Morphologic and Metabolic Alterations in Hepatic Cell Nucleoli Induced by Varying Doses of Actinomycin D

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SUMMARY

The effect of varying doses of actinomycin D on the nucleolar structure and RNA metabolism of hepatic parenchymal cells of the rat has been investigated. Maximal dissociation of the nucleolar components as well as maximal inhibition of RNA synthesis was seen at dose levels of the drug above 0.75 μg/gm body weight. Some gradation in the morphologic effect was seen, particularly at dose levels below 0.3 μg/gm body weight. Below this level portions of the nucleolonema persist, particularly in the periphery of the nucleolar mass, all nucleoli being affected. A dose of 0.01 μg/gm body weight produces no alteration in synthetic ability and does not produce a discernible morphologic change. Although breakdown of newly synthesized RNA is documented, and this too appears to be dose dependent, it was not possible to separate this effect from inhibition of synthesis. The exact mechanism of the morphologic alteration is still unknown. However, it is unlikely that inhibition of RNA synthesis or failure to transfer newly synthesized RNA from nucleus to cytoplasm is responsible, since regeneration of the nucleolus takes place while marked inhibition of synthesis persists and because ethionine, which produces inhibition of synthesis and prevents transfer to a similar degree, does not induce this morphologic change. Although enhanced breakdown of newly synthesized RNA remains a possible mechanism, it is postulated that the physical combination of the actinomycin molecules with the DNA molecules governing nucleolar synthesis may be more important than inhibition of synthesis, failure to transfer, or breakdown of newly synthesized RNA per se in producing this change.

INTRODUCTION

The demonstration that actinomycin D, an antibiotic known to inhibit DNA-dependent RNA synthesis (11, 13, 15, 17—19, 30, 34, 40, 41, 45, 46, 51, 52), (c) rapid acceleration of breakdown of some fraction of recently synthesized nuclear RNA (1, 55, 73), and (d) interruption of the normal transfer of ribosomal and transfer RNA from the nucleus to the cytoplasm (15, 17). The latter three effects are most likely secondary to the first. The purpose of this study was to begin to determine which cellular metabolic effects of actinomycin are intimately associated with the nucleolar alterations, in the hope that such information would enable a more penetrating analysis of the organizational pattern of the nucleolus in normal and neoplastic cells. The results of the first phase of this study are the subject of this communication.

MATERIALS AND METHODS

Animals. White female rats of Wistar strain (Carworth Farms), weighing between 160 and 200 grams, were used in all experiments. The animals, maintained on Wayne Lab Block diets ad libitum, were fasted overnight prior to all experiments, except as noted below, but allowed free access to drinking water.

Injections. With one exception, all injections were administered intraperitoneally, and, except in the 12 hour experiments, the initial injection was given between 8 and 10 A.M. In one experiment, to test the rapidity of onset of the morphologic change in nucleoli, the test solutions were administered by the femoral vein under light nembutal anesthesia. An injection of saline, in a volume equal to the dose of actinomycin, was given to all controls. In earlier experiments, actinomycin D (gift of Dr. G. Boxer, Merck, Sharp and Dohme, Rahway, New Jersey) was dissolved in saline at a concentration up to 250 μg per ml. Because of light sensitivity, solutions were made fresh, and tubes and syringes were made light tight by wrapping in aluminum foil. In later experiments, the actin-
omycin was obtained as Cosmegen® (Merck, Sharpe and Dohme, West Point, Pennsylvania) in 500-μg Lyovac’s®, and appropriate dilutions were made with sterile water.

Biochemical Analyses

Solutions. Solution A. 0.35 M sucrose, 0.05 M Tris (tris(hydroxymethyl)aminomethane)-HCl buffer, pH 7.6, 0.025 M potassium chloride, and 0.004 M magnesium chloride.

Heavy Sucrose. 2.1 M sucrose, 0.001 M MgCl2, 0.0007 M ATP, and 0.0035 M potassium dibasic phosphate (K2HPO4), pH 6.8.

Scintillation Mixture. 5 gm PPO (2,5-diphenyloxazole) and 0.3 gm POP (1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, q.s. to 1 liter with toluene.

RNA and DNA Determination. RNA was estimated by modifications of the Schmidt and Tannhauser (53) procedure suggested by Munro and coworkers (12, 22). Rapidly excised livers were minced in a given volume (2.3 times liver weight) of solution A and homogenized by six up-and-down strokes with a loosely fitting Teflon-stainless steel pestle rotating at an approximate speed of 1,350 rpm. Duplicate aliquots of 0.4 ml of the total cellular homogenate were removed and suspended in 4.0 ml of ice-cold distilled water, to which were added 4.0 ml of 0.3 gm POP (1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene, q.s. to 1 liter with toluene.

Estimation of Radioactivity. The 5 mg of dry sample removed for assay of radioactivity were dissolved in 1 ml of hyamine by heating at 90°C for two minutes. After cooling, 15 ml of the toluene-scintillation mixture were added to each vial, and counts were made for 10 minutes in a Packard liquid scintillation counter.

Isolation of Nuclei. Nuclei were isolated essentially by the method of Chaveau et al. (7). The animals were killed by decapitation, and the livers were rapidly excised and placed in a beaker containing ice-cold solution A (2.3 times the liver weight). The livers were homogenized by six up-and-down strokes with a loosely fitting, Teflon-stainless steel pestle rotating at an approximate speed of 1,350 rpm. The samples were centrifuged (Spinco model L centrifuge, rotor no. 30) at 15,000 rpm for 15 minutes. The pellet was suspended in 60 ml of heavy sucrose solution by three up-and-down strokes of the Teflon pestle, and the samples were centrifuged at 25,000 rpm for two hours. The pellet was resuspended in 4 ml of ice cold-distilled water by ten up-and-down strokes of a glass-steel homogenizer rotating at approximately 1,000 rpm. Four milliliters of ice cold 20% TCA were added to this suspension and the lipid extraction and washing procedure was carried out as described for the total homogenate.

Experimental Plan. RNA metabolism was assessed in the following ways: To test synthetic ability, varying doses of actinomycin or saline were given at 0 time, followed in 60 minutes by a 20-minute period of labeling with 5 μc orotic acid-6-14C (Nuclear Chicago or New England Nuclear) administered intraperitoneally. The animals were decapitated, their livers rapidly excised, minced, and homogenized in ice-cold solution A, and RNA, DNA, and radioactivity of the samples were determined as outlined above (Chart 1). To detect synthetic ability after varying durations of actinomycin treatment, three doses were selected from the experiments above—1.25, 0.3, and 0.1 μg per gram body weight given at time 0—and they were allowed to act for 10 and 30 minutes, and one, two, four, twelve, and twenty-four hours, though not all time intervals were tested for each dose (see Chart 2). After the appropriate time interval, 5 μc of radioactive orotate were injected intraperitoneally, the animals were killed 20 minutes later, and their livers were handled as outlined above for determination of radioactivity as well as DNA and RNA content. To investigate the loss of prelabeled RNA, 3 μc of radioactive orotate were injected intraperitoneally, followed in two hours by an intraperitoneal injection of actinomycin in a dose of 1.25, 0.3, or 0.1 μg per gram body weight. Controls again received labeled orotic acid without injection of saline or actinomycin. At one and two hours after the injection of actinomycin or saline (three and four

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**Chart 1. Inhibition of orotate incorporation into hepatic RNA by various doses of actinomycin D acting for 60 minutes prior to a 20-minute pulse of 5 μc of orotic acid-6-14C. Each point represents 6 to 10 animals, and all determinations were made in duplicate. The values are calculated from specific activities of RNA preparations and are shown as a percentage of the values of their saline-injected controls, with the range of percent indicated by the bars.**
hours after orotate), pairs of animals were decapitated and the livers rapidly excised, weighed in an ice-cold tared beaker containing solution A, and minced and homogenized as indicated above. Radioactivity in nuclear and total cellular RNA, as well as the DNA and RNA content, was determined on duplicate samples.

Electron Microscopy. Tissue for electron microscopy was obtained from the left lateral lobe of the liver, either from animals used for biochemical determinations or from separate animals treated solely for morphologic study. Tissue from at least two experimental and one control rat was examined at each of the following dose levels: 0.01, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 1.0 μg/gm body weight at 80 minutes following the injection of actinomycin or saline. Additional doses and times examined are summarized in Table 1.

The tissue was prepared by mincing in 2% osmium tetroxide (OsO₄) buffered to pH 7.4 with 0.2 M s-collidine buffer (2) and was fixed for two hours at 0 to 4°C. Dehydration through a graded series of alcohols was carried out at room temperature, and tissue was embedded in Epon-Araldite mixture (32.5 ml Epon 812; 5 ml Araldite 502; 67.5 ml dodecenylsuccinic anhydride (DDSA) and 3 ml DMP-30®; D. Svoboda, personal communication). Sections 0.5- to 1.0-micron thick were cut on a Porter-Blum MT1 microtome and stained with toluidine blue (68). From these thick sections, tissue from portal, central, and indeterminate areas of the liver lobule was selected for electron microscopy. Thin sections were cut with a Cambridge-Huxley or Reichert O MU2 ultramicrotome, with glass or diamond knives, picked up on uncoated 300-mesh copper grids, and stained with lead (47) or uranyl acetate (71) and lead. With the exception of a few micrographs which were taken on the Philips EM 200, sections were examined in a Philips 100 B electron microscope with a 25-micron apertures and an accelerating voltage of 60 kv, and photographed at negative magnifications of 1,300 to 7,500 times.

RESULTS

Biochemical Analysis

Dose Response in Inhibition of RNA Synthesis. The effect of incorporation of orotate-¹⁴C into total rat liver RNA 60 minutes after graded doses of actinomycin D is shown graphically as percent of control values in Chart 1. Because later experiments showed a decrease in nuclear RNA, the values were calculated as percent of control counts per minute per milligram of DNA as well as RNA. However, since the difference was found to be negligible for total cellular values, we continued to express them in terms of the counts per milligram RNA. The data are expressed in terms of percentage of their own control values, since daily fluctuations in total incorporation of radioactivity were great enough for controls to make it difficult to average all control and experimental values. In a given experiment, however, there was internal consistency for control and experimental values. At a dose of 0.01 μg per gram body weight, there was no significant inhibition of orotate incorporation; however, a sharp increase in inhibition of RNA synthesis subsequently occurred, and, at a dose of 0.3 μg per gram body weight, approximately 50 percent inhibition of RNA synthesis was observed (Chart 1), as compared to controls injected with saline. Above a dose of approximately 0.75 μg per gram body weight, RNA synthesis was maximally inhibited (80—90%), over a 20-minute labeling period.

Time Response in Inhibition of RNA Synthesis. To determine the evolution and recovery of inhibition of RNA synthesis following the injection of actinomycin, three doses were examined: 1.25 μg per gram body weight, which had been found to inhibit synthesis maximally, after 60 minutes: 0.3 μg per gram body weight, which produced about 50 percent inhibition, and 0.1 μg per gram body weight, which produced only about 30 percent inhibition of RNA synthesis. The results are summarized in Chart 3. In ten minutes, the largest dose (1.25 μg/gm) produced a maximal effect and, although there was a slight rise over the next three hours, the inhibition remained virtually constant, as far as was tested, i.e., until twelve hours after the intraperitoneal injection of the drug. At this time, the animals were sick, and most of them had severe diarrhea. Previous experiments have shown that the maximal

**Table 1**

<table>
<thead>
<tr>
<th>Doses of actinomycin D</th>
<th>Times examined</th>
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<tbody>
<tr>
<td>0.01</td>
<td>80 min (2)</td>
</tr>
<tr>
<td>0.1</td>
<td>80 min (1), 140 min (4), 4 hours (2)</td>
</tr>
<tr>
<td>0.2</td>
<td>80 min (3)</td>
</tr>
<tr>
<td>0.3</td>
<td>30 min (2), 80 min (4), 4 hours (2)</td>
</tr>
<tr>
<td>0.4</td>
<td>12 hours (4), 24 hours (3), 48 hours (2)</td>
</tr>
<tr>
<td>0.5</td>
<td>80 min (2)</td>
</tr>
<tr>
<td>0.6</td>
<td>80 min (3)</td>
</tr>
<tr>
<td>1.0</td>
<td>80 min (2)</td>
</tr>
<tr>
<td>1.15</td>
<td>12 hours (2)</td>
</tr>
<tr>
<td>1.25</td>
<td>5 min (2)</td>
</tr>
<tr>
<td>2.3</td>
<td>4 hours (2), 6 hours (2)</td>
</tr>
<tr>
<td>Saline-injected controls</td>
<td>5 min (1), 30 min (1), 80 min (9)</td>
</tr>
<tr>
<td></td>
<td>120 min (1), 4 hours (3), 6 hours (1)</td>
</tr>
<tr>
<td></td>
<td>12 hours (2), 24 hours (2), 48 hours (1)</td>
</tr>
</tbody>
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Doses and times examined by electron microscopy.

*Micrograms per gram body weight.

*Number of animals examined in parentheses.

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doses are lethal beginning at about 16 to 18 hours; therefore we did not prolong this part of the study. With 0.3 μg/gm, there was a similar slight enhancement of the inhibition over the first few hours, followed by a slight recovery of synthetic ability at 12 hours and somewhat greater return towards control levels at 24 hours, although recovery was not complete even at this latter time. With the lowest dose tested (0.1 μg/gm), synthesis appeared to return to control levels within four hours after an intraperitoneal injection (Chart 2).

**Loss of Radioactivity from RNA.** In prelabeling experiments, the radioactivity of the nuclear fraction increased over the first three hours and then by four hours fell to the two-hour level in control animals, whereas total cellular label increased over the entire four-hour period (Chart 3). When a dose of actinomycin of 1.25 μg per gram body weight was given intraperitoneally two hours after the orotate injection, the radioactivity of both nuclear and total cellular RNA fell within one hour after the actinomycin injection to about 50% of the value of control animals and remained constant over the remaining hour (Chart 3). Following a dose of 0.3 μg/gm, the fall in radioactivity was not great but similarly remained constant at one and two hours. The radioactivity in the nuclear fraction was virtually the same in control and treated animals at four hours. If a dose of 0.1 μg/gm was given, the rise in nuclear label seen in controls did not occur, but the activity remained constant at about the level of the two-hour control. That is, after two hours of actinomycin treatment, at a dose of 0.1 μg/gm, treated animals had levels of activity in both the nuclear and total cellular RNA fractions which were almost identical to those of controls (Chart 3).

**Electron Microscopy.** Since our attention in these studies was centered on the morphology of the nucleus, and in particular the nucleolus, our description will be confined to these organelles. In each experimental group, portal, central, and intermediate zones of the liver lobule were examined in both control and treated animals. No objective difference was discernible in the morphology of the nucleus or of the nucleolus from different areas of the lobule, in the animals treated either with saline or actinomycin, at any of the doses examined. Changes were observed in the nucleoli of supporting cells as well as in parenchymal cells, but our description will be confined to the alterations in morphology of hepatocytes.

**Morphology of Parenchymal Cell Nuclei and Nucleoli.** Control. The fine structure of the nucleus and nucleolus (4, 6, 33, 67), particularly of rat liver, has been described previously (5, 35, 36), and our observations on animals injected with saline agree closely with these observations (Figs. 1, 2). The nomenclature of the components of the nucleolus has recently been revised (9), and we shall adhere to these terms. In thin sections of hepatic parenchymal cells, the nuclei are usually round or oval, centrally placed, and predominantly single, though cells with two nuclei may be seen. In general, the nuclei present a smooth contour, bounded by a double membrane, the outer one of which is studded with ribosomes at intervals and connected through nuclear pores with a smooth inner membrane. In tissue fixed primarily in osmium tetroxide, there is a diffuse distribution of chromatin, with only faint aggregation along the inner nuclear membrane and around the periphery of the nucleolus. The chromatin fibers are embedded in a matrix of low electron density, the nucleoplasm, which fills the interchromatinic space. In interchromatinic areas, aggregates of dense granules 100—250 Å in diameter, the interchromatinic granules, are seen. Individual granules of larger size, surrounded by a clear halo, the perichromatin granules (72), are also seen infrequently. Occasionally, also, inclusions of various sorts, such as those shown in Figure 3a, nuclear bodies (26) and lipid droplets, may be present within the nucleus.

The nucleolus is less regular in outline and is not membrane-bound. Frequently, two or more nucleoli may be seen in an hepatic cell nucleus. Even in osmium-fixed material, there is at least faint aggregation of chromatin surrounding the nucleolus, and this appears to interdigitate with the nucleolar substance (Fig. 2). The substance of the nucleolus is made up of at least two components: a particulate component consisting of dense granules about 150 Å in diameter and a fibrillar component composed of less dense filaments 40—60 Å in diameter (Fig. 2). These two components are arranged in a network, the nucleolonema, which surrounds areas resembling the interchromatinic substance, the so-called nucleolar vacuoles. Chromatin is often seen within the nucleolar vacuoles, and it seems likely

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**Chart 3.** The loss of counts from hepatic RNA prelabeled by intraperitoneal injection of 3 μC of orotic acid-6-14C two hours prior to injection of 0.1, 0.3, or 1.25 μg of actinomycin D per gram body weight. The upper (solid) line represents radioactivity of RNA of control animals decapitated one or two hours after a saline injection. Controls were done with each set of experiments, and slight variation in incorporation is reflected by somewhat greater radioactivity of controls for the higher doses. The dashed line represents the radioactivity in the actinomycin-treated animals at the same intervals following injection. Each point represents the average of determinations on four animals, and the total cellular values were determined in duplicate. The bars represent the range of specific activities.
that this connects with the peripheral nucleolus-associated chromatin (70). Perichromatin granules, when seen in our material, were somewhat more frequent in the area of the nucleolus-associated chromatin.

**Experimental.** Intraperitoneal doses of actinomycin D higher than 0.4 µg/g body weight produced fully developed nucleolar segregation within 80 minutes (Figs. 3, 8). This segregation or "nucleolar capping" consisted of apparent condensation of all nucleoli within a single cell, forming a smooth, more-or-less circular outline, with loss of the skin-like arrangement of the nucleolone. In addition, the particulate and fibrillar components separated to form individual portions of the nucleolar mass (Figs. 3, 8). As previously described (23, 50, 54, 69), at least three components were seen: aggregates of 150-200 Å particles similar to the particulate component of the normal nucleolus, dense clumps of fibrillar material (Fig. 8), and a dense component composed of fibrils with occasional granules (Figs. 3, 8, 10), previously designated microspheres (69) or plaques (20, 43, 65, 66). At high magnification, these plaques appeared to be made up of fibrils disposed in a tubular arrangement, which could be appreciated better in cross-section (Fig. 3). These plaques appeared five minutes after an intravenous dose of 1.25 µg/gm and were disposed adjacent to the nucleolone, both in the nucleolar vacuoles and in the region of the nucleolus-associated chromatin. No other change in nucleolar structure was obvious at this time (Fig. 9). Occasionally, electron dense granules approximately 150 Å in diameter were superimposed on these tubules. This tubulogranular component (the plaque) was less constant than the dense and light fibrils and the granular aggregates but was present in a large proportion of nucleoli. From the experiment using intravenous injection, it appeared to be the earliest morphologic change following a high dose, but we have not as yet observed these plaques in the nucleoli of animals treated with the lower doses (0.3 µg/gm or below) of actinomycin. Nucleoli in all parts of the liver lobule, and all nucleoli in a single cell, showed this dissociation of components. At this time period (80 minutes), changes in the remainder of the nucleus were subtle or absent. Occasionally, there was an accentuation of the chromatin pattern and there may have been more frequent aggregates of interchromatinic granules, but recognition of these changes is subjective at best, and they did not appear in all nuclei.

If tissue was examined 12 hours after the intraperitoneal injection of 1.15 µg/gm of actinomycin, a dose which results in the death of the animals, beginning around 24 hours (42, 55), nucleoli were difficult to identify (Fig. 10). Images consisting of aggregates of large electron-dense granules were seen, and occasional clumps of fibrillar material, usually without granules and suggesting remnants of altered nucleoli, were observed (Fig. 10). There was definite clumping of the chromatin, predominately against the inner nuclear membrane but also in association with presumed nucleolar masses. At times, chromatin clumps surrounded the dense granular accumulations (Fig. 10).

An intermediate dose, 0.3 µg/gm, produced a qualitatively similar, but slightly less complete, nucleolar rearrangement, 80 minutes after the actinomycin D injection. The evolution and recovery of this alteration will be described in detail elsewhere, but, as can be seen in Fig. 6, all the components of the nucleolus are altered. However, there seemed to be a more frequent preservation of the nucleolar vacuoles, a somewhat less complete aggregation of fibrillar components, which remained more in the configuration of a nucleolonema, particularly at the periphery, and plaques were not evident. It is interesting that all nucleoli showed a similar change. There appeared to be no lobular distribution, all parts of the liver lobule being equally affected. In contrast to the higher doses, at 12 hours the nucleolus showed substantial return to its normal configuration after a dose of 0.3 µg/gm. As illustrated in Fig. 7, there was reconstitution of the nucleolone with both particulate and fibrillar components disposed in the usual manner surrounding nucleolar vacuoles. However, this reconstitution appeared to be zonal within the nucleolus with the outer portion regaining normal form and the core consisting almost entirely of the 150-Å particulate component.

The lesion produced by a dose of 0.1 or 0.2 µg/gm was different from that seen with the maximal dose, when viewed at 80 minutes after the injection (Fig. 5). It differed only slightly from the lesion following 0.3 µg of actinomycin per gram body weight (Fig. 6) in that the nucleolone was more clearly defined, particularly in the periphery of the nucleolar mass. However, all the nucleoli had assumed a circular or oval outline, and the irregular appearance was lost. There did seem to be aggregation of the particulate component which was no longer randomly applied to the fibrillar portions of the nucleolus. Nucleolar vacuoles were present, often surrounded by the nucleolone, but the plaques were not seen. These changes too were present in all nucleoli, although the degree varied somewhat.

No objective change in the nucleolar structure was seen 80 minutes after an intraperitoneal dose of actinomycin D of 0.01 µg/gm per gram of body weight (Fig. 4).

**DISCUSSION**

It is evident from the results of this combined biochemical-ultrastructural study that no selection can be made as to which of the two metabolic effects of actinomycin D measured, inhibition of RNA synthesis and breakdown of preformed RNA, is most intimately related to nuclear segregation, when the inductive phase of this nucleolar alteration is observed. The smallest dose of actinomycin producing segregation also induced some inhibition of RNA synthesis and altered the labeling pattern of prelabeled RNA. Significantly, the two biochemical parameters and the morphology of the nucleolus showed a gradation of effects with varying doses of the antibiotic.

However, a distinct clue to a selection is evident from the study of the reparative or regenerative phase of the phenomenon with two doses of actinomycin. With a dose of 0.1 µg per gram body weight, nucleolar morphology substantially returned to normal after 140 minutes, although orotate incorporation was still 30% inhibited. This degree of inhibition during the inductive phase was associated with a clearcut but incomplete dissociation of nucleolar components. More convincingly, with a dose of 0.3 µg/gm, a substantial reformation of the nucleolonema and restoration of normal nucleolar morphology had taken place at 12 hours, despite the persistence of a considerable degree of inhibition of orotate incorporation (40 to 50%) at this time period. Thus, the nucleolar compo-
nents are able to become reorganized or reformed to produce an essentially normal-appearing organelle, even in the presence of evidence of continuing inhibition of RNA synthesis. These observations suggest that the nucleolar segregation is not due mainly, or exclusively, to interference with RNA synthesis, but is more likely associated with some other metabolic consequence of the binding of actinomycin D to DNA.

This tentative conclusion, which is in essential agreement with that of Simard and Bernhard (58), is further supported by a comparison of the morphologic consequences of inhibition of RNA synthesis in the liver with ethionine (35, 56) with those of actinomycin. Under conditions in which each agent inhibits RNA synthesis about 90%, actinomycin leads to obvious segregation of nucleolar components while ethionine does not (35, 56). The latter not only interferes with RNA synthesis but also interrupts the transfer of RNA from the nucleus to the cytoplasm (64). It does not, however, lead to the rapid breakdown of recently labeled nuclear RNA, as does actinomycin, and does not interfere with the loss of newly synthesized RNA induced by actinomycin, (G. A. Stewart and E. Farber, personal communications). Thus, the available evidence, from studies with actinomycin and ethionine, favors the hypothesis that nucleolar segregation is a reflection of the ability of the agent to bind to DNA and possibly to trigger the breakdown of some moiety of nuclear RNA as a consequence. It does not support the hypothesis that the nucleolar change is the result of either inhibition of RNA synthesis per se or the interruption of the transfer of RNA from the nucleus to the cytoplasm (28).

This tentative conclusion is also consistent with the results of studies on aflatoxin (8, 10, 14, 29, 61) and on several agents such as 4-nitroquinoline-N-oxide, (32), proflavin (57), and daunomycin and ethidium bromide (58), all of which induce a similar dissociation of nucleolar components and many of which become bound in one way or another to DNA (32, 61). In the case of aflatoxin, inhibition of hepatic RNA synthesis (8, 29) and RNA polymerase (14) and a rapid loss of nuclear RNA (61) have been reported.

A possibility that must be considered in the analysis of the reappearance of an organized nucleolus after segregation is the de novo regeneration at a different locus. If all the biochemical and structural effects of actinomycin are due to its binding to DNA, the persistence of the inhibition of RNA synthesis for many hours must indicate the continued binding of the antibiotic. Assuming that the initial binding was the trigger for the nucleolar dissociation, how can the reassembly of the components occur at the same locus? Presumably either the binding has been reversed at that locus or a new site on the DNA for regeneration or reorganization of the nucleolus has been opened up. Although this is at present highly speculative, its possibility has obviously important implications in regard to nuclear control mechanisms and genomic expression; this could be relevant to neoplastic transformation.

An observation of considerable importance is that the morphologic alteration with actinomycin was seen in all liver cells examined, even at the lowest dose at which a definite change was observable (0.1 μg/ml). This finding suggests that any level of inhibition of RNA synthesis in the whole liver (e.g., 20 to 30% at this dose) is a reflection of that degree of inhibition in all cells, rather than 100% inhibition in only a small proportion of cells. If this is truly so, it indicates that an analysis in depth of the response pattern of the liver to an agent such as actinomycin is theoretically feasible. A similar observation was reported by Kume et al. (28).

The observation that gradations of morphologic response can be produced by varying doses may well indicate that specific species of RNA are being selectively affected. That synthesis of specific types of nuclear RNA are affected by differing doses of actinomycin has been reported previously (19, 30, 38, 40, 41, 44, 51, 52, 62, 74), and it is possible that varying doses will yield further information about the identity of the various components of the nucleolus. It is interesting that the plaque component, which appears to be the earliest nucleolar alteration following ethionine (56) or high doses of actinomycin and which has been seen in a wide variety of circumstances (28, 31, 43, 48—50, 58, 65, 66) was not seen following the lower doses of actinomycin. This component, which has been shown to be composed of RNA and protein (69), may well reflect an alteration in a rather specific nucleolar component, which, however, is susceptible to many agents (28, 31, 43, 48—50, 58, 65, 66).

On the basis of the available evidence, we are in agreement with Simard and Bernhard (58) that nucleolar segregation is probably a response or a reflection of some alteration of DNA at or near the nucleolus, rather than the consequence of inhibition of RNA synthesis. Whether it is the triggering of breakdown of some essential RNA species consequent to this alteration or some other metabolic consequence has yet to be established. Hopefully, the further analysis of this type of model system in depth may clarify this important problem and may concomitantly lead to new insight into the organizational pattern of this organelle in relation to its important function in the synthesis and regulation of ribosomes.

ACKNOWLEDGMENTS

The authors are grateful to Miss Gloria DiLuiso and Mrs. Susan Porter for expert technical assistance, to the photographic staff of the Department of Pathology for excellent reproduction of electron micrographs and drawing of charts, and to Dr. Kenneth Shull for allowing us to use his facilities.

REFERENCES

Hepatic Cell Nuclei Alterations


Figs. 1—10. All figures represent tissue from the left lobe of the liver of female rats, fixed in 2% osmium tetroxide buffered with s-collidine. Thin sections of tissue embedded in Epon-Araldite were stained with lead citrate or uranyl acetate followed by lead citrate.

Fig. 1. Nucleus of hepatic parenchymal cell from saline-injected control rat. The round nucleus is separated from the cytoplasm by a double membrane which is interrupted at intervals by nuclear pores. There are faint aggregates of chromatin (Chr) disposed near the inner nuclear membrane and surrounding the nucleolus (Nuc). Scattered groups of interchromatic granules (Ig) and an occasional perichromatin granule (arrow) are visible. Clearer areas of interchromatinic substance are seen, especially adjacent to nuclear pores (double arrows). × 23,500.

Fig. 2. A higher power view of the nucleolus seen in Fig. 1. Chromatin (Chr) fibrils surround and interdigitate with the nucleolar mass which is made up of fibrillar (F) and particulate (P) elements arranged in a skein-like pattern, the nucleolonema, surrounding less dense nucleolar vacuoles (V). The particles measure approximately 150 Å in diameter, while the fibrillar areas can be seen in several areas to consist of dense fibrils approximately 40—60 Å in diameter separated by a less dense 60—80 Å core (arrow) which gives a tubular appearance when cut in cross section. The nucleolar vacuoles contain chromatin or material with low electron density similar to the interchromatinic substance. × 46,100.

Fig. 3. Nucleolus of hepatic parenchymal cell from rat injected with 0.6 µg of actinomycin D per gram body weight eighty minutes prior to decapitation. The nucleolenuma and nucleolar vacuoles are no longer seen, and the nucleolar mass has assumed a rounded contour. The fibrillar (F) and particulate (P) components have separated. Dense fibrillar plaques (PL) are present at the periphery of the fibrillar mass. Chromatin (Chr) is distinctly visible. The plaque component appears to consist of filaments (arrow) measuring approximately 40 Å in width and are separated by a space of approximately 50 Å. × 74,150. Insert a. Dense nuclear inclusions (arrow) from saline-injected control rat. They are composed of dense fibrils approximately 100 Å in diameter surrounding a less dense 70-Å core, which in cross section suggests a tubular arrangement (arrow). These inclusions, which do not show the characteristics of so-called nuclear bodies, have been seen in several nuclei of a number of control animals but have not been seen in association with the nucleolus. × 28,800.

Fig. 4. Nucleolus of hepatic parenchymal cell of rat injected with 0.01 µg of actinomycin D per gram body weight eighty minutes prior to decapitation. The fibrillar (F) and particulate (P) elements of the nucleolenuma are disposed about nucleolar vacuoles. Chromatin (Chr) is seen in association with the normal appearing nucleolar mass. × 28,500.

Fig. 5. Nucleolus of hepatic parenchymal cell of rat injected with 0.2 µg of actinomycin D per gram body weight eighty minutes prior to decapitation. The nucleolenuma has assumed a more oval outline, though distinct chromatin (Chr) masses are visible at the periphery. The particulate element (P) is aggregated, though a suggestion of the nucleolonema composed entirely of fibrillar material is still seen (arrow) and nucleolar vacuoles (V) are still present. × 30,100.

Fig. 6. Nucleolus of hepatic parenchymal cell of rat injected with 0.3 µg of actinomycin D per gram body weight eighty minutes prior to decapitation. The nucleolenuma has assumed a more regular oval outline and the nucleolonema is no longer distinctly seen. The fibrillar (F) and particulate (P) components have separated, though the fibrillar elements appear to be grouped mainly in the periphery and to surround areas of lower density resembling nucleolar vacuoles (V). The dense plaque component is not present, though distinct chromatin (Chr) masses are visible. × 29,900.

Fig. 7. Nucleolus of hepatic parenchymal cell of rat injected with 0.3 µg of actinomycin D per gram body weight twelve hours prior to decapitation. Orotate incorporation was still inhibited approximately 40% at this time. The nucleolonema composed of both fibrillar (F) and particulate (P) elements has reformed surrounding less dense nucleolar vacuoles (V). However, one central zone consisting entirely of 150-Å particles persists. × 26,200.

Fig. 8. Nucleolus of hepatic parenchymal cell of rat injected with 0.6 µg of actinomycin D per gram body weight eighty minutes prior to decapitation. The nucleolenuma, which lies adjacent to the nuclear membrane, has an oval outline and is composed of five distinct zones: a light fibrillar (LF) and a dark fibrillar (DF) component partially separated by a lucent area resembling a nucleolar vacuole (V), an area consisting entirely of 150-Å dense particles (P), and a dense plaque (PL) made up of fibrils disposed in a tubular manner. × 33,000.

Fig. 9. Nucleolus of hepatic parenchymal cell of rat injected with 1.25 µg of actinomycin D per gram body weight intravenously five minutes prior to decapitation. The nucleolenuma has an irregular outline and is composed of both fibrillar (F) and particulate (P) elements arranged in a nucleolonema disposed about lucent nucleolar vacuoles (V). However, a small dense plaque-like component is also present adjacent to the nucleolenuma either in the region of nucleolar vacuoles or in association with the nucleolus chromatin (arrow). × 28,300.

Fig. 10. Nucleus of hepatic parenchymal cell of rat injected with 1.15 µg of actinomycin D per gram body weight twelve hours prior to decapitation. There is distinct aggregation of the chromatin (Chr), particularly adjacent to the nuclear membrane and in association with a presumed nucleolar remnant consisting entirely of the fibrillar (F) and dense plaque-like (PL) elements. A mass composed of large granules (250—300 Å) and plaque-like material is also present (GP). × 17,700.
Hepatic Cell Nuclei Alterations
Morphologic and Metabolic Alterations in Hepatic Cell Nucleoli Induced by Varying Doses of Actinomycin D

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