# THE EFFECTS OF ACTINOMYCIN D ON THE ULTRASTRUCTURE OF THE NUCLEUS OF THE AMPHIBIAN EMBRYONIC CELL

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ABSTRACT

Observations have been made of ultrastructural modifications induced in the nuclei of differentiating amphibian embryonic cells cultured in the presence of Actinomycin D. Of particular interest are regions within the nucleus (regions otherwise rather empty) containing loose groupings of uniform threads having a diameter of around 200 A. These threads have been observed in continuous lengths up to 0.5 $\mu$, and appear to be composed of subfilaments. It is suggested, after taking account of some recent work on lampbrush chromosomes, that these threads are lengths of uncoiled chromosome in a condition of heterosynthetic inhibition. It is further suggested that active and inactive portions of the genome may be distinguishable by the facility with which they can be induced to undergo this ultrastructural modification.

Actinomycin D (AMD) inhibits RNA synthesis dependent upon DNA (1–3), but RNA synthesis dependent upon RNA is unaffected by it (3). DNA synthesis is not affected materially by low concentrations of AMD (4). Actinomycin apparently binds to DNA (5), probably specifically with guanine (6,7), in bringing about its biological effects. These properties of AMD have led us to study its effects on cell differentiation *in vitro* using the technique of small aggregate cultures of amphibian embryonic cells developed in this laboratory (8). In the course of this work, we are studying the effects of AMD administration on the ultrastructural appearance of developing cells. The present report concerns the appearance of structural changes in the nucleus following treatment of the cells. These changes are described and also discussed in relation to nuclear function in the light of the known effects of AMD.

## MATERIAL AND METHODS

*Rana pipiens* embryos were obtained by induced breeding (9), and reared to neural folds, Shumway stage 14. Regions of prospective muscle cells were excised in the first experiment, neural crest in the second. These fragments were disaggregated in calcium- and magnesium-free Barth's solution following a 20-minute pretreatment in 0.05 per cent Bacto Trypsin (Difco, Detroit). Single cells and small groups were cultured in a medium consisting of 0.1 per cent Bovine Plasma Albumen Crystalline (Armour, Chicago) in Barth's solution as previously described (8). When the cells had begun to differentiate, but before the formation of striations or pigment, the culture fluid was changed. Control cultures received the same medium as before; the remainder received the same with the addition of either 0.1 $\mu$g per ml or 2 $\mu$g per ml of AMD. The cultures were maintained at 21 °C for a further 16 to 24 hours. At the end of this period the cultures were photographed in the living state under the phase contrast microscope, and fixed in cold, buffered osmium tetroxide for 20 minutes. Myoblasts were embedded in Araldite, neural crest cells in methacrylate, and sectioned at 500 to 700 A. The sections were mounted on carbon Formvar filmed grids, stained in uranyl acetate for 30 minutes, and observed in an AEI EM6 electron microscope.

## OBSERVATIONS

### *Phase Microscopy*

At the time of fixation some of the myoblasts in control and AMD-treated cultures had acquired visible striated regions, and the pigment cell component in the neural crest cultures had started to accumulate perinuclear pigment in control cultures but not in those treated with AMD. During the treatment period some of the neural epithelial cells included in the treated neural crest cultures formed actively elongating growth cones. In the case of the myoblasts and pigment cells, therefore, the beginning of AMD treatment coincided with the formation of the first definitive products of cytodifferentiation in control cells. The neural epithelial cells were at an earlier stage in their morphological differentiation.

The phase microscope revealed no differences between treated and control cells in the appearance of cytoplasmic detail. The nuclei in the treated cells, however, were distinctly different from those in the untreated cells. The nuclei in control cultures were rich in phase-dense areas of irregular shape. Such areas were, in general, reduced in the nuclei of AMD-treated cells to a smaller number of evenly spaced isodiametric blobs. The nucleoli in control cells were large and amorphous. Nucleoli in AMD-treated cells were only half the diameter of those in the controls, and were considerably more phase-dense, and spherical.

### *Electron Microscopy of the Nucleus in Control Cells*

Excluding the nuclear membrane and the nucleolus, the bulk of the nucleus (Fig. 1) appears to be composed largely of material of indeterminate structure. Granular components having a definable and measurable structure may, however, be discerned. These fall roughly into two groups which may be referred to as large granules and small granules.

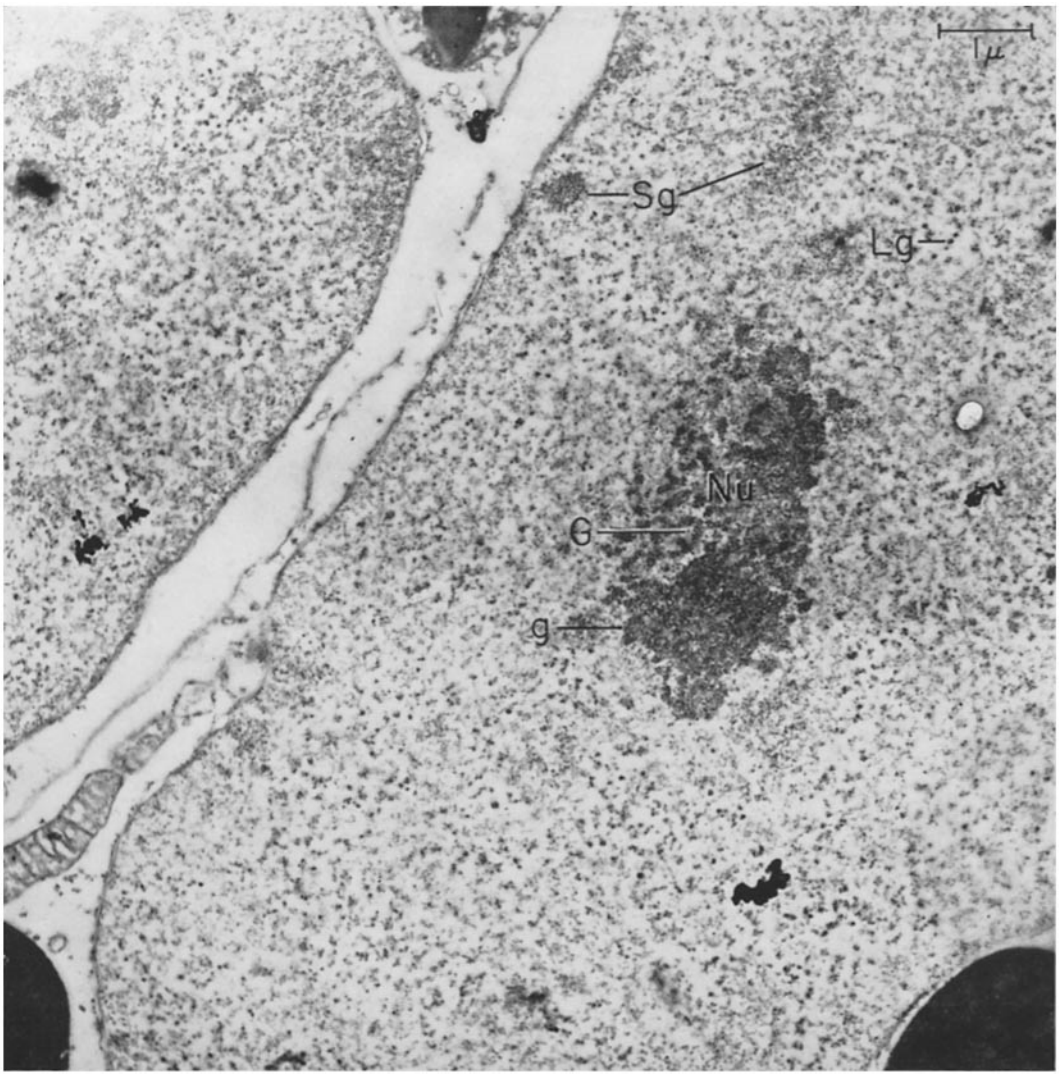
The large granules have diameters varying between approximately 150 and 250 A. The smaller granules vary roughly between 30 and 100 A in diameter. Both kinds of granules appear to be present in occasional dense clumps in addition to being scattered throughout the nucleus.

The nucleoli (Fig. 1) appear to be composed of large and small granules in greater concentration than in the surrounding chromatin. The more central portion of the nucleolus is composed of the smaller granule component, apparently to the exclusion of the larger granules, whereas the peripheral region of the nucleolus is composed of both kinds of granule, with the larger granules more in evidence. Both types of granule occasionally appear diploform. The peripheral zone of the nucleolus also contains regions empty of electron-opaque material.

### *The Nucleus in AMD-Treated Cells*

The general appearance of the nucleus in treated cells suggests a structural simplification. The chromatin now consists mainly of a fuzz of indistinct filaments (Fig. 2), the larger and smaller granules having largely disappeared. This is not evenly distributed throughout the nucleus, denser accumulations being found in some regions compared to others. These nuclei also contain some extremely dense masses of substance whose structure is difficult to discern but which appears to be granular (Figs. 2 and 3). The granular bodies range in size from 1000 to 5000 A and are irregular in outline. These masses have a density of the same order as that of the nucleolar remnant (Fig. 2), but they are irregular in outline. The nucleolar remnant is roughly spherical and composed of a compact mass of dense substance of almost crystalline appearance, but the granules characteristic of the untreated nucleolus cannot be clearly discerned. Around the remnant fine radial threads may be seen.

Within the nucleus of AMD-treated cells, threads are observed which are quite unlike any component in the normal nucleus. They occur in loose groupings within circumscribed areas of the nucleus comparatively empty of other structures (Figs. 2 and 3). These areas measure from 0.25 to 2 $\mu$ across, and one or two of them are encountered in about two-thirds of the nuclear sections. (The nuclei themselves in the cultured living cell are usually oval in shape with a longer diameter, around 30 $\mu$, and are flattened by the deformation of the cell consequent upon its adhesion to the culture vessel. The plane of section, being parallel to the substratum, passes through the greatest possible area of the nucleus.) These particular areas do not appear to bear a special relationship to the nuclear membrane or the nucleolar remnant. They are frequently




All illustrations from $OsO_4$-fixed, methacrylate-embedded, uranyl acetate-stained material.

FIGURE 1 Low power view of the greater part of a nucleus in control culture. The section passes through the more peripheral parts of the nucleolus (*Nu*) which contains large (*G*) and small (*g*) granules. The rest of the nucleus is composed of material of rather indeterminate structure within which large (*Lg*) and small (*Sg*) granules may be seen. × 12,500.

surrounded by several of the dense 1000 to 5000 A granular bodies mentioned above. The threads are about 200 A thick and present a uniform, unbranched appearance at higher magnifications (Fig. 4); individual threads have measured up to 0.5 μ in length in sections 700 A thick. Their appearance in transverse section at high magnification occasionally suggests that they are composed of a small but unknown number of subfilaments. It is not clear from the longitudinal aspect whether these subfilaments are coiled. The regions between the 200 A threads are seen in some sections to be occupied by filaments (Figs. 2 and 4) which vary in diameter from around 40 A to around 100 A. In other sections such finer filaments are not seen (Fig. 3). Those

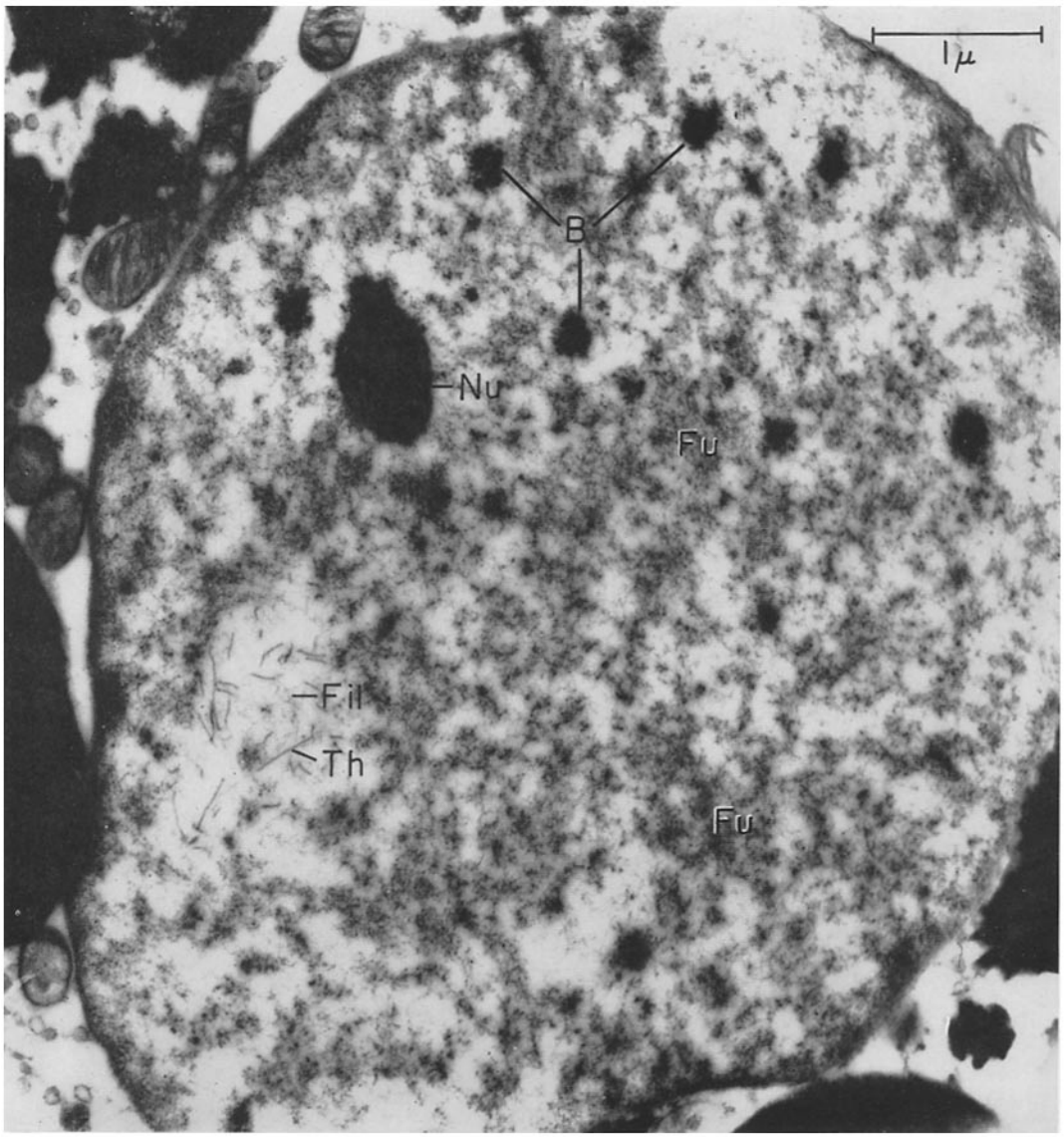



FIGURE 2 Low power view of nucleus from treated culture. Section passes through the nucleolus (*Nu*) which is denser and less voluminous than in controls. The chromatin parts of the nucleus have largely lost the granular appearance they have in controls and show indeterminate fuzzy structure (*Fu*) containing denser accumulations of substance (*B*) of a possibly granular nature. Well defined threads (*Th*) have appeared in one region and are surrounded by less well defined filaments (*Fil*). × 23,000.

regions showing the fine 40 to 100 A filaments tend to show less of the larger 200 A threads, and *vice versa*.

The uniformity of presentation of these 200 A threads within comparatively empty regions of the nucleus is underlined by the fact that identical observations have been made on two cell types (myoblasts and neural crest) exposed to two different concentrations of AMD, different by a factor of 20, in preparations embedded both in Araldite and in methacrylate.

## DISCUSSION

Previous authors have dealt, in more or less detail, with the ultrastructural aspects of the nucleoplasm in untreated amphibian cells (10)

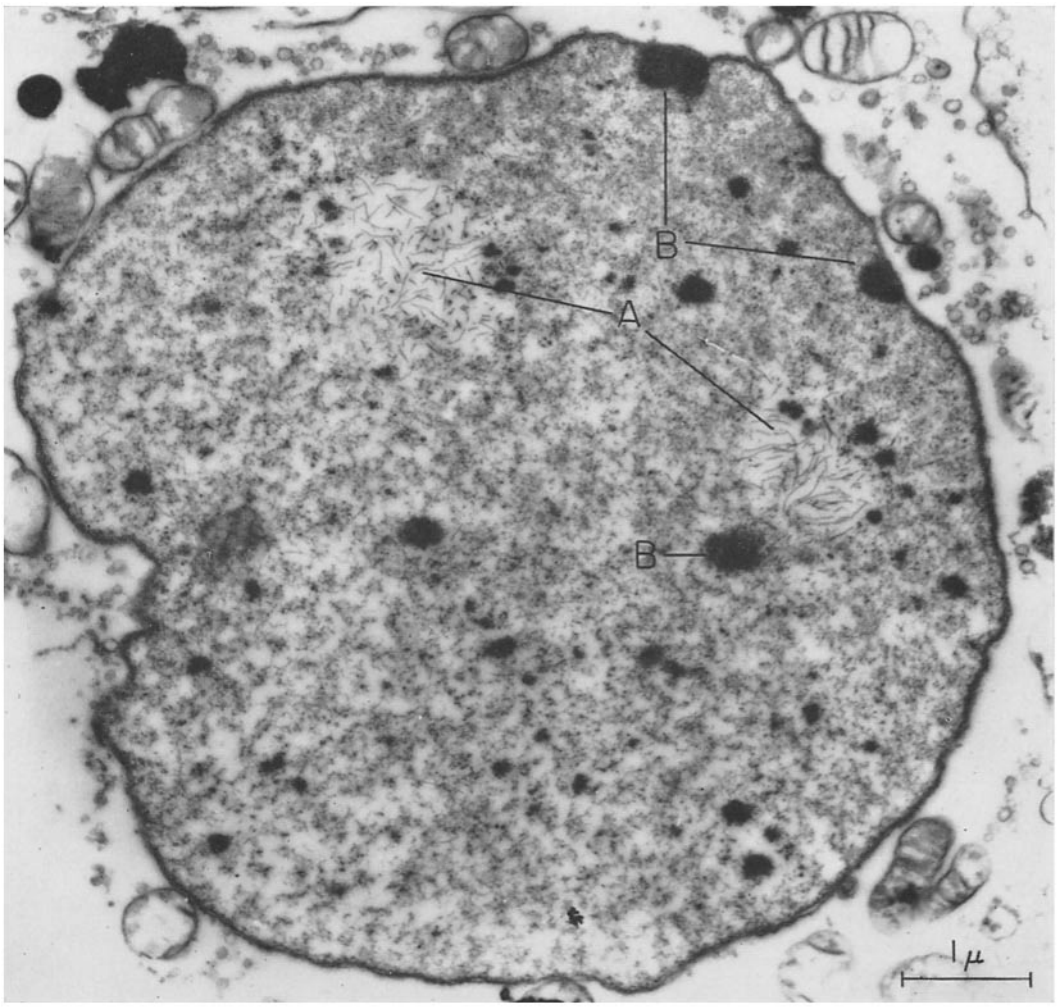



FIGURE 3 Low power view of nucleus from treated culture, showing two quite discrete regions (*A*) containing many threads of the kind shown in Fig. 2. In these regions no filaments appear and the threads have a comparatively greater density. Regions of accumulation of granular substance (*B*) are well defined. × 17,300.

and in Actinomycin-treated HeLa cells (11). No previous account, of which we are aware, however, has described the 200 A threads which we find in the present material. The following discussion will, therefore, be largely confined to considering the possible nature of these structures.

A previous account of the ultrastructure of AMD-treated nuclei in HeLa cells (11) was based on material receiving treatment almost identical with that described here. It is of considerable interest, therefore, that similar threads were not described, and appear to be absent from the illustrations published. The possibility that technical factors may influence the appearance of the threads in treated materials seems to us remote, and therefore the remaining possibility, that the threads reflect something unique to certain cells, is being investigated.

Three possibilities suggest themselves in interpreting the nature of the threads described in the AMD-treated interphase nuclei. These are as follows: (i) the threads are brought about by the AMD denaturation of DNA (12), or are to be considered as an artifact caused directly by AMD

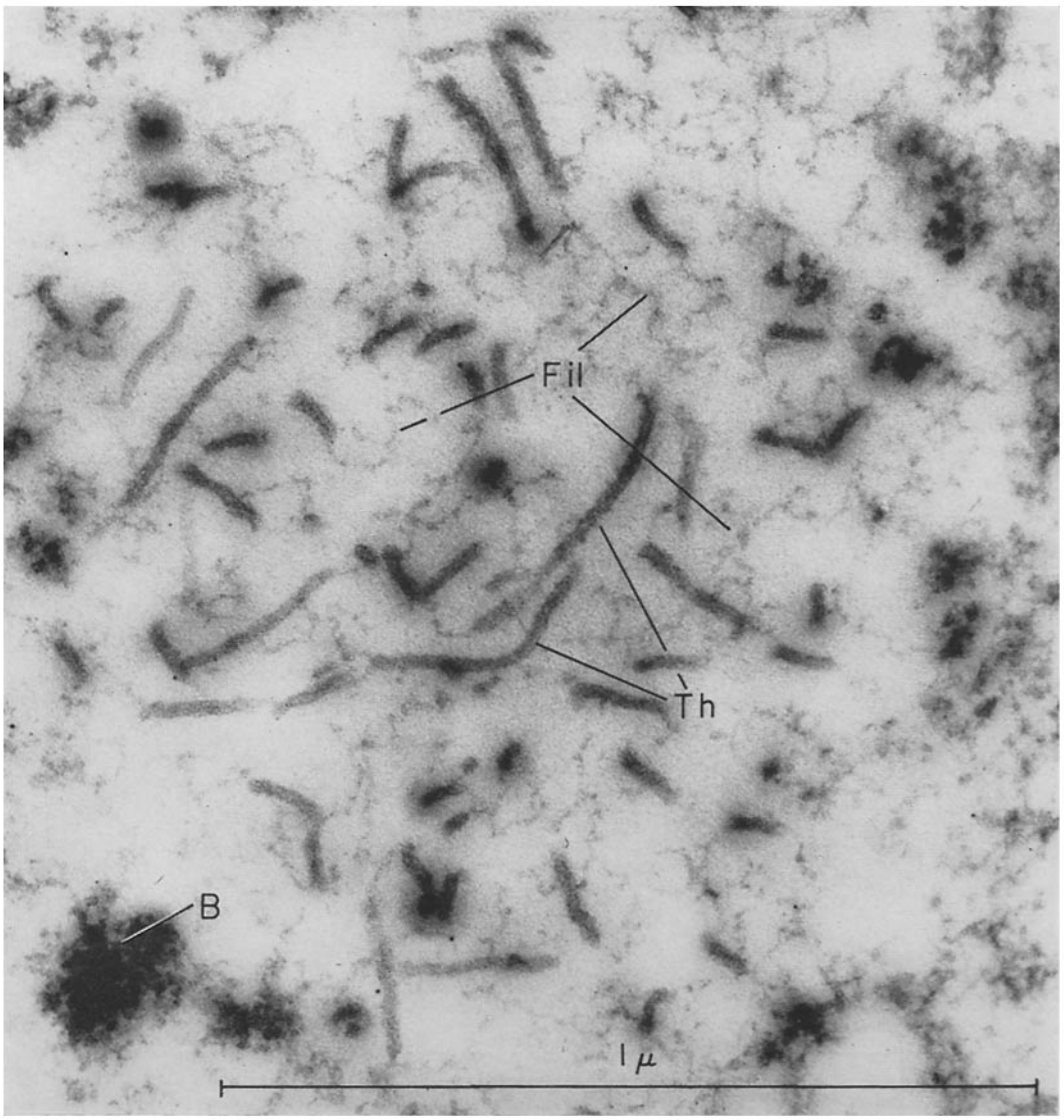



FIGURE 4 High power view of a region of the nucleus in a treated cell, showing threads (*Th*) and filaments (*Fil*). At lower left is a region (*B*) of dense accumulation of granular material. × 108,000.

binding to DNA; (ii) they are present in the untreated nucleus and become visible only because AMD, by interfering with RNA metabolism, causes a reduction in the amount of the chromosomal (heterosynthetic) products of the nucleus and renders the residual material clearer; (iii) they are produced by chromosomal structural changes consequent upon the loss of certain chromosomal functions following binding of AMD.

Possibility (i) suggested itself following a recent brief mention of the denaturing action of AMD on DNA (12). We consider this action to be unlikely to provide an explanation of the threads, since denaturation would be most unlikely to produce such ordered structures of these dimensions. DNA is, furthermore, highly resistant to denaturation, which would not, we feel, be reconciled with the low-dose response observed. The absence of a dose-response effect when the dose is increased by a factor of 20 is a further argument

against this proposal. In consideration of a recent account of the stereochemical stability of DNA, when binding about one molecule of AMD for each 18 DNA nucleotides (13), we think it also unlikely that the chromosomal nucleohistones would suffer direct alteration of such a magnitude as to be supramolecularly visible in the present form, particularly at the low doses employed. We consider this to be unlikely even in the event of preferential binding at these sites. Thus, while the slight possibility remains, we consider that direct chemical activity of the kinds discussed contributes least to the present findings.

In favour of explanation (ii), attention may be directed to the undoubted reduction in the amount of granular substance, regarded as ribonucleoprotein, in the nucleus, particularly in the vicinity of the nucleolus and the 200 A threads. However, we consider it unlikely that such well defined threads would escape detection through being obscured by the presence of this material. It is, nevertheless, certain that the absence of granules must contribute to the extremely uncluttered nature of the surrounding regions, thus affording the best possible view of the threads.

As for possibility (iii), a recent report of the effects of AMD on the loop structures of the amphibian oocyte chromosomes, which are thought to reflect the metabolic activity of these chromosomes (14), demonstrated that these regions are structurally modified following AMD treatment. The authors of that report interpreted their findings in favour of the view that the structural modification they observed followed the inhibition of RNA synthesis by AMD, as demonstrated by radioautographic studies. They pointed out that binding to DNA by a modifie AMD which did not interfere with RNA synthesis did not produce the observed change in morphology. This evidence is strongly suggestive, but does not conclusively demonstrate, that chromosomal morphology is, in this case, a direct expression of physiologic activity. This view of the present observations (*i.e.*, that the ultrastructural modifications are caused by the inhibition of RNA synthesis) means that the relatively clear regions about the threads are occupied in the untreated nucleus by the homologues of the lampbrush lateral loop structures, whatever form these may take in the interphase nucleus. Therefore, we are tentatively of the opinion that the threads observed in this study are chromosomal in nature and that they are rendered visible in the treated nucleus by a modification of the chromosomal structure following reduced heterosynthesis.

The main evidence for this view is, of course, circumstantial at present. It may strengthen the case, however, to draw attention to the fact that the thickness of these threads compares well with published accounts of the dimensions and, in some cases, the appearance of non-interphase chromosomes, to which the present threads bear comparison, in the physiological sense, if one allows the present tentative conclusions. For example, Ris and others (see 15, for review) have suggested, on the basis of many measurements made from electron micrographs, that the basic morphological unit of the chromosome is a fibre 100 A thick. The micrographs of sperm heads and of nuclei in certain stages in spermatogenesis published by this author (15) show groups of filaments which resemble the threads described herein but which are half their diameter, corresponding more closely to the greatest apparent diameter of some of the finer filaments in their immediate vicinity. It is claimed that two fibrils are associated in the interphase chromosome to produce fibres around 200 A (15, p. 101). It may, therefore, be suggested that the threads observed in the present study are derived from the finer filaments. This suggestion is reinforced by the finding that regions showing more fine filaments have less threads, and *vice versa*. This would be explained if the finer filaments were incorporated into the 200 A threads. At present, there is no indication of the number of filaments composing one of the 200 A threads, if such is, indeed, the case. Tomlin and Callan (16) consider the main axis of the lampbrush chromosome in amphibians to be 200 A in width (see also 17). Other estimates (18) do not agree quite so closely with those quoted. The fact remains that there is good evidence that amphibian chromosomes may, at some periods, be of the dimensions of the threads we have observed.

The status of the threads depends, to some extent, upon whether they are envisaged as having been engaged in activities metabolically distinct from those of the surrounding chromatin at the time of treatment. Evidence supporting this view is as follows. If the effect is due to an equal reactivity of all of the chromatin, then it must be explained in terms of likelihood of random con-

tact between any chromatin and AMD. In this event, we should expect the effect either to extend to all of the chromatin or to be greatest immediately within the nuclear membrane. This is not the case in any observation so far. Furthermore, we consider it unlikely, in these circumstances, for discrete areas to show the effect; a more diffuse presentation would be expected. Also, one might reasonably expect the proportion of the chromatin showing the effect to vary according to the dose level of the treatment. We have observed no obvious variation ascribable to differences in dose levels of 20 times. It is, perhaps, not too speculative to discuss the status of the threads in relation to the large amount of work which demonstrates that differential effects occur within, and among, the chromosomes in response to agents which cause breakage (19), and with respect to synchrony of duplication as demonstrated by isotope uptake (20). The chromosomal regions differentially affected are sometimes morphologically distinct and identifiable with heterochromatin (21). As a working hypothesis, we therefore suggest that the threads described in the nuclei of our present material are chromosomal, and represent differentially active portions of the genome at the time of AMD treatment. This implies the possibility that AMD, and perhaps other substances, can be employed to distinguish differentially active portions of the chromatin through induced ultrastructural modifications.

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