NUCLEOLAR NECKLACES IN CHICK EMBRYO FIBROBLAST CELLS

I. Formation of Necklaces by Dichlororibobenzimidazole and Other Adenosine Analogues That Decrease RNA Synthesis and Degrade Preribosomes

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ABSTRACT

A number of chemicals, mostly adenosine analogues, cause the nucleolus of the chick embryo fibroblast to lose material and unravel over a period of several hours into beaded strands termed nucleolar necklaces (NN). The results of analyses of the fibroblasts, treated with the NN-forming chemical dichlororibobenzimidazole (DRB), suggest that the following biochemical alterations occur: DRB almost completely prevents the increase in both messenger RNA (mRNA) and heterogeneous nuclear RNA. It interferes with ribosome synthesis by decreasing the rate of 45S ribosomal RNA (rRNA) accumulation by 50%, slowing the rate of 18S rRNA appearance by 50%, and causing an extensive degradation (80%) of the 32S and 28S rRNA-containing preribosomes. Most of this preribosome degradation probably occurs at or before the 32S rRNA preribosome stage. The degradation of these preribosomes appears to be due to the formation of defective 45S rRNA preribosomes rather than to a direct DRB interference with preribosome processing enzyme action. DRB inhibits total cellular RNA synthesis in less than 15 min, suggesting a direct interference with RNA synthesis. DRB also inhibits the uptake of nucleosides into the cell. DRB in the concentrations used does not appear to directly interfere with the translation of mRNA (i.e., protein synthesis). Other NN-forming adenosine analogues and high concentrations of adenosine (2 mM) cause biochemical alterations similar to those produced by DRB. To explain the preribosome degradation, we propose the hypothesis that DRB inhibits the synthesis of mRNA; as a consequence, some of the preribosomal proteins that normally coat the 32S rRNA portion of the 45S precursor RNA become limiting, and this defective portion is then subject to degradation by nucleases.

A number of adenosine analogues, and even adenosine itself in high concentrations (2 mM), interfere with ribosome synthesis and cause fragmentation of the nucleolus which is the ribosome synthetic center of the cell. Toyocamycin in HeLa cells (29) and 5-bromotubercidin in chick embryo

fibroblast (CEF)[1] cells (2) have been shown to decrease the rate of 45S ribosomal RNA (rRNA) accumulation and almost completely halt its subsequent "processing" or conversion to 32S, 28S, and 18S rRNAs.[2] Tavitian et al. (29) suggested that toyocamycin caused these biochemical alterations by being inserted into the growing rRNA chains. The effects of these chemicals vary somewhat with cell type.

In CEF cells we have found that the alterations in the nucleolar morphology caused by these adenosine analogues are particularly striking: the round CEF cell nucleoli lose material and the remaining fragments unravel into long (20 μm) beaded strands termed nucleolar necklaces (NN) (9). NN formation in CEF cells was initially observed after the addition of high concentrations (2 mM) of adenosine (16, 17). These chemically produced NN are similar in appearance to those formed when CEF cells are grown in a medium deficient in the amino acid arginine (11).

In this paper we report biochemical studies on a number of the NN-forming chemicals in CEF cell cultures. The chemicals investigated were: toyocamycin, 5-bromotubercidin, 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB), 2-mercapto-1-(2-[4-pyridyl]ethyl)benzimidazole (MPB), 2 mM adenosine, and α-amanitin. Although the biochemical actions of some of these chemicals such as toyocamycin and bromotubercidin have been well characterized (29, 2), less is known about the action of the others. Our investigations place special emphasis on reactions which are directly or indirectly related to the synthesis of ribosomes such as: the synthesis of messenger RNA (mRNA), heterogeneous nuclear RNA (HnRNA), transfer RNA (tRNA), protein synthesis, and the synthesis of the 45S ribosomal precursor RNA and its subsequent processing to the 18S and 28S RNAs found in the completed ribosomes.

DRB was chosen for detailed examination because its structure would not permit it to be inserted into the rRNA via the normal RNA polymerase base pairing mechanism; it was, therefore, probably interfering with ribosome production by a mechanism different than that suggested for toyocamycin (29). Early work with this inhibitor demonstrated that DRB was able to decrease total cellular RNA labeling (3). Later studies by Egyhazi et al. (7) in Diptera salivary glands indicated that DRB inhibited both rRNA synthesis and HnRNA synthesis. In this paper, we extend these studies with DRB to cover all types of RNA synthesis and investigate the disruption in rRNA synthesis and processing in more detail.

In the accompanying paper (9) I discuss light microscope studies on the process of NN formation and attempt to correlate them with the biochemical data.

MATERIALS AND METHODS

Cell Culture

A chick embryo fibroblast cell inoculum was prepared as described by Granick (9). Approximately 5×10^5 cells were inoculated when the cells were to be grown on cover slips in vials containing 1.0 ml medium. When the cells were to be grown in 100-mm Falcon tissue culture dishes containing 10 ml medium, $1.0\text{–}2.0 \times 10^7$ cells were inoculated. The cultures were maintained at 37°C in Eagle's minimal essential medium containing 10% fetal bovine serum. The medium was changed 1 day after inoculation of the cultures and every other day subsequently. Biochemical experiments were generally carried out with 2- or 3-day cultures (approximately 10^7 cells/dish).

Primary chick embryo liver cell cultures were prepared according to the method of Granick (10) and were grown in Ham's medium containing 10% fetal bovine serum.

Chemicals

DRB and MPB were gifts from the Merck Chemical Div., Merck & Co., Inc., Rahway, N. J. 5-Bromotubercidin was a gift from Dr. B. Brdar of The Rockefeller University, New York. Toyocamycin was a gift from Dr. R. J. Suhadolnik, Albert Einstein Medical Center,

[1] *Abbreviations used in this paper:* AIA, allylisopropylacetamide; ALA, Δ-aminolevulinic acid; CEF, chick embryo fibroblast; DRB, 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole; HnRNA, heterogeneous nuclear RNA; MPB, 2-mercapto-1-(2-[4-pyridyl]ethyl)-benzimidazole; mRNA, messenger RNA; NN, nucleolar necklaces; rRNA, ribosomal RNA; tRNA, transfer RNA; 45S rRNA preribosome, preribosome containing 45S rRNA.

[2] The HeLa cell nucleolus synthesizes a "preribosome" consisting of a 45S rRNA strand which is coated with ribosomal proteins (1). The processing of the 45S rRNA, according to Wellaur and Dawid (36), proceeds as follows: first, a piece of rRNA is cleaved off at the 3' end and discarded, leaving a 41S RNA. The latter is then cleaved into two preribosomes, one containing 32S RNA and the other containing 20S RNA. The 20S RNA in the preribosome is trimmed to become the cytoplasmic ribosome containing 18S RNA, and the 32S RNA in its preribosome is trimmed to become the cytoplasmic ribosome containing 28S RNA.

Philadelphia. α-Amanitin was a gift from Dr. Th. Wieland of the Max-Planck-Institut, Heidelberg, Germany. [5-^{3}H]Uridine and L-[1-^{14}C]leucine were purchased from the New England Nuclear Boston, Mass. Ethylene diacrylate was purchased from the Monomer-Polymer Div., Bordon Chemical Div., Philadelphia, Pa. Seakem agarose is distributed by Bausch and Lomb Inc., Rochester, N. Y.

RNA Isolation

The RNA isolation was essentially that of Tiollais et al. (31) which was a modification of the one devised by Scherrer and Darnell (23). We scaled down the procedure to handle CEF cells from two 100-mm tissue culture dishes, or approximately 1.2–4.0 × 10^7 cells with a packed cell volume of 0.1–0.2 ml. Volumes of the solutions were selected so that narrow 3.0-ml test tubes (1 × 7.5 cm) could be used for centrifugation of the phenol emulsion. After the first phenol extraction, the aqueous phase was transferred to a new 3-ml test tube and the phenol extraction was repeated twice by adding 0.5 vol of phenol solution, covering the test tube tightly with Parafilm, and shaking it at 5°C for 15 min. Two ethanol purification steps were used and the final RNA precipitate was redissolved in the smallest possible volume of glycerol electrophoresis buffer.

Gel Electrophoresis

The RNA samples were electrophoresed on composite acrylamide-agarose gels similar to those developed by Peacock and Dingman (19). We substituted ethylene diacrylate for the bisacrylamide for easier hydrolysis (4) and modified the concentrations of various components to get better slicing characteristics in an egg slicer-type gel slicer. The gels were made with the electrophoresis buffer of Weinberg et al. (34). The preparation of this modified gel solution has been described in detail by Granick (8). Quartz gel tubes (0.7 cm inside diameter × 10.0 cm in length) were used. In order to form flat upper gel surfaces, cylindrical plastic plugs (about 0.65 cm in diameter) scored with two vertical grooves on their sides were allowed to sink 1.5 cm into the gel-filled tubes. A small rubber band around the plug prevented the plugs from slipping farther. The RNA samples were dissolved in electrophoresis buffer containing 15% glycerol (vol/vol) and were electrophoresed at 5 mA/gel (approximately 40 V) as described by Weinberg et al. (34). In this paper all RNA samples were electrophoresed for 6 h at room temperature.

Gel Scanning and Quantitation

The optical density of the gels was determined by scanning them directly inside their quartz tubes at 260 nm with a specially designed motorized gel holder in a Cary no. 14 spectrophotometer. Three blank gels were run to determine an average base line for the OD curves.

To determine the cpm of the gels, they were sliced in an egg slicer-type gel slicer (0.91 mm/slice) and two slices were added per scintillation vial. Soluene (Packard Instrument Co., Inc., Downers Grove, Ill.) (0.7 ml) was added to each vial and the vials were tightly closed with plastic lined caps and incubated at 60°C for 2–3 h. Toluene-Omnifluor (New England Nuclear) (10 ml) and 0.05 ml of absolute ethanol were then added. The vials were counted in a Packard scintillation counter.

The cpm curves for a series of gels were normalized to equal amounts of RNA sample per gel by using the 18S cytoplasmic rRNA peak from the gel optical density scans as a measure of cell number.

EXPERIMENTAL PROCEDURES

Effects of NN-Forming Chemicals on Total Cellular RNA and Total Protein Synthesis

The effects of NN-forming chemicals were investigated in a series of vial labeling experiments (Fig. 1). CEF cells were grown as monolayer cultures on cover slips in vials containing Eagle's medium. DRB or adenosine was added to the medium at concentrations which had been found to produce good NN formation as seen by microscope examination (9). Then [^{3}H]uridine or [^{14}C]-leucine was added and the cells were harvested after increasing periods of time. The cover slips were then fixed in glutaraldehyde, washed, and their cpm determined.

The NN-forming chemicals were seen to greatly inhibit [^{3}H]uridine incorporation (Fig. 1 *a*) but to have little effect on [^{14}C]leucine incorporation (Fig. 1 *b*) for at least a 2-h period. Only a small decrease in protein synthesis (10–20%) was observed after 4 h of [^{14}C]leucine incorporation in the presence of the NN-forming chemicals. Similar results have been reported for toyocamycin (29) and for bromotubercidin (2).

Effect of NN-Forming Chemicals on the Rate of Uridine Uptake by the Cells

Most of the NN-forming chemicals are nucleosides or nucleoside analogues. Some of these, such as MPB or high concentrations of adenosine, have been reported to be competitive inhibitors of nucleoside uptake into the cell (18, 26, 28). For this reason any decrease in [^{3}H]uridine incorporation observed in the presence of these inhibitors will

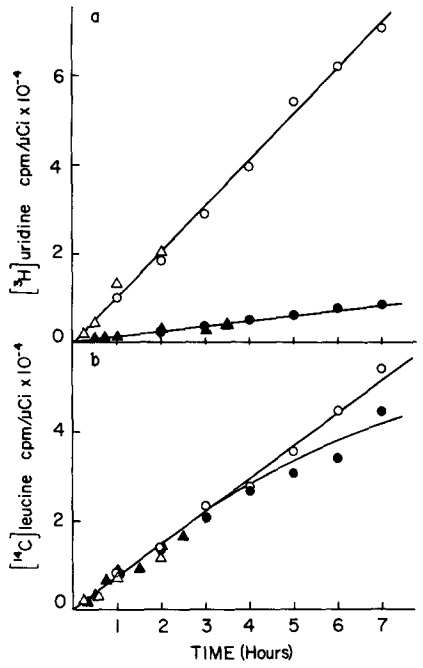


FIGURE 1 Inhibition of total RNA synthesis and non-inhibition of overall protein synthesis by DRB and adenosine. A separate experiment with its own control was used for each compound. (*a*) [³H]uridine incorporation. (*b*) [¹⁴C]leucine incorporation. O——O, control for DRB experiment; Δ——Δ, control for adenosine experiment; ●——●, DRB (50 μg/ml); ▲——▲, adenosine (2 mM). For DRB: Vials containing monolayers of cells growing on cover slips were preincubated for 30 min with 50 μg/ml DRB per ml of medium. At zero time, one series of vials received [³H]uridine (1.0 μCi/ml medium and a final concentration of 0.3 μM) and a second series of vials received [¹⁴C]leucine (0.25 μCi/ml medium and a final concentration of 0.87 μM). The control cell series were given the same amounts of label but no DRB. For adenosine: Both adenosine and labeled compounds were given together at zero time. One series of vials received adenosine to a final concentration of 2 mM and [³H]uridine (1.0 or 2.0 μCi/ml medium and a final concentration of 0.12 or 0.24 μM) and a second series of vials received adenosine (2 mM) and [¹⁴C]leucine (0.5 or 1.0 Ci/ml medium and a final concentration of 2 or 4 μM). Control vials received the same concentration of label but no adenosine. Labeling was stopped at the indicated times by placing the vials in an ice bath. The cover slips of labeled cells were washed in their vials with three changes of ice-cold Earle's solution and were fixed for 10 min in 2.5% glutaraldehyde 0.1 M phosphate buffer, pH 7.4. They were then washed with several changes of physiological saline over the next 1–2 h to remove small radioactive molecules which might have been trapped inside the cells. After dipping the cover slips in distilled water, they were air dried. They were digested in scintillation vials with 0.5 ml Soluene at 60°C for 2 h. Toluene-Omnifluor was then added and the vials were counted in a Packard scintillation counter. Data were plotted as cpm/μCi/cover slip vs. time of incubation with label. Each point represented the average cpm of four cover slips. The average deviation from this mean value was ±10%. The cpm data for the adenosine experiment were scaled to those of the DRB experiment so that they could be plotted on the same graph. This was done by multiplying the adenosine cpm by a factor to superimpose the control curves for the two sets of data.

reflect both a decreased rate of RNA synthesis and a decreased rate of [³H]uridine uptake into the cell. Therefore, to determine the actual effect of an NN inhibitor on the rate of RNA synthesis, it is necessary to determine a correction factor for the inhibition of [³H]uridine transport.

The correction factor for the inhibition of [³H]-uridine uptake was obtained in a vial-labeling experiment by comparing the amount of [³H]uridine found inside the cells in the presence and absence of each inhibitor (Fig. 2). In one series of vials, cells growing on cover slips were labeled, then fixed in glutaraldehyde, and extensively washed so that all the soluble nucleotides were removed. The cpm remaining on the cover slips represented [³H]uridine fixed into the cellular RNA. In a second series of vials the cover slips of labeled cells were washed with medium, just to remove contaminating nucleosides from the cell surface, and were then air dried so that the acid-soluble pool of nucleotides remained inside the cells. These cpm represented label in both "RNA" + "pool." A subtraction of the cpm from these two vial series yielded the cpm of the [³H]uridine pool.

Values for the inhibition of uptake of [³H]uridine into the cell for various NN-forming chemicals have been summarized in Table I. Adenosine, MPB, and DRB were found to inhibit [³H]uridine uptake but toyocamycin had no effect.

Rate of Total RNA Synthesis

The values of [³H]uridine incorporation in the presence of NN-forming chemicals were then corrected to represent the actual rate of total RNA synthesis (Table I). Toyocamycin (2 μg/ml) and DRB (50 μg/ml) were found to be the most efficient inhibitors of total RNA synthesis, depressing it to 40% of the control level.

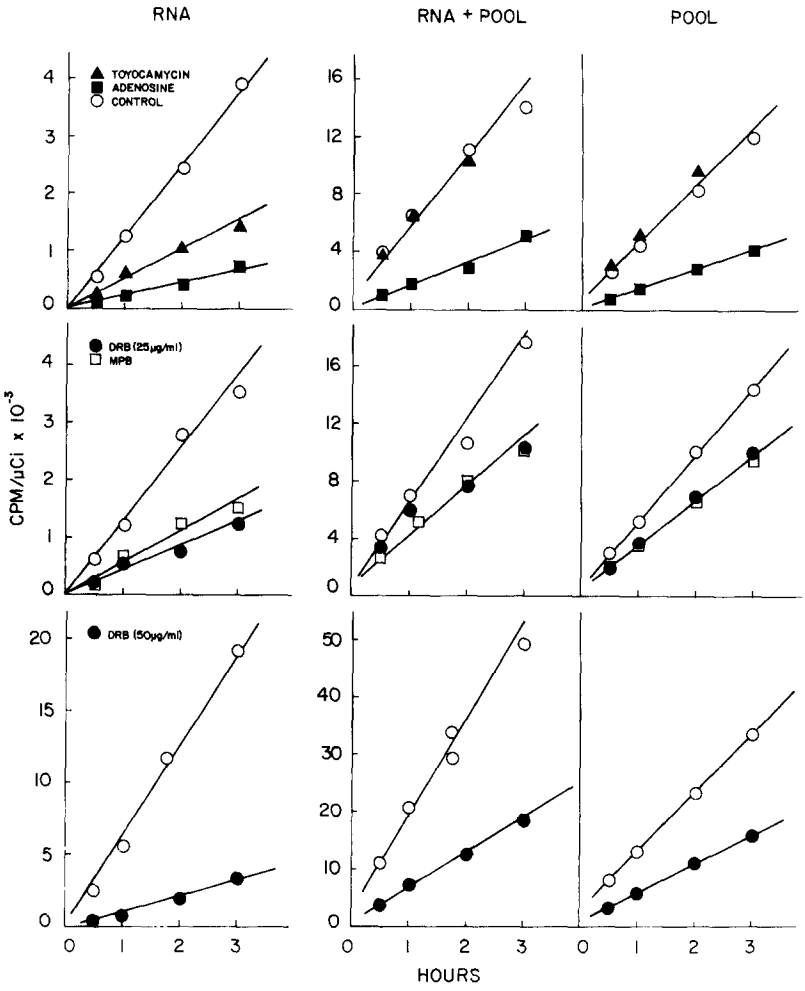


FIGURE 2 Inhibition of [^{3}H]uridine uptake and inhibition of total RNA synthesis by various NN-forming chemicals in CEF cells. O——O, control; ▲——▲, toyocamycin; ■——■, adenosine; ●——●, DRB; □——□, MPB. Two series of vials, one for RNA and one for RNA + pool, were assayed for each inhibitor. Cover slips, with approximately one layer of CEF cells growing in Eagle's medium, were preincubated with one of the following inhibitors for 30 min: toyocamycin (TCM) (2 μg/ml); adenosine (2 mM = 0.53 mg/ml); DRB (25 μg/ml); and MPB (25 μg/ml). Both series of vials then received the same concentration of [^{3}H]uridine (2 μCi/ml; 14 μM). A second experiment with DRB was done with 50 μg/ml DRB and concentrations of [^{3}H]uridine similar to those used in the later gel experiments. In this vial experiment, the cells were preincubated for 30 min with 50 μg/ml DRB. Vials from the RNA series were the given 4 μCi/ml [^{3}H]uridine; vials from the RNA + pool series received 0.8 μCi/ml [^{3}H]uridine. Control cells received label only: 2 μCi/ml for the RNA series; 0.4 μCi/ml for the RNA + pool series. Although the concentration of [^{3}H]uridine was varied, the total uridine concentration was kept at 0.16 μM by adding cold uridine. In all other respects this second DRB experiment was identical with the first. Vials were harvested at various times after addition of label (t = 0) throughout a period of 3 h. The cover slips were washed, fixed, and counted as described in the legend to Fig. 1. The resulting cpm were expressed as cpm/μCi/cover slip. All vials were run in quadruplicate and the average deviation from the mean for these points was ±10%. The cpm/μCi/cover slip for the RNA vial series, the RNA + pool vial series and their arithmetical difference or pool were plotted vs. time.

TABLE I

Inhibition of [³H]Uridine Uptake and Corrected Total RNA Synthesis by NN-Forming Chemicals in CEF Cells

Inhibitor	Inhibitor concn	Uridine concn	Ratio cpm inhibitor/control		
			"Uncorrected" [³H]uridine incorporated into RNA	[³H]uridine uptake into cell pool	"Corrected" total RNA synthesis
		μM			
Adenosine	2 mM	14	0.15	0.33	0.51
Toyocamycin	2 μg/ml	14	0.38	1.0	0.38
MPB	25 μg/ml	14	0.44	0.65	0.68
DRB	25 μg/ml	14	0.33	0.65	0.51
DRB	50 μg/ml	0.16	0.19	0.47	0.40
DRB*	50 μg/ml	0.3	0.12	—	—

These calculations were made with the cpm values obtained from Fig. 2. The "corrected" values for RNA synthesis were obtained by dividing the "uncorrected" RNA ratios by the [³H]uridine uptake ratio. The ratios have an approximate error of ±20%.

* Indicates that the data were obtained from the experiment of Fig. 1.

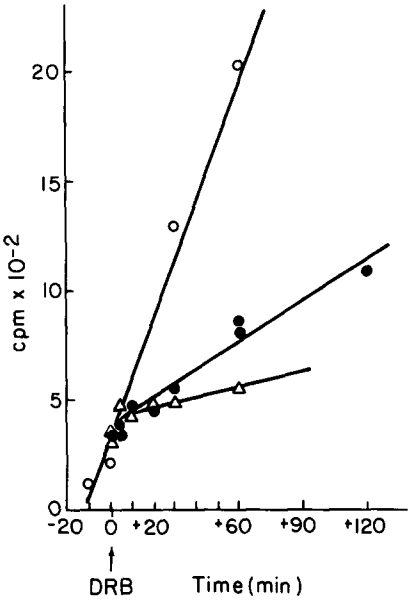


FIGURE 3 Rate of onset of DRB inhibition of total RNA synthesis. O——O, control; ●——●, DRB 25 μg/ml; Δ——Δ, DRB 50 μg/ml. Vials of cells growing on cover slips were preincubated with [³H]uridine (1 μCi/ml; 0.04 μM final medium concentration) for 20 min. Then either 25 or 50 μg/ml DRB in aqueous solution was added at zero time, and the vials were harvested at various times afterwards. Controls did not receive DRB. Cover slips of cells were washed, fixed, and counted as described in Materials and Methods. Data are plotted as cpm/μCi/coverslip vs. time of labeling. Each point represents the average of four cover slips, deviation from the mean being ±10%.

DRB (50 μg/ml) Maximally Inhibited Total RNA Synthesis within a 10–15-Min Period

This was determined by allowing vials with cells to incorporate [³H]uridine for 20 min, then adding 50 μg/ml DRB, and following the decrease in [³H]uridine incorporation into the cover slips over a short time period (Fig. 3). This rapid action suggested that DRB was interfering directly with RNA synthesis.

Inhibition of HnRNA Synthesis

The inhibition of HnRNA synthesis by DRB was demonstrated indirectly by measuring total cellular RNA labeling over a short time period in the presence of low concentrations of actinomycin D. This experiment took advantage of two observations on cellular RNA synthesis: (*a*) After short labeling periods (1 h), approximately 50% of the [³H]uridine label is found in the HnRNA and approximately 50% is found in the rRNA species (27). (*b*) Low concentrations of actinomycin selectively inhibit rRNA synthesis. At concentrations of actinomycin which inhibit rRNA synthesis by 80–90%, other types of RNA synthesis are practically unaffected (22). Therefore, in the presence of low concentrations of actinomycin, the [³H]uridine label, incorporated into total cellular RNA after a 1-h period, will be essentially that incorporated into the HnRNA.

This experiment (Fig. 4) was done in two stages.

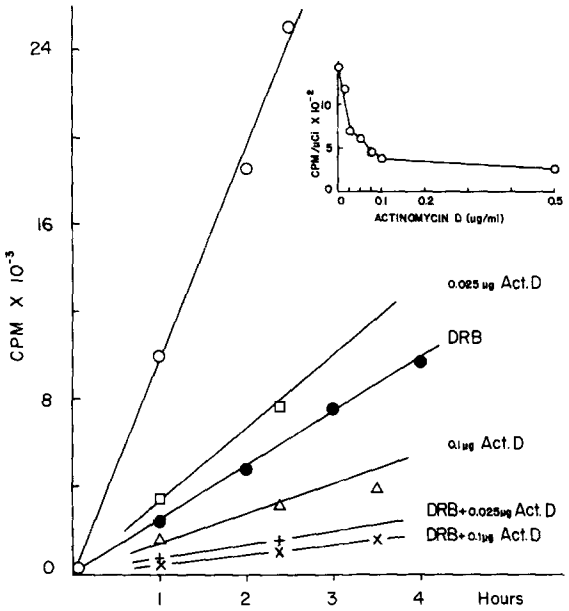


FIGURE 4 The effect of DRB on total RNA synthesis when rRNA synthesis has been selectively inhibited with low concentrations of actinomycin D. CEF cells growing on cover slips in vials were given 0.025 or 0.1 μg/ml of actinomycin D. Control cells received no actinomycin D. Some vials from each of these three groups also received 50 μg/ml DRB. After 30 min of preincubation with the inhibitors, 1.0 μCi/ml [^{3}H]uridine (0.3 μM) was given and the cells were harvested over a period of 3.5 h. The cover slips of labeled cells were washed, fixed in glutaraldehyde, and counted as described in Fig. 1. All points were done in quadruplicate and the average cpm of the four cover slips were plotted vs. time. The average deviation from the mean for these points was ±10%. The DRB cpm were corrected for inhibition of [^{3}H]uridine uptake (Table I). O——O, control. The *inset* shows the inhibition of total cellular RNA synthesis caused by increasing amounts of actinomycin D. The cells were preincubated for 30 min with different concentrations of actinomycin. [^{3}H]Uridine (2 μCi/ml; 0.04 μM) was then added and the incubation was terminated after another 60 min as described above. The average cpm per cover slips were plotted vs. actinomycin concentration.

First, the inhibition of total RNA labeling by increasing concentrations of actinomycin D was determined for the CEF cells (inset, Fig. 4). From these data two actinomycin D concentrations (0.025 and 0.1 μg/ml) were chosen. Then both DRB (50 μg/ml) and actinomycin D (0.025 or 0.1 μg/ml) were given simultaneously, 30 min before the addition of [^{3}H]uridine (Fig. 4). Even at the lowest concentration of actinomycin D, DRB caused an almost complete cessation (95%) of total cellular RNA synthesis. One can conclude therefore that DRB is a very effective inhibitor of HnRNA synthesis. Labeling experiments in Diptera salivary glands strongly support the conclusion that HnRNA synthesis is almost completely inhibited by DRB (7).

Synthesis of tRNA

The synthesis of tRNA was only slightly inhibited (20%) by DRB (50 μg/ml) over a 3-h period as shown by RNA labeling and gel electrophoresis of the tRNA (for experimental details see reference 8).

Extensive Inhibition of mRNA Synthesis by NN-Forming Chemicals

This was suggested by experiments in chick embryo liver cells which showed that the synthesis of the enzyme Δ-aminolevulinic acid (ALA) synthetase was inhibited to the same extent by NN-forming chemicals as it was by high concentrations of actinomycin D. ALA synthetase is the first and rate-limiting enzyme of the porphyrin biosynthetic pathway. The enzyme is relatively short lived with a half-life of about 3 h and its mRNA has a half-life of 4–6 h (10).

In whole cells, ALA synthetase activity can be assayed indirectly by measuring the fluorescence of the porphyrins generated from ALA (12). This

assay is sensitive enough to be used with chick embryo liver cells grown on a cover slip in a vial (0.1 mg protein/cover slip). In the presence of the inducer, allylisopropylacetamide (AIA), the level of ALA synthetase is increased 10- to 20-fold over the normal basal level. The difference between these levels can be made even greater by addition of the iron chelating agent desferrioximine along with the AIA and by growing the cells in a modified Ham F12 medium containing insulin instead of a Ham-fetal bovine serum medium (24).

Over a 5-h period, if mRNA synthesis has been stopped by a certain concentration of an adenosine analogue, the assay will show lower levels of enzyme activity than if mRNA synthesis has been occurring during this time. The inhibition of ALA synthetase production by various inhibitors is summarized in Table II. The protein synthesis inhibitor acetoxycycloheximide completely stopped the synthesis of new enzyme. In contrast, inhibitors known to block mRNA synthesis, such as actinomycin (0.25 μg/ml) and α-amanitin (2 μg/ml), decreased the ALA synthetase activity by only 50% during the 5-h period. This 50% level represents enzyme synthesized from the mRNA already present in the polysomes at the time of inhibitor addition. Cordycepin (3 μg/ml) appeared to decrease the enzyme levels even further but this may have been due to some cell damage.

TABLE II

Inhibition of ALA Synthetase Production in Chick Embryo Liver Cells by NN-Forming Chemicals and Other Inhibitors

Inhibitor	Inhibitor concn	Protoporphyrin per cover slip, % control
	μg/ml	
Control		100
Acetoxycycloheximide	0.6–0.2	10
Actinomycin	0.25	50*
α-Amanitin	2–20	55
Cordycepin (3'-deoxyadenosine)	3	35
DRB	5–20	45
DRB	50	30
Toyocamycin	0.5–2.0	50
Toyocamycin	4.0	40
Bromotubercidin	0.5–4.0	55
Adenosine	100–500	50
Guanosine	100–300	40*
Adenosine + guanosine	100 + 100	40*
Acetoxycycloheximide + DRB	0.2 } 50 }	10

Chick embryo liver cells were grown in a monolayer in vials on cover slips in 1 ml of modified Ham's medium + 10% fetal bovine serum for 24 h; the medium was changed to Ham's + insulin (0.25 μg/ml) and desferrioximine (500 μg/ml), and the cells were allowed to grow 2 h more. At zero time of the experiment, the medium was replaced by 1 ml of the Ham + insulin + desferrioximine medium and the inducing chemical AIA (300 μg/ml) and the various inhibitors were added. At this time the cells were not multiplying. After 5 h, the medium was removed and the porphyrins were extracted from the cover slips of cells with 1 N perchloric acid, methanol (1:1). The porphyrin extracts were then assayed fluorimetrically (8). Control cells, given only AIA and desferrioximine, contained 22.2 ± 1.6 pmol protoporphyrine/cover slip (the cover slips contained about 0.1 mg protein). The vials were run in quadruplicate and the average deviation from the mean was ±15%.

* Indicates that the value was determined in a second experiment.

DRB at concentrations of 5–20 μg/ml inhibited porphyrin synthesis about 55%, or about the same as seen with 0.25 μg/ml actinomycin, suggesting that the inhibition by DRB was probably occurring at the transcription or posttranscription level. Increasing DRB up to 50 μg/ml inhibited porphyrin synthesis 70%; this additional inhibition might have been due to inhibition of ALA synthetase at the translational level.

A control experiment demonstrated that DRB (50 μg/ml) did not interfere with the conversion of ALA to porphyrin, therefore a decrease in porphyrin formation reflected a decrease in ALA-synthetase activity.

The other NN-forming chemicals, toyocamycin, bromotubercidin, and adenosine also inhibited ALA-synthetase activity by 50–60%.

A more detailed comparison between the effects of actinomycin D and DRB on ALA-synthetase production was made to confirm the possibility that NN-forming chemicals were completely inhibiting mRNA synthesis. The procedure was similar to that in the previous experiment except that the time of addition of inhibitor and inducing chemicals was varied. Actinomycin (0.25 or 0.5 μg/ml) or DRB (25 or 40 μg/ml) was given 2 h before, simultaneously with, or 2 h after the inducing chemical AIA. The experimental plan and data are detailed in my thesis (8). Here I summarize the experimental results.

DRB (25 μg/ml) was found to inhibit ALA synthetase production to the same degree as 0.5 μg/ml actinomycin. As expected, the inhibitors were most effective when added before the inducing chemicals. The close correspondence between the action of the two inhibitors added at different times with respect to the inducing chemical AIA over a 4-h period strongly supports the hypothesis that DRB and actinomycin were acting similarly, probably to completely inhibit mRNA synthesis.

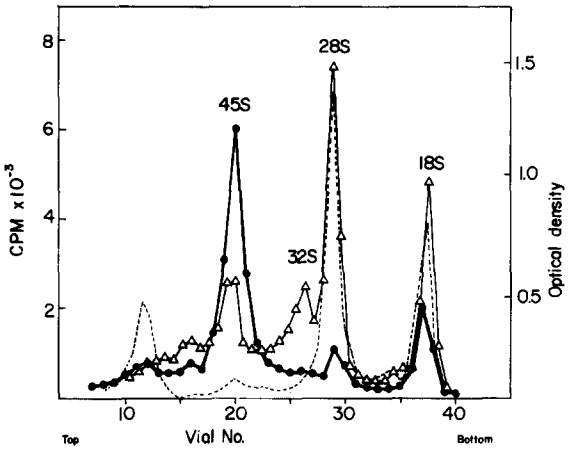


FIGURE 5 Inhibition of rRNA synthesis and processing by DRB. Radioactivity determined at the end of 3 h of continuous labeling with [^{3}H]uridine. CEF cells were grown in 100-mm diameter tissue culture dishes to a cell density of approximately 1–2 × 10^7 cells/plate. Two plates were used per point. They were allowed to equilibrate for 1 h with 5 ml fresh Eagle's medium and were then preincubated for 30 min with 50 μg/ml DRB before addition of 4.0 μCi/ml [^{3}H]uridine. Cold uridine was added to make the final uridine concentration 0.3 μM. (The addition of cold uridine does not affect the conclusions drawn from this experiment.) Control cells were given 1.0 μCi/ml [^{3}H]uridine (final uridine concentration 0.3 μM). The cells were allowed to incorporate label for 3 h. After harvesting the cells, the RNA was isolated and electrophoresed on 2.5% acrylamide-0.6% agarose gels. The gels were scanned at 260 nm and were then sliced, their cpm determined and the cpm values normalized to equal [^{3}H]uridine concentration in the medium (2.0 μCi/ml) and to equal RNA sample size (using the 18S OD peak as described in the text). The DRB cpm were corrected for inhibition of uridine uptake by DRB (Table I). The large OD peak (dashed curve) seen to the left of the 45S RNA peak, i.e., closer to the top of the gel, is actually DNA because it is heavily labeled when the CEF cells are exposed to [^{3}H]thymidine for a 1-h period (D. Granick, unpublished observations). (45S RNA cpm measured before any correction factors were applied: DRB = 1.9 × 10^4, controls = 4.3 × 10^3.) - - - OD 260 nm; Δ——Δ cpm, control cells; ●——● cpm, DRB cells.

rRNA Synthesis

The effect of DRB and other NN-forming chemicals on rRNA production was examined in a series of gel experiments. Studies on the action of two of these chemicals, toyocamycin (29) and bromotubercidin (2), had already demonstrated that synthesis of the 45S precursor rRNA was partly inhibited and that the subsequent rRNA processing steps were almost completely blocked. We undertook similar types of experiments with toyocamycin and other NN-forming chemicals using the CEF cell system.

In these experiments labeled RNA was isolated from whole cells, allowing all of the rRNA species, both nuclear and cytoplasmic, to be separated on the same gel. This procedure had two major advantages: (*a*) Degradation of any rRNA species, especially during the later stages of the processing sequence (e.g., 32S to 28S RNA), could be quantitated more precisely than if nuclear and cytoplasmic RNA fractions were assayed separately. (*b*) During a labeling experiment, results from a series of gels could be normalized, using the 18S cytoplasmic rRNA peak from the gel optical density scans as a measure of cell number. Such a correction assumes a constant cytoplasmic ribosome content which is approximately true for an experimental period of 3 h or less.[3] This procedure eliminated the necessity of tedious double-labeling experiments used by other authors to scale their data to constant amounts of RNA recovery.

Comparison of the rRNA Gel Patterns Produced by NN-Forming Chemicals

The effect of five NN-forming chemicals on rRNA synthesis and processing was examined during a 3-h period of labeling with [^{3}H]uridine. Concentrations of the NN-forming chemicals were

[3] The RNA of these CEF monolayer cultures doubles every 36 h. Therefore the amount of rRNA synthesized in a 3-h period is less than 10% of the total cellular RNA or within the other experimental errors of the system.

used that were known to produce good NN formation in CEF cells as seen by microscope examination. In Fig. 5, the cpm and OD gel scans are shown for CEF cells treated with DRB (50 μg/ml). Cpm gel scans are presented in Fig. 6 for CEF cells treated with toyocamycin (2 μg/ml), MPB (25 μg/ml), α-amanitin (20 μg/ml), or 2 mM adenosine (0.53 mg/ml). All cpm curves were normalized to equal RNA sample size (using the 18S OD peaks of the gels as a reference) and were corrected for inhibition of [^{3}H]uridine uptake, using the factors of Table I.

In the control curves of Figs. 5 and 6, by the end of a 3-h labeling period with [^{3}H]uridine, the labeling in the 45S RNA peak had reached a steady-state level (as shown by Fig. 8 *b*), a slightly smaller peak of labeled intermediate 32S RNA was present, and large peaks of completely processed [^{3}H]uridine 18S and 28S rRNAs were evident.

In contrast, when NN-forming chemicals were present during the 3-h labeling period, marked changes from the control curves were noted (Figs. 5, 6). (*a*) In all inhibitor-treated cells, the sum of the cpm in the (32S + 28S) RNA peaks was much less than the cpm of the 18S peak; this fact suggested that extensive degradation of the 32S and 28S species had occurred. (*b*) The decreased size of the 45S RNA and 18S RNA peaks in Fig. 6 suggested that there had been a decreased rate of 45S RNA synthesis and that a steady-state in the labeling of the 45S RNA pool had probably not

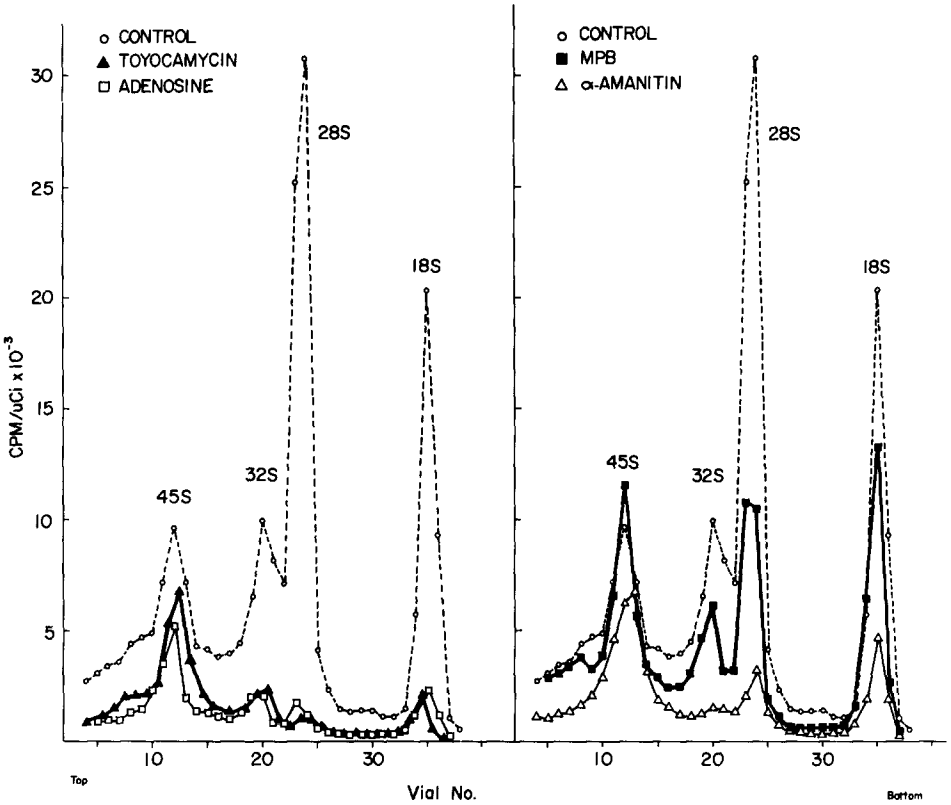
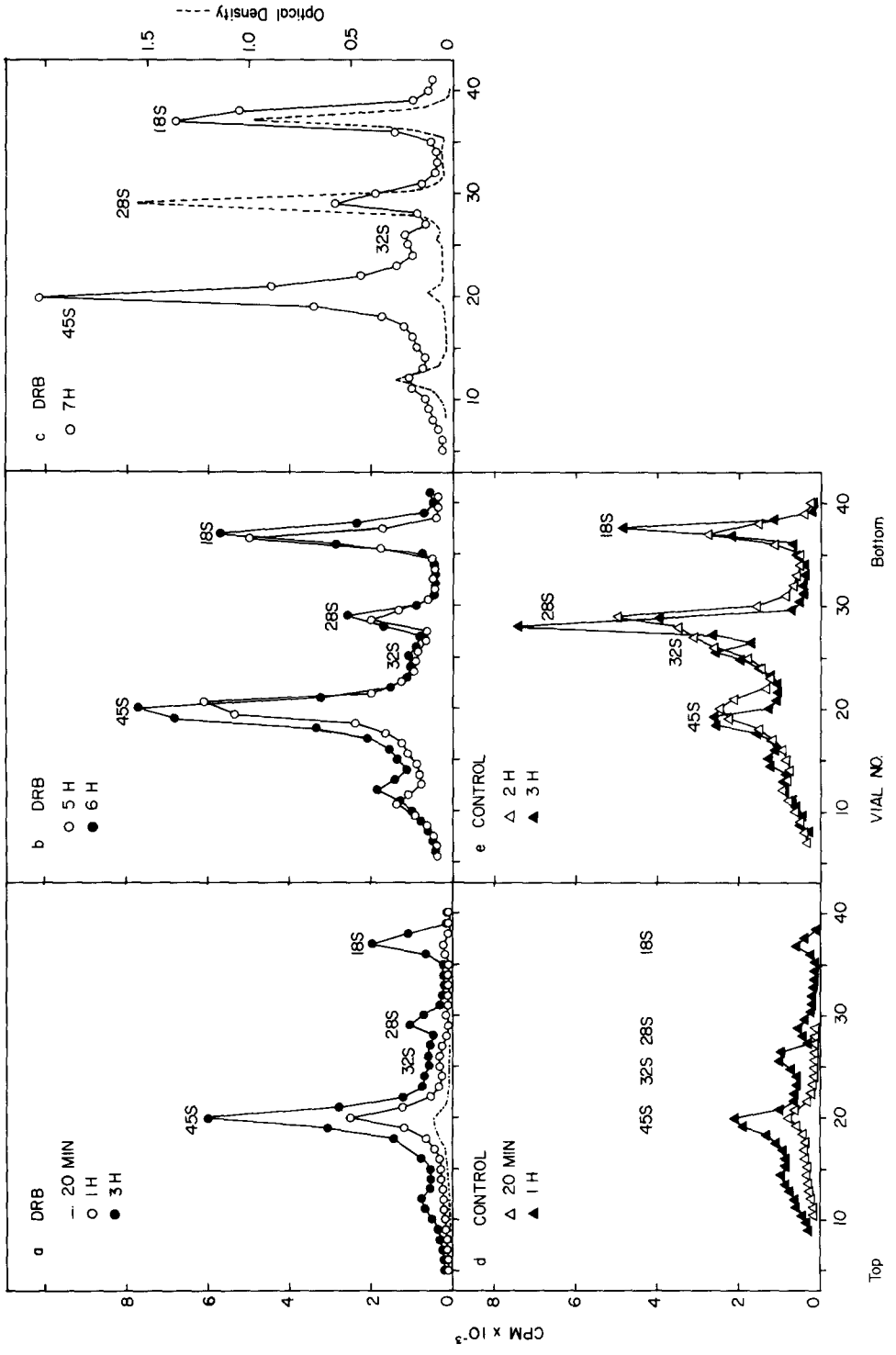


FIGURE 6 Inhibition of rRNA synthesis and processing by the NN-forming chemicals toyocamycin, adenosine, MPB, and α-amanitin. Procedure was the same as in Fig. 6. The CEF cells received one of the inhibitors to make the following concentrations: adenosine (2 mM = 0.53 mg/ml), MPB (25 μg/ml), toyocamycin (2 μg/ml). 30 min later, all plates then received 4.0 μCi/ml of [^{3}H]uridine (sp act 27.8 Ci/mmol; final concentration = 0.04 μM; no cold uridine was added). α-Amanitin (20 μg/ml) was given 4 h before addition of 4.0 μCi/ml uridine. Control cells also received 4.0 μCi/ml [^{3}H]uridine. All plates were labeled for 3 h. Gel cpm were corrected to equal RNA sample size and for inhibition of uridine uptake (Table I) as described in the text. (45S RNA cpm before any correction factors were applied (× 10^3): adenosine = 4.8, toyocamycin = 2.6, MPB = 2.0, α-amanitin = 6.2, control = 26.)


a DRB
20 MIN
1 H
3 H
b DRB
5 H
6 H
c DRB
7 H
d CONTROL
20 MIN
1 H
e CONTROL
2 H
3 H
45S
32S
28S
18S
Optical Density
CPM x 10⁻³
VIAL NO.
Top
Bottom

yet occurred. In the case of DRB (Fig. 5) the 45S RNA peak was higher than in the control cells, suggesting that this inhibitor caused an alteration in the normal balance of the rates of 45S RNA synthesis and processing.

Action of DRB on rRNA Synthesis and Processing with Time

To attempt to analyze more fully the various changes that were revealed by the above experiments, a time study of rRNA labeling in the presence of DRB was undertaken (Fig. 7). The CEF cells were continuously labeled with [^{3}H]uridine in the presence of DRB (50 μg/ml) for periods of time up to 7 h. The cpm for each individual rRNA species was estimated from the gel curves of Fig. 7, and some of these cpm were plotted vs. time in Fig. 8.

From these data four major conclusions were derived: (*a*) In the presence of DRB, the recovery of labeled 32S and 28S rRNA species was greatly (75–80%) decreased. To quantitate the loss of the 32S and 28S RNA, the assumption was made that the accompanying labeled 18S RNA was not degraded; this approximation was confirmed in a later experiment (Fig. 9; Table III). The extent of loss was then calculated from the molecular ratio (32S + 28S)/18S of the labeled RNA species recovered from each gel. A number proportional to the number of these rRNA molecules was calculated by dividing the [^{3}H]uridine cpm of each chick rRNA species by its molecular weight (21).

The (32S + 28S)/18S ratio was nearly 1.0 for the control cells (confirming that the recoveries of the rRNA species on the gel and base-line correction for the gel were good). However, this ratio was only 0.25 to 0.20 after 5–7 h labeling in the presence of DRB. These data suggest that when 45S RNA is labeled in the presence of DRB and is then processed in the presence of DRB, 75–80% of the resulting 32S and 28S RNA species are lost (perhaps to undetectable fragments).

This loss of the 32S and 28S RNA species is reflected in Fig. 8 *a* which shows the total increase in rRNA cpm with time. The increase in rRNA cpm is linear for the control cells, but gradually drops off with the DRB-treated cells.

(*b*) In the presence of DRB, 45S RNA accumulates at half the control cell rate. The rate of 45S RNA synthesis was assumed to be proportional to the increase in total rRNA cpm (Pig. 8 *a*) after correction had been made for the loss of 32S and 28S RNA cpm. The cpm of the degraded (32S + 28S) RNA were estimated by calculating the theoretical yield of 28S RNA from the measured 18S RNA cpm multiplied by the molecular weight ratios of the 28S and 18S RNA species. When these lost cpm were added back to the total rRNA cpm measured for the DRB cells (dotted circles, Fig. 8 *a*), the buildup of total rRNA cpm was found to be nearly linear and proceeded at a rate approximately 50% of the rate observed in the control cells. (The initial rate of 45S rRNA accumulation can also be approximated from Fig. 8 *b*, but such a calculation is less accurate because the early time points are the least accurate of this experiment.)

(*c*) In DRB-treated cells, the rate of appearance of labeled 18S rRNA products was half that found in the control cells (Fig. 8 *c*), in parallel with the 50% decrease in 45S rRNA accumulation.

(*d*) In DRB-treated cells the cpm in the 45S rRNA pool, during a 6-h exposure to DRB, built

FIGURE 7 Inhibition of 45S RNA synthesis and processing by DRB: 7-h continuous labeling with [^{3}H]uridine. (*a–c*) Radioactivity of DRB-treated cells: (*a*) -·-·- 20 min, O——O 1 h, ●——● 3 h; (*b*) O——O 5 h, ●——● 6 h; (*c*) O——O7 h, Optical density of DRB cells ---, 7 hr. (*d*, *e*) Radioactivity of control cells: (*d*) Δ——Δ 20 min, ▲——▲ 1 h; (*e*) Δ——Δ 2 h, ▲——▲ 3 h. The experimental procedure was similar to that described for Fig. 5. Cells were grown to approximately 1.2 × 10^7 cells per 100-mm plate. Plates containing 5 ml of Eagle's medium were preincubated for 30 min with 50 μg/ml DRB given as a dimethylsulfoxide solution (50 μg DRB/μl DMSO). Controls received DMSO only. [^{3}H]Uridine was then added. DRB cells received 2 or 4 μCi/ml of [^{3}H]uridine; control cells received 1 or 2 μCi/ml. The final uridine concentration for all plates was brought up to 0.3 μM by the addition of cold uridine. Plates were then harvested over a period of 7 h. Two plates were used per point. RNA was extracted, separated by gel electrophoresis, and the OD and cpm curves for each gel were determined. The cpm curves for each gel were normalized to equal [^{3}H]uridine concentration in the medium (2.0 μCi/ml), to equal RNA sample size, and the DRB cpm were corrected for inhibition of uridine uptake by DRB (Table I) as described in the text. (A correction for increased cytoplasmic 18S RNA content after 7 h of growth was not considered necessary for the DRB-treated cells since they make 18S RNA at only half the rate of the control cells)

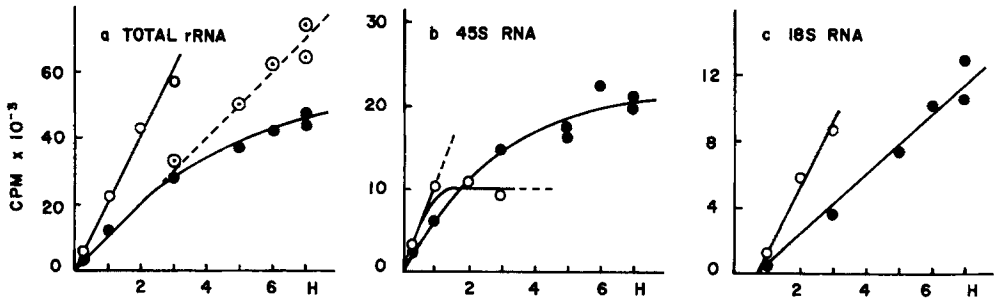


FIGURE 8 Changes in the cpm of various rRNA species during continuous [^{3}H]uridine labeling in the presence of DRB. Measured cpm: O——O control cells, ●——● DRB cells. The cpm of the individual rRNA species were estimated from the gel curves of Fig. 8. (*a*) Nonlinear increase in total rRNA cpm in the presence of DRB due to the degradation of 32S + 28S RNA. When the cpm of the degraded 32S + 28S RNA is calculated (see text) and added back to the total rRNA cpm of the DRB cells, a straight line (- -⊙- -) is obtained. (*b*) Accumulation of 45S RNA cpm. (*c*) Inhibition of 18S RNA cpm appearance.

up and leveled off to a value about twofold higher than that seen in the control cells (Fig. 8 *b*).

Tests of Mechanisms for Degradation of 32S and 28S RNA Preribosomes

There were two possible explanations for the loss of the 32S and 28S rRNA preribosomes seen in the presence of DRB: (*a*) A normal 45S rRNA-containing preribosome was made and DRB interfered with the function or synthesis of the enzyme(s) which normally process it to 32S and 28S RNA. (*b*) DRB caused the formation of a defective 45S rRNA preribosome which could not be processed properly; but the processing mechanism itself was unaffected by DRB and could convert normal 45S rRNA into normal products even in the presence of DRB. These two possibilities were distinguished by using a pulse-chase experiment.

Methionine Pulse-Chase Experiment

PROCESSING OF 45S rRNA SYNTHESIZED IN THE PRESENCE OF DRB: After 30 min of preincubation with DRB (50 μg/ml), CEF cell 45S rRNA was labeled in the presence of DRB for a 30-min period with [*methyl*-^{3}H]methionine. The radioactive medium was then replaced with fresh medium containing a large excess of cold methionine as chase. The processing of the labeled 45S rRNA was followed with time during the chase period. Cpm gel curves are presented in Fig. 9. The cpm of the individual rRNA species were estimated and plotted vs. time of chase (Fig. 10). All cpm curves were normalized to equal RNA sample size (using the 18S OD peaks as reference). A correction for [^{3}H]methionine uptake into the cell in the presence of DRB was unnecessary since DRB does not seem to appreciably affect the transport of amino acids (Fig. 1). For short-time rRNA labeling experiments, [^{3}H]methyl labeling gives more accurate results than [^{3}H]uridine labeling (14).

From Figs. 9 and 10 *a*, it can be observed that the total rRNA methyl cpm decreased during the chase period in both the DRB-treated cells and the control cells. Theoretically, the number of rRNA methyl groups should remain constant during the processing. A slow loss in methyl label was observed for the control cells. In the DRB-treated cells the degradation of total rRNA methyl cpm was much more pronounced.

32S AND 28S rRNA: The extensive loss of 32S and 28S rRNA cpm in the DRB-treated CEF cells during the chase period is demonstrated in Figs. 9 *a* and 10 *e*. The extent of degradation was quantitated by calculating the cpm ratios of (32S + 28S) RNA recovered to the amount of 18S RNA produced during the chase. Table III compares these calculated cpm ratios to the theoretical [^{3}H]methyl ratios calculated from the HeLa cell data of Weinberg and Penman (35). The data in Table III indicate that when 45S RNA which was synthesized in the presence of DRB is processed after DRB is removed, 75–80% of the resulting (32S + 28S) RNA is lost.

18S rRNA: In contrast, there was only a slight loss (approximately 20%) of 18S rRNA in the DRB-treated cells. This loss in 18S cpm was reflected in the Δ18S/Δ45S ratio of Table III, which compares the amount of 18S produced to

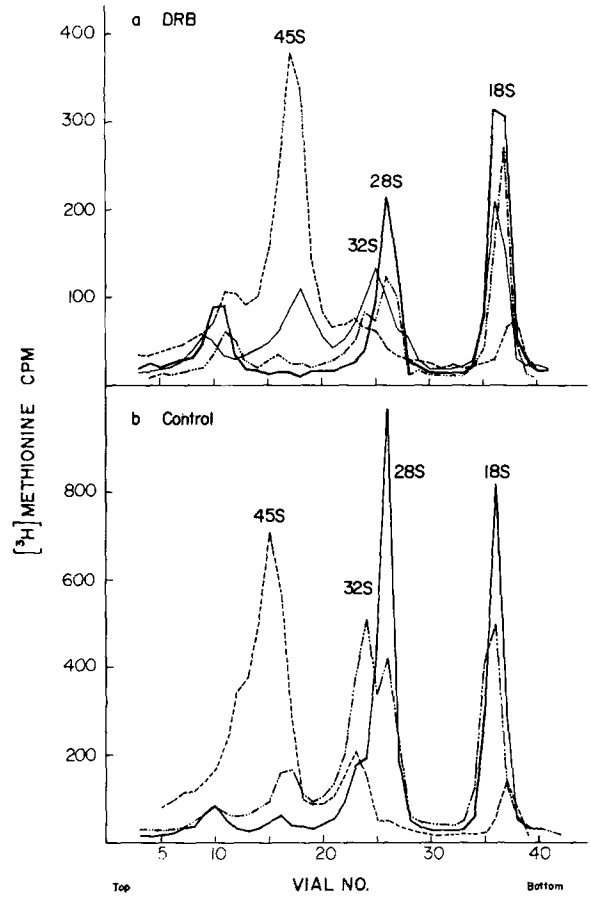


FIGURE 9 Processing of [³H]methyl-labeled "DRB 45S RNA" after removal of the inhibitor. (*a*) Processing of DRB 45S RNA. Time of chase: --- 0 min, —— 30 min, —·— 1 h, —— 2 h. (*b*) Processing of normal 45S RNA. Time of chase: - - - 0 min, —·— 30 min, — 1 h. Cells were grown to a monolayer density of approximately 10^7 cells per 100-mm diameter plate. The medium was then changed to 5 ml Eagle's medium minus methionine and supplemented with 0.02 mM adenosine and 0.02 mM guanosine to prevent use of the methyl label during purine biosynthesis (14). Cells to be inhibited were given 50 μg/ml of DRB in 5 μl of DMSO. Control cells received 5 μl of DMSO only. After 30 min of incubation at 37°C, [*methyl*-³H]methionine (6 μCi/ml; sp act 4 Ci/mmol) was added and the cells were returned to the incubator for a 30-min labeling period. The radioactive medium containing DRB was then replaced with 10 ml of the frash Eagle's medium containing 0.1 mg/ml of cold methionine (500-fold the original [³H]methionine concentration). Cells were harvested from the plates immediately after the medium change as a "zero time" sample. Other plates were harvested after 30 min, 1 h, and 2 h of chase. Two plates were used per point. RNA was extracted and separated by gel electrophoresis and the cpm data were normalized to equal sample size (see text).

that of the 45S RNA which disappeared. The increase in this ratio with time suggests that there may have been some delay in the processing of an intermediate species (20S?) into the completed 18S molecules.

The loss of 32S and 28S rRNA in the DRB-treated cells suggested that extensive degradation of the DRB preribosomes was occurring. It is probable that this degradation occurred before completed 32S rRNA-containing preribosomes appeared in the 45S to 32S rRNA processing sequence. This has been concluded because the fraction of the total cpm which entered the 32S rRNA pool was never as high in the DRB-treated cells as it was in the control cells (Fig. 10 *f*). However, one cannot eliminate the possibility that

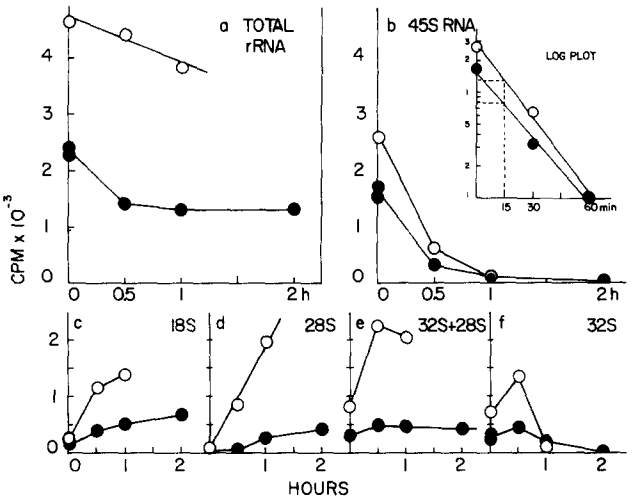


FIGURE 10 Changes in the cpm of various rRNA species during the processing of [^{3}H]methyl-labeled DRB 45S RNA after the inhibitor has been removed. O——O control cells, normal labeled 45S RNA; ●——● DRB cells, 45S RNA labeled in the presence of DRB. The cpm of the individual rRNA species were estimated from the gel curves of Fig. 9 and were plotted against time of the cold methionine chase. (*a*) Total rRNA cpm present in the gel. (*b*) cpm of the 45S RNA peak. *Inset* shows log plot of these data with a graphic determination of the half-life of the 45S RNA. (*c*) 18S RNA cpm. (*d*) 28S RNA cpm. (*e*) The sum of the 32S RNA + 28S cpm. (*f*) 32S RNA cpm.

TABLE III

Cpm Ratios of Various rRNA Species When [Methyl-^{3}H]DRB 45S RNA is Processed after the Inhibitor Has Been Removed

		Ratios of [*Methyl*-^{3}H]cpm			
		$\frac{\Delta(32S + 28S)}{\Delta 18S}$		$\frac{\Delta 18S}{\Delta 45S}$	
Sample	Time of chase		% of theoretical		% of theoretical
			%		%
Theoretical*		1.7		0.37	
50 μg/ml DRB	30 min	0.72	42	0.21	57
	1 h	0.46	27	0.24	65
	2 h	0.26	15	0.34	92
Control	30 min	1.66	98	0.43	116

The cpm of the individual rRNA species were estimated from the gel curves in Fig. 9. The change in labeling of the various rRNA species from time zero of the chase was calculated for each time point. The ratios of these cpm differences were calculated and compared to the theoretical methyl group distributions.
* Calculated from HeLa cell data of Weinberg and Penman (35).

the 32S rRNA preribosomes were made but were very unstable.

DRB Does Not Directly Modify Processing Enzyme Function to Cause This Degradation of Preribosomes

This conclusion has been suggested by two pieces of experimental evidence: (*a*) Fig. 10 *b* with its semilog inset indicates that the half-life of [*methyl*-^{3}H]45S RNA in both the DRB-treated and control cells is nearly the same (approximately 15 min). It appears therefore that, for the 45S RNA preribosomes made in the presence of DRB, processing is initiated at the same rate as in control cells after DRB has been removed.

(*b*) In another pulse-chase experiment (data reported in my thesis [8]), normal prelabeled 45S

RNA was processed in the presence of DRB. The sequence of labeling and DRB addition in this experiment was the reverse of that reported in Fig. 9. Normal 45S RNA preribosomes were prelabeled by pulsing CEF cells with label for a time just sufficient to label the 45S RNA; DRB was then added and further labeling of newly synthesized RNA was stopped by adding a cold chase. After 1 h of chase in the presence of DRB, when appreciable amounts of normal prelabeled 45S RNA had been processed to labeled 32S, 28S, and 18S RNAs, it was found that little 32S and 28S RNA (20% at most) had been lost. In other words, DRB did not directly cause the processing enzyme(s) to make abnormal products from the normal 45S RNA preribosomes.

These two experiments suggest that the processing enzyme action itself is unaffected by DRB. One may conclude therefore that the degradation of preribosomes which have been synthesized in the presence of DRB must be caused by some defect in the preribosomes themselves.

In both the [^{3}H]uridine experiment (Fig. 7) and the [*methyl*-^{3}H]methionine experiment (Fig. 9) it was observed that labeled 45S rRNA accumulated in the presence of DRB at only half the rate seen in the control cells. This close correspondence in rates between [^{3}H]methyl labeling and [^{3}H]uridine labeling suggests: (*a*) that the defective preribosomes synthesized in the presence of DRB were probably normally methylated, and (*b*) that the factor used for calculation of inhibition of [^{3}H]uridine uptake into the cell by DRB (Table I) was probably correct.

DISCUSSION

A number of adenosine analogues, including a high concentration of adenosine itself, act in a similar fashion to cause dispersal of nucleoli in chick embryo fibroblast cells into beaded strands or NN (9). Reversal back to the normal nucleolar state occurs after removal of the chemicals from the medium. The biochemical basis of this action was studied because it was considered that such an investigation might reveal details of the normal mechanism by which the nucleolus generates ribosomes and maintains itself in a steady-state. It was also hoped that these compounds might prove to be useful inhibitors of specific biochemical steps for cell and viral growth.

Of the five NN-forming chemicals studied in this report, the action of the compound DRB was chosen for detailed investigation because this adenosine analogue could not form hydrogen bonds in the RNA polymerase base-pairing mechanism and therefore could not be inserted into growing RNAs.

Effects of DRB

DRB appears to interfere primarily with the production of mRNA and rRNA in CEF cells. In the presence of 50 μg/ml DRB the following effects were produced: (*a*) Inhibition of total RNA synthesis occurred within 10–15 min after DRB addition (Fig. 3), suggesting that there was a direct interference with RNA synthesis; (*b*) HnRNA synthesis was almost completely inhibited (Fig. 4); (*c*) mRNA synthesis (as determined by following the production of an inducible enzyme in chick embryo liver cells) appeared to be almost completely inhibited (Table II); (*d*) the rate of accumulation of 45S rRNA was inhibited by 50% (Figs. 7 and 8); (*e*) the rate of appearance of the final 18S rRNA ribosome product was inhibited 50% (Fig. 8 *c*); (*f*) extensive (80%) degradation of the 32S and 28S rRNA species occurred (Fig. 7; Table III); (*g*) only small amounts of 18S RNA were degraded (approximately 20%) (Table III).

The data in this paper also suggest that most of the preribosome degradation caused by DRB occurs at or before the 32S RNA preribosome stage (e.g., 41S intermediate stage?) (Fig. 10 *f*). It also appears that degradation of these preribosomes is due to the formation of defective 45S RNA preribosomes containing 45S RNA and not to a direct DRB interference with the rRNA processing enzyme action (Fig. 10 *b*; reference 8).

The defect in the 45S rRNA preribosomes produced in the presence of DRB is interpreted to reside in the protein content of the particle rather than in the composition of its 45S rRNA. It is unlikely that DRB causes major defects in the 45S rRNA molecules because our experiments have shown that the 45S rRNA appears to be normal with respect to size and methyl content. It is also unlikely that DRB is directly incorporated into the 45S rRNA, in contrast to toyocamycin which was shown to be incorporated by Tavitian et al. (29, 30), because the DRB cannot base pair in the manner required for the normal operation of the rRNA polymerase. We therefore propose that the 45S rRNA preribosomes which are synthesized in the presence of DRB lack a proper protein coat.

Evidence that protein-deficient preribosomes can be subject to degradation during the subsequent processing steps is supplied by studies with cycloheximide and with hypertonic medium. These inhibitors of protein synthesis cause the synthesis

of protein-deficient preribosomes (6, 20). Accompanying this synthesis of defective preribosomes is an extensive degradation of 28S rRNA and a lesser loss of 18S rRNA. The production of protein-deficient preribosomes occurs well before the 2 h required to deplete the bulk of the preribosomal protein pool (33), suggesting that a fraction of the preribosomal proteins is more limited in supply (6, 20).

Effects of Other NN-Forming Chemicals

The compounds toyocamycin and 2 mM adenosine were found to affect mRNA synthesis, rRNA synthesis, protein synthesis, and the processing of preribosomes, in a fashion similar to that of DRB (Figs. 1 and 6; Table II; reference 8). They may therefore inhibit ribosome production in CEF cells via a mechanism similar to that of DRB.

Actions of DRB

There are two possible ways in which DRB and the other NN-forming chemicals could be acting to decrease the supply of preribosomal proteins: they could decrease mRNA production at the transcriptional levels, causing those polysome proteins made from short-lived mRNAs to gradually disappear from the cytoplasm; or the NN-forming chemicals could decrease all preribosomal protein synthesis directly at the translational level by inhibiting the rate of initiation of protein synthesis on the polysomes.

Evidence for direct inhibition at the translational level by actinomycin D, MPB, and cordycepin, i.e., inhibitors previously considered to act only at the transcriptional level, has recently been presented by Singer and Penman (25) and by Craig (5) for the HeLa and L cell systems. However, other data suggest that the mode of action of these RNA synthesis inhibitors may vary with cell type and the particular spectrum of proteins examined. For example, when L cells were treated with actinomycin D or MPB, total [^{3}H]leucine incorporation was appreciably inhibited after 2 h and decreased by 50% after 4-h exposure to the inhibitor (5). This relatively rapid drop in the rate of protein synthesis was consistent with a mechanism of inhibition at the translational level. In contrast, in more specialized types of cells, protein synthesis was often markedly insensitive to actinomycin D, as in the case of the protein secreted by the colleterial gland tubules of the Cecropia moth (13) and in the case of the production of serum albumin in cultured chick embryo liver cells (15). In rat liver, actinomycin D caused a rapid decrease in the synthesis of catalase, but almost no decrease in the synthesis of ornithine transcarbamylase (32). In a longer incubation period of 20 h, using the chick embryo liver culture system of this paper, actinomycin D (0.25 μg/ml) caused an inhibition of total RNA synthesis of 82 $\pm$ 6%, whereas the inhibition on protein synthesis was only 24 $\pm$ 16% (S. Sassa, personal communication). Such data imply that there are classes of polysomes which are relatively insensitive to actinomycin D inhibition at the translational level and that protein synthesis may continue uninhibited when the mRNAs have a long half-life.

Our data suggest that in the CEF cells the inhibition of ribosomal protein synthesis by DRB and the other NN-forming chemicals probably occurs as the indirect result of the inhibition of mRNA production and is not due to a general inhibition of protein synthesis at the translational level. After DRB addition to CEF cells, the inhibition of total RNA synthesis was rapid (within 15 min) and extensive, the accumulation of rRNA and HnRNA being decreased 50 and 95%, respectively. Protein synthesis, however, was only slightly affected by DRB. [^{14}C]Leucine incorporation was approximately normal after 2-h exposure to 50 μg/ml DRB or to 2 mM adenosine (Fig. 1). By this time large amounts of 28S and 32S RNAs had already been lost (Fig. 7). After 4 h of DRB treatment, protein synthesis had still only slightly decreased (10–20%). Our postulate that DRB interference with preribosome production results from the inhibition of mRNA synthesis is also supported by the observation that α-amanitin, a known inhibitor of the mRNA polymerase, causes NN formation in CEF cells with an accompanying degradation of 32S RNA preribosomes in a manner very similar to that observed in the presence of DRB (reference 9; Fig. 7).

Although our data indicate that actinomycin D and DRB do not inhibit the bulk of CEF cell protein synthesis directly at the translational level, we cannot at this time rule out the possibility that these chemicals may be slowing the translation of only a limited number of proteins, such as preribosomal proteins. Nor have we eliminated the possibility that the rates of RNA synthesis may be normal in the presence of DRB but that DRB may be causing an increased rate of RNA degradation thus decreasing HnRNA and 45S RNA pools. It is hoped that the use of cell-free systems may permit

a further, more specific analysis of the inhibitory action of DRB.

Model for DRB Interference with Ribosomal Production in CEF Cells

We propose a model for DRB action which, although not proven by the data in this paper, may be useful for planning future studies. My model (Fig. 11) assumes that the primary sites of inhibitory action of DRB and related analogues are at the rRNA polymerase and mRNA polymerase levels. As a consequence of the partial inhibition of the rRNA polymerase, the rate of 45S RNA synthesis and the rate of appearance of 18S products are slowed by 50%. Furthermore, as a consequence of the almost complete inhibition of mRNA synthesis, a certain number of the preribosomal protein species may become limiting, resulting in the production of protein-deficient preribosomes. DRB would stop the production of these special proteins by inhibiting the production of their mRNAs. I propose that these missing proteins are (*a*) normally limited in supply and (*b*) have relatively short lived mRNAs, so that after the addition of DRB the supply of these proteins rapidly decreases, i.e. in less than 30 min.

The protein-deficient 45S rRNA-containing preribosomes, synthesized in the presence of DRB, are stable because they have the same half-life as normal 45S RNA preribosomes (Fig. 10 *b*). The 18S rRNA preribosomes cleaved from them are also stable. However, the larger preribosome particle is highly susceptible to degradation either at the 32S RNA stage or at some time during the processing steps which lead to the production of the 32S RNA preribosome particle. One might envision that, after the cleavage of the 45S RNA preribosome by the endoribonuclease processing enzyme, protein-deficient regions of the precursor

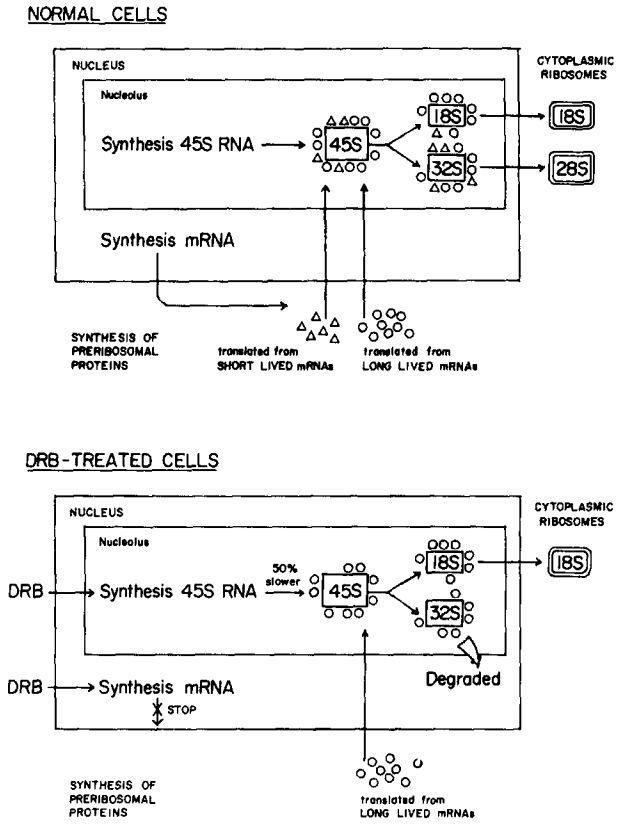


FIGURE 11 Model of DRB action. DRB inhibits rRNA synthesis by 50%. It inhibits mRNA synthesis almost completely; only long-lived mRNA is translated on the polysomes. As a consequence, the protein coat of the 32S RNA preribosomes is deficient in the proteins translated from short-lived mRNAs. This protein-deficient 32S RNA preribosome is then susceptible to degradation.

32S RNA preribosome may be exposed to random nuclease attack, perhaps by the same endoribonuclease which specifically cleaves normal preribosomes.

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Note Added in Proof: Green and Chan (Green, H., and T. S. Chan. 1973. Pyrimidine starvation induced by adenosine in fibroblasts and lymphoid cells. *Science [Wash. D. C.]*. **182**:836), have suggested that in their mammalian fibroblast and lymphoid cells adenosine inhibited the *de novo* synthesis of pyrimidines. However, in our CEF cell system given 2 mM adenosine it is unlikely that a decrease in the intracellular pyrimidine level was the primary factor responsible for NN formation. This is inferred because high concentrations (2 mM) of cytidine and uridine, added along with the 2 mM adenosine to the CEF cells did not overcome NN formation (8).

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