likewise the analog and control 32S peaks are

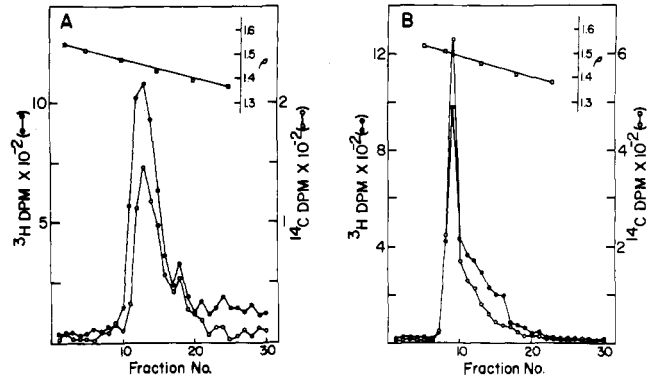
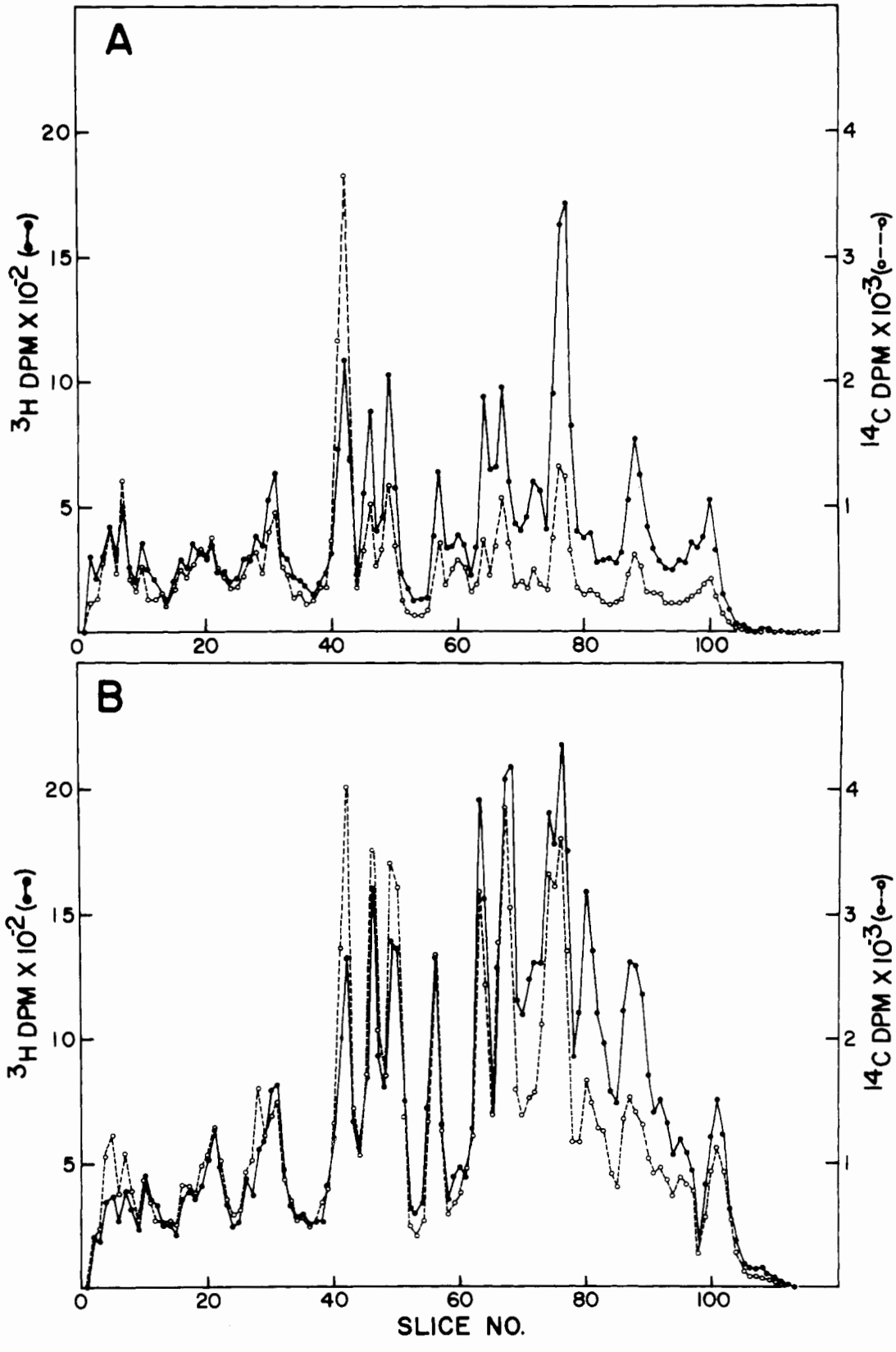


FIGURE 7: Buoyant density analysis of 80S and 55S RNP particles from 5-azacytidine-treated and control Novikoff cells. Two cultures of 2×10^8 Novikoff cells in 100 ml of media were incubated with 5×10^{-4} M 5-azacytidine or 5×10^{-4} M cytidine (control) for 3 hr. During the last 50 min of incubation, [^{3}H]uridine (2 μCi/ml, 28 Ci/mmol) was added to the analog-treated culture and [^{14}C]uridine (1 μCi/ml, 101 Ci/mol) was added to the control culture. Nucleolar RNP particles were prepared; 80S and 55S samples were separately fixed with glutaraldehyde. Fixed analog and control 80S samples were then mixed; likewise the two 55S samples were mixed. The combined samples were then centrifuged to equilibrium on CsCl gradients and assayed for radioactivity as described in Methods. (A) 80S RNP particles: 5-azacytidine (●); control (○). (B) 55S RNP particles; 5-azacytidine (●); control (○).



coincident. The lack of differences in electrophoretic mobility in this conformation-independent system suggests that 5-azacytidine incorporation alters the secondary structure of the 45S and 32S ribosomal precursor RNAs.

Inhibition of Protein Synthesis by 5-Azacytidine. 5-Azacytidine has been shown to be a potent inhibitor of protein synthesis in *E. coli* (Doskocil *et al.*, 1967) and in various eukaryotic systems (Reichman and Penman, 1973; Raska *et al.*, 1966; Zain *et al.*, 1973; Shutt and Krueger, 1972). Figure 5 shows the effect of 5-azacytidine on protein synthesis in cultured Novikoff cells. Cells were exposed to 5×10^{-4} M 5-azacytidine, the same analog concentration at which the preceding RNA studies were carried out. As can be seen detectable inhibition of cellular protein synthesis is not found during the first hour of exposure to the analog. Subsequently there is abrupt reduction in radioactive amino

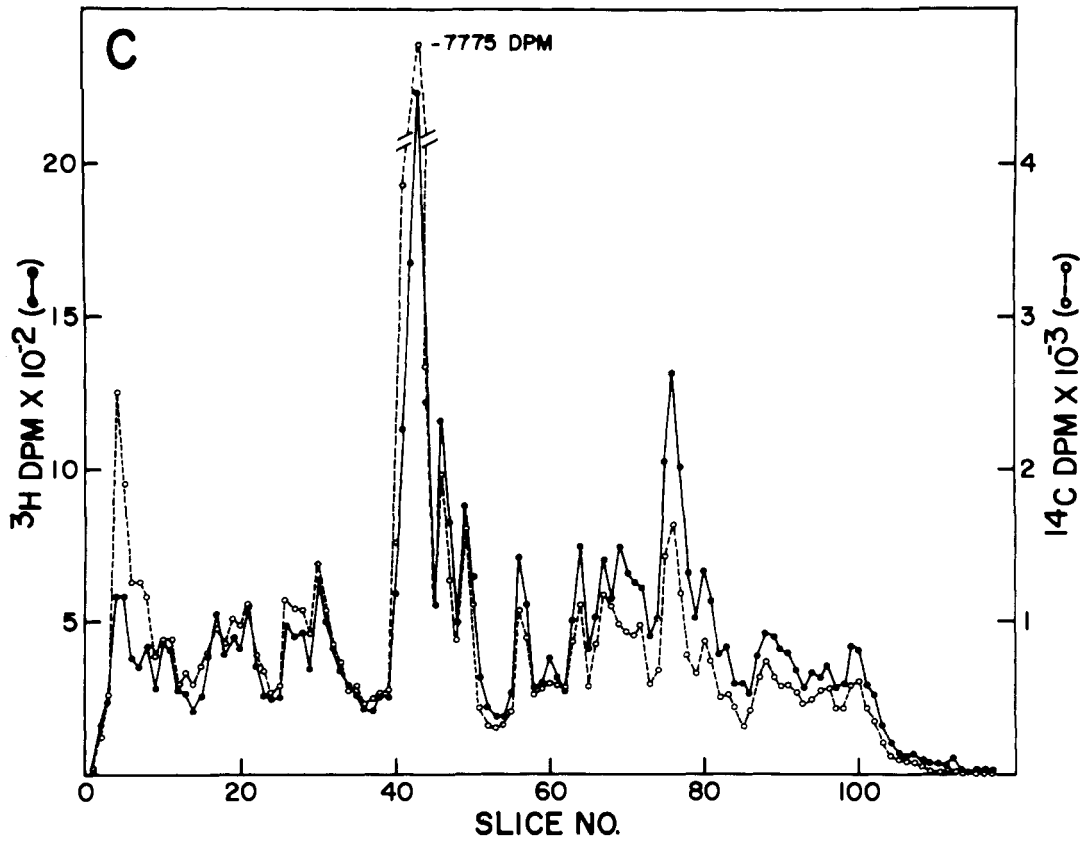


FIGURE 8: Coelectrophoresis of 80S, 55S, and nucleolar proteins from 5-azacytidine-treated and control Novikoff cells. Two cultures of 4×10^8 Novikoff cells in 200 ml of media were treated with 5×10^{-4} M 5-azacytidine or 5×10^{-4} M cytidine (control) for 3 hr. During the last 50 min, L-[^{3}H]leucine (3 μCi/ml, 67.6 Ci/mmol) was added to the analog-treated culture and L-[^{14}C]leucine (1 μCi/ml, 280 Ci/mol) was added to the control. Proteins from the nucleolar RNP particles and from washed nucleoli were prepared. Appropriate samples of 80S and 55S proteins and also nucleolar proteins from 5-azacytidine-treated cells were coelectrophoresed with corresponding samples from control cells. Electrophoresis was for 10 hr on 7.5% acrylamide gels in the presence of 0.1% SDS. Origins are at the left. 5-Azacytidine treated (●); control (○). (A) (left-hand page) 80S protein mixture; (B) (left-hand page) 55S protein mixture; (C) (above) nucleolar protein mixture.

acid incorporation such that after 3 hr of exposure there appears to be nearly complete inhibition of protein synthesis.

Buoyant Density of the Preribosmal RNP Particles. To determine whether inhibition of protein synthesis by 5-azacytidine was an important aspect of the analog's ability to inhibit rRNA maturation, the proteins of the preribosomal ribonucleoprotein particles were studied. RNP particles were isolated from the nucleoli of both normal and 5-azacytidine-treated cells by the method of Warner and Soeiro (1967). Figure 6 shows typical profiles of nucleolar RNP particles from preparative sucrose gradients. Extracts from control cells (Figure 6A) contain 80S and 55S RNP particles which by electrophoretic analysis are found to contain 45S RNA and 32S RNA, respectively, in agreement with previous investigations (Warner and Soeiro, 1967; Craig and Perry, 1970). Also sizable populations of 50S and 30S ribosomal subunits, containing 28S and 18S RNA, respectively, are found as noted in earlier work (Craig and Perry, 1970; Mirault and Scherrer, 1971). Predominantly 80S and 55S RNP particles are found in nucleoli from 5-azacytidine-treated cells (Figure 6B). While these particles are present in slightly less amounts than in control nucleoli, the populations of 50S and 30S ribosomal subunits are markedly reduced in nucleoli from analog-treated cells. This pattern reflects the known rRNA inhibition pattern for 5-azacytidine.

In order to assess the effect of 5-azacytidine on the proteins of the nucleolar RNP particles, buoyant density studies in CsCl gradients were performed. The buoyant densities of RNP particles reflect their RNA/protein ratios (Spirin *et al.*, 1965; Perry and Kelley, 1966). If 5-azacytidine treatment reduced available ribosomal proteins, the resulting nucleolar RNP particles should have greater buoyant densities because of their lower protein contents. Both 80S and 55S RNP particles were isolated from 5-azacytidine treated and control cells labeled with [^{3}H]uridine and [^{14}C]uridine, respectively. Samples of 80S RNP particles from analog and control cells were separately fixed with glutaraldehyde freshly neutralized with $NaHCO_3$, then mixed and analyzed on a preformed CsCl gradient. Analog and control 55S RNP particles were handled in the same manner. The resulting density profiles are shown in Figure 7. The 80S RNP particles form a rather wide density band which is similar to previous findings (Craig and Perry, 1970). No detectable difference in density between 5-azacytidine and control 80S RNP particles is noted. Likewise the radioactivity profiles of the 55S RNP particles, which display a sharper density band, do not differ between analog and control. Thus, 5-azacytidine does not appear to alter the protein contents of the 80S and 55S preribosomal RNP particles.

Electrophoretic Analysis of Proteins from Nucleoli and RNP Particles. While the buoyant density studies revealed no quantitative difference in the overall protein contents of the 80S and 55S preribosomal particles caused by 5-azacytidine, the proteins from these particles and also from total nucleoli were studied directly by SDS–acrylamide gel electrophoresis. If 5-azacytidine's effect on rRNA maturation

were mediated by an inhibition of protein synthesis the analog would have to act by limiting short-lived or rapidly utilized proteins since the rRNA inhibition is already maximal after 3 hr of analog treatment. Thus Novikoff cells exposed to 5×10^{-4} M 5-azacytidine for 3 hr were pulsed with [^{3}H]leucine during the last 50 min of incubation while control cells were pulse labeled with [^{14}C]leucine during this period. Proteins from 80S and 55S RNP particles and proteins from nucleoli were isolated as described under Materials and Methods. Corresponding analog and control protein samples were mixed and coelectrophoresed on 7.5% acrylamide gels in the presence of 0.1% SDS. Staining with Coomassie Blue showed that the one-dimensional gel system used resolved the protein mixtures into more than 40 discreet bands.

Figure 8 shows the radioactivity profiles of the various protein mixtures. Labeling patterns of the 80S proteins (Figure 8A), 55S proteins (Figure 8B), and total nucleolar proteins (Figure 8C) from the 5-azacytidine-treated cells are quite similar to those of the corresponding control samples. If 5-azacytidine were inhibiting rRNA maturation by a generalized inhibition of ribosomal protein synthesis significant differences in the proteins of the preribosomal particles might be expected, yet examination of the protein profile from the 55S RNP particles whose processing is the most severely inhibited by 5-azacytidine, shows it to be nearly identical with that of the control 55S RNP particles.

Discussion

Electrophoretic analysis of nucleolar RNA isolated from Novikoff hepatoma cells treated with 5×10^{-4} M 5-azacytidine shows that the initial steps of rRNA processing of analog-substituted 45S RNA do occur, resulting in considerable 32S RNA being formed. The studies further show that 28S RNA formation is completely inhibited. The formation of the 20S intermediate appears inhibited but because it is present normally in very low amounts (Weinberg *et al.*, 1967), it is difficult to assess the degree of inhibition. The amount of 5-azacytidine that is incorporated into the 45S RNA increases with the length of analog treatment. After 3 hr of exposure to 5×10^{-4} M 5-azacytidine, approximately 37% of cytidine in the 45S RNA is replaced by 5-azacytidine. The degree of incorporation compares with the values in *E. coli* where 20–30% of cytidine in newly synthesized RNA was replaced by 5-azacytidine (Paces *et al.*, 1968).

As postulated in studies with other base analogs, 5-azacytidine may inhibit rRNA maturation by causing physicochemical changes in the ribosomal precursor RNA into which it is incorporated. 5-Azacytidine has been shown to reduce temperature-dependent hyperchromicity and T_m values of DNA in *E. coli* (Zadrazil *et al.*, 1965) and of RNA in AKR mice (Cihak *et al.*, 1965). The finding that the analog measurably reduces the electrophoretic mobilities of the 45S and 32S RNAs lends stronger support for this contention.

Both molecular weight and conformation influence the electrophoretic mobility of RNA molecules (Boedtker, 1971; Fisher and Dingman, 1971, Staynov *et al.*, 1972). If the mobility differences of the rRNA precursors were due to an increase in molecular weight it would have to result from the synthesis of an initial rRNA precursor with a longer nucleotide sequence. Thus it seemed more likely that the altered mobilities reflected conformational differences. To test this possibility formaldehyde denaturation was employed. Destruction of secondary structure by formaldehyde is found to abolish the mobility differences suggesting that 5-azacytidine incorporation does in fact alter the secondary structure of the 45S and 32S RNA species.

A number of factors have been found to influence the electrophoretic mobility of RNA molecules such as temperature (Fisher and Dingman, 1971; Grivell *et al.*, 1971), pH (Ro-Choi *et al.*, 1973; Grivell *et al.*, 1971), and ionic strength (Loening, 1969; Grivell *et al.*, 1971). Also incomplete deproteinization can affect RNA mobility. Hence the possibility exists that the electrophoretic mobility differences between the analog and control rRNA precursors could in part reflect the influence of one or more of these factors. While the present experiments were performed by mixing the RNA samples together and coelectrophoresing them on the same gel in order to reduce any subtle variations in electrophoretic conditions, we cannot say with absolute certainty that the reduced mobilities of the analog-containing rRNA precursors reflects only differences in secondary structure.

While 5-azacytidine incorporation appears to alter the electrophoretic mobility of the ribosomal precursor RNAs, it is possible that the analog's ability to inhibit rRNA maturation is a consequence of its inhibition of protein synthesis. Complete inhibition of protein synthesis by 5-azacytidine treatment occurred after 3 hr in HeLa cells (Reichman and Penman, 1973) and after 4 hr in phytohemagglutinin-stimulated lymphocytes (Zain *et al.*, 1973). An initial lag period of 60–90 min between the addition of analog and the onset of inhibition of protein synthesis has been observed (Raska *et al.*, 1966; Zain *et al.*, 1973; Shutt and Krueger, 1973). In our studies with Novikoff cells inhibition of protein synthesis was not detected until 1 hr after addition of analog but by 3 hr this inhibition appeared complete. Evidence indicates that 5-azacytidine must be incorporated into RNA to exert its inhibitory effect (Doskocil *et al.*, 1967; Reichman and Penman, 1973). Thus the delay before the onset of inhibition probably reflects the time required for the synthesis of analog-containing RNA.

A number of findings do not support the possibility that 5-azacytidine is acting by inhibiting protein synthesis. Inhibition of rRNA maturation by the analog is already apparent by 1 hr. Since there exists a pool of ribosomal proteins which as shown in HeLa cells allows assembly of ribosomes for 1–2 hr in the presence of cycloheximide (Warner *et al.*, 1966), it would seem that the inhibition of rRNA processing is occurring before ribosomal proteins could become limiting.

The preribosomal RNP particles from nucleoli do not reveal any marked changes in their protein complements under similar conditions of analog treatment that produce an inhibition of rRNA maturation. Buoyant density studies on CsCl gradients show no density differences between the 80S and 55S RNP particles from 5-azacytidine-treated and control cells suggesting that the analog does not alter the protein contents of these particles. Also the electrophoretic protein profiles of the 80S and 55S RNP particles and whole nucleoi from 5-azacytidine-treated cells are similar to those from control cells. Reduction of protein synthesis in HeLa cells by incubation in hypertonic media resulted in 80S and 55S RNP particles with decreased protein contents as judged by the increased buoyant densities of these particles (Pederson and Kumar, 1971). Buoyant density measurements of the 80S and 55S RNP particles from cycloheximide-treated L cells revealed altered protein compositions of these particles (Craig and Perry, 1970). Thus, if 5-

azacytidine has an effect on the ribosomal proteins in the 3-hr period of treatment, some changes in the buoyant densities of the preribosomal RNP complexes might be expected.

5-Azacytidine completely inhibits 28S and 18S RNA formation and the ribosomal precursor RNA synthesized in the presence of the analog does not mature once the analog is removed (Weiss and Pitot, 1974a). In contrast some 28S and 18S RNA is still synthesized when protein synthesis is inhibited by cycloheximide (Willems *et al.*, 1969; Warner *et al.*, 1966) or by starvation for the essential amino acid, L-valine (Maden *et al.*, 1969). Also ribosomal precursor RNA formed in the presence of the inhibitor of protein synthesis, puromycin, is subsequently processed into the mature rRNA after the antibiotic is removed (Soeiro *et al.*, 1968).

The results reported in this paper and elsewhere do no exclude the possibility that the inhibition of rRNA maturation by 5-azacytidine is due to the absence of proteins essential for processing. The buoyant density analysis and one-dimensional protein electrophoresis system used may not be sensitive enough to detect subtle changes in the protein complements of the preribosomal particles. Prestayko *et al.* (1974) have shown that nucleolar preribosomal particles from Novikoff cells display a total of 60 distinct protein spots by two-dimensional gel electrophoresis, while our system resolved the proteins into approximately 40 bands. Hence if a few or even one essential protein were missing in the analog-containing preribosomal particles, their absence could go undetected. Further studies are needed to establish conclusively the mechanism by which 5-azacytidine inhibits rRNA maturation.

At present, however, a primary effect on the structure of the rRNA precursors seems a more likely explanation for 5-azacytidine's inhibition. Other studies have indicated the importance of secondary structure in the processing of ribosomal precursor RNA. In an *E. coli* mutant deficient for RNase III a common initial precursor RNA of the 23S and 16S rRNA has been found to accumulate (Dunn and Studier, 1973; Nikolaev *et al.*, 1973). This pre-rRNA is cleaved *in vitro* by RNase III into RNA molecules resembling the 16S precursor RNA and the 23S precursor RNA (Dunn and Studier, 1973). RNase III is an enzyme which has a specificity for double-stranded RNA (Robertson *et al.*, 1968). In HeLa cells several intercalating agents were found to inhibit 45S RNA processing, suggesting that a double-stranded region in the structure of the precursor RNA is required for normal processing (Snyder *et al.*, 1971). Since the structure of rRNA molecules in solution is very similar to the structure of the rRNA molecules within ribosmes (Zubay and Wilkins, 1960; Klug *et al.*, 1961; Blake and Peacocke, 1965), it is likely that the alterations of the isolated RNA molecules indicated in the present studies exist in the RNP complexes within the cell.

Electron microscopic mapping experiments with 5-azacytidine substituted RNA could confirm that the analog alters the secondary structure of the 45S and 32S RNA. Electron microscopic analysis by Wellauer and Dawid (1973) showed that very reproducible regions of secondary structure are retained by the ribosomal precursor RNAs and the 28S RNA during formamide fixation. Examination of rRNA precursors containing 5-azacytidine by such techniques may give insight into the importance of secondary structure in regulating the various steps in the rRNA maturation process.

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Thermal Denaturation of the DNA–Ethidium Complex. Redistribution of the Intercalated Dye during Melting†

Stelios Aktipis,* William W. Martz, and Antonis Kindelis

ABSTRACT: The temperature dependence of the circular dichroism of the DNA–ethidium bromide complex at elevated temperatures provides evidence that the optical activity of the complex near 307 nm originates from interactions between intercalated dye molecules while the optical activity near 515 nm results from singly intercalated ethidium bromide molecules. The behavior of the circular dichroism of the complex at elevated temperatures also explains the higher ellipticities near 307 nm which characterize complexes formed between ethidium bromide and denatured DNA. Finally the circular dichroism data indicate that the melting of the complex takes place in a stepwise manner with some DNA regions, probably AT-rich regions, dissociating first. The implications of these findings regarding the inhibiting effect of ethidium bromide on the function of DNA polymerase are examined.

Ethidium bromide is an antitrypanosomal drug which is known to inhibit nucleic acid synthesis both *in vivo* (Tomchick and Mandel, 1964) and in cell-free systems (Elliott, 1963; Waring, 1964). It has been suggested (Waring, 1964) that the pharmacological properties of ethidium are at least partly due to inhibition of polymerases involved in nucleic acid synthesis. This inhibition may be a direct consequence of the physicochemical interaction between DNA templates and the planar phenanthridinium ring structure of the drug (Waring, 1965a).

The interaction between DNA and ethidium bromide results in pronounced changes in the physical properties of both components such as an increase in the fluorescence quantum efficiency of ethidium (LePecq and Paoletti, 1967; Wahl *et al.*, 1970) and an increase in the viscosity of DNA (Cohen and Eisenberg, 1969). In terms of a physical model, the interaction may be described as an insertion of the planar phenanthridinium ring between adjacent nucleotide pairs in DNA. The binding depends primarily on hydrophobic interactions occurring between ethidium bromide and

† From the Department of Biochemistry and Biophysics, Loyola University of Chicago, Stritch School of Medicine, Maywood, Illinois 60153. *Received August 1, 1974.* This investigation was supported by National Institutes of Health Grant No. CA10346 and by a General Research Support Grant to Loyola University.