LIGHT AND ELECTRON MICROSCOPIC
RADIOAUTOGRAPHY OF HEPATIC CELL NUCLEOLI
IN MICE TREATED WITH ACTINOMYCIN D

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ABSTRACT
Nucleolar partition induced by actinomycin D was used to demonstrate some aspects of nucleolar RNA synthesis and release in mouse hepatic cells, with light and electron microscopic radioautography. The effect of the drug on RNA synthesis and nucleolar morphology was studied when actinomycin D treatment preceded labeling with tritiated orotic acid. Nucleolar partition, consisting of a segregation into granular and fibrillar parts was visible if a dosage of 25 µg of actinomycin D was used, but nucleolar RNA was still synthesized. After a dosage of 400 µg of actinomycin D, nucleolar RNA synthesis was completely stopped. If labeling with tritiated orotic acid preceded treatment with 400 µg of actinomycin D, labeled nucleolar RNA was present 15 min after actinomycin D treatment while high resolution radioautography showed an association of silver grains with the granular component. At 30 min after actinomycin D treatment all labeling was lost. Since labeling was associated with the granular component the progressive loss of label as a result of actinomycin D treatment indicated a release of nucleolar granules. The correlation between this release and the loss of 28S RNA from actinomycin D treated nucleoli as described in the literature is discussed.

Fine-structural alterations in nucleolar morphology as an effect of actinomycin D are well known. They are described as a segregation of nucleolar material into three components, i.e. granular, fibrillar, and amorphous (1–4).

Apart from a general blocking effect on DNA-RNA transcription, low dosages of this drug are known to have a rather selective inhibitory effect on RNA synthesis in the nucleolus, as Perry (5) showed in light microscopic radioautographic studies.

Biochemically, Muramatsu et al. (6) found a precursor-product relationship between 45, 35, and 28S RNA's in the nucleolus of rat liver by employing actinomycin D to suppress new RNA synthesis in the nucleolus. They found a half-life of 8 min for the 45S. A decrease of the radioactivity of the newly synthesized 28S RNA was observed after a 20 min treatment with actinomycin D.

On the basis of these morphological and functional data, it seemed worthwhile to study the effect of actinomycin D on the nucleolus by means of high-resolution radioautography, with the following questions in mind: (a) can a specific, morphologically visible, fine-structural disturbance exclusively located in the nucleolus be demonstrated in our material deriving from mice injected intraperitoneally with actinomycin D? (b) is all RNA labeling absent in the nucleolus when such a disturbance becomes visibly manifest? (c) will the
RNA release from the actinomycin-treated nucleolus be impaired, or will the nucleolus still be able to release RNA?

**MATERIAL AND METHODS**

Female O20 mice weighing 20 g and aged about 2 months were given a standard laboratory diet and tap water ad lib. All experiments were done at the same time of the day, between 11.00 and 12.00 a.m., in order to exclude diurnal variation. Orotic acid-5-3H (specific activity 2300–4000 mc/mmole, The Radiochemical Centre, Amersham, England) was injected intraperitoneally in a dosage of 0.150 mc. Actinomycin D (kindly provided by Merck & Co. Inc., Rahway, New Jersey) in saline was injected intraperitoneally.

In one set of experiments the animals were injected with actinomycin D in dosages of 25, 100, 200, and 400 µg 80 min before decapitation, and with orotic acid-5-3H 20 min before death.

In a second set of experiments the animals were first injected with orotic acid-5-3H, and 20 min later with 400 µg of actinomycin D. They were sacrificed 15, 30, and 60 min after the actinomycin D treatment. Control mice of the same age and weight were injected with orotic acid-5-3H and sacrificed after 20 min. Tissue processing consisted of cutting small (approximately 1 mm³) blocks from the left lobe of the liver, then fixation for 1 hr in a mixture of two parts osmium tetroxide (2%) and one part collidine buffer (7). After dehydration in alcohol, the blocks were embedded in Epon 812 (8). Sections 0.9 µm thick were made on a Porter-Blum MT-2 microtome and mounted on glass slides. For both light and electron microscopic radioautography, Ilford L-4 emulsions (10 g diluted in 20 ml distilled water) prepared by the loop method according to Caro and van Tubbergen (9) were used. This type of emulsion was especially suitable since the grain size is large enough to be visible with the light microscope, and since in electron microscopic radioautography the emulsion gives a localization which is sufficiently accurate for the purposes dealt with in this paper. Silver grains were counted according to the method already described in a previous paper (10).

From some of the liver samples, ultrathin sections were made for high-resolution radioautography. Electron microscopic radioautography was done with Ilford L-4 emulsion prepared according to methods that have already been reported (10, 11). In addition, Kodak NTE emulsion was prepared according to a method described by Salpeter and Bachmann (12), and the sensitivity of this emulsion was enhanced with the gold latensification method as described by these authors (12). After exposure and development of radioautographs, ultrathin sections were stained with lead cacodylate (13).
RESULTS

To establish the effect of different dosages of actinomycin D on the RNA synthesis in the liver cell, the following experiment was done.

The effect of actinomycin D treatment at 60 min before labeling with orotic acid-5-3H on the incorporation of the tritiated orotic acid into RNA as measured by grain counting in radioautographs of 0.9 μm sections is shown in Fig. 1. For comparison, normal values are included in the graph. It will be seen that a dosage of 25 μg of actinomycin D decreases the number of grains in the nucleus, whereas the number of grains in the cytoplasm is not significantly changed. With higher dosages of 100 and 200 μg the number of grains in both the nucleus and the cytoplasm decreases progressively, and with the highest dosage used the number of grains in the nucleus and the cytoplasm becomes negligible.

Fine-structural changes induced by actinomycin D are especially prominent in the structure of the nucleolus. Normally, the nucleolus shows a folded structure, the nucleolonema, consisting of finely granular and fibrillar electron-opaque components through which numerous, larger (about 180 Å) granules with the appearance of ribosomes are randomly scattered. In high-resolution radioautographs of normal nucleoli the silver grains are randomly scattered over the nucleolus (Fig. 4). In liver cells of animals treated with actinomycin D, however, there is a striking demarcation between the finely fibrillar component and a coarsely granular ribosome-like component, the latter being distributed in crescents at the periphery of the structure. After prolonged treatment with higher dosages of actinomycin D (Figs. 9 and 10), these changes are more conspicuous. Strongly electron-opaque clusters of coarsely granular material are then visible at the outer boundary of the nucleolus. In many instances, loosely arranged agglomer-
tions of ribosome-like particles are found in the surrounding nucleus. These agglomerations appear to be connected with the granular component of the nucleolus. Although these alterations were especially marked after the use of higher dosages of actinomycin D, partition was also visible to some extent when a dosage of 25 μg was used for 80 min (Fig. 6). The radioautographs in this latter instance were interesting because they showed that although radioactivity was very low in these nucleoli a few grains were occasionally found (Figs. 5 and 6). These grains were located in the granular part of the nucleolus (Fig. 6), thus indicating that with a dosage of 25 μg some RNA labeling still occurs in spite of prolonged (80 min) treatment with actinomycin D.

The second set of experiments was designed with the purpose of establishing the effect of actinomycin D on the release of labeled RNA from the nucleolus. Therefore, actinomycin D treatment was initiated after a previous labeling with orotic acid-5-³H for 20 min. Actinomycin D was used in a dosage of 400 μg, since the preceding experiments had shown that with this dosage RNA synthesis is virtually blocked. Figures 7 and 8 show that at 15 min after treatment with actinomycin D several silver grains occur over the nucleoli, whereas at 30 min after treatment labeling is absent. This indicates that labeled RNA is lost from the nucleolus. Contrary to this loss of nucleolar labeling, silver grains are still present over other parts of the nucleus and the cytoplasm. High-resolution
radioautographs of the liver cells treated with actinomycin D for 15 min are interesting because they show that silver grains are associated with the coarsely granular components of the nucleolus (Figs. 2 and 3).

**DISCUSSION**

In general, the actinomycin D-induced alterations of the nucleolus agree well with the extensive descriptions in the literature, although an amorphous component was infrequently found. In our material the extent of the changes shows a dose-dependency. It is interesting that the minimal structural changes seen in nucleoli of animals treated with 25 μg of actinomycin D seem to be compatible with some RNA labeling in the nucleolus (Figs. 5 and 6). It is also clear that if a high dosage (400 μg) is given the extent of the changes increases with the duration of treatment.

Whereas in the normal nucleolus the ribosome-like particles are randomly distributed throughout the structure, as a result of actinomycin D treatment the ribosome-like particles are moved to the periphery of the nucleolus. The occurrence of loosely packed agglomerations of ribosome-like particles in the surrounding nucleus, which are, however, continuous with the granular part of the nucleolus proper (Figs. 9 and 10), even seem to suggest a release of such particles. This phenomenon runs parallel with the finding of a loss of
FIGURES 5 and 6. Light microscopical (Fig. 5) and high-resolution (Fig. 6) radioautographs of mouse liver cells treated for 80 min with 22 µg of actinomycin D. Labeling started 20 min before death. Silver grains in Fig. 5 are shown to be occasionally associated with nucleoli (arrow). Figure 6 shows the granular component being shifted to the periphery of the nucleolus. Silver grain is associated with granular part. Fig. 5, × 1200; Fig. 6, × 21,000.

FIGURES 7 and 8. Light microscopical radioautographs of mice treated for 15 min (Fig. 7) and 30 min (Fig. 8) with 400 µg of actinomycin D after an initial labeling for 20 min. Some of the silver grains in Fig. 7 are associated with the nucleoli (arrow). No such association of silver grains with the nucleoli is found in Fig. 8. Figs. 7 and 8, × 1200.
FIGURES 9 and 10. Electron micrographs of nucleoli of mouse hepatic cells treated for 30 min (Fig. 9) and 60 min (Fig. 10) with 400 \( \mu \)g of actinomycin D. Both micrographs show that the nucleoli have a centrally located fibrillar component and a peripheral granular part. Note continuity between the loosely arranged agglomerations of ribosome-like particles in the surrounding nucleus and the granular component of the nucleolus. Figs. 9 and 10, \( \times 21,600 \).

labeling of the nucleolus between 15 and 30 min after treatment with 400 \( \mu \)g of actinomycin D. The loss of labeling is probably related to the release of the ribosome-like particles, since in Figs. 2, 3 and 6 it can be seen that the silver grains are associated with the granular part of the actinomycin-D treated nucleolus. This assumption is further supported by the work of Muramatsu et al. (6). They found a decrease of radioactivity of newly synthesized 28S RNA in the nucleolus between 20 and 40 min after actinomycin D treatment. Since 28S RNA is associated with 60S ribosomal particles, it seems justified to consider the nucleolar granules as 60S ribosomal particles. It may be assumed that at 15 min after a high dose of actinomycin D which stops all new synthesis of RNA the conversion of precursor RNA to 28S RNA which runs parallel with a migration of radioactivity from fibrillar to granular material (14) can be expected to be practically completed since according to Muramatsu (6) the half-life of precursor RNA is about 8 min. Geuskens' finding (3) of an impaired release of newly synthesized RNA from actinomycin D-treated nucleoli in cultured monkey kidney cells may be attributed to differences in the experimental material and techniques used.

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REFERENCES


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