

EFFECTS OF ACTINOMYCIN D ON THE ASSOCIATION OF NEWLY FORMED RIBONUCLEOPROTEINS WITH THE CISTRONS OF RIBOSOMAL RNA IN *TRITURUS* OOCYTES

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ABSTRACT

The effect of actinomycin D (AMD) on the association of the nascent ribonucleo-protein (RNP) fibrils containing the precursors of ribosomal RNA (pre-rRNA) with their template deoxyribonucleoprotein (rDNP) strands has been studied in lampbrush stage oocytes from *Triturus alpestris*. Ovary pieces were incubated in vitro either in media containing radioactive ribonucleosides and then, for various times, in solutions containing 25 $\mu\text{g}/\text{ml}$ AMD, or were directly exposed to the drug. The ultrastructure of the nucleoli and the nuclear periphery was studied by electron microscopy of thin sections and positively stained spread preparations of isolated nuclear contents, and by light and electron microscope autoradiography. The fate of the labeled pre-rRNA was followed by gel electrophoresis of RNA extracted from manually isolated nuclei.

Our results show that the growing fibrils which contain the nascent pre-rRNA progressively detach from the DNP strands, the majority being released between 45 and 180 min after application of the drug. The release pattern seems to be random and does not show preference for regions close to the initiator or terminator sites of the transcribed rDNP units. There is a pronounced tendency to removal of groups of adjacent nascent fibrils. The effect of the drug is very heterogeneous. Even after 3 h of treatment with AMD the nucleoli exhibit several individual transcriptional units which appear almost completely covered with lateral fibrils. Autoradiography revealed that most of this released RNP remains within the confinements of the nucleoli which show some foci of aggregation and condensation of fibrillar components but no clear "segregation" phenomenon. In the gel-electrophoretic analysis, a significant but moderate decrease of labeled pre-rRNA was noted only in the first stable pre-rRNA component, whereas pre-rRNA classes of lower molecular weight are very stable under these conditions.

The results are discussed in relation to the stability of rDNA transcription complexes and as a basis for an explanation of the ultrastructural changes which are generally observed in nucleoli of AMD-treated cells. It is postulated that

inhibition of transcription results in a slow but progressive release of the arrested incomplete RNP fibrils from the template.

Actinomycin D (AMD) is a drug which represents an important biochemical tool, especially in studies of RNA metabolism, and is also clinically used as a very potent antitumoral agent (for a review on the latter aspect see, e.g., 24). It inhibits DNA-directed transcription by binding to the DNA helix, apparently by preventing the translocation of the RNA polymerase along the template (for reviews see, e.g., 25, 55). In vitro AMD does not significantly affect the formation of the initiation complex between DNA and RNA polymerases but drastically reduces the chain elongation rate of the nascent RNA by lowering the "rate terms" for CTP and GTP incorporation (34). It is not known whether, under the influence of AMD, the arrested RNA chains remain attached to their template or are released as premature molecules. Besides its primary effect of binding to DNA, preferentially to G + C rich regions, and its inhibition of transcription, AMD interferes with several other metabolic processes. For example, it reduces the initiation rates in translation (12, 31), it causes nonphysiological breakdown of rapidly labeled RNA in pro- and eucaryotes (1, 61, 65, 74; see, however, also the report of a prolonged half-life time of messenger RNA [mRNA] by Endo et al. [16]), and impairs the processing of the ribosomal precursor RNA (pre-rRNA) molecules, concomitant with a reduced or even inhibited transfer of mature rRNA to the cytoplasm (e.g., 14, 27, 28, 33, 36, 41, 42, 53, 73, 75). How these differential effects of AMD relate to the characteristic morphological alterations in nucleolar structure induced by this drug (e.g., the "nucleolar segregation" sensu Bernhard et al. [5]; for reviews see 4, 9, 67, 68) is not understood. Several authors assume that it is the binding of AMD to the DNA which contains the rRNA cistrons (rDNA) and the inhibition of transcription which is responsible for the sorting out of the nucleolar components and not the secondary effects on RNA metabolism and protein synthesis (e.g., 4, 30, 54, 68). In this context it seemed especially desirable to us to study the primary effect of AMD in vivo in greater detail, in particular its influence on the stability of the transcriptional complex. For this study, we have chosen the amphibian oocyte in the lampbrush stage since in this cell type a greatly amplified number of cistrons for rRNA occur in numerous

nucleoli and are in a transcriptionally active state (e.g., 6, 8).

MATERIALS AND METHODS

Preparation and Treatment of Oocytes

Females of *Triturus alpestris* were collected in ponds in the Black Forest area from March to July and were kept in aquaria at about 15°C. Parts of ovaries were removed under anesthesia (0.1% MS-222, Serva Feinbiochemica, Heidelberg, Germany) and were placed in Eagle's minimal essential medium diluted 1:1 with distilled water. Small ovary pieces freed from mature oocytes were incubated at 18°C in the same medium containing either 200 μ Ci/ml of [³H]uridine (26 Ci/mmol) for autoradiography or, for RNA extraction, 100 μ Ci/ml each of tritiated uridine, cytidine (29 Ci/mmol), adenosine (12.1 Ci/mmol), and guanosine (12.5 Ci/mmol; all from the Radiochemical Centre, Amersham, England). After 4 or 12 h of preincubation the ovary pieces were washed with nonradioactive Eagle's medium and further incubated with an additional 25 μ g/ml AMD (Serva Feinbiochemica) for periods ranging from 1 to 5 h. Controls were incubated in the same medium, but without AMD.

Electron Microscopy

After the incubation lampbrush stage oocytes with a diameter of ca. 800 μ m were isolated from the ovary pieces and fixed in 4% glutaraldehyde buffered with 0.05 M Na cacodylate to pH 7.2 for 4 h at 4°C, washed several times in cold buffer, and postfixed in 2% OsO₄ for 2 h at 4°C. Nuclei manually isolated from oocytes of the same stage were fixed in the same way. After dehydration through a series of graded ethanol solutions and in propylene oxide the specimens were embedded in Epon 812. Sections were prepared with a Reichert ultramicrotome OmU-2. For application of Bernhard's differential staining method (3) oocytes and isolated nuclei were fixed in 4% glutaraldehyde (same buffer), dehydrated, and embedded as described. The ultrathin sections were stained in aqueous uranyl acetate, treated with neutral EDTA solution, and washed and poststained with lead citrate (for details see 18). Micrographs were taken with a Siemens Elmiskop IA or 101 or with a Zeiss EM 10. Nuclei were isolated from pretreated and control oocytes and nuclear contents were prepared and spread according to the technique of Miller and Beatty (52) and Miller and Bakken (49) as previously described (64). Length measurements were performed as described elsewhere (64, 72).

Light and Electron Microscope Autoradiography

Light microscope autoradiographs were prepared using 1- μ m thick sections and Kodak AR 10 stripping film. For electron microscope autoradiography we followed the method of Granboulan (32) using Ilford L4 emulsion and Kodak D19 developer. Exposure times were 10 or 30 days.

Gel Electrophoresis of Nuclear RNA

After the incubation lampbrush stage oocytes were removed from the ovary pieces and transferred into the "5:1-medium" (0.083 M KCl and 0.017 M NaCl; c.f. 10). The nucleus was manually isolated, cleaned from visible cytoplasmic contaminations (c.f. 62), and was fixed in ice-cold ethanol-acetic acid (3:1). 20 nuclei were washed in 70% ethanol and stored at -20°C . For RNA extraction the alcohol was removed from the nuclear pellet, and the pellet was immediately dissolved in 0.5 ml of a predigested pronase solution (1 mg/ml, Calbiochem San Diego, Calif., ribonuclease-free, in 20 mM Tris-HCl, pH 7.4, with 0.5% sodium dodecyl sulfate). After 10 min of incubation at room temperature 30 μ g of *Xenopus laevis* rRNA (prepared from ovarian ribosomes) were added, the NaCl concentration was raised to 0.1 N, and RNA was precipitated by adding 2.5 vol of ethanol. After storage at -20°C the material was centrifuged and the pellet was resuspended in electrophoresis buffer (20 mM Tris-HCl, pH 8.0, 20 mM NaCl, 2 mM EDTA) containing 0.2% sodium dodecyl sulfate. The RNA was analyzed on gel slabs (0.5% agarose-2.25% acrylamide) as described by Ringborg et al. (57) and Scheer et al. (64).

RESULTS

Incubation in vitro of oocytes contained in ovary pieces from various amphibia represents, for several hours after removal from the animal, conditions in which a great many processes continue as in vivo, proper preparation and incubation procedures provided. This holds in particular for incorporation of nucleosides into RNA (26, 38-40, 59, 60, 63, 71). When *T. alpestris* lampbrush stage oocytes were incubated for 1-12 h in media containing tritiated nucleosides, an incorporation of radioactivity into nuclear RNA, mostly into pre-rRNAs, is noted (Fig. 1). The molecular weight pattern of the nuclear RNA is identical with that observed after injection of the label into the body cavity (unpublished data), with a predominance of pre-rRNA classes of $2.6-2.7 \times 10^6$, $1.9-2.0 \times 10^6$, and 1.5×10^6 daltons molecular weights (for almost identical patterns in other Amphibia see 22, 26, 38, 45, 59, 60, 63). As mentioned in previous publications of studies on

quite diverse cell types (e.g., 26, 53, 58; for references see also 14, 21, 22, 66, 72) it is obvious that the nucleus does not contain considerable amounts of labeled RNA of the same electrophoretic mobility as the mature large (28S) cytoplasmic rRNA. Subsequent incubation of the labeled oocytes in media containing amounts of AMD which in preliminary studies have been shown to completely inhibit RNA synthesis (see also 35, 40, 71) leads to a significant decrease of radioactivity contained in the 2.6-2.7 million daltons component, the putative primary transcript of rDNA (for review see 44). On the contrary, the components with molecular weights of about 2 million and 1.5 million daltons show only slightly reduced amounts of radioactively labeled RNA, if any reduction at all (Fig. 1 b). As a consequence of this change in the RNA pattern the relative amount of radioactivity contained in the 1.5×10^6 daltons molecular weight nuclear 28S pre-rRNA increases by a factor of about 1.5 after 3 h of influence of AMD. This reduction of label in early stages of pre-rRNA processing resembles similar findings in many other cell types (e.g., 9, 28, 43, 53, 66, 73) but differs from the marked pre-rRNA stability in the macronuclei of AMD-treated *Tetrahymena pyriformis* cells (14). However, we do not regard these observations in *Triturus* oocytes as conclusively indicating the continuation of processing of primary transcription products in the presence of AMD, concomitant with an inhibition of later stages of nucleolar rRNA processing (e.g., 53), since the change could as well merely reflect the higher susceptibility of the large (ca. 2.6×10^6 daltons) molecule to degradative processes. In this connection, however, it is also important to note that radioactivity was detected in the 2.6×10^6 daltons pre-rRNA component even after prolonged influence of AMD (up to 5 h). We never did observe a complete disappearance of this RNA component under our conditions.

The incubation in the AMD-containing media did not result in a marked change of the distribution of nuclear radioactivity as revealed by autoradiography; the preferential labeling of the nucleoli was always detectable (Fig. 2). The silver grain density over nucleolar structures was consistently lower in the AMD-treated cell but there was no notable shift of radioactivity to the nucleolar periphery and into the cytoplasm in the course of AMD treatment. Marked labeling was also noted in a distinct class of intranuclear granular aggregates (Fig. 2), at the nuclear envelope, and over the

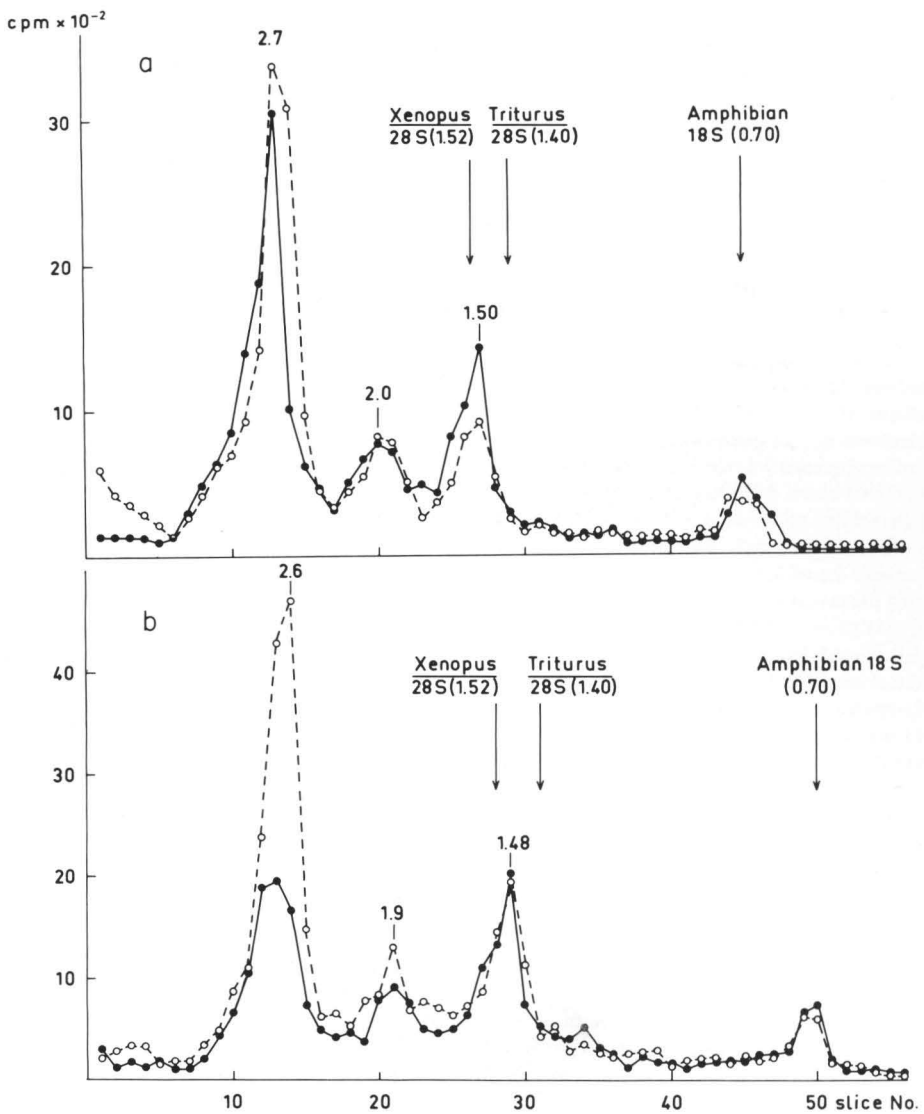


FIGURE 1 Electrophoretic separation of nuclear RNA from *T. alpestris* oocytes in polyacrylamide-agarose gels. Ovary pieces were incubated for 12 h at 18°C in medium containing 100 $\mu\text{Ci/ml}$ of each [^3H]uridine, [^3H]cytidine, [^3H]guanosine, [^3H]adenosine, and then in nonradioactive medium containing 25 $\mu\text{g/ml}$ AMD for 1 h (a) and 3 h (b). For controls (dashed lines) the labeled ovary pieces were incubated for the same time in medium without AMD. For each separation 20 nuclei of midlamprbrush stage oocytes were manually isolated. Molecular weights were calculated from the positions of coelectrophoresed reference RNAs, namely cytoplasmic rRNAs isolated from ovaries of *X. laevis* and *T. alpestris* (indicated by the vertical arrows). Molecular weights are given in million daltons. Note the reduction of radioactivity contained in the first stable pre-rRNA component ($2.6\text{--}2.7 \times 10^6$ daltons) with increasing exposure to AMD.

fibrillar tangles connecting the nucleolar periphery with the pore complexes of the nuclear envelope (for morphological details of the nucleolus-nuclear envelope relationship in amphibian oocytes see 19, 20, 22, 39).

We did not observe a clear AMD-induced nucleolar segregation (e.g., Fig. 3), in contrast to a report of Lane (40) in *Triturus viridescens*, but in agreement with observations of Snow (69) in *Triturus cristatus*. However, we gained the impres-

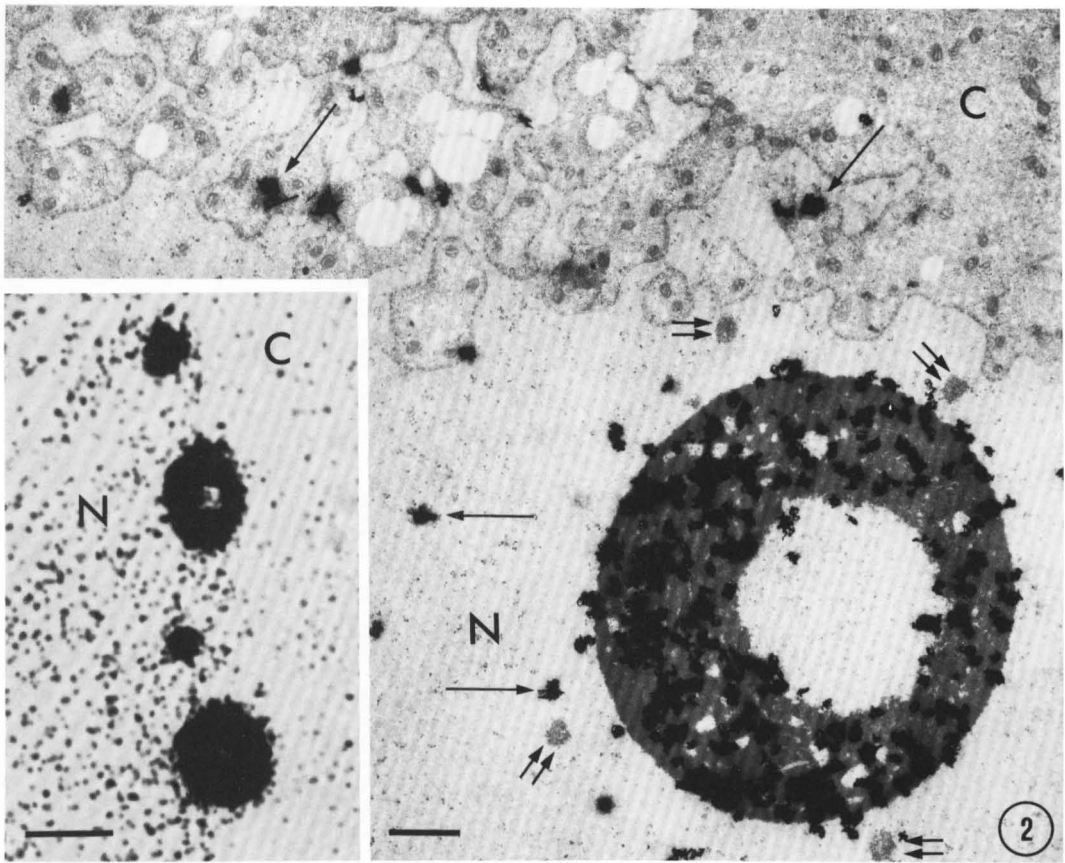
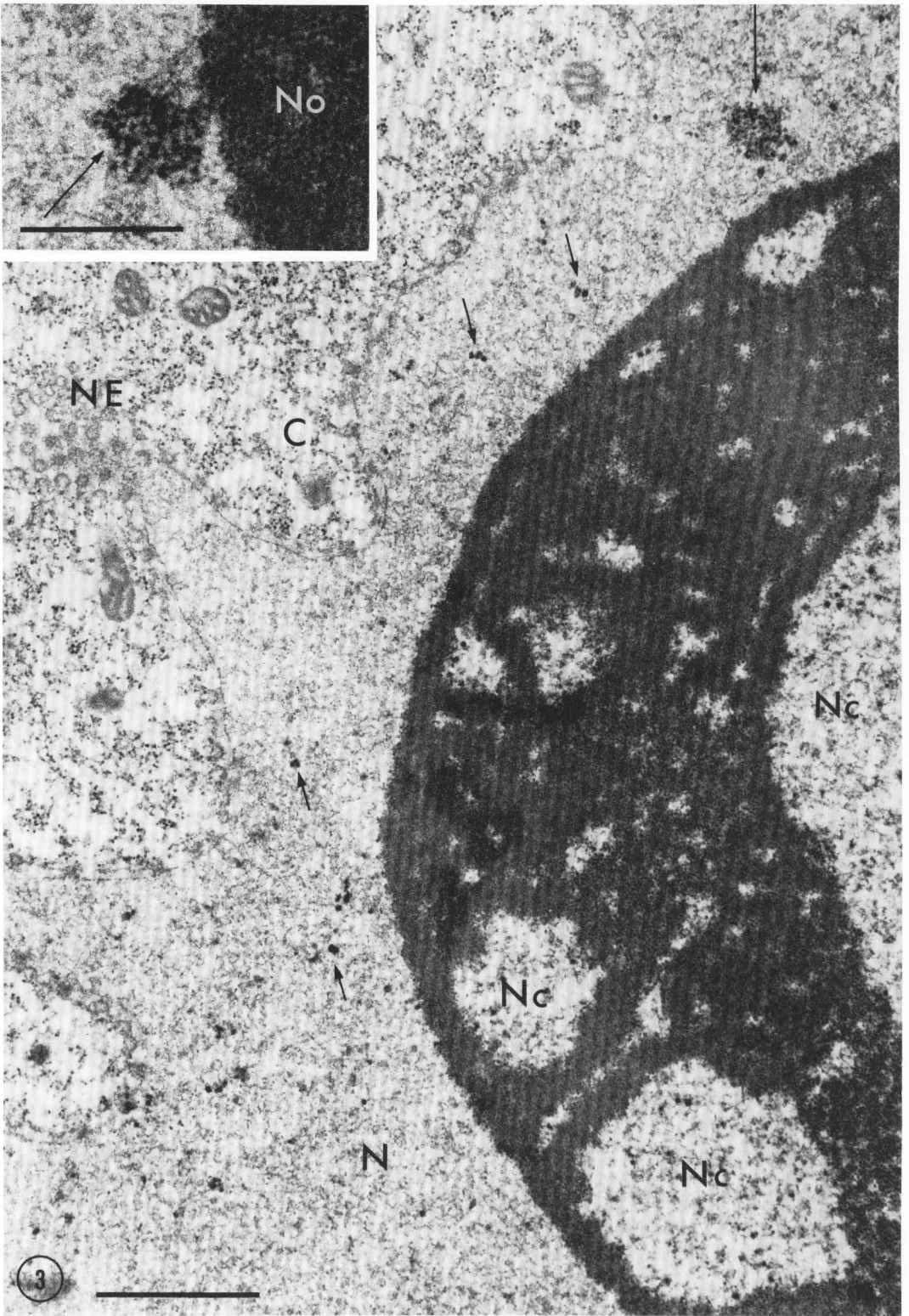


FIGURE 2 Autoradiogram of a 1- μ m thick (light microscope; *inset*) and an ultrathin section (electron microscope) through the nuclear periphery of a *T. alpestris* lampbrush stage oocyte which had been incubated for 4 h in a medium containing [3 H]uridine, followed by an incubation for 3 h in medium containing AMD. The grain density is by far highest in the nucleoli. The silver grains show an almost uniform distribution over the nucleolus proper, but the central cavity is essentially free of radioactivity. Many of the characteristic electron-dense aggregates that occur in the nucleoplasm as well as in the juxtannuclear cytoplasmic zone are also labeled (arrows). Note, however, that some aggregates are distinctly unlabeled (double arrows). *N*, nucleoplasm; *C*, cytoplasm. $\times 4,700$, scale indicates 2 μ m; *inset*, $\times 580$, scale indicates 20 μ m.

sion of a progressive formation of intranucleolar cavities (Figs. 2, 3) and a more marked nucleolonema-like organization (Fig. 4). The predominance of such nucleolonemata, however, can not be specifically contributed to the influence of AMD since it can be sometimes observed in untreated amphibian oocytes (50) as well as after application of quite different metabolic inhibitors including those of oxidative phosphorylation (R. Berezney and U. Scheer, unpublished observations). Staining with the EDTA procedure of Bernhard (3) showed marked retention of uranyl ions in the nucleolar substructures as well as in the characteristic, about 0.5–0.8- μ m large granular aggregates, which appear to derive from the nu-

cleolar periphery and to migrate to the cytoplasm (Fig. 3) in the about 400- \AA large dense granules (e.g., denoted by the small arrows in Fig. 3), and in the cytoplasmic ribosomes. This might be indicative of the preserved RNA content of all these structures. At closer inspection we noted that with prolonged influence of AMD the relative amount of granular components in the nucleolus was reduced concomitant with the predominance of densely aggregated, relatively indistinct fibrillar masses (Fig. 4 *a, b*) which might represent a phenomenon equivalent to the AMD-induced "segregation" in chromosomal nucleoli (for reviews see 4, 9, 68).

When nuclear contents manually released from



the AMD-treated oocytes were prepared according to the Miller and Beatty technique (52) using relatively short times for incubation in the low salt borate buffer, a nucleolonema-like organization of the nucleoli was demonstrated which appeared as coils of about 2–3- μm broad, sausage-shaped threads (Fig. 5), which were not observed in untreated nuclei. Sometimes the composition of these sausages by fine, about 120- \AA thick fibrils was revealed, in particular in regions in which the coarse threads were partially dispersed and the fibrils had become unravelled (Fig. 5 *b*). The appearance of nucleolar core material after AMD treatment as revealed in more intensely spread preparations is demonstrated in Figs. 6, 7, 9 (as to controls the reader is referred to our previous article [64] on the structure of actively transcribing nucleoli of *T. alpestris*). Significant changes in the ultrastructural organization of the transcriptional complexes were not noted until 45 min after addition of AMD, i.e., long after complete cessation of RNA synthesis (about 15 min at the concentrations used in this study). After 45 min in the presence of AMD, one still notes the composition of the nucleolar core by extended axial deoxyribonucleoprotein (DNP) fibrils that contain regions covered with lateral fibrils of increasing lengths, the “matrix units,” and fibril-free intercepts, the “spacer units” (for principles of interpretation of such positively stained nucleolar preparations see 49–52, 64, 72, 76–78). Although several situations suggest the removal of lateral ribonucleoprotein (RNP) fibrils from matrix units (some are denoted by the arrows in Fig. 6) complete matrix units are still frequent. A detailed analysis of the incomplete matrix units which increase in frequency from this time on (see also Fig. 8 *a*) showed a tendency of detachment of intramatrix groups of fibrils, i.e., a cooperative behavior in removal, rather than a progressive homogeneous dilution of such fibrils

(insets of Figs. 6 and 7). The change in the pattern of the lengths of fibril-covered intercepts as obtained after 45 min of AMD treatment is particularly clear from the quantitative evaluation shown in Fig. 8 *a*. There was no preference for a removal of fibrils in either start or terminal regions of the matrix units (Fig. 8 *b*). Survey micrographs of the typical appearance of such spread nucleolar structures obtained after short AMD treatment are presented in Fig. 7 and suggest, in addition to the lack of intramatrix fibril groups, the (almost) total absence of lateral fibrils in long rDNA intercepts, suggesting the removal of the vast majority of the nascent RNP fibrils from some of the matrix units. We would like to emphasize, though, that at this duration of AMD treatment there is a marked heterogeneity in the effect between the matrix units within one nucleolus since regions with clusters of almost complete matrix units are recognized besides other regions from which RNP fibrils obviously have detached.

The appearance of spread nucleoli from *T. alpestris* oocytes after prolonged action of AMD is illustrated in Fig. 9. Matrix units or related groups of lateral fibrils are much less frequent and conspicuous and long extended fibrils, most probably representing the “naked” rDNP axes, predominate. Occasionally, however, individual matrix units of remnants thereof can still be recognized, thus facilitating the identification of the whole fibril aggregate as a nucleolar structure. We noted in such nucleolar preparations from AMD-treated oocytes a pronounced tendency of the axial fibrils to coil formation and lateral aggregation as well as an increased indistinctiveness of both classes of fibrils, coincident with the appearance of isolated fibrils in the vicinity of the extended continuous rDNP axes. Perhaps these reflect the detachment of some lateral fibrils that takes place “on the grid,” i.e., in the final stages of the preparation.

FIGURE 3 Electron micrograph presenting an EDTA-treated section showing the appearance of nucleoli and nuclear periphery in *T. alpestris* oocytes incubated for 1 h with AMD. The nucleolonema-like organization of the nucleoli is apparent. Note the numerous nucleolar cavities (*Nc*) which are filled with loosely packed granulofibrillar material. The large dense aggregates in the nucleoplasm (*N*; large arrows), which are often in direct structural continuity with the nucleolar periphery (*inset*), appear frequently to be composed of distinct electron-dense granules with diameters of about 400 \AA , and have retained the uranyl stain. Granules of similar size also occur isolated or in small clusters (denoted by small arrows) in the nucleoplasm as well as within the nucleolar cavities (*Nc*). Note also the retention of stain in the cytoplasmic ribosomes. *NE*, nuclear envelope; *No*, nucleolus. $\times 25,000$, scale indicates 1 μm ; *inset*, $\times 50,000$, scale indicates 0.5 μm .

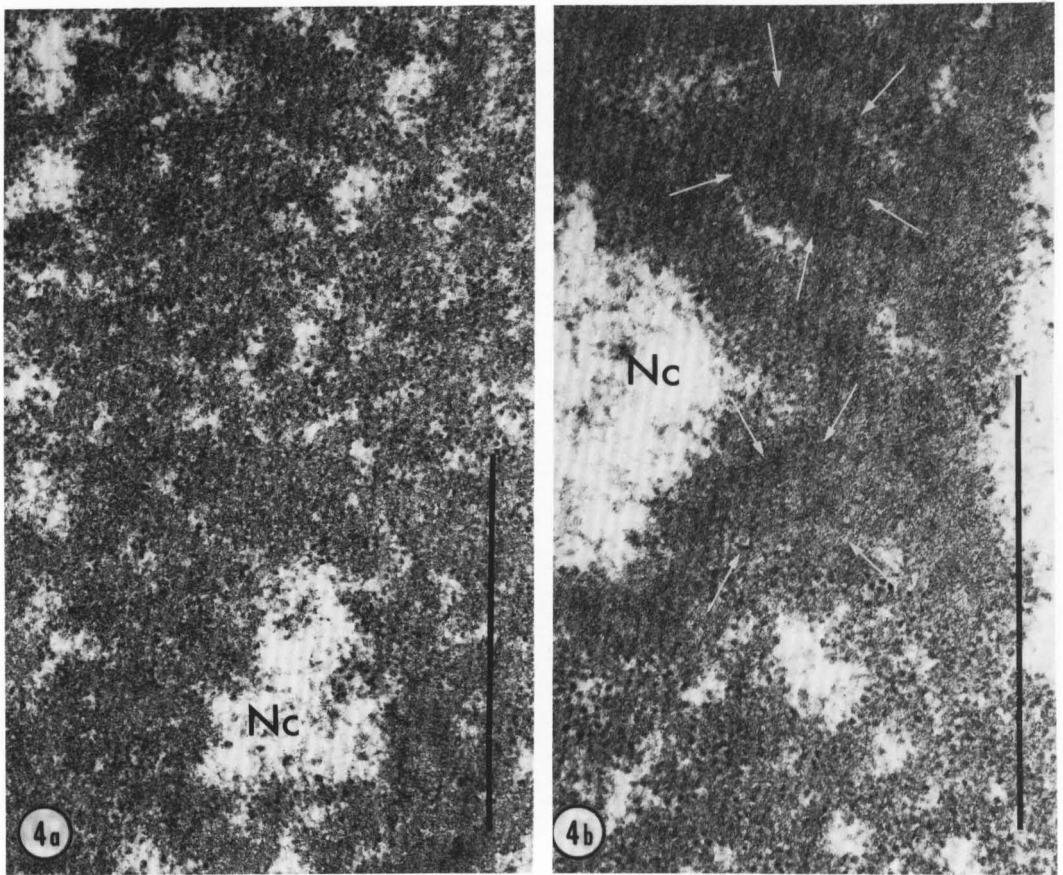


FIGURE 4 With prolonged incubation of the *T. alpestris* oocytes in AMD a segregation of the nucleolar granules from purely fibrillar areas (two examples are denoted by the circles of arrows in Fig. 4 *b*) is recognized. These micrographs show examples of oocytes incubated for 1 h (*a*) and 3 h (*b*) in Eagle's medium containing AMD. EDTA method. *Nc*, nucleolar cavity. *a*, $\times 50,000$; *b*, $\times 60,000$, scales indicate 1 μm .

DISCUSSION

The present study demonstrates that AMD, in addition to its inhibition of transcription, also has an effect on the binding of the transcriptional complex to the template-containing DNP strand, although at a much lower rate. In oocytes, as probably in all cells, normally only fibrils which contain mature pre-rRNA molecules are released from the rDNA, that is, after termination of transcription. This is demonstrated by the characteristic "Christmas tree" formations of the transcriptional units and the predominance of only one stable class of pre-rRNA molecules, the putative primary product of transcription. From the average rates of pre-rRNA synthesis and chain elongation in lampbrush stage oocytes of *X. laevis* (63)

and *T. alpestris* (U. Scheer, unpublished data) one can estimate that the complete pre-rRNP fibril is released within 2.5 s. In view of our finding that during the incubation in vitro the rate of nucleoside incorporation into RNA is not considerably reduced (see also 38) it seems reasonable to assume that the rate of release of the rDNA transcript is of the same order of magnitude under our experimental conditions.

With the relatively high AMD concentration used in the present study, which almost immediately and completely inhibits further incorporation of nucleoside triphosphates into RNA (35, 40, 70, 71) striking alterations are observed in the binding of the nascent pre-rRNP fibrils to the template, but only after 45 min after addition of the drug. In contrast to the normal release of pre-rRNP fibrils,

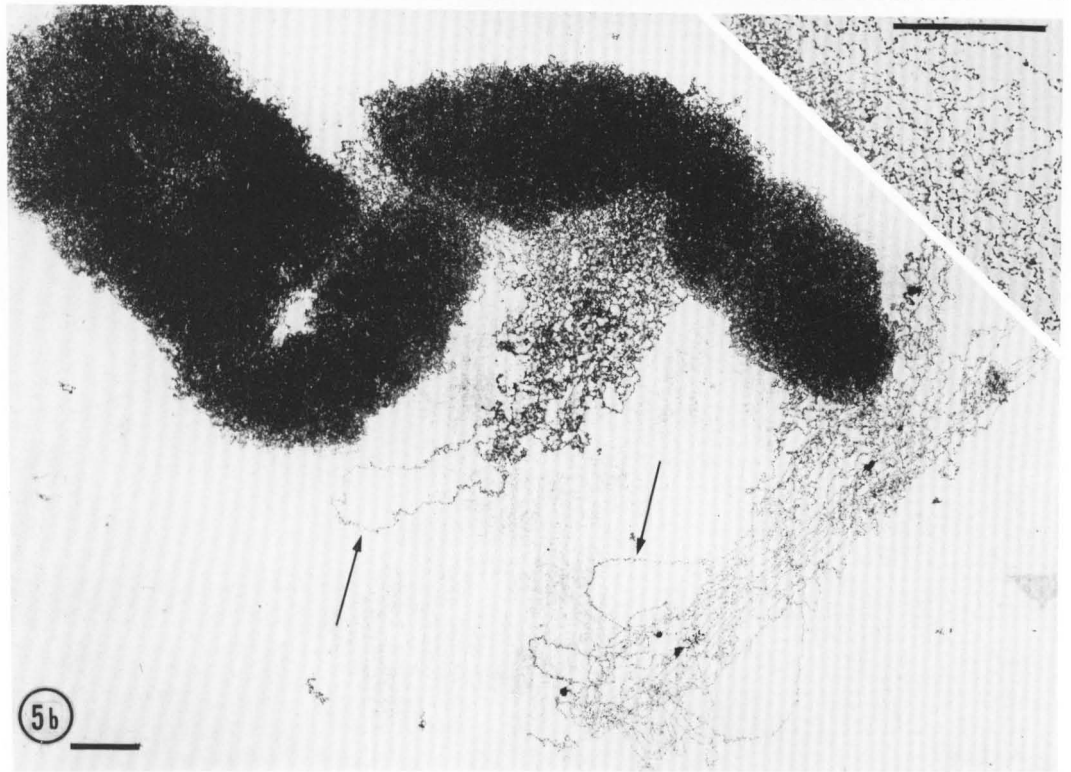
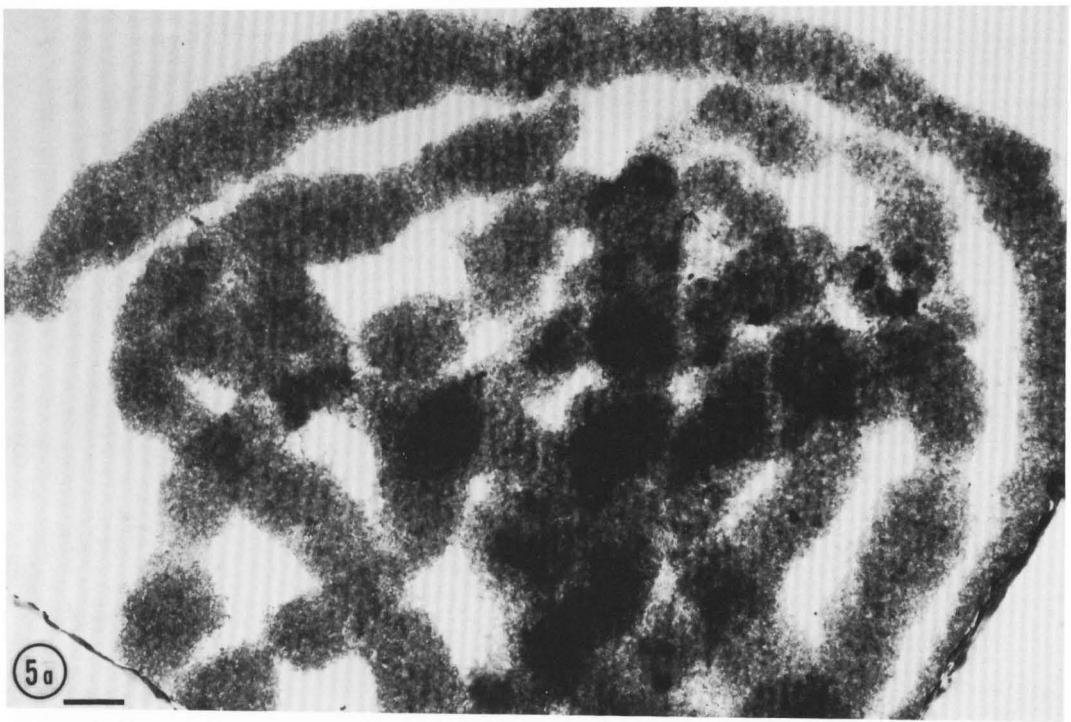


FIGURE 5 Positively stained (phosphotungstic acid) spread preparation of a nucleolus isolated from *T. alpestris* oocytes treated for 1 h with AMD. Here the nucleolus appears as a coil of coarse, mostly 2–3- μm thick threads (a). A composition of these threads by fine fibrils is suggested after more extensive spreading, in particular in the peripheral regions (b). Extended fibrillar loops (arrows in Fig. 5 b) are identified. The inset in Fig. 5 b demonstrates a fine granularity along the well-spread fibrils. a, $\times 4,000$; b, $\times 4,400$, scales indicate 2 μm ; inset, $\times 20,000$, scale indicates 1 μm .

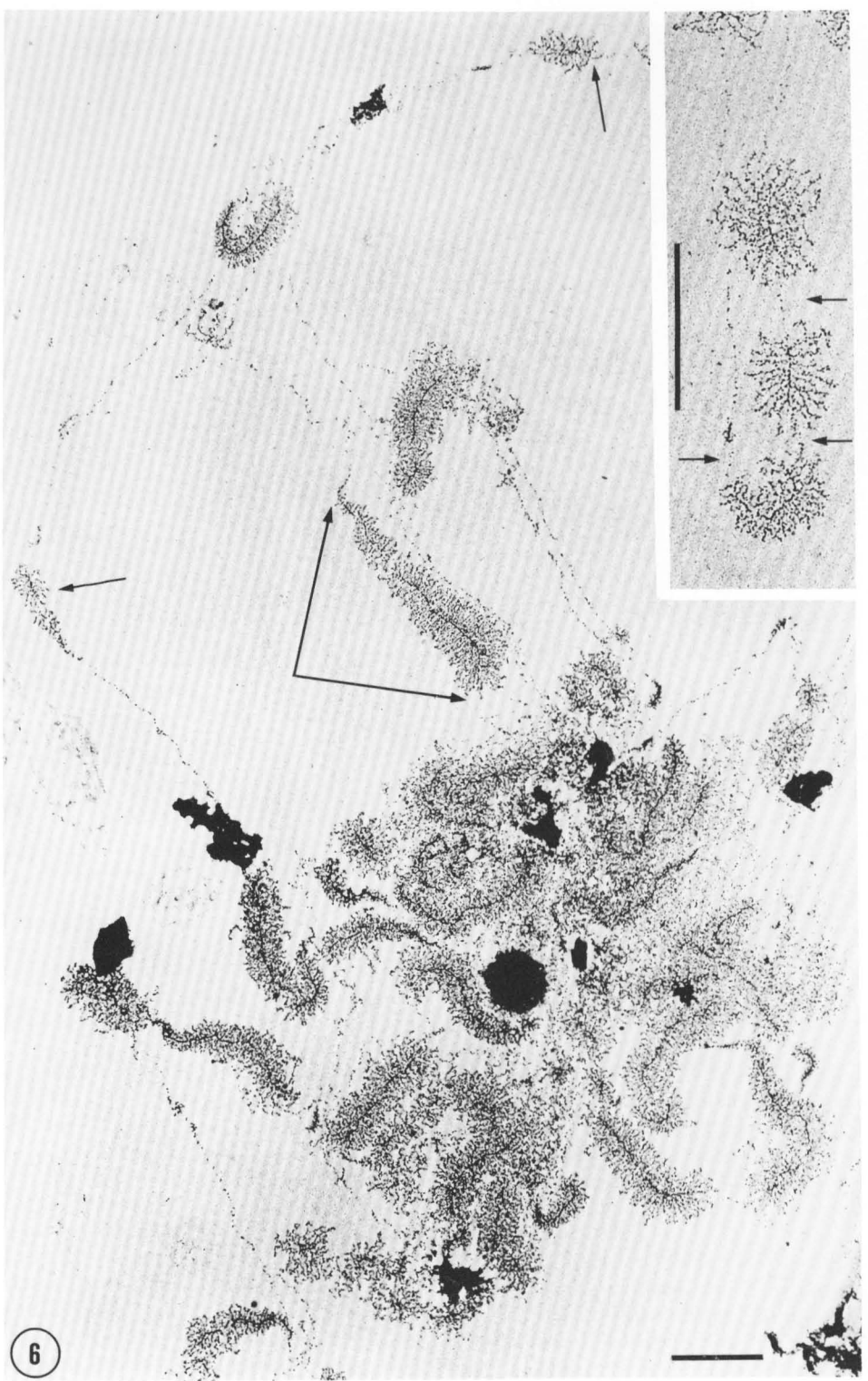


FIGURE 6 Nucleolar (core) material as seen in spread and positively stained preparations from *T. alpestris* oocytes which were exposed for 45 min to AMD. One notes the normal nucleolar arrangement showing, on extended axial fibrils, alternations of regions covered with lateral fibrils (matrix units *sensu* Miller and Beatty [51]) and fibril-free intercepts (spacer units). Some of the matrix units (one is denoted by the pair of arrows) have the same length as those in control oocytes. One notes, however, an increased frequency of matrix units from which lateral fibrils are partially removed (some are denoted by the arrows). Note in particular the absence of groups of fibrils within individual cistrons (arrows in the *inset*). $\times 13,500$; *inset*, $\times 24,000$, scales indicate $1 \mu\text{m}$.

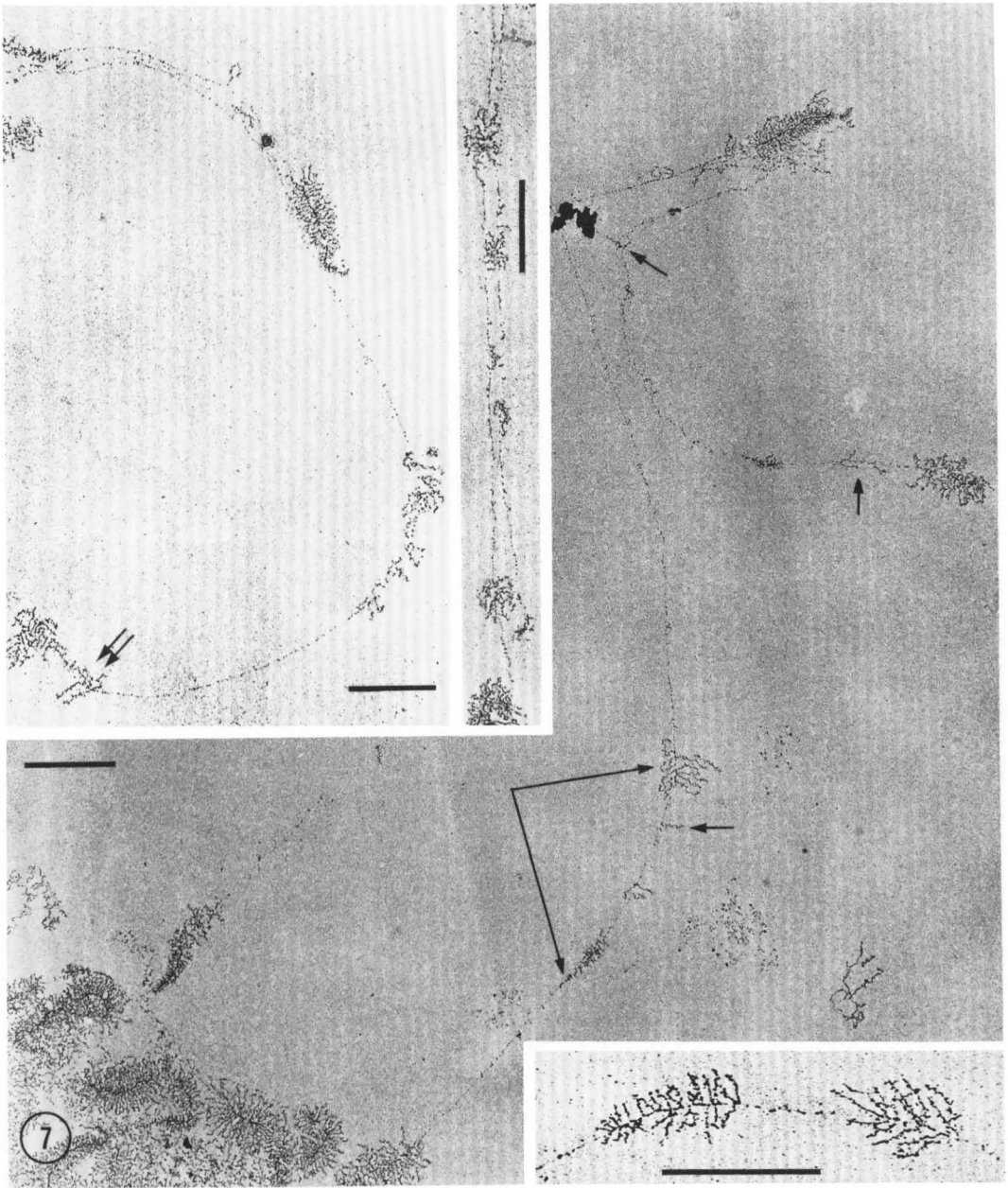


FIGURE 7 Spread preparation from nucleoli of oocytes treated for 45 min with AMD showing very extended rDNP axes. Note that in some regions sizeable matrix units are still recognized (e.g., in the lower left) whereas in other regions matrix unit intercepts are still only identified by few remaining lateral fibrils which, however, sometimes still illustrate the typical length gradient (pair of arrows). Note also the lateral adherence of rDNP fibrils (e.g., in the upper insets). While there is a marked tendency for the retention of groups of adjacent fibrils (e.g., lower inset) there are also examples notable of isolated matrix unit fibrils (e.g., at the small arrows). $\times 13,000$; left upper inset, $\times 12,500$; right upper inset, $\times 13,200$; lower inset, $\times 22,000$; scales indicate $1 \mu\text{m}$.

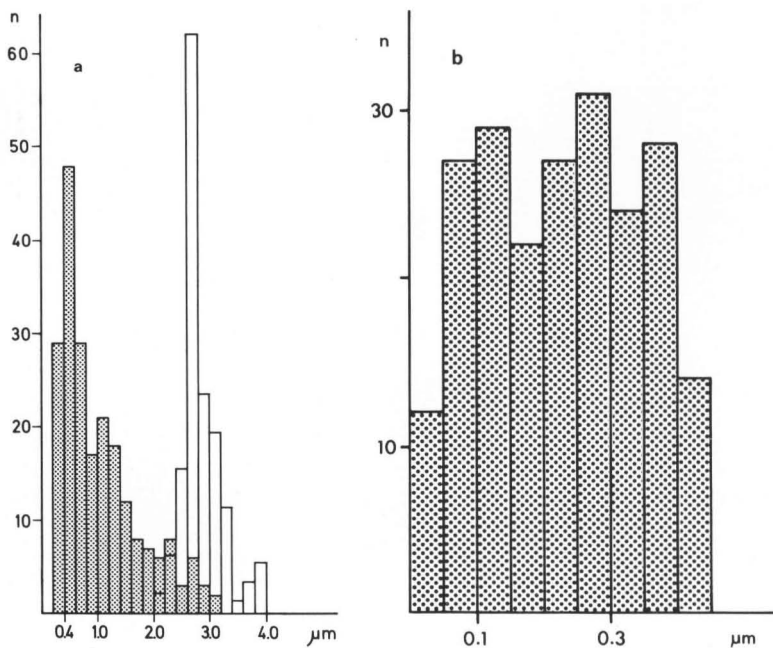
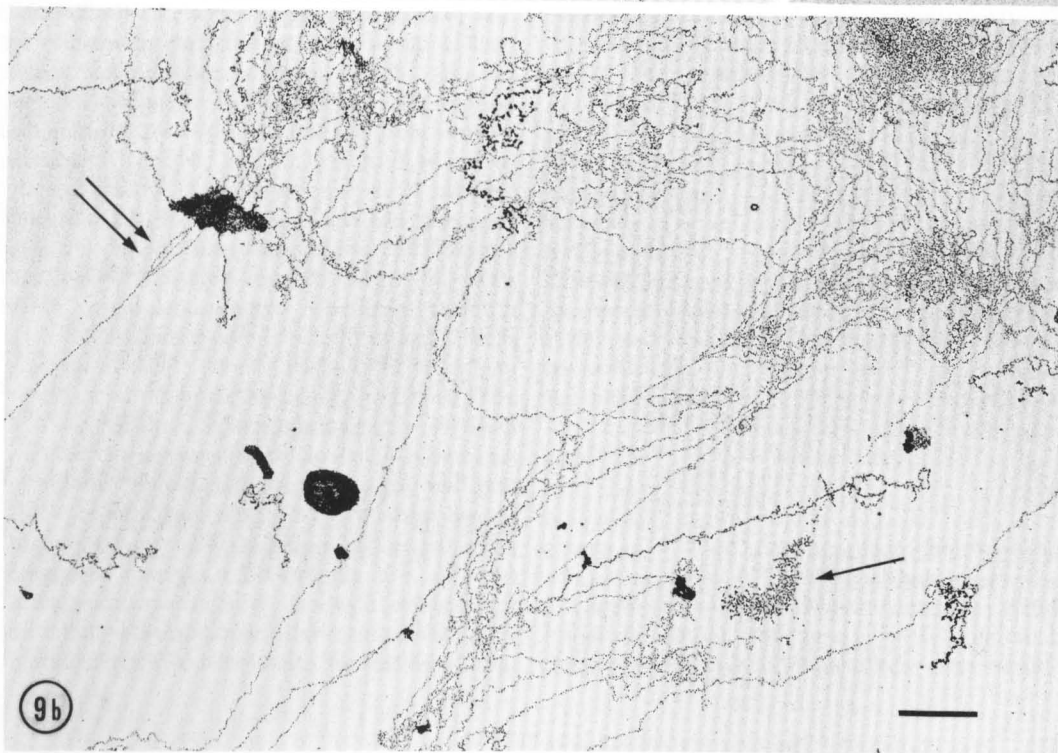
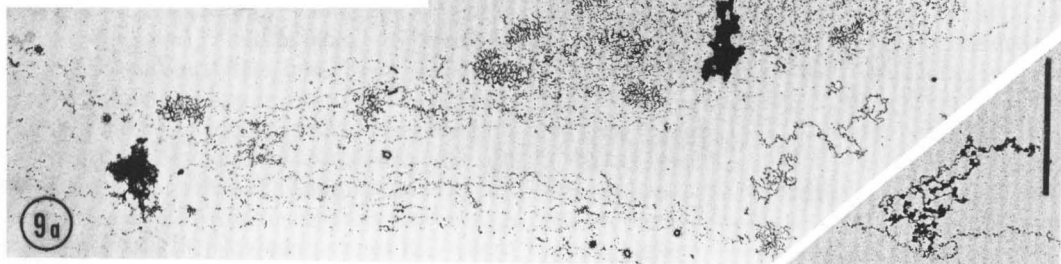
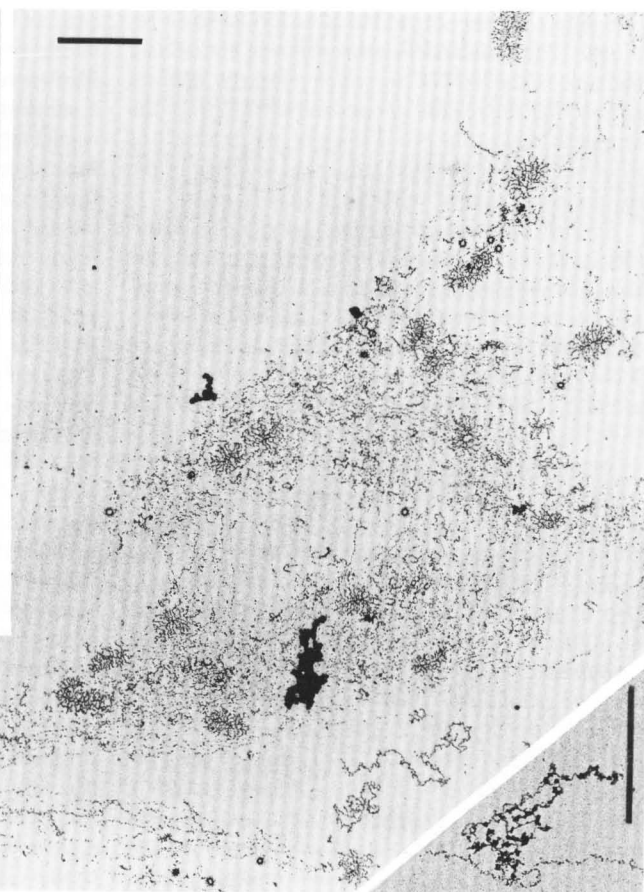
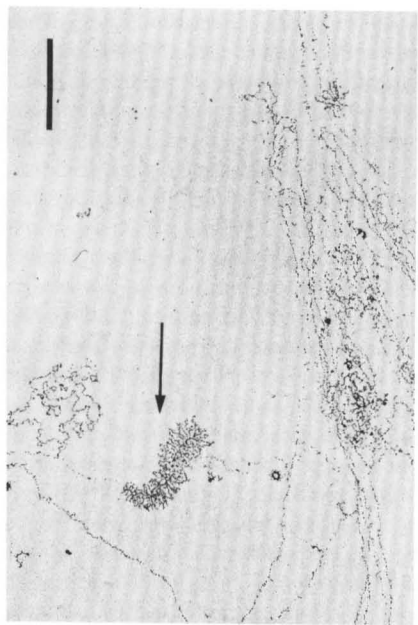


FIGURE 8 Fig. 8 *a* presents the distribution of lengths of uninterrupted groups of matrix unit fibrils after 45 min of incubation of *T. alpestris* lampbrush stage oocytes in medium containing AMD (hatched blocks) and without AMD (open blocks). The control (in the absence of AMD) shows the normal distribution of complete matrix units. Fig. 8 *b* presents the frequency (*n*, number) of fibril groups vs. the mean length of the lateral fibrils (abscissa) contained in the specific group as identified after the AMD treatment. There is no obvious enrichment for groups with short or with long lateral fibrils, indicating that preferential release of fibrils does not take place in initial or terminal regions of matrix units.

the AMD-induced release is not exclusive or preferential for completed fibrils. The pattern of release seems to be random, since the probability of removal of a growing fibril appears to be the same irrespective of its specific distance from the cistron initiation site. The pattern is also markedly heterogeneous in the sense that some transcriptional (matrix) units are less affected than others. Occasionally, even after prolonged influence of AMD, almost complete matrix units can still be recognized. A similar reduction in matrix unit

length, also with a heterogeneity among the individual units, has recently been noted in spread preparations of spermatocyte nuclei from fruit flies *Drosophila hydei*, that had been injected with AMD (47; these authors, however, also noted a preferential preservation of fibrils in the start region of matrix units). The reason for such differences in sensitivity of RNP fibril attachment to rDNP among the nucleolar matrix units after AMD-induced cessation of transcription is unclear. There is, however, also a marked coopera-

FIGURE 9 Spread *T. alpestris* nucleoli after 150 min of incubation of oocytes in medium containing AMD. The axial fibrils show a high tendency to aggregate and, over long distances, exhibit only very few lateral fibrils attached, indicative of their rDNP nature. Note that, even after such prolonged exposure, occasionally rather complete matrix units can be encountered (arrows), besides the small groups of fibrils typical for AMD-treated nucleoli. Some isolated irregularly coiled fibrils, often with a beaded appearance (*inset* in the lower right in Fig. 9 *a*), are found in these preparations. Note also the extremely close aggregation and entanglement of fibril-free axes which is obvious at sites of "branching" (double arrow in Fig. 9 *b*), and the occurrence of isolated fibrils of up to 3 μm in length, sometimes in circles, between the extended rDNP axial fibrils. *a*, $\times 11,300$; left *inset*, $\times 12,500$; lower right *inset*, $\times 18,000$; *b*, $\times 10,500$; scales indicate 1 μm .



tive effect in the inducible pre-rRNP fibril removal. The structural organization of the spread nucleoli after AMD treatment clearly demonstrates that the release of one lateral fibril from the template raises the probability of the removal of neighboring fibrils, thus frequently resulting in the formation of "gaps" within the matrix units.

Several different though not necessarily mutually exclusive mechanisms can be envisaged for this AMD-induced dissociation of the fibrils containing incompleting pre-rRNA molecules. *First*, the whole transcriptional complex including the RNA polymerase is detached from the template; *secondly*, the nascent RNP fibrils are released from the still bound polymerase; *thirdly*, proteins dissociate from the nascent pre-rRNA and thus render the RNA molecule undetectable by the phosphotungstic acid stain; or *fourthly*, the pre-rRNA is processed or degraded in the still attached RNP fibrils, which results in the disintegration of the latter. The observation of Goldberg et al. (29) that AMD bound to calf thymus DNA displaces RNA polymerases lends some support to the first possibility. Such a mechanism could also serve as a basis for explaining the cooperative effect in fibril release if one assumes that the binding strength of AMD is enhanced at DNA sites that are not occupied by RNA polymerases (e.g., 2). This would facilitate, perhaps by a local alteration of the physical state of the DNA (e.g., 55), the dissociation of adjacent RNA polymerase molecules. Other authors, however, did not observe such an AMD-induced dissociation of polymerase molecules from DNA (2, 7). In favor of the second possibility, that the lateral fibrils are released from the polymerase molecules which remain bound to the DNP axis, is our observation of distinct electron-dense granules with a diameter of ca. 150 Å still attached to the fibril-free rDNP axes (e.g., Figs. 6 and 7) in the AMD-treated oocytes. Similar granules have been tentatively identified by Miller and Beatty (52) as RNA polymerases. These authors have also discussed the possibility that RNA polymerases might not leave the template after termination of transcription but might slide between two "read" intercepts (cistronic rDNA segments) along the spacer segment (51). By this interpretation, termination under normal conditions would result in the release of only the finished pre-rRNA molecule but the RNA polymerase would remain on the template-containing DNP strand. The fourth possibility mentioned is very unlikely in view of our present

data. It should primarily affect the lengths of the lateral fibrils but not reduce the axial lengths of the fibril-covered matrix unit, provided one does not assume that this RNA degradation preferentially occurs at the basal region of the nascent fibrils. Moreover, our biochemical studies show no indication of a rapid breakdown of the diverse pre-rRNA classes.

In a previous article (64) we have shown that, in untreated amphibian oocytes, sometimes matrix units can be found from which lateral RNP fibrils, single or in groups, were lost from the rDNP axis. This may reflect the *in vivo* situation in the specific oocyte or nucleolus or could be merely a preparative artifact. Such observations, however, do not detract from the significance of the present finding of the AMD-induced dissociation of RNP fibril material, since such incomplete matrix units are very infrequent and isolated compared to the AMD-treated samples.

Our observation of an AMD-induced detachment of RNP fibrils from the template might also offer an explanation for the "condensing" effect of AMD on intranucleolar DNP (e.g., 13, 37, 54), since the rDNP after stripping off of the RNP fibril material, under the ionic conditions present (see 11, 56), should be expected to change to a "condensed chromatin" state, i.e., assume a greater packing density of DNA. Such a structural change of the intranucleolar DNP also might contribute to the segregation of the fibrillar and granular portions (see introductory paragraph). In the case of the amplified nucleoli in *Triturus* oocytes, the condensation of the rDNP and its asymmetrical localization has been clearly demonstrated by Ebstein (13) using [³H]AMD autoradiography. Recher and co-workers (54) have recently examined the effect of AMD on the nucleolar ultrastructure in a human cell line. By analogy with the model of the AMD-inactivated and retracted lampbrush chromosome loops, which show a similar release of matrix material (70, 71), they proposed that the primary effects of the drug are a condensation and retraction of the nucleolar DNP (for related findings in plant nucleoli see 37). An especially instructive illustration of the view that the segregated fibrillar moiety in AMD-treated nucleoli contains the condensed rDNP is presented in the article of Fakan and Bernhard (e.g., Fig. 9 in reference 17). It may well be that the reported "segregations" which occur during natural nucleolar inactivation processes (for literature see, e.g., 9, 23) are also caused by a condensation

of the rDNP, subsequent to the release of associated pre-rRNP. The many demonstrations of nucleolar segregation during natural processes and in the presence of drugs such as α -amanitin (e.g. 46, 48) that do not specifically bind to nucleolar components speak against the generalized postulate by Goldblatt et al. (30) that nucleolar segregation is due to the binding of AMD rather than to inhibition of RNA synthesis or impaired nucleocytoplasmic transfer. In our opinion, it is the release of the pre-rRNP fibrils, irrespective of the method of induction, which results in the condensation and segregation phenomena.

Our biochemical and autoradiographic data show that the RNP fibrils released from the rDNP in the presence of AMD do not leave, to any considerable extent, the nucleus even after prolonged chase periods (in agreement with the reports of an impaired intranuclear and nucleocytoplasmic translocation of nucleolar RNA in AMD-treated cells; for references see introductory paragraph). In contrast to the findings of Snow in *T. cristatus* (69), our autoradiographic results indicate that the majority of newly synthesized nucleolar RNA is retained, in the presence of AMD, within the confinements of the nucleolus, although no longer attached to the rDNP. Therefore, we assume that the released RNP fibrils tend to loosely adhere to the various nucleolar structures, especially within the granular portion. This effect of AMD on the released RNP fibrils of the nucleolus is obviously in contrast to the effectively unimpaired nucleocytoplasmic translocation of other classes of nuclear RNA, including those containing mRNA sequences (for recent discussion see 15).

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