INTRANUCLEAR FIBRILLAR BODIES IN ACTINOMYCIN D–TREATED OOCYTES

NANCY J. LANE. From the Department of Biology, Yale University, New Haven, Connecticut 06520. Dr. Lane's present address is the Department Zoology, Downing Street, Cambridge, England.

INTRODUCTION

Earlier investigations have shown that actinomycin D, which inhibits DNA-dependent RNA synthesis (Reich et al., 1961) by binding to DNA (Kirk, 1960), has a profound effect on the nuclear structure of cells. Some of the most striking consequences are visible in the nucleoli which exhibit both a decrease in size and a variety of fine structural changes (Journey and Goldstein, 1961; Reynolds et al., 1964; Stevens, 1964; Schoefl, 1964; Jacob and Sirlin, 1964; Jézéquel and Bernhard, 1964; Boloukhère Presburg, 1965; Stenram, 1965; De Man and Noorduyn, 1967). These alterations commonly involve segregation of nucleolar material into granular, fibrillar, and amorphous components, leading to degranulation of the nucleolar matrix and ultimately resulting in a disintegration of the nucleolar substance. In the present series of experiments, in addition to changes in nucleolar structure, fibrillar bodies have been observed in the nuclei of oocytes after inhibition of RNA synthesis with actinomycin D. Intranuclear fibrils have been previously reported after actinomycin D treatment in a study on cultured amphibian embryonic cells (Jones and Elsdale, 1964; Jones, 1967). The fibrillar bodies reported in this study, however, are very different from those found by Jones and Elsdale; they are composed of many more fibrils and appear more numerous. They are also much larger; in fact, they are sufficiently sizeable so to be readily visible by light microscopy which makes possible the application of cytochemical tests.

MATERIALS AND METHODS

Maturing oocytes of the newt, *Triturus viridescens*, were used in these investigations. The oocytes were removed from the animals according to the procedure outlined by Gall (1965) and were incubated in vitro in actinomycin D. In order to test biochemically that the actinomycin D was inhibiting RNA synthesis, the cells were subjected to simultaneous application of actinomycin D and uridine-^{3}H. The radioactivity in the RNA extracted from the nuclei of oocytes after such treatment should be low or negligible if the actinomycin is affecting an inhibition. In addition, other oocytes were allowed to incorporate uridine-^{3}H prior to actinomycin D treatment. Counting the radioactivity in the nuclear RNA of these oocytes before and after their subjection to actinomycin D gives an estimate of the amount of isotope being incorporated into RNA; decreasing values would suggest that uptake of uridine-^{3}H had ceased and either that the newly synthesized RNA was being transported into the cytoplasm or that it was being broken down due to the actinomycin.

Oocytes were incubated in vitro in culture medium consisting of 0.6 ml of commercial medium 199, 0.1 ml of calf serum, and 0.3 ml of water, with added streptomycin (0.1 mg/ml) and penicillin (0.06 mg/ml), and containing 100 μc of uridine-^{3}H (specific activity, 25.0 c/mmole; obtained from Schwarz Bio Research Inc., Orangeburg, N. Y.). After 24 hr, these oocytes were removed, washed in culture medium, and transferred to a solution of actinomycin D in mammalian culture medium (50–100 μg/ml) for 12 or 24 hr. Following incubation in actinomycin D, the oocytes were either transferred to culture medium for 12 hr, their nuclei removed and then fixed, or their nuclei were isolated and fixed immediately after removal from the solution. For biochemical assay, oocytes were also allowed to remain in culture medium for longer intervals, those incubated in uridine-^{3}H plus actinomycin remained in the medium for up to 4 days. Other control oocytes were incubated in uridine-^{3}H alone, with no subsequent actinomycin treatment.

The nuclei for biochemical assay were isolated manually and fixed in 0.1 M sodium acetate at pH 5.0 with added polyvinyl sulphate (4 μg/ml). Their RNA was dissolved in 0.5 ml hydroxide of hyamine (10-X; Rohm & Haas Co., Philadelphia, Pa.) diluted with 10 ml of Bray's solution, and the radioactivity of the samples was measured in an Ansitron scintillation counter. The oocytes to be studied for structural changes were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 at 4°C (Sabatini et al., 1963), washed in 0.1 M phosphate buffer with added 0.2 M sucrose, and then treated with Veronal acetate-buffered osmium tetroxide for 60 min at 4°C. The cells were washed, carried through an ascending series of ethanols to propylene oxide, and embedded in Araldite. Thin sections were cut on a Porter-Blum MT 2 ultramicrotome and examined in a Philips EM 200.

Sections were also cut from the Araldite blocks at

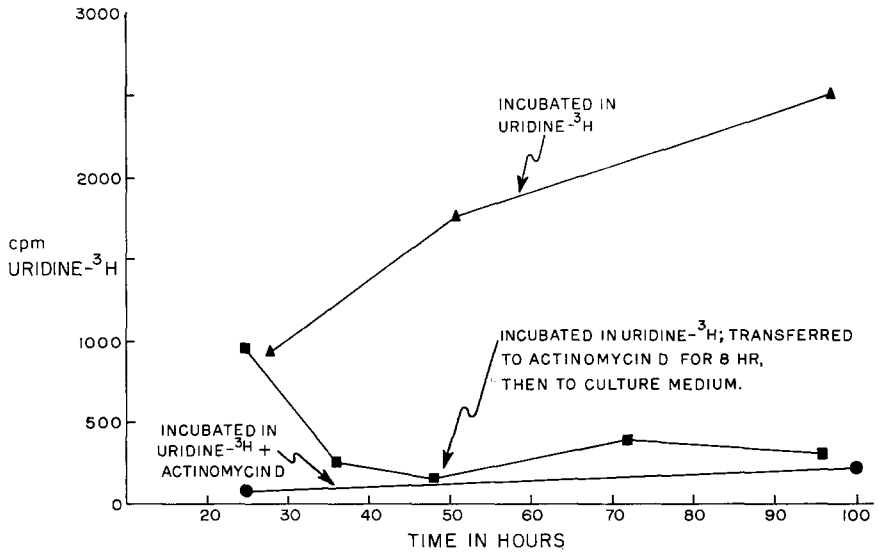

FIGURE 1 Graph summarizing the results of experiments on oocytes incubated in vitro in uridine-^{3}H (—▲—▲—), in uridine-^{3}H followed by actinomycin D incubation (—■—■—), or in uridine-^{3}H plus actinomycin D (—●—●—). Each point on the graph represents the radioactivity of the RNA from the nuclei of 10 oocytes. The nuclear RNA extracted from the oocytes transferred to actinomycin D shows a progressive decrease in counts per minute (cpm) in comparison with oocytes not treated with the antibiotic; the latter continue to incorporate uridine-^{3}H.

1–2 μ and were stained with 1% toluidine blue in 1% borax. Other serial sections were tested for the presence of DNA by the Feulgen technique and for RNA by staining with azure B (0.25 mg/ml in 0.1 M citrate buffer at pH 4) at 40°C (Flax and Himes, 1952). Further serial sections were mounted on slides and dipped in Kodak NTB 2 photosensitive emulsion; these preparations were dried, exposed in the dark for 1–2 wk, and the radioautographs were subsequently developed with D-19 to determine the sites of uridine-^{3}H incorporation into RNA. The ultrathin sections to be studied by electron microscopy were cut serially with respect to these 2-μ sections.

OBSERVATIONS

Measurements of the radioactivity of the RNA extracted from nuclei after uridine-^{3}H incorporation and actinomycin D inhibition show that the actinomycin inhibits uptake of radioactive uridine into RNA (Fig. 1). Other experiments have shown that the actinomycin takes effect almost immediately after commencing treatment. Similar measurements of the RNA from control nuclei removed from oocytes incubated only in uridine-^{3}H indicate that these nuclei continue to incorporate uridine (Fig. 1). Concomitant examination of spread preparations of actinomycin-treated nuclei revealed that the loops of the lampbrush chromosomes had retracted, a phenomenon symptomatic of a cessation of RNA synthesis (Izawa et al., 1963).

When electron microscopic examination is made of nuclei from oocytes subjected to actinomycin D, it can be seen that the nucleoli display structural changes similar to those reported by other investigators; segregation of nucleolar material, resulting in peripheral concentration of the granular component, was observed. An additional fine structural change also occured, however; unusual fibrillar bodies were found in the nucleoplasm.

These long, thin fibrillar structures were observed in certain of the nuclear sections examined after treatment of the oocytes with actinomycin D (Fig. 2). Similar inclusions were never observed in untreated nuclei or in nuclei from oocytes incubated only in uridine-^{3}H. These bodies were found in nuclei from immature oocytes, the nucleoli of which are spheroidal, are associated with the nuclear envelope, and have not yet assumed the "necklace" form (Lane, 1967); they were also found in more mature oocytes whose

nucleoli have reaggregated to form spheroids subsequent to necklace formation. That these structures were not observed in every oocyte subjected to actinomycin D inhibition does not prove their absence, as serial sectioning through each oocyte thus treated would be required to do so.

The fibrillar bodies measure up to 65 μ in length in 2-μ thick sections (Fig. 2). Their average diameter is 2–3 μ, although some measure up to 4 μ, and in cross-section they are roughly circular in outline (Figs. 2, 3, and 6). The number of these structures seen in any given section ranged from 3 to 18 per nucleus. These structures stain only faintly with toluidine blue/borax, much less intensely than the nucleoli (Fig. 2), and react negatively to the tests for DNA and RNA. If they should contain nucleic acids, the amounts are so small as to be undetectable by the cytochemical procedures used here. In radioautographs they show no labeling, which indicates that they do not incorporate radioactive uridine.

When examined by electron microscopy, these fibrillar bodies were found to be bundles composed of numerous filaments ranging from about 50 to 70 A in diameter (Figs. 4–7). The filaments are arranged roughly parallel to one another as can readily be seen in longitudinal sections (Figs. 4 and 5), but they are not always equidistant from one another, as illustrated in cross-sections (Figs. 6 and 7). In longitudinal section the bundles have a somewhat ellipsoidal shape, tapering off with pointed ends (Figs. 3 and 4); the filaments composing the bundles seem to terminate blindly in the nucleoplasm (Figs. 4 and 5). The fibrillar bodies are frequently found in close spatial association with clumps of granular material (Figs. 2–4) or intranuclear granules (Figs. 4–6) and sometimes lie fairly near a nucleolus (Fig. 2).

DISCUSSION

In 1964, Jones and Elsdale reported intranuclear threads occurring in the nuclei of some actinomycin-treated embryonic cells of the frog, *Rana pipiens*. These threads measured 200 A in diameter, up to 0.5 μ in length, and each appeared as a separate entity rather than in bundles; they were frequently surrounded by dense, granular bodies somewhat similar to the clumps of granular material described here. Jones and Elsdale (1964) suggested that the threads might represent lengths of uncoiled chromosomes in a condition of chromosomal inhibition. More recently Jones (1967) has

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All the micrographs are sections through the nuclei of oocytes (from the newt *Triturus viridescens*) incubated in vitro first in uridine-^{3}H for 24 hr and subsequently in actinomycin D for 12 hr.

FIGURE 2 2 μ grazing section stained with toluidine blue/borax solution. Note the deeply staining nucleoli around the periphery of the nucleus and the less intensely staining fibrillar bodies (arrows). Lampbrush chromosomes with retracted loops can be seen at *C*, and small dense granular bodies are seen at *G*. × 350.

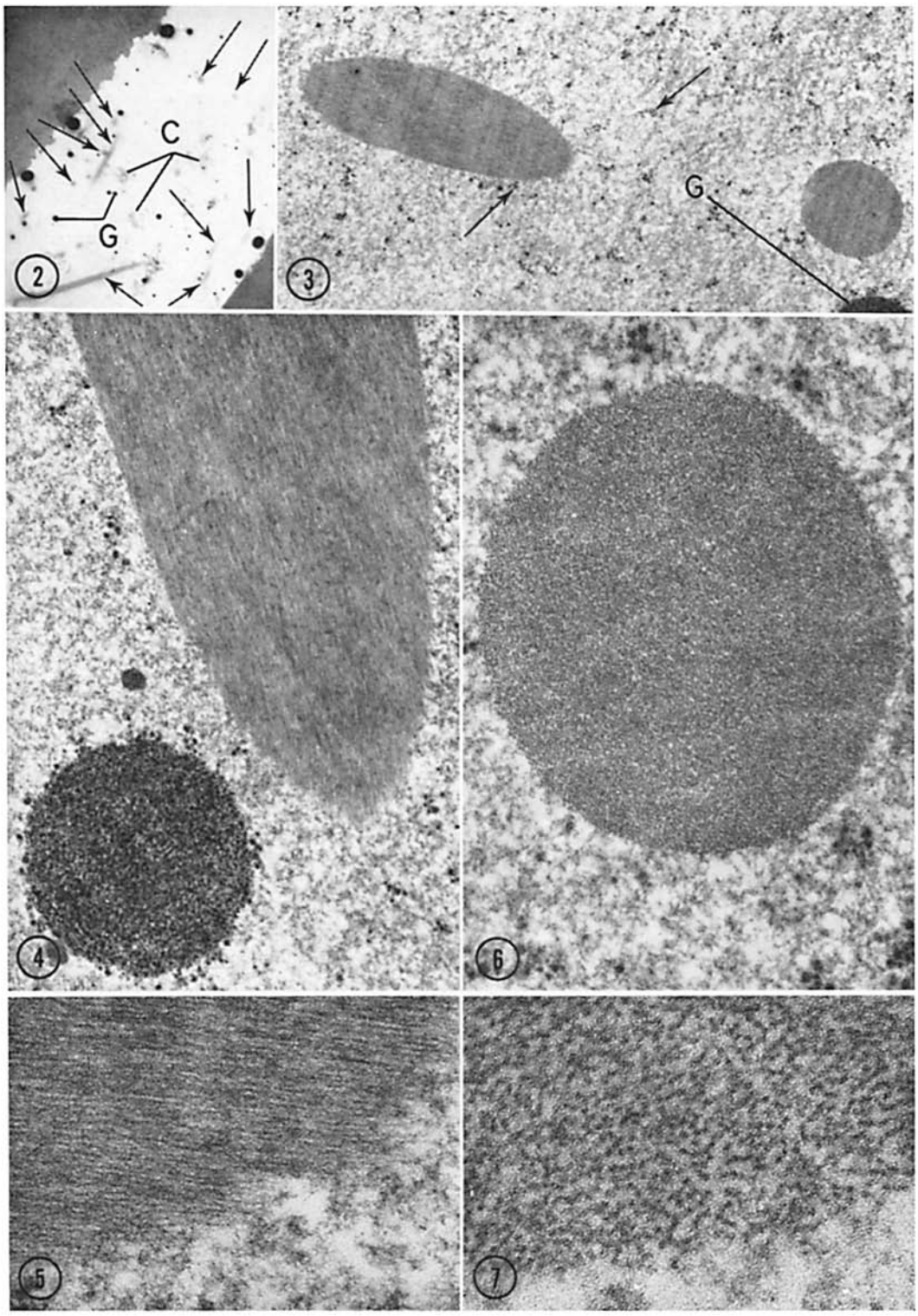
FIGURE 3 Low-power electron micrograph showing fibrillar bodies cut in longitudinal section and cross-section. Note the somewhat ellipsoidal shape of the former, the dense granular body at *G*, and the intranuclear granules (arrows). × 9,000.

FIGURE 4 Tapering end of a fibrillar bundle cut in longitudinal section, showing the component fibrils. Note the dense granular inclusion lying in proximity to the bundle. × 22,500.

FIGURE 5 Higher magnification of a fibrillar bundle such as that in Fig. 4 cut in longitudinal section. Note almost parallel alignment of the component fibrils. × 78,500.

FIGURE 6 Cross-section through a fibrillar bundle showing the random distribution of the fibrils. Note the intranuclear granules lying nearby. × 35,500.

FIGURE 7 Higher magnification of a bundle such as that in Fig. 6 cut in cross-section. × 163,000.



presented more extensive information on these threads. He has shown that they may occur in bundles with a regular hexagonal packing and be up to 2 μ in length. Although looked for in four other amphibians, they have so far been seen only in *R. pipiens* and *R. temporaria*. Some other DNA-binding antibiotics can induce their formation. Waddington (1966) stressed that these threads were most commonly found near nucleoli and that they had not been seen in pregastrula cells in which a nucleolus had not yet been formed nor in adult tissues where nucleoli are poorly developed. If this association with the nucleoli is meaningful, their presence in amphibian oocytes, which contain hundreds of nucleoli, could be significant. However, it seems unlikely that the bodies described here represent uncoiled chromosomes, since not only are they Feulgen-negative but they attain a very large size and contain many component fibrils. For the same reasons, it is also doubtful that they represent either the sites of actinomycin binding to DNA or virus particles.

In actinomycin-treated cultured HeLa cells, intranuclear inclusions have also been described (Reynolds et al., 1964); these inclusions, however, appear as vacuoles or areas of decreased density and contain RNA, so that they are unlike the fibrillar bodies reported here.

Fibrillar or tubular structures have been found to occur in nuclei elsewhere such as the macronuclei of *Paramecium* (Vivier and André, 1961) and in the nuclei of insects (Smith and Smith, 1965), while intranuclear crystalline inclusions have also been reported (Fawcett, 1966). Such structures, present normally in these cells, have been considered to represent protein arrays. None of these inclusions are exactly similar to the fibrillar bodies seen here, but it is nevertheless possible, if the actinomycin D inhibition of RNA synthesis also interferes with intranuclear transport of protein, that the bundles represent protein material. Perhaps the actinomycin prevents the ribosomal protein from joining the nucleolus-associated ribosomal RNA precursor molecules (40S and 30S) (Rogers, 1968), so that the protein accumulates in the nucleoplasm as an artifact in the form of bundles. On the other hand, the fibrillar bundles could represent protein ordinarily associated with the chromosomes or nuclear sap. At present, it is possible merely to speculate as to the nature of these fibrillar bodies; however, the fact that they are observed only in oocytes treated with actinomycin D suggests that this antibiotic is in some way responsible for their formation.

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