

NUCLEOLAR NECKLACES IN CHICK EMBRYO FIBROBLAST CELLS

II. Microscope Observations of the Effect of Adenosine Analogues on Nucleolar Necklace Formation

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ABSTRACT

The round nucleoli of chick embryo fibroblast cells, when exposed to adenosine (2 mM) or to a number of adenosine analogues, lose material and unravel over a period of several hours to become beaded strands, 20 μm in length, termed nucleolar necklaces (NN). Light microscope observations on this process are described. Biochemical experiments have revealed that most of these analogues interfere with both messenger RNA synthesis and ribosome synthesis, causing extensive degradation of the preribosome species containing 32S RNA although most of the preribosomes containing 18S RNA survive. We suggest that it is the depletion from the nucleolus of the adhesive 32S and 28S RNA preribosomes which allows the remaining nucleolar apparatus to spread apart into the NN configuration. Also required for the maintenance of the NN structure is the synthesis of some ribosomal RNA (rRNA) possibly present as rRNA "feathers" on the DNA. The addition of inhibitors of rRNA synthesis such as actinomycin D to the NN-containing cells causes loss of rRNA. Then a contraction and collapse of the NN structure into small dense spheres is observed.

A number of chemicals, mostly adenosine analogues, have been shown to interfere with ribosome synthesis and to cause fragmentation of the nucleolus which is the ribosome synthetic center of the cell. Chemicals which belong to this category include the adenosine analogues toyocamycin (8, 14, 18, 22), 5-bromotubercidin (2), and 5,6-dichloro-(β -D-ribofuranosyl)benzimidazole (DRB)¹ (4, 5). In addition, high concentrations of

adenosine (2 mM) (9, 11, 13, 15), α -amanitin (10, 17, 21), and arginine starvation (7) cause morphological changes which are similar to those produced by the adenosine analogues.

In chick embryo fibroblast (CEF) cells these alterations of nucleolar morphology are particularly striking: the round nucleoli lose material and unravel into beaded strands termed nucleolar necklaces (NN) which are readily observable with a phase-contrast microscope. These NN are approximately 20 μm long and contain about 25 beads per strand. They are similar to those formed when CEF cells are grown in a medium deficient in the amino acid arginine (7). They also resemble the beaded chain nucleoli observed in

¹ *Abbreviations used in this paper:* CEF, chick embryo fibroblast; DRB, 5,6-dichloro-(β -D-ribofuranosyl)-benzimidazole; mRNA, messenger RNA; NN, nucleolar necklaces; rRNA, ribosomal RNA; 45S rRNA preribosome, preribosome containing 45S rRNA.

late-stage amphibian oocytes (12). A report of electron microscope studies of CEF NN formed after treatment with DRB, or after amino acid starvation is in preparation.²

In the accompanying paper (5), we investigated the biochemical action of some of the NN-forming chemicals in CEF cells and suggested that these compounds interfered primarily with the production of messenger RNA (mRNA) and ribosomal RNA (rRNA). The adenosine analogue DRB appeared to completely inhibit the synthesis of both mRNA and heterogeneous nuclear RNA, but to decrease the rate of 45S rRNA accumulation by only 50%. Along with this decrease in RNA synthesis was an extensive (80%) degradation of the 32S + 28S rRNA-containing preribosome species. Most of this preribosome degradation probably occurred at or before the 32S rRNA preribosome stage. Experimental evidence suggested that the degradation of these preribosomes in the presence of DRB occurred as a result of the synthesis of defective preribosomes containing 45S rRNA.³

The biochemical evidence for preribosome degradation in the presence of these adenosine analogues has also been confirmed by electron microscope studies. The 32S + 28S RNA preribosomes probably correspond to the 200-Å granules which make up a large fraction of the normal nucleolus. The fragmentation of the nucleolus seen after exposure to DRB or toyocamycin is usually accompanied by an appreciable loss of these 200-Å granules² (8, 13, 14).

In this paper we describe light microscope studies on the development of the CEF NN after the addition of adenosine analogues and investigate the effects of other types of inhibitors on this process. Then we attempt to correlate both microscope and biochemical studies on the NN-forming

chemicals in order to explain the phenomenon of NN formation. On the basis of these data we postulate that NN formation occurs primarily as a result of the degradation of the preribosomes containing 32S rRNA.

MATERIALS AND METHODS

Materials

5,6-Dichloro(β -D-ribofuranosyl)benzimidazole, 4,5,6-trichloro(β -D-ribofuranosyl)benzimidazole, and 2-mercapto-1-(2-[4-pyridyl]ethyl)benzimidazole were gifts from the Merck Chemical Div., Merck & Co., Inc., Rahway, N. J. Tubercidin was obtained from the David W. Mann Co., Burlington, Mass. 5-Fluorouridine and 5-bromotubercidin were gifts from Dr. B. Brdar of The Rockefeller University. α -Amanitin was a gift from Dr. Th. Wieland of the Max-Planck-Institute, Heidelberg, Germany. Toyocamycin was a gift from Dr. R. J. Suhadolnik, Albert Einstein Medical Center, Philadelphia. Bovine crystalline trypsin (grade 5) was purchased from Miles Seravac Co. Fetal bovine serum was purchased from the Microbiological Associates, Bethesda, Md.

CEF Cell Cultures

A 10- or 11-day old chick embryo was decapitated and its internal organs were removed. After washing in Earle's salt solution, the remainder was chopped up finely with a razor blade in a small Petri dish containing 5 ml of 1% crystalline trypsin. After 10 min at 37°C, Varidase (a minimal amount <0.1 mg) was added to eliminate the stringiness due to released DNA. The suspension was occasionally sucked gently back and forth in a wide mouth pipette to help break up the tissue lumps. After 30–35 min, when the suspension was cloudy and most of the tissue particles had disappeared, it was transferred to a 30-ml test tube and was diluted with Eagle's minimal essential medium (MEM) (10 ml Eagle's was used for a 10-day embryo; 15 ml Eagle's was used for an 11-day embryo). Large particles were permitted to settle and the supernatant cell suspension (approximately 10^7 cells/ml) was used as an inoculum.

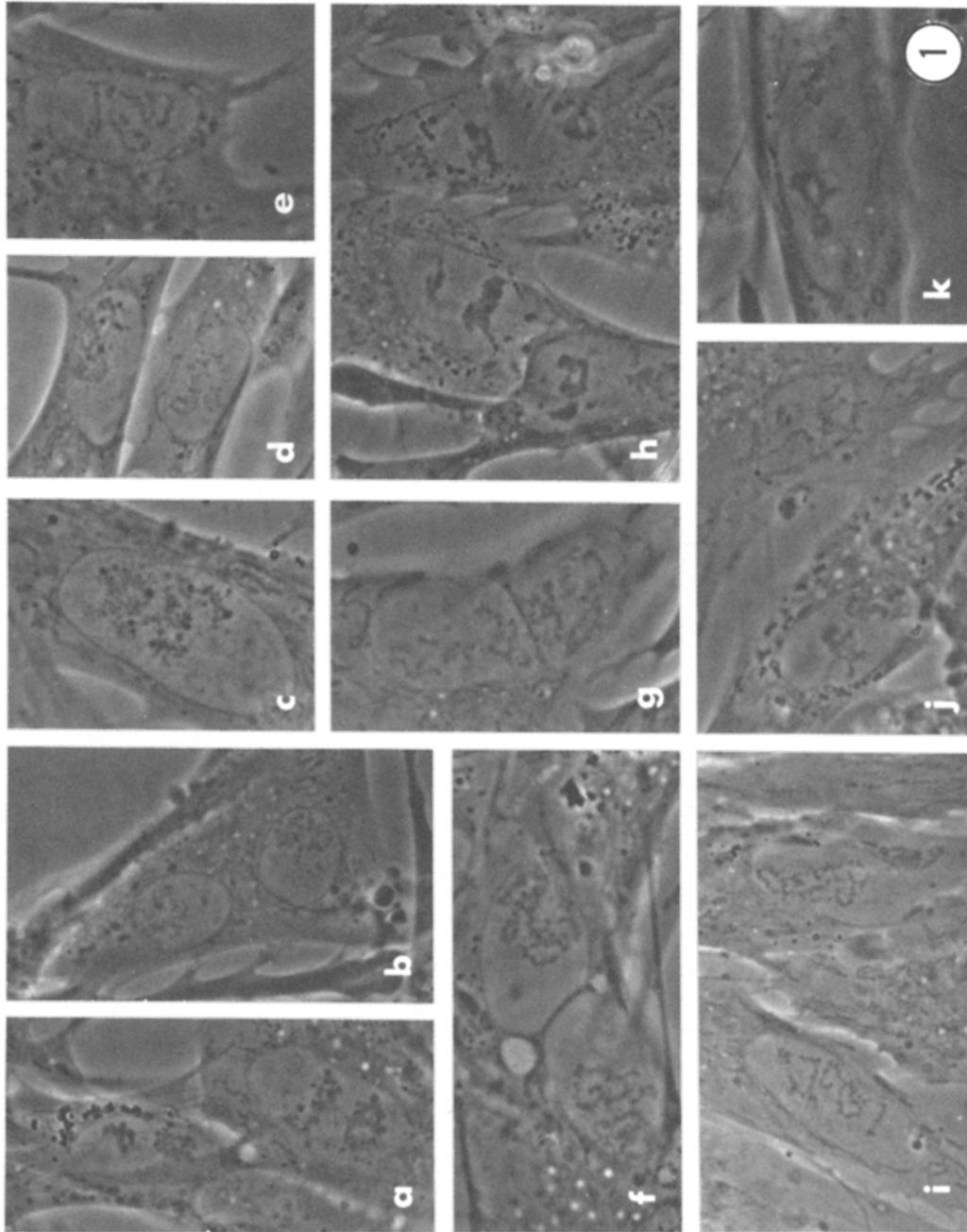
When the cells were to be grown on cover slips in vials (18×60 mm) containing 1.0 ml of Eagle's medium supplemented with 10% fetal bovine serum, approximately 0.05 ml of cell inoculum (5×10^5 cells) was added to each vial. When the cells were to be grown in 100-mm Falcon tissue culture dishes containing 10 ml of Eagle's medium, 1.0–2.0 ml of cell inoculum ($1-2 \times 10^7$ cells) was added to each dish. The medium was changed on the next day and every other day subsequently.

Culture of Other Cell Types

Chick embryo liver cell cultures were prepared according to the method of Granick (6). HeLa cells were grown

² Granick, D., and A. K. Kleinschmidt. 1975. Structural studies on the nucleolar necklaces of chick embryo fibroblast cells. Manuscript in preparation.

³ 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 (24), 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.



as monolayer cultures in Eagle's MEM with 10% calf serum. L cells were grown as monolayer cultures in Eagle's MEM with 10% fetal bovine serum.

Light Micrographs

Cover slips of cells were mounted wet onto glass slides and were photographed with phase-contrast optics in a Zeiss Ultraphot microscope with a 100 \times oil immersion lens. Polapan type 52 film was used for Figs. 1, 3, 4. Tri-X 120 film was used for Fig. 2.

EXPERIMENTAL PROCEDURES

Light Microscope Observations on the Effect of NN-Forming Chemicals

To test for the ability of various types of chemicals to form NN, they were added to CEF cells growing in monolayers on cover slips in vials containing Eagle's medium. The morphological changes were followed by removal of the cover slips at various times for examination with a phase-contrast microscope. Completed NN were formed within 6 h. The chemicals found to cause NN formation were adenosine (2 mM) and the adenosine analogues: toyocamycin (2 μ g/ml); 5-bromotubercidin (2.5 μ g/ml); DRB (25–50 μ g/ml or approximately 0.1–0.2 mM); 4,5,6-trichloro(β -D-ribofuranosyl)benzimidazole (25 μ g/ml); 2-mercapto-1-(2-[4-pyridyl]ethyl)benzimidazole (25 μ g/ml). α -Amanitin (20 μ g/ml) and 5-fluorouridine (5 μ g/ml) caused somewhat different patterns of nucleolar alteration.

The steps in NN development caused by the adenosine analogues were similar and are presented in Fig. 1. After approximately 30 min, the CEF nucleoli enlarged and became full of holes as if they were losing material. By 1 h, the nucleoli had fragmented into aggregates of large

fuzzy dots (Fig. 1 *a–c*). After 2–3 h, discrete dots were present in more than 95% of the cells. They were more spread out, and already there were some linear arrangements of beads. The stringing out of the beads into necklaces occurred after 6–8-h exposure to the inhibitors (Figs. 1 *e, f, i; 2*). Combinations of the various NN-forming adenosine analogues produced additive effects, as judged by microscope observations, suggesting that these inhibitors had the same site of action.

Recovery from the NN morphology back to a normal nucleolar morphology also occurred about 6 h after the NN-forming chemical was removed and the CEF cells were given fresh Eagle's medium. The NN beads fused into thin chains. These chains then became swollen and condensed together into normal nucleolar spheres (Fig. 1 *h*).

Two other inhibitors, α -amanitin and 5-fluorouridine, caused a somewhat different pattern of nucleolar alterations suggesting a somewhat different mechanism of action. α -Amanitin (20 μ g/ml) made NN in 90% of the CEF cells (Fig. 1 *g*). However, different cells started NN formation at different times over a 2–6-h period although the actual time for NN formation appeared to be approximately the same for all of them. α -Amanitin inhibition was irreversible. 5-Fluorouridine (5 μ g/ml) caused only partial NN formation. About 50% of the nucleoli were opened up into looped structures but only 10% were in thin chains and no NN were seen (Fig. 1 *j*).

Other Cell Types

Other cell types were also checked for their ability to produce NN. Chick embryo liver cells responded in essentially the same way to NN-

FIGURE 1 Conversion of CEF nucleoli to NN after addition of various chemicals. Primary CEF cell monolayer cultures were grown on cover slips for 2 or 3 days in Eagle's MEM supplemented with 10% fetal bovine serum. The following chemicals were then added to the medium and after the indicated times the living cells were examined by phase-contrast microscopy: (*a*) 60 min, toyocamycin (1.0 μ g/ml). (*b*) 70 min, bromotubercidin (5.0 μ g/ml). (*c*) 70 min, adenosine (2 mM). (*d*) 3 h, toyocamycin (2.0 μ g/ml). (*e*) 5 h, bromotubercidin (3 μ g/ml). (*f*) 6.5 h, adenosine (3 mM). (*g*) 6 h, α -amanitin (20 μ g/ml). (*h*) Partial reformation of nucleoli: NN were developed by 2.5 h with toyocamycin (1.0 μ g/ml) treatment and the cells were then changed to fresh Eagle's medium and incubated for an additional 1.5 h. (*i*) 8.5 h, toyocamycin (2.0 μ g/ml). (*j*) 5 h, fluorouridine (5 μ g/ml). The thin chainlike structure seen in the right-hand nucleus was seen only in 10% of the cells. Most of the nucleoli were more normally shaped than those of the left-hand nucleolus. (*k*) 6 h, acetoxycycloheximide (1.0 μ g/ml). These thin looped nucleolar forms were seen in only 20% of the cells. The nucleoli of most cells were more regularly shaped. Fig. 1 *a, b, d, h, and i*, \times 840; Fig. 1 *c, e–g, j, and k*, \times 675.

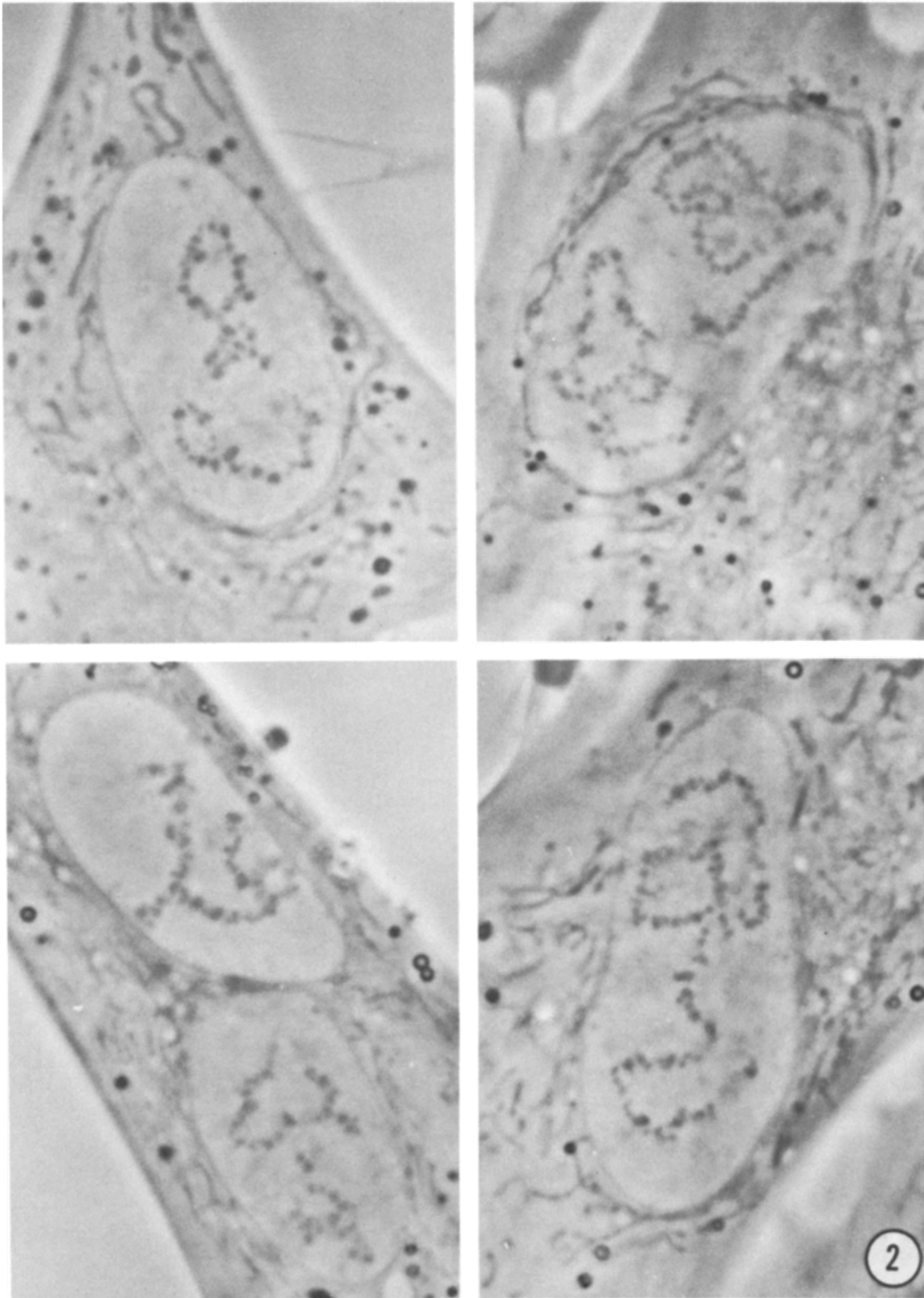


FIGURE 2 NN in CEF cells formed after 4 h of incubation with DRB (50 $\mu\text{g}/\text{ml}$). Primary CEF cells were grown on cover slips in vials for 2 days in Eagle's MEM supplemented with 10% fetal bovine serum. DRB (50 $\mu\text{g}/\text{ml}$) was then added to the medium. 4 h later the living cells were photographed with phase-contrast optics. The cells in the two right-hand micrographs are probably tetraploid. $\times 2,700$.

forming chemicals as the CEF cells did (Fig. 3 *b*). Although a certain fraction of the HeLa and L cell nucleoli were fragmented by the same chemicals, a necklace-like chain pattern was never seen (Fig. 3, *c-i*). After several hours of exposure to toyocamycin, 5 mM adenosine, or DRB, only 25% of the HeLa and L cell nucleoli were fragmented. The nucleolar fragments varied in size and were often connected by trails of light material but these fragments were randomly dispersed throughout the nucleus. With α -amanitin, 90% of the HeLa and L cell nucleoli were fragmented over a 20-h period but these fragments were also randomly dispersed throughout the nucleus.

Arginine Starvation

NN develop in primary cultures of chick embryo cells (fibroblast, myoblast, liver) after 3-5 days of growth in a medium which lacks the amino acid arginine (Fig. 3 *a*; also reference 7). Arginine starvation did not cause nucleolar fragmentation in HeLa or L cells (unpublished observations). Starvation of CEF cells for any other amino acid did not result in NN formation (7). Arginine may become especially limiting in the chick cells because some of the urea cycle enzymes required for its synthesis are present only in trace amounts (20). Addition of arginine did not prevent NN formation by adenosine analogues.

Morphological Effects of Inhibitors of Transcription and Translation on Normal Nucleoli and on NN

Some insights into the biochemical mechanism of NN formation were obtained by observing the influence of other types of inhibitors on this process.

Drugs which completely block both mRNA and rRNA synthesis at the RNA polymerase level such as actinomycin D (0.01-0.25 μ g/ml) or miracil D (leucanthone) (5-10 μ g/ml), caused a contraction or collapse of normal CEF nucleoli into dense spheres. In a similar fashion the long beaded necklaces, which had been produced by the adenosine analogues (drugs that only partially inhibited rRNA synthesis), contracted into one or several dense spheres after the addition of actinomycin or miracil (drugs that inhibited rRNA synthesis completely) (Fig. 4 *c, d*). After the NN had been caused to contract, they could be reformed back to NN (although they could not be spread out to the original extent) by removing the collapsing

inhibitor and giving the cells fresh medium containing the original NN-forming chemical (Fig. 4 *e*). Reformation of NN structure was only possible if the low concentration ranges of the collapsing inhibitors had been used.

Cordycepin (3'-deoxyadenosine) (5-20 μ g/ml) caused collapse of NN structure in a manner similar to actinomycin D and miracil D although cordycepin inhibits rRNA synthesis by a different mechanism. This adenosine analogue can be inserted into the growing 45S rRNA molecules causing premature termination of their synthesis by the rRNA polymerase (16).

In conclusion, this collapse of NN structure in the presence of an rRNA synthesis inhibitor and the reformation of NN structure after the collapsing chemical is removed and rRNA synthesis is allowed to resume suggest that (*a*) the NN dots are tied into a continuous structure, and (*b*) some rRNA synthesis (or at least the presence of incompletely synthesized rRNA sitting on the ribosomal DNA) is required to maintain NN structure.

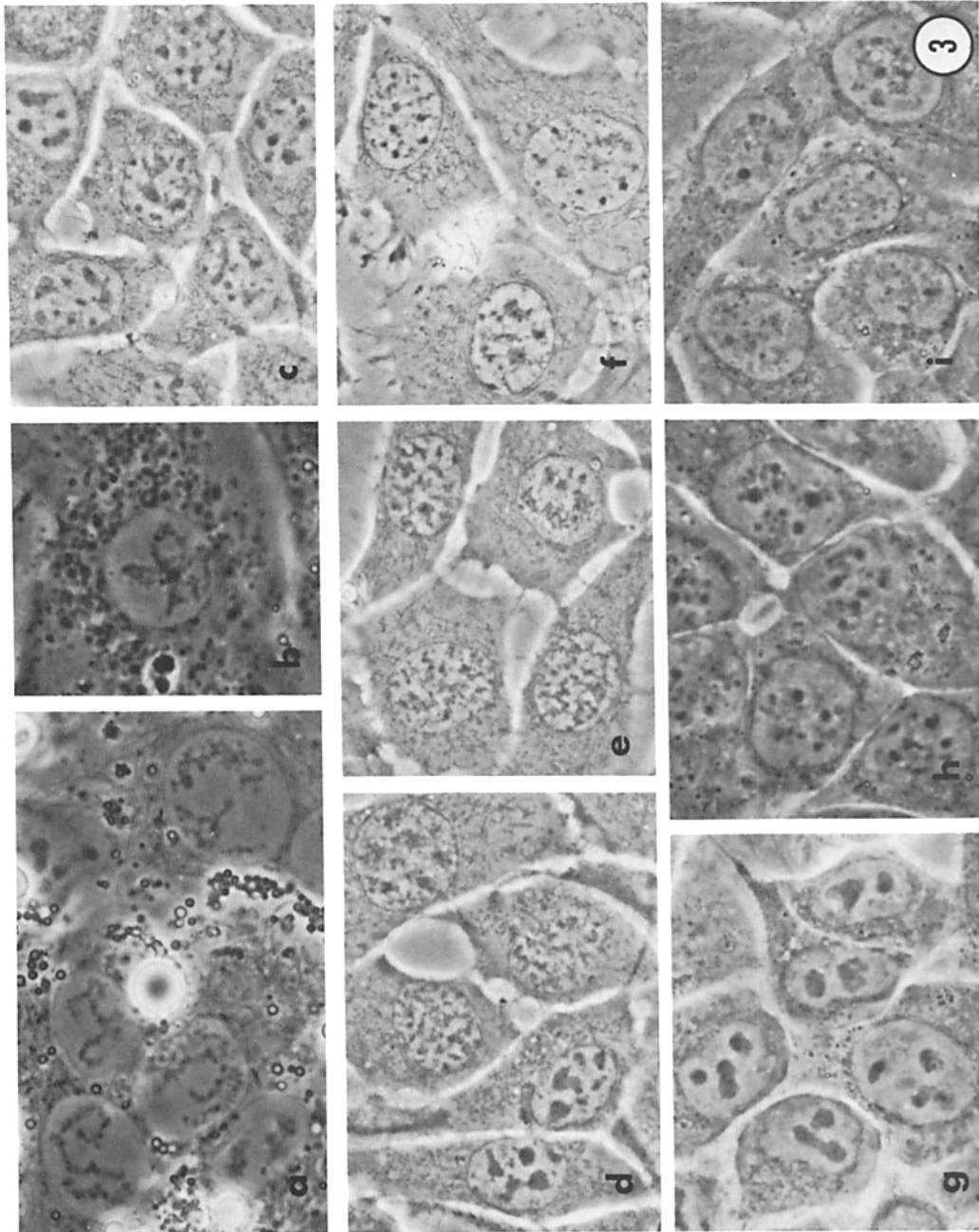
INHIBITORS OF PROTEIN SYNTHESIS: Inhibitors such as acetoxycycloheximide (1-2 μ g/ml) did not cause NN formation. Fig. 1 *k* shows some of the most affected nucleoli. These were seen in only 20% of the cells. This result suggests that NN formation is probably not caused by an inhibition of general protein synthesis.

OTHER INHIBITORS: Inhibitors of methylation (amethopterin, trimethoprim, ethionine), or of purine or pyrimidine synthesis (8-azaguanine, 6-mercaptopurine riboside, 6-azauridine), or of oxidative phosphorylation (dinitrophenol, oligomycin, antimycin) did not cause NN formation.

High concentrations of guanosine, uridine, and cytidine (1-2 mM), either singly or combined, did not cause NN formation or inhibit NN formation by 2 mM adenosine. These results suggest that the ratios of nucleosides (or nucleotides) in the cell are not a critical factor in causing NN formation.

DISCUSSION

The round nucleoli of CEF cells when exposed to adenosine (2 mM) or adenosine analogues (e.g., toyocamycin, bromotubercidin, DRB) lose material and unravel to become beaded strands termed NN. The first morphological effects of these compounds were observed in about 30 min. After 1 h the nucleoli were already highly fragmented. NN formation was complete in less than 6 h. Combinations of these inhibitors gave additive effects



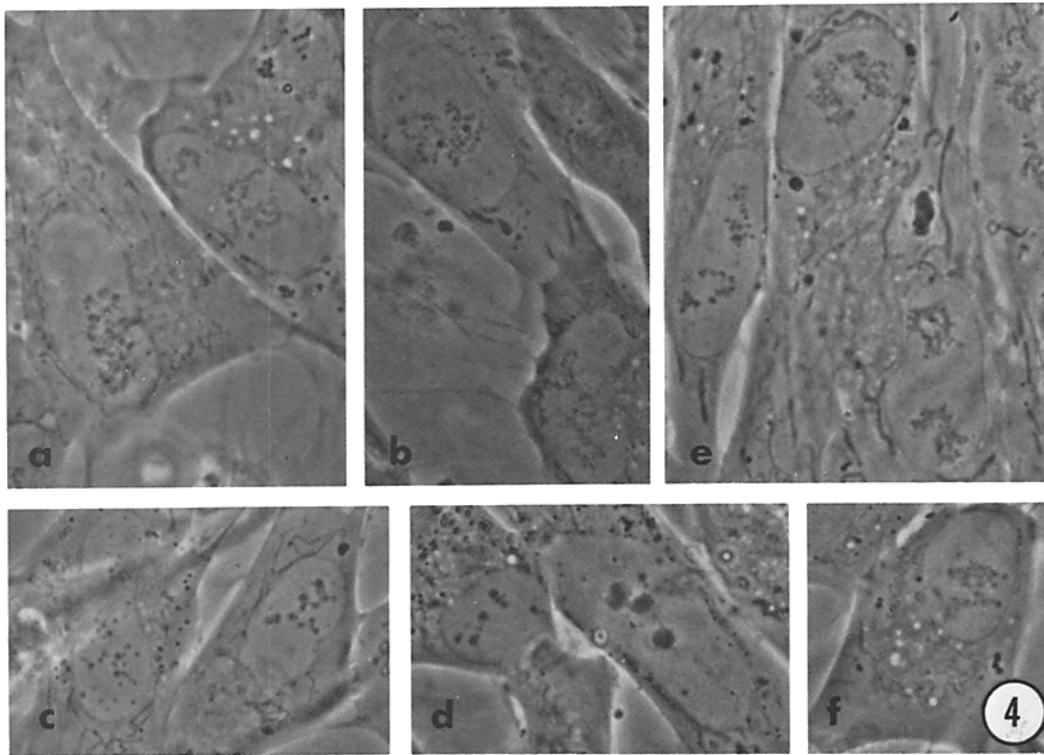


FIGURE 4 Collapsing of CEF NN by miracid D and recovery back to the NN state after miracid D has been removed. (a) NN formation after 3 h of toyocamycin (1.0 $\mu\text{g}/\text{ml}$). (b) NN formation after 2.5 h of DRB (25 $\mu\text{g}/\text{ml}$). (c, d) Collapse of NN by miracid D: NN were made with DRB as in Fig. 4 b; then 5 $\mu\text{g}/\text{ml}$ miracid D was added and incubation was continued for 3 h more. (e, f) Recovery from the collapsed nucleolar state to the NN state after miracid D has been removed: (e) NN were made with 2.0 $\mu\text{g}/\text{ml}$ toyocamycin for 2.5 h and were then collapsed with miracid D (5 $\mu\text{g}/\text{ml}$) for 3.7 h; the medium was then replaced with Eagle's medium containing 2 $\mu\text{g}/\text{ml}$ toyocamycin and the cells were incubated for another 1.5 h. (f) NN were made with 25 $\mu\text{g}/\text{ml}$ DRB for 2.5 h and were then collapsed with miracid D (5 $\mu\text{g}/\text{ml}$) for 3 h; the medium was replaced with Eagle's medium containing 25 $\mu\text{g}/\text{ml}$ DRB and the cells were incubated for 1.5 h more. Phase-contrast microscopy. $\times 960$.

suggesting that these inhibitors have the same site of action.

In the accompanying paper (5) adenosine and a number of the NN-forming adenosine analogues were shown to interfere with CEF nucleolar biochemical function, i.e., ribosome production, in

two major ways. (a) They caused the loss of a large fraction of the 32S + 28S rRNA-containing preribosomes. In the case of DRB this loss (80%) was due to preribosome degradation which probably occurred at or before the 32S rRNA preribosome stage (5). In contrast, only small amounts

FIGURE 3 Morphological changes in the nucleoli of chick embryo liver cells, HeLa, and L cells caused by amino acid starvation and NN-forming chemicals. (a) Chick embryo liver cells starved 6 days in medium lacking amino acids. (b) Chick embryo liver cells exposed to toyocamycin (5.0 $\mu\text{g}/\text{ml}$) for 4 h. (c) Normal L cells. (d) L cells exposed to adenosine (5 mM) for 2.5 h. (e) L cells exposed to toyocamycin (4.0 $\mu\text{g}/\text{ml}$) for 3 h. In Fig. 3 d and e only 25% of the cells had highly fragmented nucleoli. These cells were often seen in clusters. (f) L cells exposed to α -amanitin (20 $\mu\text{g}/\text{ml}$) for 24 h. (g) Normal HeLa cells. (h) HeLa cells exposed to DRB (50 $\mu\text{g}/\text{ml}$) for 3 h. (i) HeLa cells exposed to toyocamycin (4 $\mu\text{g}/\text{ml}$) for 5 h. Phase-contrast microscopy. $\times 830$.

(20%) of the 18S rRNA preribosomes were degraded in the presence of DRB. (b) The NN-forming chemicals slowed the rate of 45S rRNA accumulation (or synthesis?).

In the following discussion we attempt to correlate each of these changes with the morphological changes leading to NN formation.

Loss of 32S rRNA Preribosomes

Electron microscope studies of nucleoli in cells which have been exposed to amino acid starvation,² or to 2 mM adenosine (11, 13) or to the adenosine analogue DRB² and toyocamycin (8, 13, 14), have shown that the fragmentation or uncoiling of the nucleolus is usually accompanied by an appreciable loss of the 200-Å nucleolar granules. These 200-Å granules probably correspond to the 32S and 28S rRNA preribosomes which make up a large fraction of the normal nucleolus. When the normal biosynthesis of the 32S rRNA preribosomes is interrupted, a large fraction of the granular material inside the nucleolus disappears.

The close correspondence observed in the timing of the biochemical and morphological changes (as seen with light microscopy) after addition of DRB supports the idea that depletion of the 32S RNA pool is indeed an important event in NN formation. After 1 h of DRB treatment, the CEF nucleolus has fragmented into a cluster of fuzzy dots. By this time the level of the 32S rRNA pool has greatly decreased because (a) over half of the preribosomes synthesized before DRB addition have been processed and sent out to the cytoplasm, and (b) only small amounts (20%) of the new 45S rRNA preribosomes (synthesized in the presence of DRB) have survived degradation and come to take their place in the 32S + 28S RNA pool (5). After 6 h, when the NN are fully formed, the 32S + 28S rRNA preribosome pool (or "granular component") remaining in the nucleolus is only 20% of its original size (5).

45S rRNA Synthesis

45S rRNA synthesis must continue in order to develop and maintain NN morphology. When fully formed NN (where some 45S rRNA synthesis continues) were given an inhibitor which completely stopped 45S rRNA synthesis (e.g., actinomycin D or miracil D), the NN contracted into a few dense spheres (Fig. 4 c, d). After miracil D was removed and rRNA synthesis was allowed to resume, NN were again reformed (Fig. 4 e, f). This suggests that maintenance of the "rRNA feather" structure (i.e., the matrices of rRNA chains in the process of being transcribed from the ribosomal DNA [12]) is required for NN formation.

Comparison of the actions of cycloheximide and DRB suggests that 32S rRNA depletion is a more critical factor than the decrease in the rate of 45S rRNA synthesis in bringing about NN formation (Table I). This was concluded because the protein synthesis inhibitor cycloheximide also caused slowing of 45S rRNA synthesis but did not cause NN formation. In contrast to the adenosine analogue inhibitors which caused a disappearance of 32S rRNA preribosomes, cycloheximide did not change the levels of the 32S rRNA preribosome pool (23). We postulate that it is the depletion of the 32S and 28S rRNA preribosomes that is the primary factor responsible for NN formation.

During chemically induced NN formation, the initial biochemical changes responsible for the dispersal of the nucleolus are established fairly rapidly (30 min) but the spreading out of these fragments requires about 6 h. It is not known at the present time why the normal nucleolus stays together in such a tight mass without a bounding membrane. Perhaps the 32S + 28S rRNA preribosomes have a tendency to adhere to each other holding the nucleolus in a semicrystalline array. This idea is supported by the observations that ribosomes can crystallize in the cell cytoplasm after exposure to low temperatures (3, 19), and

TABLE I
Action of Different Inhibitors of Ribosome Production

Inhibitor	Biochemical alterations			Morphological alterations of nucleolar structure
	45S rRNA synthesis	Pool size		
		45S rRNA	32S rRNA	
Actinomycin D	Stopped	Decreased	Decreased	Collapsed
Cycloheximide	Decreased	Normal	Normal	Normal
DRB	Decreased	Normal (increase)	Decreased	NN

that ordered arrangements of preribosomes can occasionally be seen in the nucleoli of amino acid-starved CEF cells.² After removal of the "sticky" preribosomes from the nucleolus, the remaining nucleolar material could then slowly spread apart through the viscous nuclear matrix via Brownian motion.

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