

# ACTION OF DICHLOROBENZIMIDAZOLE RIBOSIDE ON RNA SYNTHESIS IN L-929 AND HeLa CELLS

IGOR TAMM, ROGER HAND, and LAWRENCE A. CALIGUIRI

From The Rockefeller University, New York 10021. Dr. Hand's present address is the Departments of Medicine and Microbiology, McGill University, Montreal H3A, 2B4 Quebec. Dr. Caliguiri's present address is the Department of Microbiology, Albany Medical College of Union University, Albany, New York 12208.

## ABSTRACT

5,6-Dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) inhibits RNA synthesis in L-929 cells (mouse fibroblast line) and HeLa cells (human epithelioid carcinoma line) within 2 min of addition of the compound to the medium. By removing DRB from the medium, the inhibition is promptly and completely reversed after treatment of cells for as long as 1 h or even longer. The inhibitory effect of DRB on the overall rate of RNA synthesis is similar in L and HeLa cells and is markedly concentration-dependent in the low dose range (5–20  $\mu$ M or 1.6–6.4  $\mu$ g/ml), but not at higher concentrations of DRB. At a concentration of 12  $\mu$ M, DRB has a highly selective inhibitory effect on the synthesis of nuclear heterogeneous RNA in L cells. At higher concentrations, there is also inhibition of 45 S ribosomal precursor RNA synthesis, but at all concentrations the effect on heterogeneous RNA synthesis in L cells is considerably greater than that on preribosomal RNA synthesis. In HeLa cells, too, DRB has a selective effect on heterogeneous RNA synthesis, but quantitatively the selectivity of action is somewhat less pronounced. In both L and HeLa cells, the inhibition of synthesis of nuclear heterogeneous RNA is incomplete even at very high concentrations of DRB (150  $\mu$ M). Thus, while DRB is a selective inhibitor of nuclear heterogeneous RNA synthesis, not all such RNA synthesis is sensitive to inhibition. It is proposed that messenger precursor RNA synthesis may largely be sensitive to inhibition by DRB. In short-term experiments, DRB has no effect on protein synthesis in L or HeLa cells. DRB has a slight to moderate inhibitory effect on uridine uptake into L cells and a moderate to marked effect on uptake of uridine into HeLa cells.

Ribosides of halobenzimidazoles are highly active inhibitors of the multiplication of RNA (3, 13, 27, 29, 30) and DNA (16, 31, 32) viruses and of cellular RNA synthesis (3, 14, 31). The inhibitory activity of such derivatives increases with multiple substitution of halogen atoms in the benzenoid ring (27, 28, 29). Of special interest is the fact that the highest inhibitory activity is obtained when the

carbohydrate moiety is identical with that present in RNA. Any departure from the  $\beta$ -D-ribofuranose structure in the benzimidazole glycoside results in reduced inhibitory activity (27–29). The influenza virus growth cycle is sensitive to inhibition by 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) (31) and  $\alpha$ -amanitin (18) only during the first 2–3 h from infection. This and other evidence

has suggested a role for host RNA in the multiplication of myxoviruses.

It was demonstrated early that DRB retards the proliferation of cells in culture (16, 29). Upon removal of the compound (73  $\mu\text{M}$  DRB), proliferation of chorioallantoic membrane cells rapidly reached the degree shown by controls (29). These and other observations suggested that DRB may reversibly reduce the rate of nucleic acid metabolism rather than cause irreversible changes in the biosynthetic machinery of the cells (29, 33). In rhesus monkey kidney cells, in vitro microscopic changes could be detected after treatment of cells with 40  $\mu\text{M}$  DRB for 48 h, but these changes did not progress with time (30). At higher DRB concentrations, the morphological changes were progressive.

The early work was followed by the demonstration that DRB inhibits RNA synthesis in isolated calf thymus nuclei (2) and in the chick chorioallantoic membrane and monkey kidney cells in vitro (31). In cell experiments in which incorporation of precursors was observed for a period of 3 h, approximately 60% inhibition of adenosine incorporation into RNA was associated with 20% inhibition of alanine incorporation into proteins. DRB was employed at a concentration of 55  $\mu\text{M}$  in the chorioallantoic membrane and at 95  $\mu\text{M}$  DRB in monkey kidney cells (31). Sirlin and Jacob (25) then observed that DRB markedly inhibits chromosomal RNA synthesis in the salivary gland cells of the chironomid *Smittia parthenogenetica* while causing little inhibition of nucleolar RNA synthesis. Previously it had been reported that, in the isolated calf thymus nuclei, DRB had a greater inhibitory effect on nucleolar than on nucleoplasmic RNA synthesis (1).

There is now considerable evidence that 60  $\mu\text{M}$  DRB selectively inhibits heterogeneous high molecular weight RNA synthesis in the salivary gland cells of *Chironomus tentans* and has no detectable effects on the synthesis of nucleolar preribosomal RNA or 4 S RNA (7, 8, 10, 11, 21). At higher concentrations, DRB also inhibits nucleolar preribosomal RNA and 4 S RNA synthesis. Egyházi (9) has presented data suggesting that at lower concentrations there is a selective effect on initiation of chains of heterogeneous high molecular weight RNA. Granick (14, 15) has observed in chick embryo fibroblast cells that 156  $\mu\text{M}$  DRB has a greater effect on the synthesis of heterogeneous nuclear RNA and messenger RNA than on ribosomal precursor RNA or tRNA synthesis.

Recently, DRB has been shown to cause a marked increase in interferon production in a strain of diploid human fibroblasts (FS-4 cells) after induction by polyinosinic:polycytidylic acid (poly I:C) (22, 23). There is a close correlation between the superinducing effect of DRB on interferon production and inhibition of RNA synthesis. DRB blocks a control mechanism which ordinarily shuts off interferon production within 6–8 h after induction of cells with poly I:C (22, 23). At the same time, evidence has been obtained that DRB is also capable of inhibiting interferon mRNA synthesis (24).

We have carried out an investigation of the action of DRB in the L-929 line of mouse fibroblasts and in HeLa cells derived from a human cervical carcinoma. We have investigated dose-response relationships and the kinetics of action of DRB. The effects of DRB on nucleoplasmic and nucleolar RNA synthesis have been determined. We have also investigated the effect of DRB on the uptake of [ $^3\text{H}$ ]uridine into cells.

## MATERIALS AND METHODS

### Cell Culture

Two continuous lines of cells were used. The L-929 mouse fibroblasts were grown in monolayer culture in Eagle's minimal essential medium (MEM) (6) with 5% fetal calf serum. For some experiments, L cells were grown in suspension culture in the spinner modification of MEM with 7% fetal calf serum. HeLa S3 human epithelioid carcinoma cells were grown and maintained in suspension culture in the spinner modification of MEM with 7% calf serum. For some experiments, HeLa cells were planted in plastic dishes in reinforced Eagle's medium with 10% calf serum.

### Chemicals and Buffers

[ $^3\text{H}$ ]Uridine (21.0–29.65 Ci/mmol) and [ $^3\text{H}$ ]leucine (47 Ci/mmol) were obtained from New England Nuclear, Boston, Mass. DRB was obtained from Merck, Sharp and Dohme Research Laboratories (Rahway, N. J.) through the courtesy of Dr. Arthur F. Wagner. Phosphate-buffered saline (PBS), contains 0.13 M NaCl, 0.0027 M KCl, 0.00082 M  $\text{Na}_2\text{HPO}_4$ , 0.0015 M  $\text{KH}_2\text{PO}_4$ , 0.00091 M  $\text{CaCl}_2$  and 0.0005 M  $\text{MgCl}_2$ , and the pH is 7.3. RSB is 0.01 M KCl, 0.0015 M  $\text{MgCl}_2$ , 0.01 M tris (hydroxymethyl)aminomethane, pH 7.4. HSB is 0.5 M NaCl, 0.05 M  $\text{MgCl}_2$ , and 0.01 M tris. SSE is 0.15 M NaCl, 0.015 M sodium citrate, 0.01 M disodium ethylenediaminetetraacetate (EDTA). SDS buffer is 0.5% sodium dodecyl sulfate, 0.1 M NaCl, 0.001 M EDTA, 0.01 M tris, pH 7.4.

### Pulse-Labeling Techniques

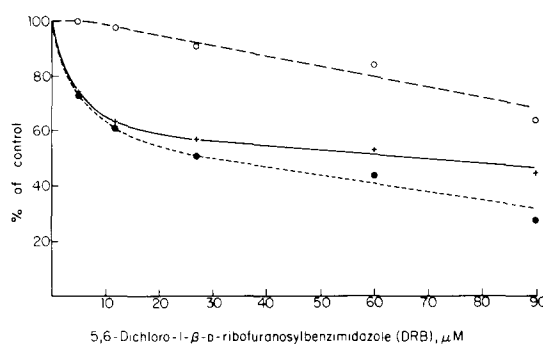
In most experiments, cells in logarithmic growth were pulse-labeled with 2.5  $\mu\text{Ci/ml}$  [ $^3\text{H}$ ]uridine for 15 min. Commonly, treatment with DRB was begun 15 min before the pulse and continued during the pulse. After the pulse, the cells were washed four times with ice-cold PBS, collected, and suspended in 5% trichloroacetic acid (TCA). Aliquots of the suspension were placed on GF/C filters for determination of total cell-associated radioactivity in a liquid scintillation spectrometer. The remainder was filtered and the precipitate was washed with 5% TCA, dried, and counted to determine acid-precipitable radioactivity. To determine the effect of DRB on RNA synthesis, the acid-precipitable counts were corrected for the effect of DRB on uptake of [ $^3\text{H}$ ]uridine into cells (22) as follows: a ratio, expressed as a percentage of precipitable to total counts was calculated for each sample, and then the ratios in experimental samples were divided by those in controls, giving estimates of rates of RNA synthesis as a percentage of control rates. An alternative approach (14), which gives the same result, is to calculate for each pair of data from a treated and a control group a ratio for precipitable counts, a ratio for counts in the acid-soluble pool, and a ratio of the two, which then gives an estimate of RNA synthesis in the treated vs. control group. It is assumed that DRB does not alter the processing of uridine after transport of the precursor into cells and that the uridine pool is not compartmentalized. In some experiments, an aliquot of the collected cell suspension from groups of three or four cultures was used for protein determination according to Lowry et al. (17).

### Isolation of Cell Nuclei and of the Nucleoplasmic and Nucleolar Fractions

Cells were harvested by centrifugation, washed with PBS, and nuclei were isolated by the procedure of Penman (19), with minor modifications. In brief, L cells were resuspended for a minimum of 10 min in 2 ml of one-half strength RSB on ice. HeLa cells were resuspended for a minimum of 15 min in RSB. The cells were ruptured in a Dounce homogenizer with 20 strokes. The nuclei were pelleted by centrifugation, washed once with RSB, and resuspended in 2 ml RSB or one-half strength RSB. A mixture of NP-40 and sodium deoxycholate was then added and the suspension shaken in a vortex-type mixer. The nuclei were collected by centrifugation, resuspended in 2 ml of HSB and digested with DNase (25  $\mu\text{l}$  of a 2 mg/ml solution) for 10 min at room temperature. The nucleoli and nucleoplasm were then separated by zonal centrifugation. The nuclear digest was layered on 2.5 ml of 30% sucrose in HSB and centrifuged at 23,000 rpm for 10 min in an SW-56 Beckman rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The nucleoplasmic components in the supernate were collected, precipitated with two volumes of ethanol, and pelleted by centrifugation. This nucleoplasmic pellet and the nucleolar pellet from sucrose gradient separation

were resuspended in SDS buffer. 10- $\mu\text{l}$  aliquots of each were placed on GF/C filters and counted after drying to obtain estimates of inhibition of nucleoplasmic vs. nucleolar RNA synthesis by DRB.

The nucleoplasmic and nucleolar fractions were brought to a final concentration of 0.01 M EDTA and shaken with phenol saturated with SSE. Then, a volume of chloroform equal to the volume of phenol was added, and the mixture shaken again. After centrifugation, the aqueous phase and the interphase were collected and extracted with chloroform. After another centrifugation, only the aqueous phase was collected, washed with ether three times, and the ether removed with  $\text{N}_2$ . RNA was



**FIGURE 1** Relation between concentration of DRB and effects on [ $^3\text{H}$ ]uridine uptake and RNA synthesis in L cells. L cells were planted in 60-mm plastic petri dishes at a concentration of  $2.5 \times 10^6$  cells/plate in Eagle's MEM with 5% fetal calf serum. 24 h later, the incomplete monolayers of L cells were washed once with warm Eagle's medium and incubated at 37°C for 15 min with DRB at varying concentrations in serum-free MEM, 5 ml/plate. Controls received no DRB. Three dishes were used per variable. The cultures were then pulse-labeled for 15 min with [ $^3\text{H}$ ]uridine added in a vol of 100  $\mu\text{l}$  to give 2.5  $\mu\text{Ci/ml}$ . In the DRB-treated cultures the compound remained present during the pulse. After the pulse, the medium was aspirated and the dishes were washed and frozen. Subsequently, 1 ml of distilled water was added per dish and the cells scraped off with a silicone square. The plates then received 1 ml of 10% TCA. Two aliquots of 10  $\mu\text{l}$  each were used for determination of total cell-associated radioactivity. The remainder was used to measure acid-precipitable radioactivity. To determine the effect of DRB on RNA synthesis, the acid-precipitable cpm were corrected for effect of DRB on uptake of [ $^3\text{H}$ ]uridine into cells as described in Materials and Methods. Mean results of two experiments are shown. In the untreated controls, the total radioactivity was 83,053 cpm and the acid-precipitable radioactivity was 5,976 cpm. (O---O), Total cellular uptake of [ $^3\text{H}$ ]uridine; (+---+), RNA synthesis; (●---●), incorporation of [ $^3\text{H}$ ]uridine into acid-precipitable material.

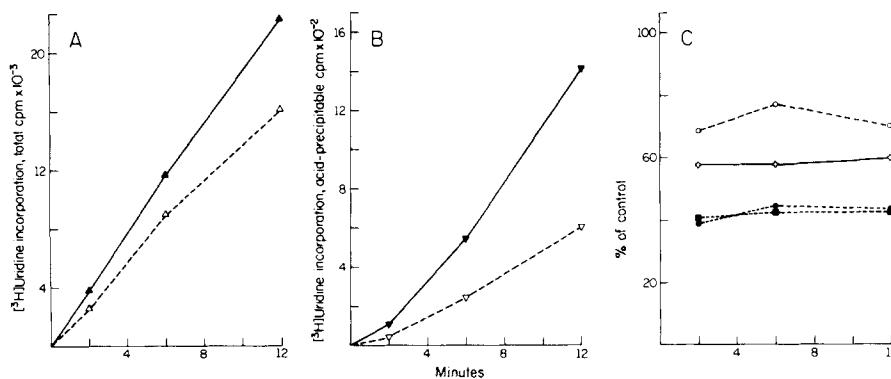


FIGURE 2 Kinetics of action DRB on [<sup>3</sup>H]uridine uptake and RNA synthesis in L cells. Monolayer cultures of L cells (see Fig. 1) were incubated with 30 μM DRB and [<sup>3</sup>H]uridine, 2.5 μCi/ml. Controls received no DRB. At intervals, three experimental and three control dishes were collected and processed for determination of total uptake of [<sup>3</sup>H]uridine into cells and of incorporation of [<sup>3</sup>H]uridine into acid-precipitable material. Mean results of four experiments are shown. (A) Total cellular uptake of [<sup>3</sup>H]uridine; (▲—▲), control; (△---△), 30 μM DRB. (B) Incorporation of [<sup>3</sup>H]uridine into acid-precipitable material; (▼—▼), control; (▽---▽), 30 μM DRB. (C) Effects of DRB expressed as % of control; (O---O), total uptake of [<sup>3</sup>H]uridine; (+---+), RNA synthesis; (●---●), incorporation of [<sup>3</sup>H]uridine into acid-precipitable material (cpm); (■---■), incorporation of [<sup>3</sup>H]uridine into acid-precipitable material (cpm/mg protein).

TABLE I  
Reversibility of the Inhibitory Effect of 60 μM DRB on Uridine Incorporation in L Cells in Monolayer

Medium		[ <sup>3</sup> H]Uridine incorporation*			
Before pulse	During 15-min pulse	cpm	μg protein	cpm/μg protein	% of control
Control	Control	2,295	89	25.8	100
DRB, 60 min	DRB	882	89	9.9	38
DRB, 60 min	Control	2,249	72	31.2	121

\* 60-mm plastic petri dishes were seeded with  $2.5 \times 10^6$  L cells per dish in MEM with 5% fetal calf serum. Approximately 22 h later, the medium was removed and the incomplete monolayers of L cells were incubated with or without 30 μM DRB in MEM without serum. The medium was then replaced with either control or DRB-containing medium, and [<sup>3</sup>H]uridine (sp act 29.65 Ci/mmol) was added to a concentration of 2.5 μCi/ml for pulse-labeling. [<sup>3</sup>H]uridine incorporated into acid-precipitable material was determined and expressed in cpm/mg protein.

then precipitated with 2.5 vol of ethanol and stored at -20°C until analyzed by gel electrophoresis.

### Gel Electrophoresis

For the analysis of RNA in the nucleoplasmic fraction, gels of 9 cm containing 1.0% polyacrylamide and 1.0% agarose were prepared according to the procedure of Watanabe and co-workers (34). During electrophoresis the gels were held in glass tubes with nylon mesh.

Electrophoresis was carried out for 3-3.5 h at a current of 5 mA per gel and a potential gradient of 20 V/cm. The runs were terminated when the tracking dye (bromophenol blue) had migrated 75% of the length of the gel. The gels were extruded from tubes directly into 1-ml tuberculin syringes. Each gel was extruded from the tuberculin syringe through a 20-gauge needle and 1.5-mm fractions were collected. For analysis of RNA in the nucleolar fraction, gels of 10 cm containing 2.0% polyacrylamide and 1% agarose were prepared. Electrophore-

sis was carried out for 4–4.5 h and the gels were sliced into 1-mm fractions with a multiple wire slicer. The fractions were prepared for scintillation counting with water and NCS solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) according to the procedure of Fromageot and Zinder (12).

## RESULTS

### Action of DRB in L Cells

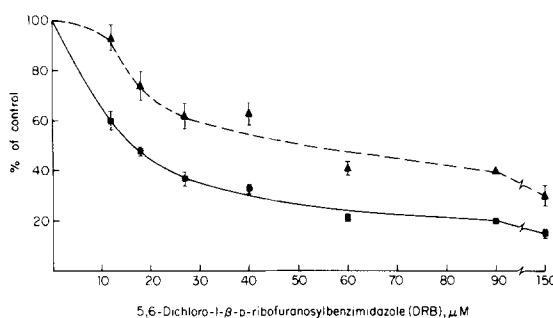
**DOSE-EFFECT RELATIONSHIPS:** The effects of DRB on total uptake of uridine and on incorporation into acid-precipitable material were determined in experiments in which L cells were treated with the compound at varying concentrations for 15 min and then pulse-labeled with [<sup>3</sup>H]uridine, 2.5 μCi/ml, for 15 min in the continued presence of DRB. Estimates of the effect on RNA synthesis were derived by correction of the data as described in Materials and Methods.

Fig. 1 shows that 30–90 μM DRB (9.6 – 28.7 μg/ml) has only a slight to moderate inhibitory effect on uptake of [<sup>3</sup>H]uridine into L cells. Inhibition of cellular RNA synthesis by DRB is markedly concentration-dependent in the very low dose range and there is little increase in effect at concentrations higher than 30 μM. It is evident that a part of RNA synthesis is highly sensitive to inhibition by DRB, as the rate is reduced to approximately 60% of control rate by 12 μM (3.8 μg/ml) DRB. However, even at 90 μM DRB the rate of RNA synthesis is 49% of control value, indicating the presence of a fraction resistant to inhibition by DRB.

In experiments on protein synthesis, using pulse-labeling with [<sup>3</sup>H]leucine, DRB did not decrease incorporation into acid-precipitable material. This is in agreement with results obtained in human foreskin (FS-4) cells, in which no inhibition was observed when protein synthesis was measured 0.5 h after DRB treatment at concentrations up to 60 μM (22). After 5 h of treatment, the rate of protein synthesis was moderately inhibited at DRB concentrations of 20 μM and higher (22). This inhibition is probably secondary to prolonged inhibition of RNA synthesis. The rate of RNA synthesis was inhibited to a similar extent at 0.5 and 5.0 h after DRB treatment of FS-4 cells; there was no significant inhibition at 5.0 μM DRB and 60–70% inhibition at 60 μM DRB concentration (22).

**KINETICS OF ACTION:** Fig. 2 summarizes results of experiments in which both the total uptake of [<sup>3</sup>H]uridine and incorporation into acid

precipitable material were followed over a 12-min period from addition of 30 μM DRB and [<sup>3</sup>H]uridine to monolayer cultures of L cells. Fig. 2 A shows that total uptake was essentially linear from 0 time and that uptake was somewhat reduced in the presence of DRB. The results in Fig. 2 B show that incorporation of uridine into acid-insoluble material by L cells attains an apparently constant and maximal rate within a few minutes after addition of uridine to the medium, as had been reported previously for Novikoff rat hepatoma cells growing in suspension culture (20). It can also be seen that DRB inhibits RNA synthesis within 2 min of addition of the compound to the culture medium. In one experiment, the effect of DRB was determined 1 min after addition, and the degree of inhibition observed at 1 min was not significantly



**FIGURE 3** Effects DRB on nucleoplasmic and nucleolar RNA synthesis in L cells. L cells from monolayer cultures were grown for 2 days in suspension culture in spinner modification of MEM with 7% fetal calf serum. Initial cell concentration was  $2 \times 10^6$  cells/ml, and the vol was in the range of 200 ml. The cell concentration was maintained between 2 and  $4 \times 10^5$  cells/ml by daily dilution. The harvested cells were washed once with warm medium and distributed among six aliquots of 50 ml each containing  $4 \times 10^5$  cells/ml ( $2 \times 10^7$  aliquot). Cells were incubated at 37°C for 15 min in serum-free MEM or in medium containing DRB at varying concentrations. [<sup>3</sup>H]Uridine, 15 μCi/ml, was then added for a pulse of 15 min. After labeling, the cells were harvested on ice, collected by centrifugation, and washed once with PBS. The nuclei and then the nucleoplasmic and nucleolar fractions were isolated as described in Materials and Methods. Incorporation of [<sup>3</sup>H]uridine into acid-precipitable material in the nucleoplasmic and nucleolar fractions was determined. Results of 12 experiments are shown. In the untreated controls, the mean radioactivity in the nucleoplasmic fraction was 974,100 cpm and in the nucleolar fraction it was 598,300 cpm. (■—■), Nucleoplasmic fraction; (▲---▲), nucleolar fraction. The vertical bars indicate probable error for data points. Results of a single experiment are given for 90 μM DRB.

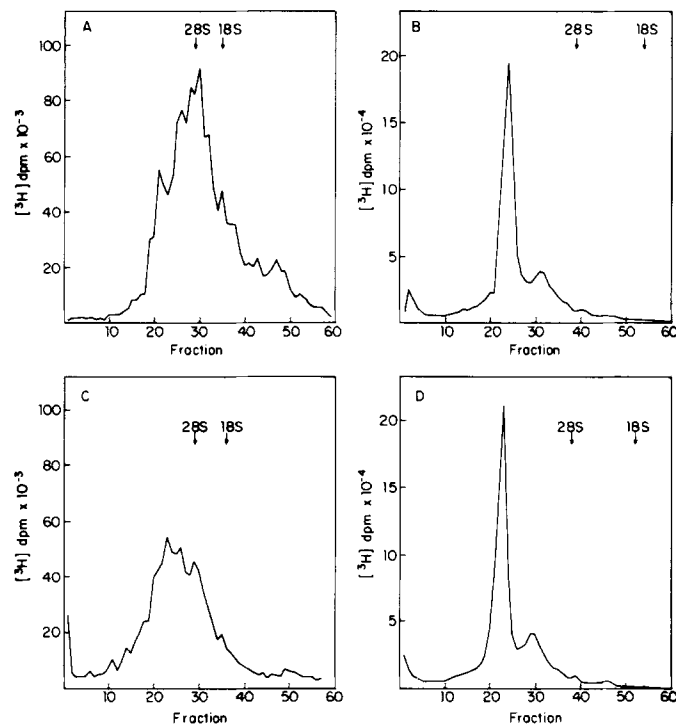


FIGURE 4 Effects of DRB on the synthesis of nuclear heterogeneous and ribosomal precursor RNA in L cells. The experimental conditions are given in Fig. 3. The isolation of nucleoplasmic and nucleolar fractions and of RNA from such fractions as well as the procedure for acrylamide gel electrophoresis are described in Materials and Methods. (A) Nucleoplasmic RNA, control; (B) nucleolar RNA, control; (C) nucleoplasmic RNA, 12  $\mu$ M DRB; (D) nucleolar RNA, 12  $\mu$ M DRB.

different from that observed at 2–12 min. Fig. 2 C illustrates the constancy of the inhibitory effects of DRB over a 2–12 min period from the beginning of treatment.

Experiments were also done to follow the kinetics of DRB action over longer periods. Monolayer cultures of L cells were incubated with 30  $\mu$ M DRB for intervals ranging from 10 to 60 min and then pulse-labeled for 10 min with [ $^3$ H]uridine. The inhibitory effect of DRB on RNA synthesis, established within 15 min from the onset of treatment, became only slightly more marked over the subsequent 50-min period. The slight effect of 30  $\mu$ M DRB on uridine uptake into cells remained essentially unchanged over the course of such a treatment period.

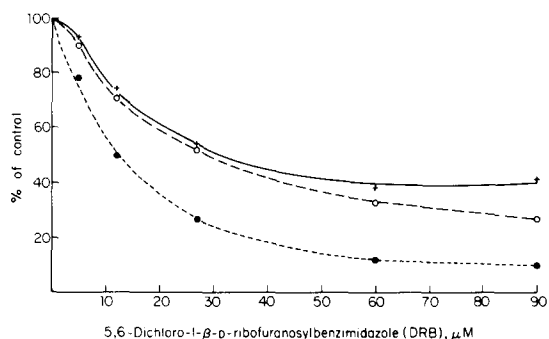
**REVERSIBILITY:** We have investigated the reversibility of the inhibitory action of DRB in experiments in which monolayer cultures of L cells were treated with DRB for varying periods and at varying concentrations. Table I shows that the

inhibitory action of 60  $\mu$ M DRB on [ $^3$ H]uridine incorporation was promptly and completely reversible upon removal of the compound from the medium after a 60-min period of treatment. It was not necessary to incubate DRB-treated cultures for a period of time in the absence of DRB to restore the normal rate of RNA synthesis. Such restoration occurred immediately upon removal of DRB, as determined by pulse-labeling carried out during the 15 min which followed the removal of the inhibitor. Similar results were obtained after a treatment period of 4 h. In some experiments, the medium contained 5% fetal calf serum during the experiment. Incorporation of [ $^3$ H]uridine was increased approximately twofold in the presence of serum, but the degree of inhibition by 60  $\mu$ M DRB was unchanged (67%) and again complete and prompt reversal of the inhibition was obtained upon removal of DRB after a 60-min period of treatment. Increased uptake of uridine upon addition of serum to confluent cultures of mouse 3T3

cells has been reported previously (4, 5). The serum stimulation observed in the present study occurred in incomplete monolayers of L cells.

**NUCLEOPLASMIC VS. NUCLEOLAR RNA SYNTHESIS:** To determine whether DRB selectively inhibits nucleoplasmic heterogeneous RNA synthesis, L cells in suspension culture were treated with DRB for 15 min and then pulse-labeled with [<sup>3</sup>H]uridine for 15 min in the continued presence of the compound. Incorporation of uridine into nucleoplasmic and nucleolar RNA was measured at DRB concentrations ranging from 12 to 150 μM.

Fig. 3 shows that 12 μM DRB had very little effect on the incorporation of [<sup>3</sup>H]uridine into nucleolar RNA while causing considerable inhibition of nucleoplasmic RNA synthesis. As was



**FIGURE 5** Relation between concentration of DRB and effects on [<sup>3</sup>H]uridine uptake and RNA synthesis in HeLa cells. Suspension cultures of HeLa cells were started at a cell concentration of  $1 \times 10^6$  cells/ml in the spinner modification of MEM with 7% calf serum. When the concentration reached  $2 \times 10^6$  cells/ml, the cells were collected by centrifugation, washed once with warm spinner medium, and distributed in 2-ml aliquots containing  $0.4 \times 10^6$  cells/ml in small plastic tubes in serum-free spinner medium with or without DRB at varying concentrations. Three cultures were used per variable. The cells were incubated on a spinner wheel or shaker for 15 min and then [<sup>3</sup>H]uridine, 2.5 μCi/ml, was added for a pulse of 15 min. The cells in each sample were harvested by centrifugation, and processed for determination of total uptake of [<sup>3</sup>H]uridine into cells and of incorporation of [<sup>3</sup>H]uridine into acid-precipitable material. The data were used to determine the inhibitory effect of DRB on RNA synthesis. Mean results of three experiments are shown, except that the 90 μM DRB data points represent results of a single experiment. (O---O), Total cellular uptake of [<sup>3</sup>H]uridine; (+---+), RNA synthesis; (●---●), incorporation of [<sup>3</sup>H]uridine into acid-precipitable material.

demonstrated above, at concentrations up to 12 μM, DRB has no significant effect on uptake of [<sup>3</sup>H]uridine into cells, and thus the results obtained at this concentration and depicted in Fig. 3 clearly demonstrate the selective inhibitory effect of DRB on nucleoplasmic RNA synthesis. At higher concentrations, there is a quantitative difference in the inhibition of nucleoplasmic and nucleolar RNA synthesis. The reduced incorporation of [<sup>3</sup>H]uridine into nucleoplasmic as well as nucleolar RNA at the higher concentrations of DRB can in part be explained on the basis of reduced uptake of the precursor into cells. However, the findings suggest that DRB at higher concentrations not only inhibits nucleoplasmic RNA synthesis but also has an effect on nucleolar RNA synthesis. Furthermore, even at very high concentrations of DRB, such as 150 μM, nucleoplasmic incorporation of [<sup>3</sup>H]uridine into acid-precipitable material is not completely inhibited.

Fig. 4 presents the results of polyacrylamide-agarose gel electrophoresis of RNA extracted from the nucleoplasmic and nucleolar fractions of control and 12 μM DRB-treated L cells after pulse-labeling for 15 min. As can be seen, the nucleoplasmic RNA is heterogeneous and shows a broad size distribution, while the predominant species in the nucleolar fraction is 45 S ribosomal precursor RNA. The amount of the heterogeneous RNA in the nucleoplasmic fraction is considerably reduced in the DRB-treated sample while the 45 S ribosomal precursor RNA shows little if any change after DRB treatment at 12 μM. In individual experiments, some apparent changes were noted in the distribution of heterogeneous nucleoplasmic RNA among different size classes after DRB treatment, but the overall results of numerous gel analyses indicated no systematic and reproducible changes in the migration profiles after 15-min treatment with DRB followed by a 15-min pulse in the continued presence of DRB.

#### Action of DRB in HeLa Cells

**DOSE-EFFECT RELATIONSHIPS:** Fig. 5 shows that, in HeLa cells, DRB has a marked effect on the uptake of [<sup>3</sup>H]uridine into cells; e.g. 60 μM DRB causes 60–70% inhibition. Thus, uptake of [<sup>3</sup>H]uridine into HeLa cells is inhibited much more than that into L cells, and, therefore, correction for this effect is essential in order to arrive at an estimate of inhibition of the rate of RNA synthesis by DRB in HeLa cells, particularly in the range above 10 μM DRB. RNA synthesis in HeLa

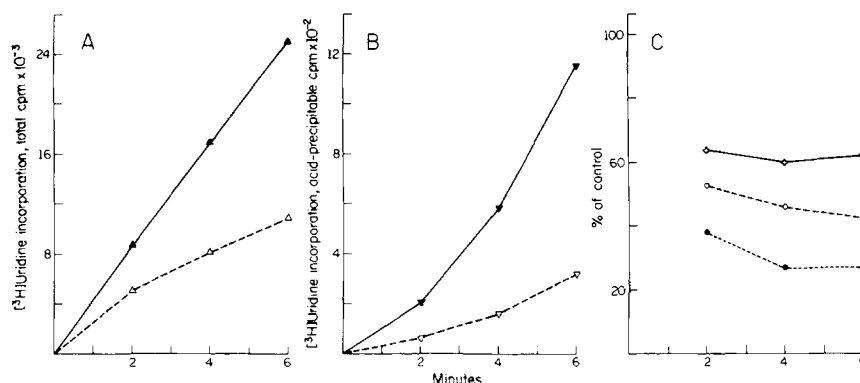


FIGURE 6 Time-course of action of DRB on [<sup>3</sup>H]uridine uptake and RNA synthesis in HeLa cells. 60-mm plastic plates were seeded with  $2.5 \times 10^5$  HeLa cells per dish in reinforced Eagle's medium with 10% calf serum. Approximately 24 h later, the medium was removed, the plates were washed once with warm reinforced Eagle's medium and the incomplete monolayer cultures incubated with 30  $\mu$ M DRB and [<sup>3</sup>H]uridine. At intervals, three experimental and three control cultures were collected and processed for determination of total uptake of [<sup>3</sup>H]uridine into cells and incorporation of [<sup>3</sup>H]uridine into acid-precipitable material. Mean results of three experiments are shown. (A) Total cellular uptake of [<sup>3</sup>H]uridine into acid-precipitable material: ( $\blacktriangle$ — $\blacktriangle$ ), control; ( $\triangle$ --- $\triangle$ ), 30  $\mu$ M DRB. (B) Incorporation of [<sup>3</sup>H]uridine into acid-precipitable material; ( $\blacktriangledown$ — $\blacktriangledown$ ), control; ( $\triangledown$ --- $\triangledown$ ), 30  $\mu$ M DRB. (C) Effects of DRB expressed as % of control; (O---O), total uptake of [<sup>3</sup>H]uridine; (+—+), RNA synthesis; ( $\bullet$ --- $\bullet$ ), incorporation of [<sup>3</sup>H]uridine into acid-precipitable material (cpm).

TABLE II  
Reversibility of the Inhibitory Effect of 30  $\mu$ M DRB on Uridine Incorporation in HeLa Cells in Monolayer

Medium		[ <sup>3</sup> H]Uridine incorporation*					
Before pulse	During 15-min pulse	Total		Acid-precipitable		Acid-precipitable	Acid-precipitable/total
		cpm	% of control	cpm	% of control	% of total	% of control
Control	Control	60,782	100	5,568	100	9.2	100
DRB, 60 min	DRB	25,069	41	942	17	3.8	41
DRB, 60 min	Control	56,493	92	5,656	101	10.0	109

\* 60-mm plastic plates were seeded with  $2.5 \times 10^5$  HeLa cells per dish in reinforced Eagle's medium with 10% calf serum. Approximately 24 h later, the medium was removed and the incomplete monolayers of HeLa cells were washed once with warm reinforced Eagle's medium. The plates were then incubated with or without 30  $\mu$ M DRB in reinforced Eagle's medium. For pulse-labeling, [<sup>3</sup>H]uridine (26.2 Ci/mmol) was used at a concentration of 2.5  $\mu$ Ci/ml. After the pulse, the cultures were processed and the uptake of [<sup>3</sup>H]uridine into cells and the incorporation into acid-precipitable material were determined. The effect of DRB on RNA synthesis was calculated.

cells is similar to that in L cells in sensitivity to inhibition by DRB (cf. Figs. 1 and 5). As in L cells, inhibition of RNA synthesis in HeLa cells appears to reach a plateau at higher concentrations, i.e. above 30  $\mu$ M DRB.

No inhibition of HeLa cell protein synthesis was observed in the concentration range of DRB investigated, i.e. 12–60  $\mu$ M. In these experiments,

HeLa cells in suspension were treated with DRB for 15 min in the presence or absence of 7% calf serum and then pulse-labeled for 15 min with [<sup>3</sup>H]leucine. Comparable cultures received [<sup>3</sup>H]uridine. While the presence of serum resulted in increases in incorporation of precursors into acid-precipitable material (1.4-fold for [<sup>3</sup>H]leucine and 1.6-fold for [<sup>3</sup>H]uridine), the inhibitory effect



of DRB on incorporation of [<sup>3</sup>H]uridine was closely similar in the presence and absence of serum.

**KINETICS OF ACTION:** Fig. 6 A shows that uptake of [<sup>3</sup>H]uridine into HeLa cells is markedly reduced after a 2-min treatment with DRB. Fig. 6 B illustrates the very marked reduction in incorporation of [<sup>3</sup>H]uridine into acid-precipitable material. Fig. 6 C demonstrates that, after correction for the effect of DRB on uridine uptake into HeLa cells, the degree of inhibition of RNA synthesis in HeLa cells is similar to that in L cells (See Fig. 2).

**REVERSIBILITY:** Table II shows that the inhibitory effect of 30 μM DRB on RNA synthesis in HeLa cells could be promptly and completely reversed by removal of the compound from the medium. The results also suggest that inhibition of uptake of uridine into cells was not completely reversed within 15 min of removal of DRB.

**NUCLEOPLASMIC VS. NUCLEOLAR RNA SYNTHESIS:** Fig. 7 shows results of determinations of incorporation of [<sup>3</sup>H]uridine into acid-precipitable material in the nucleoplasmic and nucleolar fractions of HeLa cells in suspension culture. While the effect on nucleoplasmic incorporation is greater, there is also considerable inhibition of incorporation of [<sup>3</sup>H]uridine into the nucleolar fraction, which is discussed below. These effects increase markedly in the concentration range from 5 to 30 μM, but level off at higher concentrations, as was noted above for the inhibition of the overall rate of RNA synthesis in HeLa cells (cf. Fig. 5).

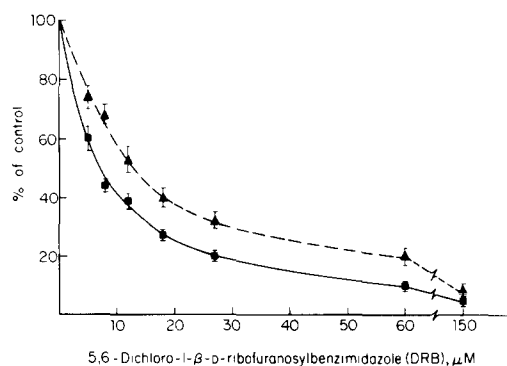
Fig. 8 shows the results of polyacrylamide gel analysis of RNA in the nucleoplasmic and nucleolar fractions from HeLa cells. In the nucleoplasmic fraction the RNA shows a broad distribution characteristic of the nuclear heterogeneous RNA, while the RNA from the nucleolar fraction is predominantly 45 S ribosomal precursor RNA. 12 μM DRB reduced the amount of labeled RNA in both fractions, but, as expected, the reduction was greater in the nucleoplasmic fraction. No significant changes were observed in the size distribution of RNA species after treatment of cells with DRB.

## DISCUSSION

An examination of the effects of DRB in the L-929 line of mouse fibroblasts and in the HeLa S3 line of human carcinoma cells has shown that the inhibitory action of this compound on RNA

synthesis is immediate and promptly reversible upon removal of the compound from the medium.

In L cells, DRB has only a minor effect on the uptake of [<sup>3</sup>H]uridine into cells. The dose-response curves show that a part of the process of RNA synthesis is highly sensitive to DRB while another part is resistant to the action of DRB. This difference arises mainly from the fact that the synthesis of nucleoplasmic heterogeneous RNA is more sensitive to inhibition than the synthesis of nucleolar ribosomal precursor RNA. At low concentrations such as 12 μM (3.8 μg/ml), DRB reduced by approximately 40% the rate of synthesis of nucleoplasmic heterogeneous RNA in L cells in the absence of a significant effect on nucleolar RNA synthesis.



**FIGURE 7** Effects of DRB on nucleoplasmic and nucleolar RNA synthesis in HeLa cells. HeLa cells were grown in suspension culture in spinner medium with 7% calf serum. The starting concentration was  $1 \times 10^6$  cells/ml, and, when the cell count had doubled, the cells were collected by centrifugation, washed once with warm spinner medium, resuspended and distributed at  $0.8 \times 10^6$  cells/ml into six tubes ( $1.6 \times 10^7$  cells/aliquot) in 20 ml of serum-free medium without DRB or with DRB at varying concentrations. The cells were incubated at 37°C for 15 min, and then [<sup>3</sup>H]uridine was added to a concentration of 15 μCi/ml. After labeling for 15 min, the cells were harvested on ice, collected by centrifugation, and washed once with PBS. The nuclei and subsequently the nucleoplasmic and nucleolar fractions were isolated as described in Materials and Methods. Incorporation of [<sup>3</sup>H]uridine into the nucleoplasmic and nucleolar fractions was measured by scintillation counting. Results of 14 experiments are shown. In the untreated controls, the mean radioactivity in the nucleoplasmic fraction was 4,044,700 cpm and in the nucleolar fraction it was 3,144,600 cpm. (■—■), Nucleoplasmic fraction; (▲---▲), nucleolar fraction. The vertical bars indicate probable error for data points.

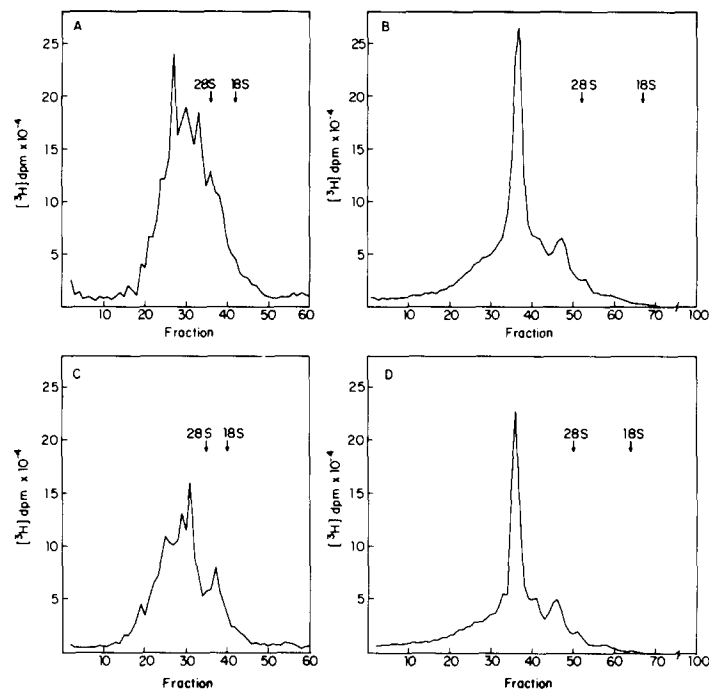


FIGURE 8 Effects of DRB on the synthesis of nuclear heterogeneous and ribosomal precursor RNA in HeLa cells. The experimental conditions are given in Fig. 7. The isolation of nucleoplasmic and nucleolar fractions and of RNA from such fractions as well as the procedure for acrylamide gel electrophoresis are described in Materials and Methods. (A) Nucleoplasmic RNA, control; (B) nucleolar RNA, control; (C) nucleoplasmic RNA, 12  $\mu$ M DRB; (D) nucleolar RNA, 12  $\mu$ M DRB.

Our results also show, however, that not all of the heterogeneous RNA in the nucleoplasmic fraction of L cells is highly sensitive to inhibition by DRB and, furthermore, that the synthesis of the 45 S preribosomal RNA is partially sensitive to the inhibitory action of DRB at concentrations higher than 12  $\mu$ M. The finding that the synthesis of a part of the heterogeneous nuclear RNA is resistant to inhibition by DRB is difficult to explain as an experimental artifact due to contamination with nucleolar material, because even at very high concentrations of DRB the pattern of size distribution of the heterogeneous RNA remains essentially unaltered. If there were present in the nucleoplasmic RNA fraction a substantial amount of 45 S ribosomal precursor RNA whose synthesis was resistant to DRB, such material should become apparent in acrylamide gel analysis of the nucleoplasmic fraction, when the synthesis of heterogeneous RNA is markedly depressed by high doses of DRB. It is more likely that among the nucleoplasmic heterogeneous RNA there is

some whose synthesis is resistant to DRB. Our data are compatible with the view that this subfraction contains RNA belonging to the broad size range of nuclear heterogeneous RNA. Work is in progress to determine the chemical properties of the DRB-sensitive and DRB-resistant nuclear heterogeneous RNA.

Recent evidence indicates that DRB can markedly inhibit interferon mRNA synthesis (24). We propose that DRB may have greatest effect on the synthesis of that portion of nuclear heterogeneous RNA which is precursor to mRNA. If DRB acts at the polymerase level, then the possibility arises that there may be more than one polymerase involved in the synthesis of nuclear heterogeneous RNA.

It is also difficult to explain the inhibition of the synthesis of some of the ribosomal precursor RNA in the molecular fraction of L cells as an experimental artifact, because this material is very clearly and sharply resolved by acrylamide gel analysis and shows a substantial decrease after

TABLE III  
*Comparison of the Effects of 150  $\mu$ M DRB in L-929 and HeLa S3 Cells; Rates of [ $^3$ H]Uridine Uptake, Incorporation into Acid-precipitable Material, and RNA Synthesis Expressed as Percent of Control Rates\**

	L-929 cells (total uptake = 51)		HeLa S3 cells (total uptake = 12)	
	Nucleo- plasmic	Nucleolar	Nucleo- plasmic	Nucleolar
Acid-precipitable incorporation	15	30	5	8
RNA synthesis	31	63	25	40

\* Cells treated with DRB for 15 min and then pulse-labeled with [ $^3$ H]uridine for 15 min in the continued presence of DRB.

treatment of L cells with DRB at high concentrations. However, the apparent reduction in the overall rate of RNA synthesis in the nucleolar fraction may in part reflect contamination with heterogeneous RNA (26).

Table III presents a summary of comparative data on the effects of 150  $\mu$ M DRB in L-929 and HeLa S3 cells. The inhibitory effect of DRB on the uptake of uridine is much greater in HeLa than in L cells. The reduction in the rate of nuclear heterogeneous RNA synthesis is similar in DRB-treated HeLa and L cells. In HeLa cells, as in L cells, there appears to be some heterogeneous nuclear RNA whose synthesis is resistant to inhibition by DRB. The apparent effect of DRB on nucleolar RNA synthesis is greater in HeLa than in L cells; however, results of acrylamide gel analysis (cf. Figs. 4 and 7) suggest that this may be due to greater contamination of the nucleolar fraction with heterogeneous RNA in HeLa cells.

The present studies show that, at the appropriate dose level, DRB is a selective inhibitor of nuclear heterogeneous RNA synthesis in mammalian cells. The compound has a similar action in insect (8, 9) and avian cells (14) and may therefore be widely useful as a probe in studies of various aspects of RNA metabolism. These include investigation of the initiation mechanism of RNA transcription, studies of processing of RNA, definition of subclasses of nuclear heterogeneous RNA, and investigation of regulation of cellular biosynthetic processes.

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