Nucleolar “Caps” Produced by Actinomycin D*

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SUMMARY
Actinomycin D produced specific morphologic alterations in the nucleoli and nuclei of Chang liver cells in tissue culture. These changes consisted of: (a) a progressive decrease in the size of the nucleoli, (b) redistribution of the components of the nucleolus to produce two types of nucleolar “caps,” and (c) production of intranuclear inclusions. These nuclear changes were identical to those produced by the carcinogen 4-nitroquinoline N-oxide. Nuclear and nucleolar changes of this type were not produced by nitrogen mustard or by 2,4-dinitrophenol. These findings suggest that 4-nitroquinoline N-oxide, like actinomycin D, may have a specific effect on the synthesis of DNA-dependent RNA.

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MATERIALS AND METHODS
Chang liver cells in tissue culture were used for all experiments. They were grown in Eagle’s basic medium1 with either 10 per cent horse or calf serum (the type of serum had no influence on the results of the experiments). The cells were grown in tissue culture chambers2 and on 1-inch round coverslips in ointment jars. The chambers received inoculations of 0.7 cc. and the jars of 3 cc. of culture medium containing 50,000 Chang liver cells per cc.

Growth medium was removed from the jars and chambers and replaced with Hanks balanced salt solution which contained either 4-nitroquinoline N-oxide,3 actinomycin D, nitrogen mustard, or 2,4-dinitrophenol. These chemicals were utilized in concentrations of $10^{-4}$ m, $10^{-5}$ m, $10^{-6}$ m, and $10^{-7}$ m. The various dilutions of test chemicals were allowed to remain on the cells for periods of time varying from 10 to 20 minutes. Following this period of exposure the test solutions were removed and replaced by growth medium. Phase-contrast microscopic images of the cells in tissue culture chambers were then recorded by means of cinematography. At intervals of 0.5, 1, 2, 4, and 6 hours the coverslip preparations were removed from ointment jars and fixed for electron microscopy and cytologic staining. Dalton’s solution and 2 per cent potassium permanganate in distilled water (14) were used as fixatives for electron microscopy. Following fixation the cells were dehydrated in graded alcohols and embedded in methacrylate. Coverslip preparations for light microscopic studies were fixed in Carnoy’s solution and stained with hematoxylin and eosin, azure “B,” or acridine-orange (1).

Additional procedures were carried out with nitrogen mustard, because the biological effects of nitrogen mustard are apparently due to the development of hydrolysis products produced when the chemical is dissolved in an aqueous medium. Golumbic et al. (10) have shown that these active hydrolysis products reach a peak concentration in 30–60 minutes after nitrogen mustard is dissolved in an aqueous medium. A $10^{-4}$ M stock solution of nitrogen mustard was prepared in Hanks balanced salt solution. This solution was allowed to remain at room temperature for periods of time varying from 5 to 60 minutes, was then diluted to $10^{-4}$ M or $10^{-5}$ M with Hanks balanced salt solution, and the diluted solution was introduced immediately into the cell cultures. Subsequent steps were identical to those described previously.

RESULTS
In living cells the principal morphologic effects produced by 4-nitroquinoline N-oxide and actinomycin D were...
found in the nuclei and nucleoli. The changes produced by these two agents were virtually identical.

*Nucleolar size.*—4-Nitroquinoline N-oxide and actinomycin D in concentrations of 10⁻⁶ M rapidly produced a marked decrease in the size of the nucleoli which was best demonstrated with the azure "B" stain. This decrease in the size of the nucleoli was complete within 2 hours after application of the chemicals (Figs. 1–3). In addition to losing a considerable proportion of their mass, the nucleoli also lost their usual irregular contour and assumed a uniform spherical shape. The number of nucleoli was slightly reduced. With 4-nitroquinoline N-oxide the reduction in the number of nucleoli was due not only to the loss of mass but also to the occasional fusion of nucleoli. This phenomenon has been demonstrated by means of time-lapse motion picture studies (19). No fusion of the nucleoli was observed with actinomycin D. Azure "B" and hematoxylin and eosin stains demonstrated that the size and contour of Chang liver cell nucleoli were unchanged following exposure to nitrogen mustard or 2,4-dinitrophenol.

*Nucleolar "caps."*—4-Nitroquinoline N-oxide and actinomycin D produced striking morphologic changes in the nucleoli of Chang liver cells. These changes involved a redistribution of nucleolar components to produce the structures designated as nucleolar "caps."

Figures 9 and 10 are pictures of normal Chang liver cells with phase and electron microscopy. With the phase microscope the nucleolus was frequently irregular and completely solid. On other occasions, as demonstrated in Figure 10, numerous small vacuoles were seen. A previous report has demonstrated that these clear areas are not simple vacuoles but consist of a definite morphologic component of the nucleolus distinct from the surrounding nucleoplasm (19).

Electron photomicrographs of the normal Chang liver nucleolus demonstrate the various morphologic patterns noted by Davis (3) in the nucleoli of normal rat liver cells. The nucleoli consist of a compact mass of particles which vary considerably in density and measure about 100 Å in diameter (Figs. 8, 9). The nucleoli may be solid, vacuolated, or show a skeinlike network in which the denser particles form strands or cords surrounding clear vacuoles. Occasionally irregular margination of the darkest particles in the nucleoli may be seen.

The alterations in nucleolar structure produced by 4-nitroquinoline N-oxide and actinomycin D could be seen as early as 30 minutes after removal of the test compounds. The first observed change consisted of a condensation of the darker particles to produce electron-dense plaques scattered irregularly throughout the nucleolus (Figs. 13, 17).

Within 1–2 hours the electron-dense plaques appeared to migrate toward the margin of the nucleoli, where they form an irregular black rim (Figs. 14, 18). Two to 4 hours after treatment the marginalized particles appear to coalesce into larger masses forming the dark nucleolar "caps" (Figs. 15, 19). These dark nucleolar "caps" have been observed with the phase microscope as well as with the electron microscope. They have a concave base and appeared to be pressed or applied to the surface of the main body of the nucleolus (Figs. 11, 12). They were generally multiple and frequently gave the nucleolus a "mulberry" appearance.

A second type of nucleolar "cap" may also be seen in Chang liver cells treated with 4-nitroquinoline N-oxide and actinomycin D. This structure has a "nipple-like" appearance. It is composed of particles lighter than those forming the dark nucleolar "cap." This structure has been designated the light nucleolar "cap" (Figs. 11, 16, 20). They were not seen as frequently as the dark nucleolar "cap" and were found occasionally in untreated cells. They were closely attached to the main body of the nucleolus and had an indistinct, convex, rather than concave, base which protrudes slightly into the nucleolus. The margin between the light nucleolar "cap" and the nucleolus was not as distinct as the margin between the dark "caps" and the nucleolus. When studied with the electron microscope the light nucleolar "caps" are generally lighter than the main body of the nucleolus, but occasionally may be composed of darker particles (Fig. 20). These light "caps" were generally single and give the nucleolus an oval shape.

Phase time lapse motion picture studies indicate that the light nucleolar "caps" originate from the light "vacuolated" areas of the normal nucleolus. Sequential photographs from a time lapse motion picture demonstrating the formation of the light and dark nucleolar "caps" may be found in a previous communication (19).

Nucleolar "caps" have not been observed in cells treated with nitrogen mustard or with 2,4-dinitrophenol. Nitrogen mustard produced an occasional small "tail-like" nucleolar structure of light particles of about the same size as the nucleolar "cap." These structures were infrequent and did not have the same distribution or morphology as the nucleolar "caps" produced by actinomycin D and 4-nitroquinoline N-oxide. It is probable that they represent a different phenomenon, since the change is not associated with a decrease in nucleolar size. No changes of this type have been found with 2,4-dinitrophenol.

*Nuclear changes.*—Figures 4 and 5 demonstrate the inclusions or vacuoles produced by 4-nitroquinoline N-oxide and actinomycin D in the nucleoplasm of cells in tissue culture. These structures were first described by Endo in studies with 4-nitroquinoline N-oxide (5, 6). Similar morphologic changes have been observed with actinomycin D in this laboratory. The morphology of these inclusions varies with the types of fixation and microscopy utilized for study. They were not visible in the living cell when studied with the phase microscope, nor were they visible with the electron microscope in cells fixed with Dalton's solution. They were visible with the electron microscope in cells which have been fixed with potassium permanganate and appeared as distinct spherical areas of decreased density (Figs. 6, 7).

Following fixation in alcoholic fixatives and staining with hematoxylin and eosin, these structures were visible as distinct intranuclear inclusions. With the aeridine-orange stain and examination with fluorescent microscopy they appeared as vacuoles containing a minute wisp of pink, fluorescing material. Endo's (6) histo-
chemical studies and studies with acridine-orange fluorescence (19) indicate that these inclusions or vacuoles contain RNA; they are not produced by nitrogen mustard or 2,4-dinitrophenol.

**DISCUSSION**

These studies were undertaken to elucidate the biochemical lesion responsible for the morphologic changes produced by 4-nitroquinoline N-oxide in Chang liver cells. The chemical agents were chosen because of morphologic or biochemical effects shared in common with 4-nitroquinoline N-oxide.

The known morphologic and biochemical effects of 4-nitroquinoline N-oxide have been reviewed by Nakahara (15) and Ono (17). 4-Nitroquinoline N-oxide is a relatively simple chemical compound in which the specific moieties responsible for its carcinogenic activity have been elucidated (16).

The known biochemical effects of 4-nitroquinoline N-oxide include: (a) a decrease in intracellular content of DPN (17, 21), (b) a strong attraction for sulfhydryl groups (4), (c) an inhibitive effect on glycolysis (8), and (d) a depressive effect on the ATP content of the cell (8). Secondary effects include inhibition of nucleic acid and protein synthesis (8).

The cytologic effects of 4-nitroquinoline N-oxide include: (a) the production of RNA-containing nuclear inclusions (5, 6, 19), (b) nucleolar exhaustion with an associated decrease in nucleolar size, and (c) reorganization of the nucleolar substance to form nucleolar "caps" (19).

Actinomycin D was chosen for these studies because of previous reports describing nucleolar alterations produced in tissue culture by this substance. It has been shown that actinomycin D, like 4-nitroquinoline N-oxide, produces a marked reduction in nucleolar size (20). Bierling (2) and Journey and Goldstein (11) have noted phase and electron microscopic changes suggesting that actinomycin D is capable of producing nucleolar changes similar to the "caps" we have observed with 4-nitroquinoline N-oxide. The present experiment demonstrates that nucleolar changes produced by actinomycin D are identical to those produced by 4-nitroquinoline N-oxide in Chang liver cells.

The major biochemical effect of actinomycin D is an inhibition of RNA synthesis brought about by a direct combination of the drug with DNA. Apparently, actinomycin D blocks the templates responsible for assembling DNA-dependent RNA (18). In addition, Levy (12) has shown that actinomycin D blocks the transfer of nucleolar RNA from the nucleolus to the cytoplasm. This latter observation could explain the RNA-containing nuclear inclusions produced by actinomycin D and 4-nitroquinoline N-oxide in Chang liver cells.

The effect of actinomycin D on RNA metabolism may be inhibited by incorporating DNA into the culture medium (9). Presumably, the actinomycin D combines with the extracellular DNA, thus reducing the actinomycin D available for combination with nuclear DNA.

Nitrogen mustard was chosen for these studies because of biochemical effects similar to those of 4-nitroquinoline N-oxide. It has been demonstrated that nitrogen mustard, like 4-nitroquinoline N-oxide, has a depressive effect on cellular DPN concentrations and glycolysis (7). The addition of nicotinamide to cultures treated with 4-nitroquinoline N-oxide prevents the decrease in DPN concentration and inhibition of glycolysis (17). Both nitrogen mustard and 4-nitroquinoline N-oxide decrease the ATP content of tissue culture cells. This effect is presumably secondary to decreased DPN levels in the treated cell. The observation that nitrogen mustard did not produce the same type of nucleolar lesions as those produced by 4-nitroquinoline N-oxide suggests that the decreased cellular DPN concentration is not responsible for the nucleolar changes described in this paper.

2,4-Dinitrophenol was used in these experiments to determine whether a decrease in ATP levels, without an associated decrease in DPN, could be responsible for the nucleolar "caps" and nuclear inclusions. 2,4-Dinitrophenol exerts its depressive effect on ATP levels by means of a specific uncoupling effect on oxidative phosphorylation (13). Since 2,4-dinitrophenol did not produce the nuclear or nucleolar changes, it would appear that decreased cellular ATP is not responsible for the morphologic changes produced by 4-nitroquinoline N-oxide and Actinomycin D.

In a previous publication the following working hypothesis was proposed as a possible explanation for the effects of 4-nitroquinoline N-oxide on the nuclei and nucleoli of Chang liver cells: (a) As a result of depressed intracellular DPN and ATP levels, protein and nucleic acid synthesis in the cell is inhibited. (b) The nucleolus continues to discharge preformed RNA to the cytoplasm in an attempt to restore adequate protein synthesis. The continued discharge of preformed nucleolar RNA without associated synthesis of RNA results in nucleolar exhaustion, decreased nucleolar size, and redistribution of nucleolar components to form nucleolar "caps." The RNA-containing nuclear inclusions were thought to represent RNA in transit from the nucleolus to the cytoplasm. Complete exhaustion of the available nucleolar RNA is accompanied by cellular death. The present experiments with nitrogen mustard and 2,4-dinitrophenol indicate that depressed cellular DPN and ATP levels are not responsible for the nuclear changes observed with 4-nitroquinoline N-oxide. The observation that actinomycin D produces nucleolar exhaustion, nucleolar "caps," and nuclear inclusions suggests that 4-nitroquinoline N-oxide, like actinomycin D, may specifically inhibit synthesis of RNA. The studies of Levy (12) suggest that the nuclear inclusions do not represent RNA in transit to the cytoplasm but, rather, RNA trapped in the nucleoplasm by an inhibiting effect of these drugs on the transport of RNA from the nucleolus to the cytoplasm. If future studies prove this to be true, then the observed changes in the nucleoli and nuclei of Chang liver cells may, in fact, represent reproducible morphologic markers for specific biochemical reactions—i.e., the inhibition of synthesis of DNA-dependent RNA and the transfer of available nucleolar RNA from the nucleolus to the cytoplasm.
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REFERENCES

Fig. 8.—Electron photomicrograph of normal Chang liver cell. Potassium permanganate fixation. Note lack of the large spherical areas of decreased density which are present in cells treated with 4-nitroquinoline N-oxide and actinomycin D (Figs. 6, 7). Mag. X11,600.

Fig. 9.—Normal Chang liver cell. Palade's fixative. Note granular pattern and vacuolization of nucleolus (N). Mag. X8,700.

Fig. 10.—Phase photomicrograph of normal Chang liver nucleolus (N). Mag. X3,500.

Fig. 11.—Phase photomicrograph of light nucleolar "caps" (LVC) and dark nucleolar "caps" (DNC) produced by 10^{-1} M 4-nitroquinoline N-oxide in Chang liver cells. Mag. X3,500.

Fig. 12.—Phase photomicrograph of dark nucleolar "caps" (DNC) produced by 10^{-1} M actinomycin D in Chang liver cells. Mag. X3,500.
Fig. 13.—Nucleolus 1 hour after exposure to $10^{-2}$ M 4-nitroquinoline N-oxide for 10-15 minutes. Note condensation of electron-dense particles to form nucleolar plaques (NP) scattered throughout the nucleolus. Mag. $\times 5,000$.

Fig. 14.—Appearance of Chang liver cells 1½-3 hours after exposure to $10^{-2}$ M 4-nitroquinoline N-oxide. Note that nucleolar plaques (NP) appear to have migrated to periphery of nucleolus to form dark nucleolar “caps” (DNC). Mag. $\times 20,000$.

Fig. 15.—Four hours after exposure to $10^{-2}$ M 4-nitroquinoline N-oxide. Note further condensation of dark nucleolar “caps” (DNC) to form bulging masses with concave bases, sharply demarcated from nucleolus proper. Mag. $\times 8,200$.

Figs. 17-20.—Electron photomicrographs of Chang liver cells treated with actinomycin D. Palade’s fixative.

Fig. 17.—Nucleolus 30 minutes after exposure to $10^{-2}$ M actinomycin D for 10-15 minutes. Note formation of nucleolar plaques (XP). Mag. $\times 8,000$.

Fig. 18.—Chang liver cell 1 hour after exposure to $10^{-2}$ M actinomycin D. Note that nucleolar plaques (XP) appear to have migrated to periphery of nucleus to form dark nucleolar “caps” (DNC). Mag. $\times 13,200$.

Fig. 19.—Two to 4 hours after exposure to $10^{-2}$ M actinomycin D. Note further condensation of dark nucleolar “caps” (DNC) to produce a “mulberry-like” nucleolus. Mag. $\times 11,000$.

Fig. 20.—Light (LNC) and dark (DNC) nucleolar “caps” produced by actinomycin D. Note the convex base of the light nucleolar “caps” (LNC). In this instance the light nucleolar “cap” is darker than the main portion of the nucleolus. Mag. $\times 19,200$. 
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