

DISTINCTIVE CHARACTERISTICS OF NUCLEOLI OF TWO ESTABLISHED CELL LINES

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ABSTRACT

Nucleoli of cultured cells of the established lines KB and L were found to possess a distinctive fine structural organization. The major portion of the nucleolar volume was composed of compact, particulate material. Spheroidal fibrillar zones about 0.4μ in diameter occurred within the particulate mass. These fibrillar zones had a central light area and a denser rim. Toyocamycin treatment, which sharply inhibited the appearance of newly synthesized RNA in the cytoplasm, caused the gradual disappearance of the fibrillar material from nucleoli. Actinomycin D treatment, which inhibited virtually all RNA synthesis, caused varying types of segregation of nucleolar components. The morphology of nucleoli of KB and L cells and the reorganization of these nucleoli in response to drugs appear to be different from those of nucleoli of freshly initiated Chinese hamster and mouse cell lines.

It has long been recognized that one of the parameters that distinguishes cancerous cells from normal cells is increased nucleolar size and prominence in stained preparations (Pianese, 1896; MacCarty and Haumeder, 1934). This increase in nucleolar prominence is a correlate of an increased rate of RNA synthesis in cancerous cells (Caspersson and Santesson, 1942), but whether or not any qualitative difference in RNA metabolism occurs in malignant cells is not clear. Cell lines of neoplastic origin are also generally characterized by large nucleoli and rapid rates of RNA synthesis. Established cell lines of malignant origin, such as HeLa, have been the most widely used material for the elucidation of cellular RNA metabolism, and data on "normal" systems comparable to data for established cell lines are generally not available (although a few comparative studies have been done, see Matsuhisa et al., 1970). Many studies have compared the ultrastructure of neoplastic or transformed and "normal" cells and very few, if any, features have been found to be characteristic only of neoplastic cells. Yet, it would be surprising if the established cell lines of malignant origin,

which are so useful for biochemical analysis, were not atypical in some ways.

We have noted consistent differences in the fine structure of nucleoli in cultured cells of two established lines and fresh cell strains. We have also found that the morphological alterations in nucleoli which are brought about by two antagonists of RNA synthesis, toyocamycin and actinomycin D, are different in the two types of cells. The fine structure of nucleoli of cell strains of nonmalignant origin (mouse and Chinese hamster fetus-derived cultures in early passages) was described in the companion paper (Phillips and Phillips, 1971). In the present paper we contrast the nucleolar structure seen in these "normal" cells with the appearance of nucleoli in two established cell lines, one derived from a malignancy and one proven to have tumorigenic potential which is expressed upon injection into immunologically incompetent hosts.

MATERIALS AND METHODS

The two cell lines used in our experiments are KB 6 and L 929. The KB cell line was initiated in 1954

by biopsy of a human oral epidermoid carcinoma (Eagle, 1955). It was shown to produce tumors, in common with other cell lines of neoplastic origin, upon injection into the cheek pouch of Syrian hamsters (Foley and Handler, 1957). The KB cells used in our experiments were obtained from St. Louis University Medical School, Institute of Molecular Virology, by courtesy of Robert Murray. They were derived from a line of KBs (KB 6) which was cloned several years ago in the laboratory of Dr. Morris Green. At the time our experiments were performed, they were quite heteroploid with a modal chromosome number of 73. The L cell line was initiated in 1943 from subcutaneous connective tissue of a mouse previously treated with the carcinogen methylcholanthrene (Earle, 1943). The L clone NCTC 929 was derived from this line 5 years later (Sanford et al., 1948). Descendants of this clone were found to produce massive sarcomas when injected into isologous mice (Earle et al., 1950; Hall et al., 1967). The L 929 cells used in this study were purchased from Grand Island Biological Co., Grand Island, N.Y.

KB and L 929 cells were maintained in monolayer cultures in Falcon plastic flasks (Falcon Plastics, Div. of B-D-Laboratories, Inc., Los Angeles, Calif.) in Joklik's modified minimal essential medium (MEM) (Grand Island Biological Co.) supplemented with 10% fetal calf serum. Cells to be used for radioautography were grown on glass coverslips in 1 ml of medium in Leighton tubes. Those to be examined with the electron microscope were grown in plastic Petri dishes. Experiments were performed on the 2nd day after seeding when the cells were growing vigorously and had not yet reached confluency. Radioautography was performed as described in the companion paper (Phillips and Phillips, 1971) except that uridine-³H, SA 0.2 Ci/mmol was used at a concentration of 0.1 mCi/ml, and the radioautographs were exposed for 3 days. Techniques for electron microscopy were also as previously described (Phillips and Phillips, 1971).

RESULTS

Nucleolar Structure of Untreated KB and L Cells

Both KB and L cells in culture normally had variable numbers of roundish nucleoli which stained intensely and uniformly for RNA with azure B (Flax and Himes, 1952). In electron micrographs, the nucleoli commonly had a compact, rounded, or oval appearance (Figs. 1-6) which was quite different from the more irregular, loosely reticular appearance characteristic of nucleoli of mouse or Chinese hamster cells from

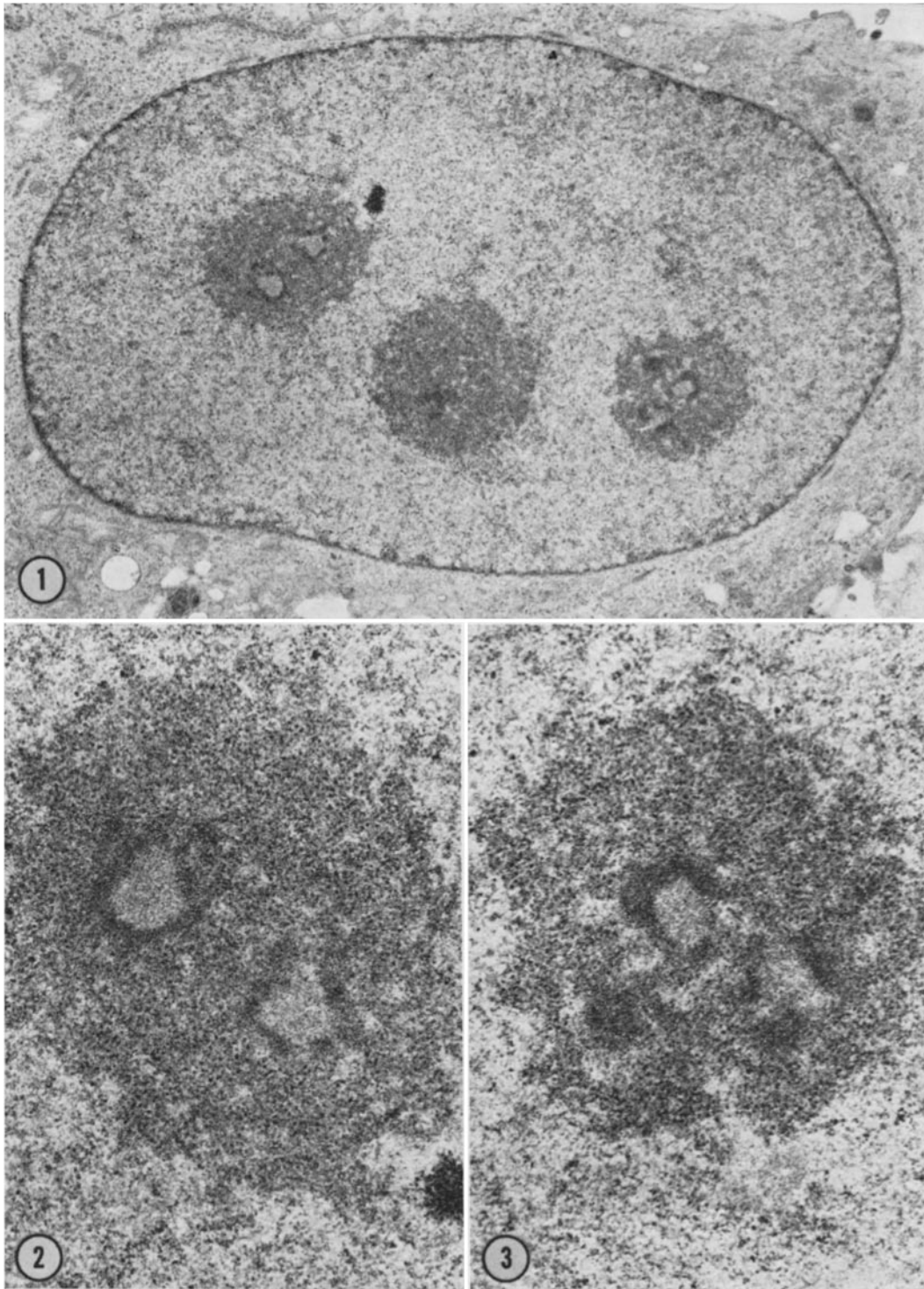
cultures of recent origin (Phillips and Phillips, 1971).

The organization of nucleolar components in KB or L cells was found to be specific and distinctive. Most of the nucleolus was made up of electron-opaque particulate material which appeared to be embedded in a fibrous matrix (Figs. 1-6). This material appeared to be rather densely packed, but its arrangement occasionally suggested that it might actually be composed of a ropelike reticulum (nucleolonema). However, a reticular arrangement was not nearly so apparent as it is in the particulate nucleolar component of many other cell types. A few small, roughly spherical fibrillar zones were generally found embedded within the particulate nucleolar component of KB or L cells. These regions consisted of an inner, light fibrous core enclosed by intimately associated, denser fibrous material. The denser material of the cortex was, in turn, generally intimately associated with the surrounding particulate material (Figs. 1-6). This arrangement was different from the organization in nucleoli of the nonmalignant Chinese hamster and mouse cells previously examined (Phillips and Phillips, 1971), in which the particulate and fibrillar zones were spatially separated.

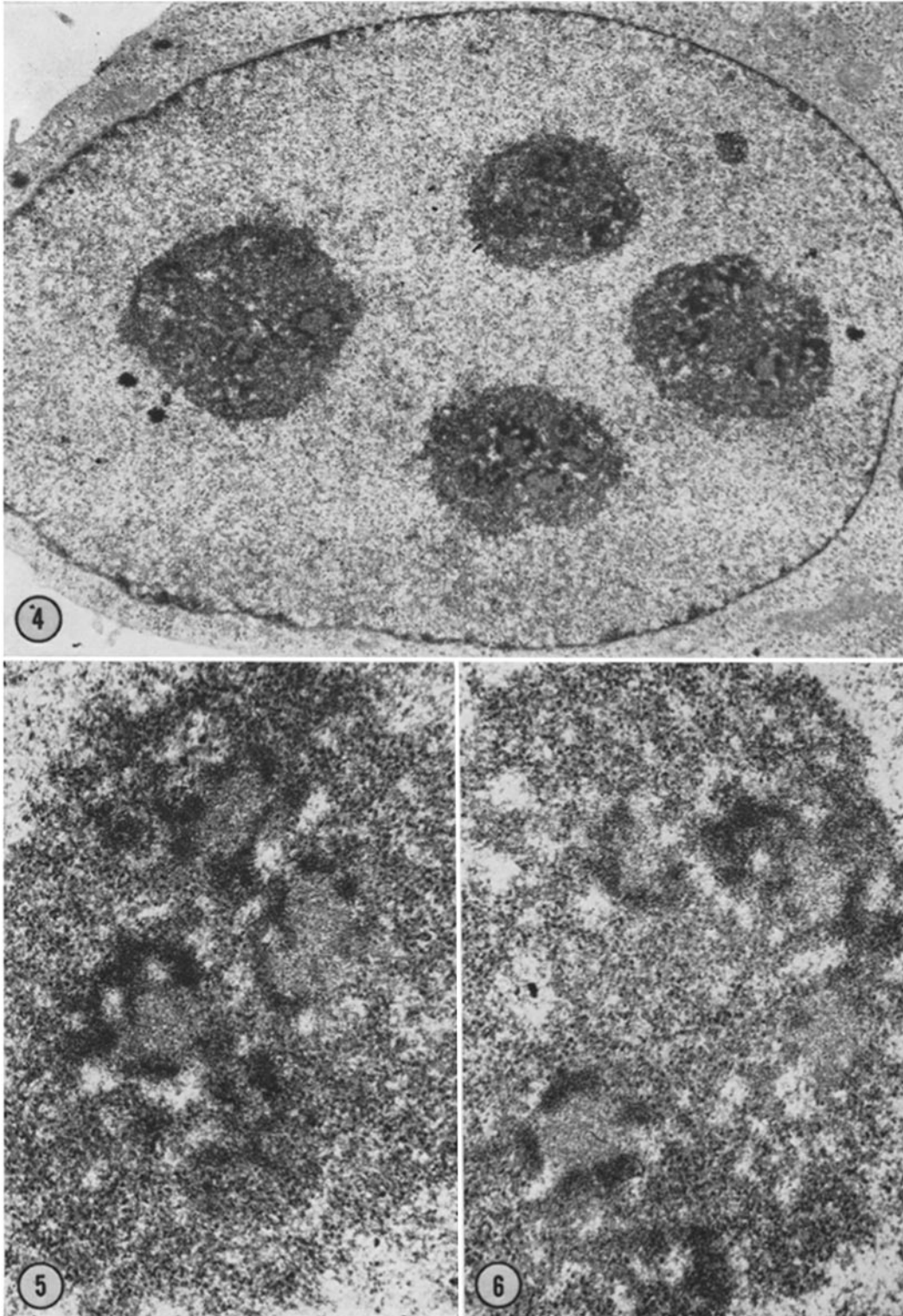
Effect of Actinomycin D on Nucleolar Structure

Alterations in nucleolar structure were clearly visible in the light microscope after 1 hr of treatment with actinomycin D at a level of 5 µg/ml. In azure B preparations nucleoli appeared segregated into stained and unstained components. The unstained material was far less abundant than the stained material and usually appeared as one or more tabs of refractile material on the periphery of the intensely stained central portion. The segregated appearance was maintained for up to 6 hr of actinomycin D treatment (the longest treatment examined). In a few cells treated with actinomycin D, the nucleoli appeared to have fragmented and the nuclei contained many tiny, ragged bodies which stained to variable extents with azure B for RNA.

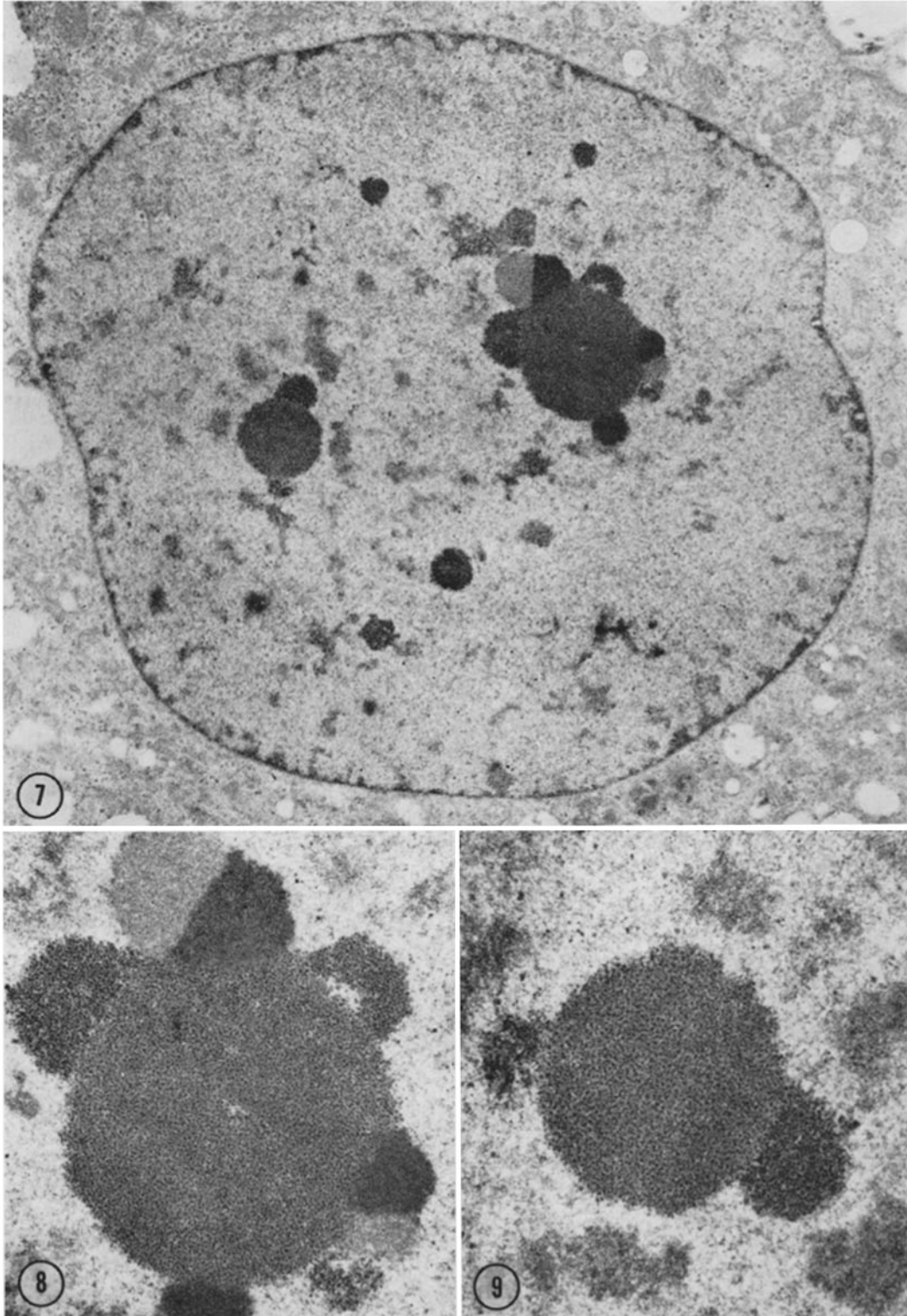
On the ultrastructural level, actinomycin D appeared to induce a range of diverse effects upon nucleolar structure in KB and L cells. Segregation of nucleolar components was clearly evident after 1 or 2 hr of actinomycin D treatment and was more pronounced after 4 hr of exposure to the



FIGURES 1-3 Untreated KB cells, and enlargements of two nucleoli. The spherical nucleoli are composed primarily of particulate material embedded in a fibrous matrix. Spherical regions about 0.3μ in diameter within the nucleoli consist of dense fibrous material enclosing a light fibrous zone. Fig. 1, $\times 9500$; Figs. 2 and 3, $\times 29,000$.



FIGURES 4-6 Untreated L cell and enlargements of two nucleoli. Nucleolar morphology is the same as seen in KB cells. Fig. 4, $\times 11,000$; Figs. 5 and 6, $\times 34,000$.



FIGURES 7-9. L cell treated with actinomycin D for 2 hr. Nucleoli of both L and KB cells appear segregated into different components after actinomycin D treatment, but the morphology and arrangement of nucleolar components are somewhat different in the two cell lines. These nucleoli have a central granular zone. Dense fibrous, light fibrous, and dense granular zones are also distinguishable. Fig. 7, $\times 6000$; Figs. 8 and 9, $\times 15,000$.

drug. Nucleoli of some cells segregated into four components of differing morphology: (a) a predominant globular mass of particulate material; (b) a peripheral knob (or knobs) of denser particulate material; (c) a small spheroidal zone of dense fibrous material; and (d) a peripheral knob of light fibrous material adjoining the dense fibrous zone (Figs. 7-9). The morphology of these components was variable. For example, the light fibrous zone in some cells was nearly as dense as the dense fibrous region, whereas in other cells the light fibrous zone was much less electron opaque than the dense fibrous region. The morphology of granular zones was also variable. In nucleoli of some cells, the denser particulate component did not form a knob, but surrounded the light particulate zone like a rim. Yet another type of nucleolus had the four commonly occurring components plus many large (roughly 400 Å in diameter), very dense particles scattered through the lighter particulate zone (Figs. 10-12). In other treated cells, nucleoli appeared to have sloughed off some of their material, and the nucleoplasm contained small, scattered, irregular pieces of varying morphology. Often cells affected in these varying ways were found side by side in the same culture. The three L cells shown in Figs. 7-15 were all treated for 2½ hr in actinomycin D. They illustrate some of the types of segregated morphology that we have observed.

Effect of Toyocamycin on Nucleolar Structure

Treatment of KB or L cells with toyocamycin at a level of 0.3 µg/ml produced little change in the appearance of nucleoli as seen by light microscopy. However, in L cells, after 4 hr or more of treatment with toyocamycin, roundish bodies appeared in nuclei in addition to nucleoli. These bodies, which occurred in numbers about equal to the numbers of nucleoli, were somewhat smaller than nucleoli. They stained very slightly with azure B, but were well stained by fast green at pH 2. Thus, they were probably principally proteinaceous.

In the electron microscope, it could be seen that toyocamycin treatment of KB or L cells caused the progressive disappearance of the spheroidal fibrillar zones of the nucleoli. In cells grown for 2 hr or less in 0.3 µg/ml of toyocamycin, the change in nucleolar morphology was not readily apparent, but after 4 hr of toyocamycin treatment, the

nucleoli appeared to be predominantly composed of a particulate component (Figs. 16-18).

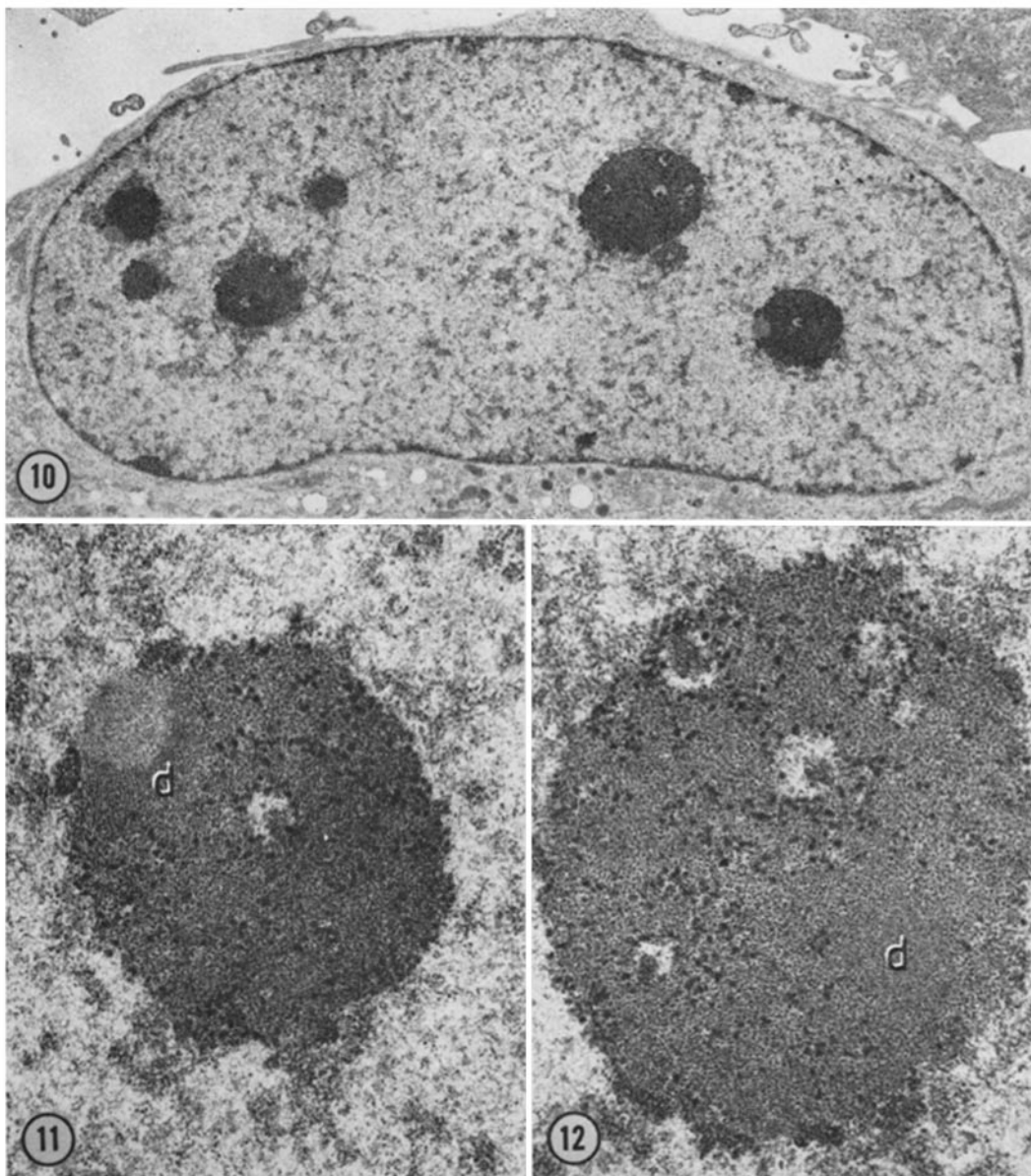
After 8 hr in toyocamycin, most nucleoli were homogeneously particulate in appearance (Figs. 19-21). Occasionally, in sections of toyocamycin-treated cells, some nucleoli appeared to contain roundish, empty-looking, lighter areas within the otherwise homogeneous particulate material. These areas were probably regions which had been occupied by fibrillar material before toyocamycin was added to the culture.

Radioautography

Quantitative radioautographic analysis was performed on the uptake of uridine-³H into nuclei, nucleoli, and cytoplasm of KB cells treated or untreated with 0.3 µg/ml of toyocamycin. As is shown graphically in Fig. 22, the amount of nucleolar and nucleoplasmic uptake of uridine-³H into trichloroacetic acid (TCA)-insoluble material was affected little, if at all, by toyocamycin during the first 4 hr of treatment. Cytoplasmic incorporation of uridine-³H into RNA was, however, greatly reduced by toyocamycin. After 4 hr of continuous growth in medium containing uridine-³H, for instance, only about 20% as much radioactivity due to newly formed RNA was found in the cytoplasm of toyocamycin-treated KB cells as in control cells.

The ability of cells to recover normal patterns of RNA synthesis after toyocamycin treatment was also tested. Cultures grown continuously with uridine-³H in the presence of toyocamycin were washed three times with Hanks' salt solution, and the medium was replaced with normal growth medium containing uridine-³H but no toyocamycin. Little recovery of RNA synthesis was apparent during the following 4 hr.

RNase treatment removed 85% (as compared to untreated controls) of radioactivity from cells of control cultures incubated for 8 hr with uridine-³H. Higher percentages of radioactivity were RNase-removable in cultures incubated for shorter times with uridine-³H. The residual radioactivity was almost entirely nuclear and probably represented incorporation into DNA. KB cells pretreated for 20 min with 5.0 µg/ml of actinomycin D and then grown continuously for up to 4 hr in the presence of uridine-³H and actinomycin D showed virtually 100% inhibition of incorporation of uridine-³H as compared with untreated controls. (Actinomycin D at 5.0 µg/ml may inhibit DNA as well as RNA synthesis, Bacchetti and Whitmore, 1969).



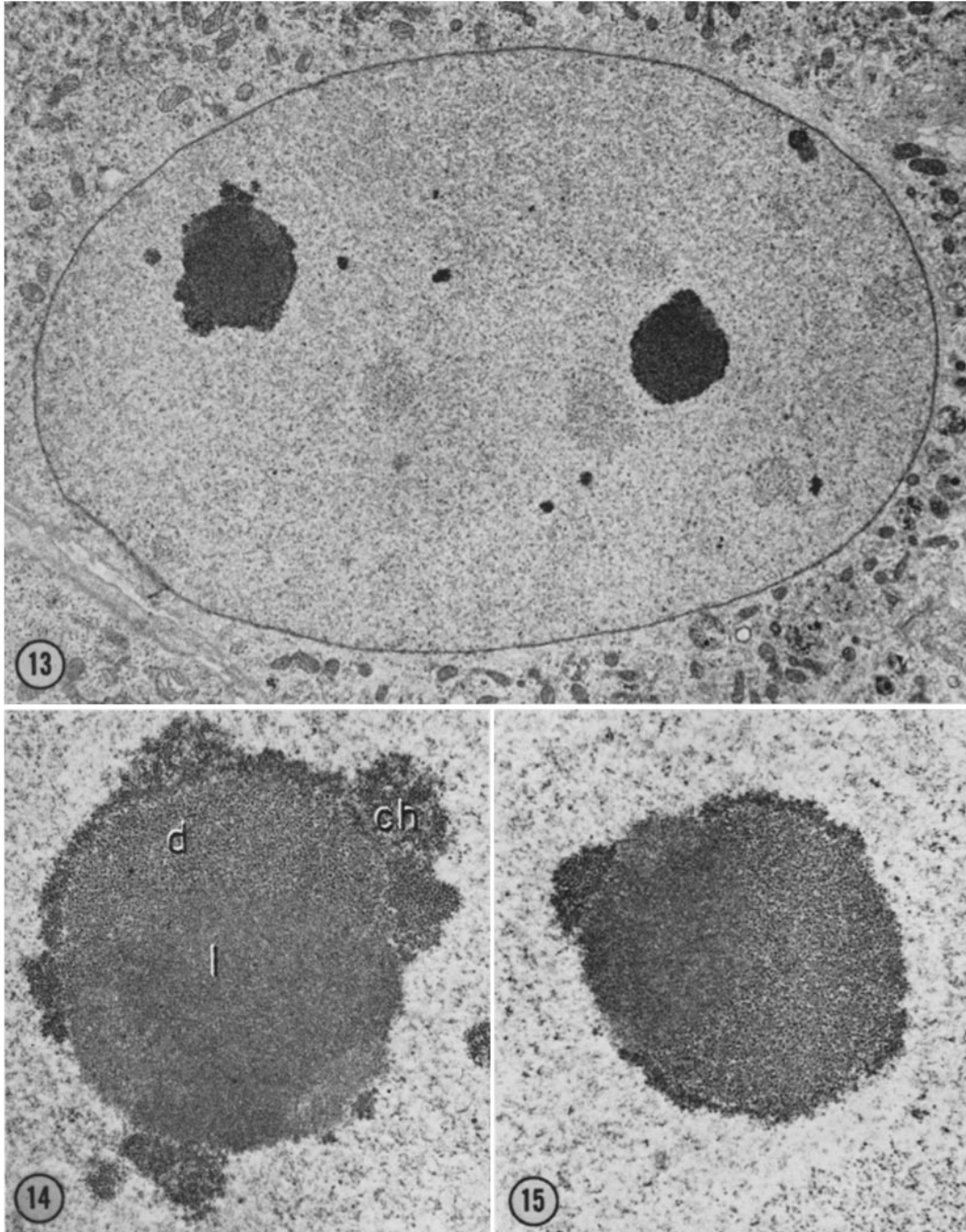
FIGURES 10-12 L cell treated for $2\frac{1}{2}$ hr with actinomycin D; from the same culture as the cell shown in Figs. 7-9. The granular zone contains very large, dense granules as well as the smaller ones. The dense fibrous zone (*d*) is less dense than in Figs. 7-9. Fig. 10, $\times 9000$; Figs. 11 and 12, $\times 40,000$.

DISCUSSION

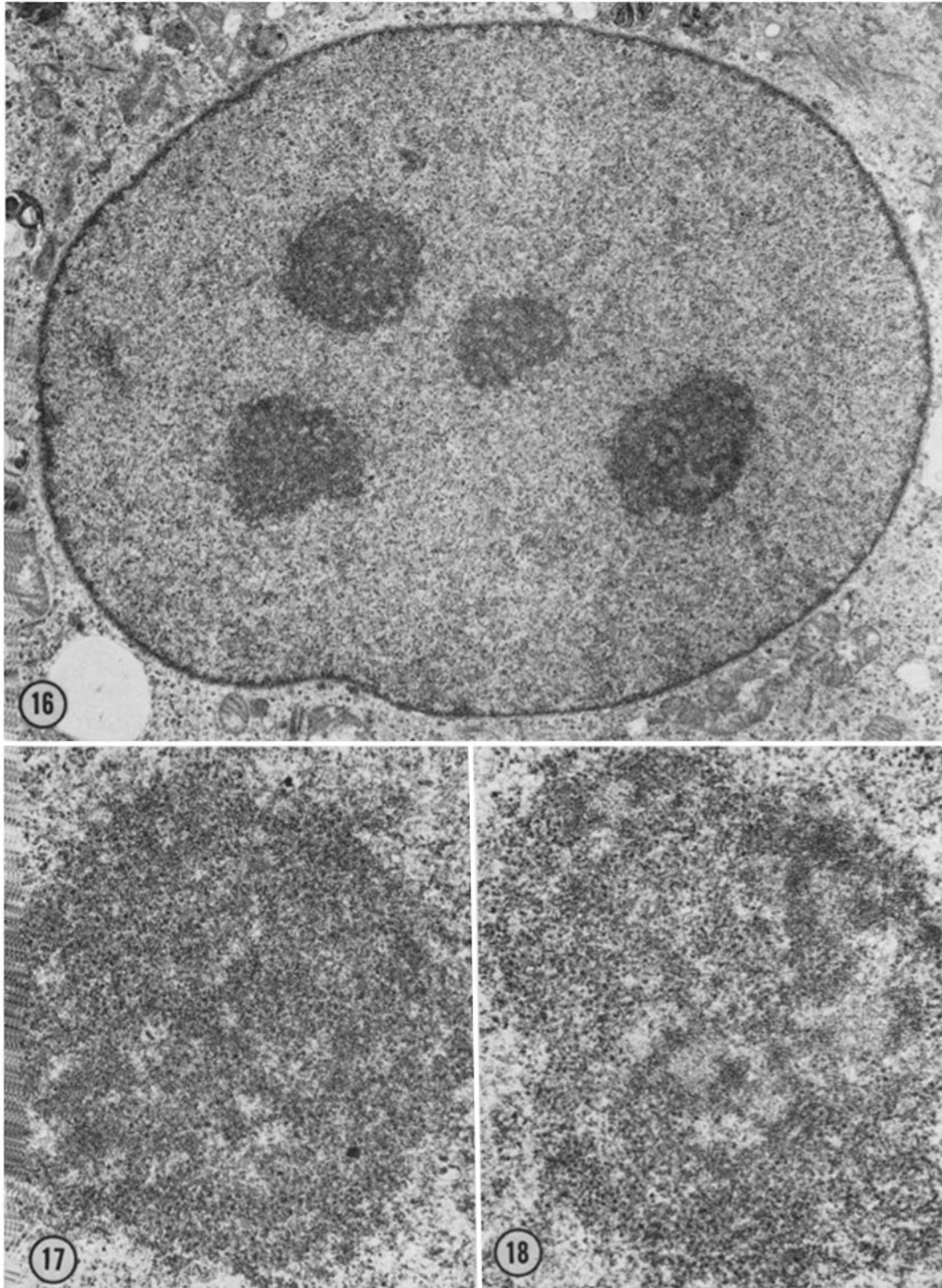
The results presented in this and the companion paper (Phillips and Phillips, 1971) suggest that a distinction may be possible between cells in short-term culture and cells of established cell lines on the basis of the morphology of their nucleoli. The

differences between nucleoli of cells of the established KB and L cell lines and of recently initiated Chinese hamster and mouse cell strains are summarized in Table I.

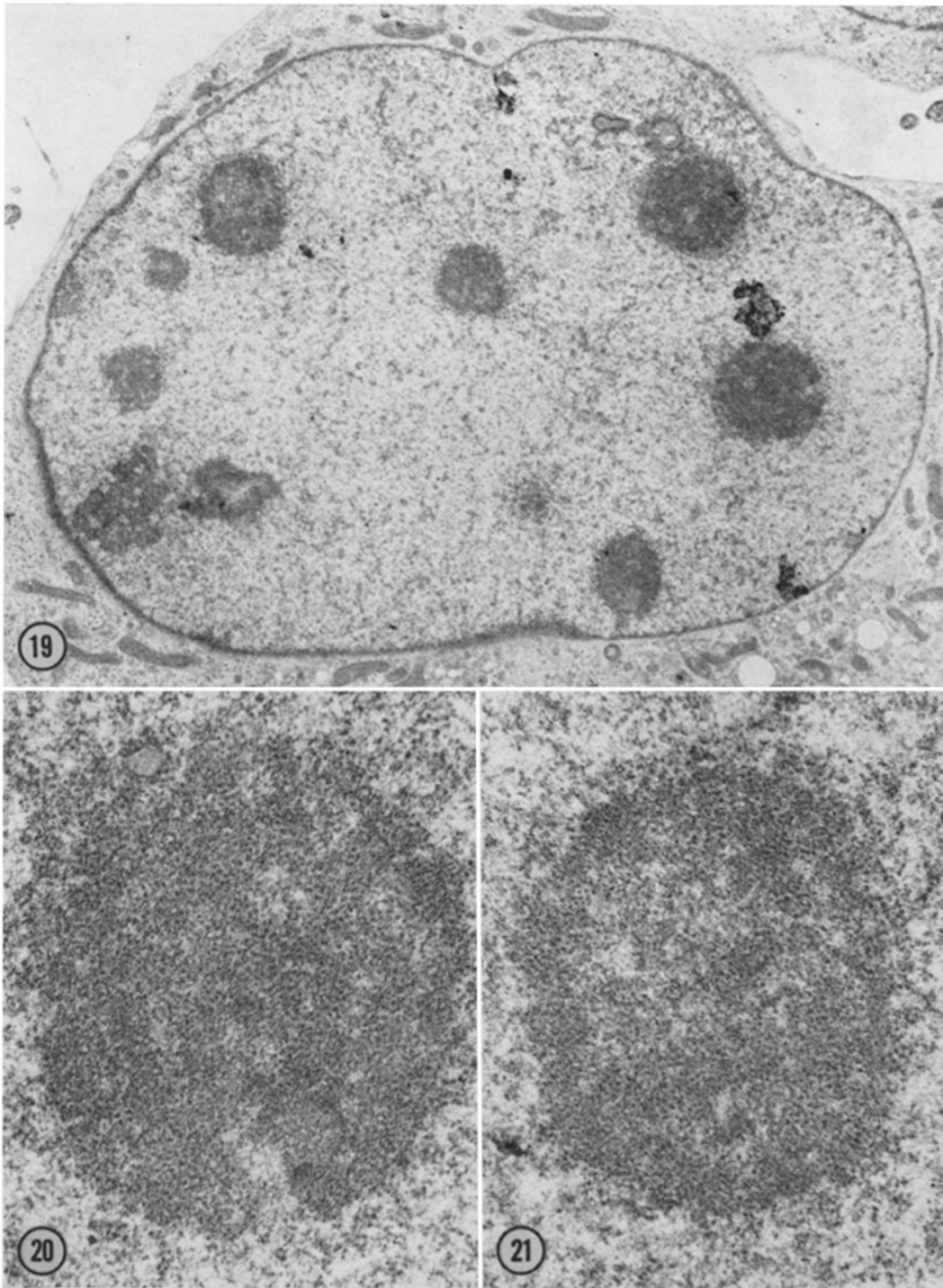
Many electron micrographs of malignant and transformed cells have been published. In examples where the nucleolar structure can be evalu-



FIGURES 13-15 Another L cell from the same actinomycin D-treated culture. The arrangement of segregated nucleoli is somewhat different from that in Figs. 7-12. Dense fibrous material (*ch*) located around the periphery of nucleoli may be chromatin. The light fibrous zone (*l*) is only slightly denser than the dense fibrous zone (*d*) in these two nucleoli. Fig. 13, $\times 6000$; Figs. 14 and 15, $\times 21,000$.



FIGURES 16-18 KB cell treated for 4 hr with toyocamycin. Dense and light fibrous zones are discernible in some nucleoli, but they seem to merge with the particulate region. Most nucleoli of toyocamycin-treated cells appear to consist of one homogeneous region which contains granules embedded in fibrous matrix. Fig. 16, $\times 1000$; Figs. 17 and 18, $\times 37,000$.



FIGURES 19-21 After treatment with toyocamycin for 8 hr the nucleoli of KB cells are composed of one zone of granules embedded in a fibrous matrix. Fig. 19, $\times 10,500$; Figs. 20 and 21, $\times 28,000$.

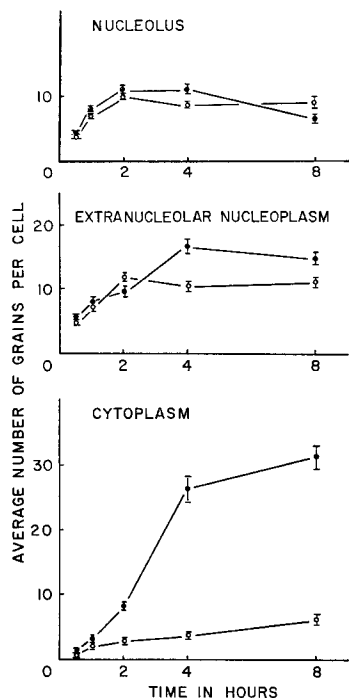


FIGURE 22 Grain counts done on radioautographs of KB cells incubated with uridine- ^3H , 0.1 mCi/ml, for the times indicated in the absence (closed circles) or presence (open circles) of toyocamycin, 0.3 $\mu\text{g}/\text{ml}$. Each point represents the average number of silver grains over nucleoli, nucleoplasm, or cytoplasm of 100 cells. The indicated intervals represent standard error of the mean.

ated, the nucleoli of such cells appear to have the structure we have described as characteristic of KB and L cells (e.g., see Recher et al., 1969; Heine, 1969; Martinez-Palomo and Granboulan, 1967). On the other hand, nucleoli of "normal," nonmalignant cells of various sorts are extremely variable in morphology, and a "typical" or "normal" nucleolus cannot be defined. Published micrographs suggest that the loosely reticular type of nucleolus, in which an electron-transparent space separates the fibrillar and particulate zones, may be characteristic of other mammalian cell types in culture (i.e., see figures in Simard and Bernhard, 1967; de Man and Noorduyn, 1967; Brinkley, 1965), mammalian liver cells (i.e., see figures in Narayan et al., 1966; Marinozzi, 1964; Shinozuka et al., 1968), and mammalian pancreas cells (Marinozzi, 1964). On the basis of our very limited sampling of cell types and the few illustrative published micrographs, we cannot generalize

confidently about the structure of nucleoli in malignant and normal cells, but it seems a worthwhile point to pursue further.

Toyocamycin treatment of KB and L cells caused the gradual disappearance of the fibrillar material of the nucleolus. This result is in direct contrast to the results obtained by toyocamycin treatment of recently initiated Chinese hamster and mouse cell cultures. In those cells, toyocamycin treatment led to the disappearance of particulate material from the nucleolus (Phillips and Phillips, 1971). However, our radioautographic analyses of toyocamycin effect on RNA synthesis indicate no difference in the inhibitory effect of the analogue. In both types of cells toyocamycin profoundly inhibited the appearance of newly synthesized RNA in the cytoplasm while inhibiting nucleolar and nuclear RNA synthesis to a much lesser extent. Though this does not necessarily mean that the toyocamycin produced the same biochemical lesion in both types of cells, it does support this idea. The differing morphological appearance of the nucleolar material accumulating in the presence of toyocamycin is puzzling; it might reflect some difference in nucleolar proteins in the different cell types. In HeLa cells, in which the structure of untreated nucleoli is apparently similar to nucleolar structure of KB or L cells, toyocamycin treatment is reported to cause a disappearance of particulate material similar to what we find in "normal" cells (Heine, 1969). Thus, the differences in the response to toyocamycin may not be related to whether the cell line is established. Since neoplastic cells sometimes lose tumorigenicity after long-term cultivation *in vitro*, further experiments are needed to determine the relationship of drug response to the cancerous state.

The change in nucleolar morphology induced by a wide variety of drugs has been tested. The most common response of the nucleolus to agents known to decrease RNA synthesis is segregation into several components. Such segregation is a well-known result of actinomycin D treatment (Schoeffl, 1964; Reynolds et al., 1964; Jézequél and Bernhard, 1964) and has also been reported to occur after treatment with aflatoxin (Bernhard, 1966), anthramycin (Harris et al., 1968), azaserine, duanomycin (Bernhard, 1966), mithramycin (Kume et al., 1967), mitomycin C (Bernhard, 1966), 4-nitroquinoline-N-oxide (Reynolds et al., 1963), nogalamycin, proflavine (Bernhard, 1966),

TABLE I
Comparison of Nucleoli of Mouse and Chinese Hamster Cells with Nucleoli of L and KB Cells

| | Short term mouse or Chinese hamster cell strain | Established L or KB cell line |
|--|---|---|
| General nucleolar shape | Irregular or loosely reticular | Spherical or oval |
| Shape of dense fibrous zones | Irregular | Hollow spheres |
| Location of light fibrous zones | Adjacent to dense fibrous zone | In center of dense fibrous zone |
| Amount of light fibrous material | Very little | Little, but generally more than in fresh cell strains |
| Relationship of fibrous to particulate zones | Space between dense fibrous and particulate zones | Dense fibrous material contiguous with particulate zone |
| Effect of toyocamycin | Gradual disappearance of granules until nucleoli appear homogeneously fibrous | Gradual disappearance of fibrillar zones until nucleoli appear homogeneously granular |
| Effect of short actinomycin D treatment, 5 $\mu\text{g}/\text{ml}$ | Regular segregation into three or four morphologically distinguishable zones | Segregation into components with variable morphology and organization |
| Effect of 4 hr actinomycin D, 5 $\mu\text{g}/\text{ml}$ | Breakdown and disappearance of nucleoli | Persistence of segregated nucleoli |

and quinocrine (Fedorko and Hirsch, 1969). The mechanism of action of many of these compounds in blocking RNA synthesis is not understood, but most of them are known to bind to DNA. Whether the nucleolar segregation is a direct result of the blockage in RNA synthesis, or of the binding of a substance to the nucleolar DNA, or both, is not known.

A second type of nucleolar rearrangement is reported to occur in response to the agents 5-fluorouracil (Stenram, 1966), excess adenosine (Stenram, 1966), and ethionine (Shinozuka et al., 1968). These agents cause nucleoli to appear spotty and loosely organized. They all cause a decrease in RNA synthesis, possibly related to their interference with adenosine triphosphate (ATP) metabolism, but the relationship of their mechanism of action to the nucleolar lesion they produce is not at all understood. Recently, it was reported (Monneron et al., 1970) that treatment with high levels of toyocamycin induces similar changes. One might suspect that this agent, like 5-fluorouracil and excess adenosine, could interfere with ATP metabolism as well as RNA synthesis.

Toyocamycin (at low levels) produces a third

type of nucleolar lesion. Nucleoli of cells treated with this agent become homogeneous in appearance. Though it seems likely that the toyocamycin-treated nucleoli contain early precursors of ribosomal RNA, one cannot draw a simple, general conclusion about the appearance of the nucleolar component containing this RNA, because the appearance of toyocamycin-treated nucleoli is different in different cell types. One is forced to conclude that neither nucleolar morphology nor drug-induced alterations in nucleolar morphology can be understood solely on the basis of ribosomal RNA metabolism. Nucleolar RNA, although it is by far the best described constituent of nucleoli, represents only some 10% of the mass of nucleoli. Better understanding of the functional significance of the morphological organization of nucleoli will have to await further elucidation of the metabolism of other nucleolar components.

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