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CYTOCHEMICAL STUDIES FOR DEMONSTRATION OF NUCLEOLI IN MITOTIC DIVISIONS OF AMPHIBIAN AND MAMMALIAN CELLS IN VITRO

By

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Introduction

Although numerous cytological and cytochemical studies of nucleoli in various kinds of cells have been done, no staining method has been found that is absolutely specific to visualize nucleoli (Busch and Smetana 1970). The identification of nucleoli in ultrathin sections by means of the electron micrscope is easier than that by the light microscope because the nucleolar ultrastructural appearance is very characteristic even in sections prepared by the commonly used electron microscopic procedures. However, cytological studies of nucleoli have to be done primarily by the light microscopy, since the number of nucleoli and their presence or absence in cells is best determined by the light microscopic methods. And fundamental problem on nucleolar morphology at mitotic phases have still been left unsolved in cells of normal and pathological conditions. Consequently the technique for specific representation of nucleoli is primarily needed for the light microscopic studies.

The present study was undertaken to compare various cytological and cytochemical techniques proposed by many investigators for the identification of nucleolus and to consider the most adequate or indispensable conditions for nucleolar detection. Trials with some modifications of the established techniques were also made in order to obtain preparations in which nucleoli are detectable in cells undergoing mitosis. If we could obtain more specific technique to visualize the nucleolus, it is to prompt us to investigate in detail the fate of nucleoli during mitotic phases with a light microscope.

Materials and Methods

The following cultured cells were used.

Long Term Monolayer Cultures

L : A fibroblast cell derived from a C3H mouse hypodermal connective tissue originally established by Earle *et al.* (1943).

HeLa : A human cervical carcinoma cell line established by Gey *et al.* (1952).

GMK : An early subculture of fibroblasts from a green monkey kidney tissue.

These cell lines were grown as monolayer cultures in the Eagle's minimum essential medium (Daigo) supplemented with 10% calf serum. Dissociated cells with 0.05% trypsin solution were transplanted on 12×32 mm coverslips in a MB flask (Miharu) or a small Petri dish and cultivated in a CO_2 incubator for three to 6 days at 37°C.

Short Term Cultures

NCL : Lung tissue cultures of newts (*Triturus pyrrhogaster*) were prepared as the method described in Seto and Pomerat (1965), and a sheet of epithelial-like cells grown out from a tissue fragment were obtained on the sixth to ninth day.

FKS : Isolated kidney tissues from a female frog (*Rana nigromaculata*) were grown in a culture bottle as monolayer cells and maintained for 12 passages as the diploid cell strain. These amphibian cells were cultivated on the coverslips in the amphibian growth medium as modified from Eagle's basal medium (Seto and Rounds 1968), and incubated at 26°C.

Cells grown on coverslips were washed quickly in a balanced salt solution before fixation. An appropriate fixative was selectively used for a certain staining as listed in Table 1. Nucleolar stain and dehydration was processed in a small Petri dish or short glass tubes, and then mounted a cover slip on a slide glass with Biolite or Canada balsam.

Various staining methods were extensively attempted in the present study. Among those, methods resulted effective visualization of nucleoli were listed in Table 1.

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TABLE 1. STAINING METHODS FOR NUCLEOLUS EMPLOYED IN THIS STUDY

STAINING METHODS	FIXATIVES	CELLS USED	LITERATURES
Giemsa stain	70% ethanol	HeLa, GMK, FKS	Munoz 1969
Wright stain	70% ethanol	HeLa, GMK	Munoz 1969
Azure B stain	50% acetic acid	HeLa, GMK	Heneen & Nochols 1966
Toluidine blue stain	Carnoy's solution	HeLa, GMK	Monroe *et al.* 1969
Methylene blue stain	Absolute methanol	L, FKS	Seman 1960
Mayer's hemalum & eosin method	10% neutral formalin	FKS, L, GMK	Menzies 1962
May-Grünwald & Giemsa method	Absolute methanol	FKS, HeLa, NLC	Jacobson & Wepp 1952
Azure II & methylene blue method	Carnoy's solution	L, HeLa	Richardson *et al.* 1960
Aniline & azure B method	10% neutral formalin	L	Menzies 1966
Methyl green & pyronin method	Carnoy's solution	HeLa, L, GMK	Kurnick 1952
Feulgen & light green method	Carnoy's solution	NLC, HeLa	Lison 1960

Extraction Procedures

Extraction of nucleic acids from fixed cells was attempted prior to the staining procedure for obtaining more distinctive contrast of nucleolus and chromatin, or nucleolus against cytoplasm by the methods indicated in Table 2.

These procedures were of enzymatic and nonenzymatic :

(1) In nonenzymatic procedure with perchloric acid, ribonucleic acid was extracted from the fixed specimen by the method of Erickson *et al.* (1949). Methanol-fixed cells were treated in $HClO_4$ solution at the concentration of 5% and 10% at room temperature for 3 to 6 minutes and washed in distilled water for 10 minutes. The cells were then stained with May-Grünwald and Giemsa method or toluidine blue stain.

(2) Enzymatic extraction of nucleic acids from the fixed cells were made independently with ribonuclease and deoxyribonuclease which were both crystallized from bovine pancreas (Sigma). Fixed cells on a cover slip were placed in a Petri dish containing a moistened filter paper, and enzymatic solution was placed directly over the cells and treatment was made at 37°C. Concentrations of enzyme solutions and treatment time are shown in Table 2. Treated cells were then washed in distilled water and stained by azure B, toluidine blue, or May-Grünwald-Giemsa method.

Results

Visualization of Nucleoli in Interphase and Mitotic Phases

Among five different cell cultures used as materials, the frog kidney cell strain (FKS) was primarily used for this experiment since it could be obtained clearer visualization of nucleoli than other mammalian cells such as HeLa, GMK, and L cells which were less extensively performed (Figs. 1-6). So far attempted in the present study, variable numbers of nucleoli were clearly detecteed in interphase cells by every method described above. Among those methods as listed in Table 1, the effectiveness of nucleolar stainings has been slightly varied in each other. Those simple methods of Munoz-Giemsa, azure B, toluidine blue and methylene blue stains exhibited well demonstration of nucleoli of all cells at interphase and prophase.

TABLE 2. EXTRACTION PROCEDURES

TREATMENT (LITERATURES)	EXTRACTED SUBSTANCE	CONCENTRATION	EXTRACTION TIME	EXTRACTION TEMPERATURE
Perchloric ($HClO_4$) (Erickson *et al*, 1949)	RNA	5%, 10%	3-6 min.	room temp.
Ribonuclease (Brachet 1953)	RNA	0.2%	2 hr.	37°C
Deoxyribonuclease (Amano 1962)	DNA	0.004%	24 hr.	37°C

TABLE 3. RESULTS OF VISUALIZATION OF NUCLEOLI IN MITOTIC CELLS

STAININGS	EXTRACTION PROCEDURES	INTER-PHASE	PRO-PHASE	EARLY META-PHASE	META-PHASE	CONTRAST WITH CHROMATIN
Munoz-Giemsa	none	++	++	−	−	−
Methylene blue	none	+++	++	−	−	−
Feulgen & light green	none	++	+	±	−	++
Aniline & azure B	none	+++	++	−	−	++
Azure II & methylene blue	none	+++	++	+	−	±
Azure B	none	+++	++	−	−	−
Azure B	RNase	++	++	++	−	±
Azure B	DNase	++	++	±	−	+
Toluidine blue	none	+++	++	−	−	−
Toluidine blue	RNase	++	++	+	±	+
Toluidine blue	$HClO_4$	++	++	++	+	−
Toluidine blue	DNase	+++	++	+	±	+
May-Grünwald & Giemsa	none	+++	++	+	±	−
May-Grünwald & Giemsa	RNase	++	++	++	++	−
May-Grünwald & Giemsa	$HClO_4$	++	++	+	+	+
May-Grünwald & Giemsa	DNase	+++	++	+	−	++

Double stainings of azure II-methylene blue, May-Grünwald-Giema, and methyl green-pyronin methods were more preferable, which have advantageously shown nucleoli as positively stained bodies against other nuclear components. The Feulgen reaction in combination with a light green stain has also shown clear contrast in color of nucleoli with chromatin at the interphase and prophase nuclei : chromatin material was positively stained with basic fuchsin and nucleoli were stained selectively with light green.

In the preparations of FKS cells as stained with May-Grünwald-Giemsa method, nucleoli obviously persist during prophase stage when they may be visible as still functioning masses (Fig. 1). Late in prophase, they were seen in association with one or more "nucleolar chromosomes" presumably linked to them in the specific sites of the "nucleolus-organizing regions" (Figs 2 & 3). However the method employed in this study did not able to analyze the chromosomes bearing nucleoli at mitotic phases since the hypotonic pretreatment for making chromosome preparation causes dissolution and in most cases complete disappearance of nucleoli. Although it was difficult to distinguish nucleoli from clumps of chromosomes at metaphase and anaphase, these nucleoli occasionally appeared as irregular shapes, faintly stained bodies on a well spreaded metaphase cytoplasm (Figs. 4-6).

Demoustration of Persistent Nucleoli at Metaphase

Nucleolar materials which persisted through mitotic stages are rarely seen in certain kind of cell cultures as well as in plant cells by specific nucleolar staining. In the present experiment, only these preparations of FKS, HeLa, and L cells were stained with methylene blue, azure II-methylene blue, and May-Grünwald-Giemsa methods. At early metaphase, chromosomes distributed relatively wide in the nuclear region and positively stained nucleolar materials were seen as droplets along the side of certain chromosomes (Fig. 5). At late metaphase, nucleolar materials were mostly enveloped within the strongly stained chromosomal mass which oriented on the equatorial plate. Cytoplasm was also liable to be stained dark by any method. This become a main reason that nucleolar materials are hardly found or undetectable at late metaphase.

To visualize nucleolar materials more clearly at mitotic stages, especially at metaphase, digestion of main chromosomal substances

and cytoplasmic RNA have been attempted before the nucleolar staining. For making optical contrast of nucleoli with metaphase cytoplasm, ribonucleic acid was tried to removed from mitotic cells with a solution of perchloric acid or ribonuclease. And changes of digestion time in combination with variety of the concentration of $HClO_4$ or RNase solution have been tested. The best result so far obtained for demonstration of persistent nucleoli were from the staining in cooperated with the extraction procedure as indicated in Table 2.

The detectability of nucleoli in mitotic cells varied depending on the contrast of nucleolar stain with other cellular structures. When RNA is fully removed with a long treatment of perchloric acid or high concentration of ribonuclease, nucleoli seem to be empty spaces surrounded by nucleolus-associated chromatin (Fig. 9), but if this extraction is moderately undertaken by using lower concentration or shorter treatment time of those extraction procedures, nucleolar RNA remains to the extent of detectable even when cytoplasmic RNA was removed (Fig. 8). This modification achieved more effective visualization of nucleoli at metaphase stages after making suitable stainings (Figs. 10-15).

Extraction of DNA from chromosomes at prophase and metaphase was attempted prior to staining for expecting to facilitate differential stain of nucleolar materials among chromosome clumps which tend to hinder the visualization of nucleolar materials. After treatment with deoxyribonuclease under the condition as indicated in Table 2, nucleolar stains were proceeded. From the limited experimental results, it was noted that nucleoli appeared as positively stained structure and stain affinity of chromosomes obviously decreased by the treatment. However persistent nucleoli have not been as preferably detected as those treated with $HClO_4$ or RNase solutions, although the contrast of nucleolus with chromosomes became relatively better (Fig. 15).

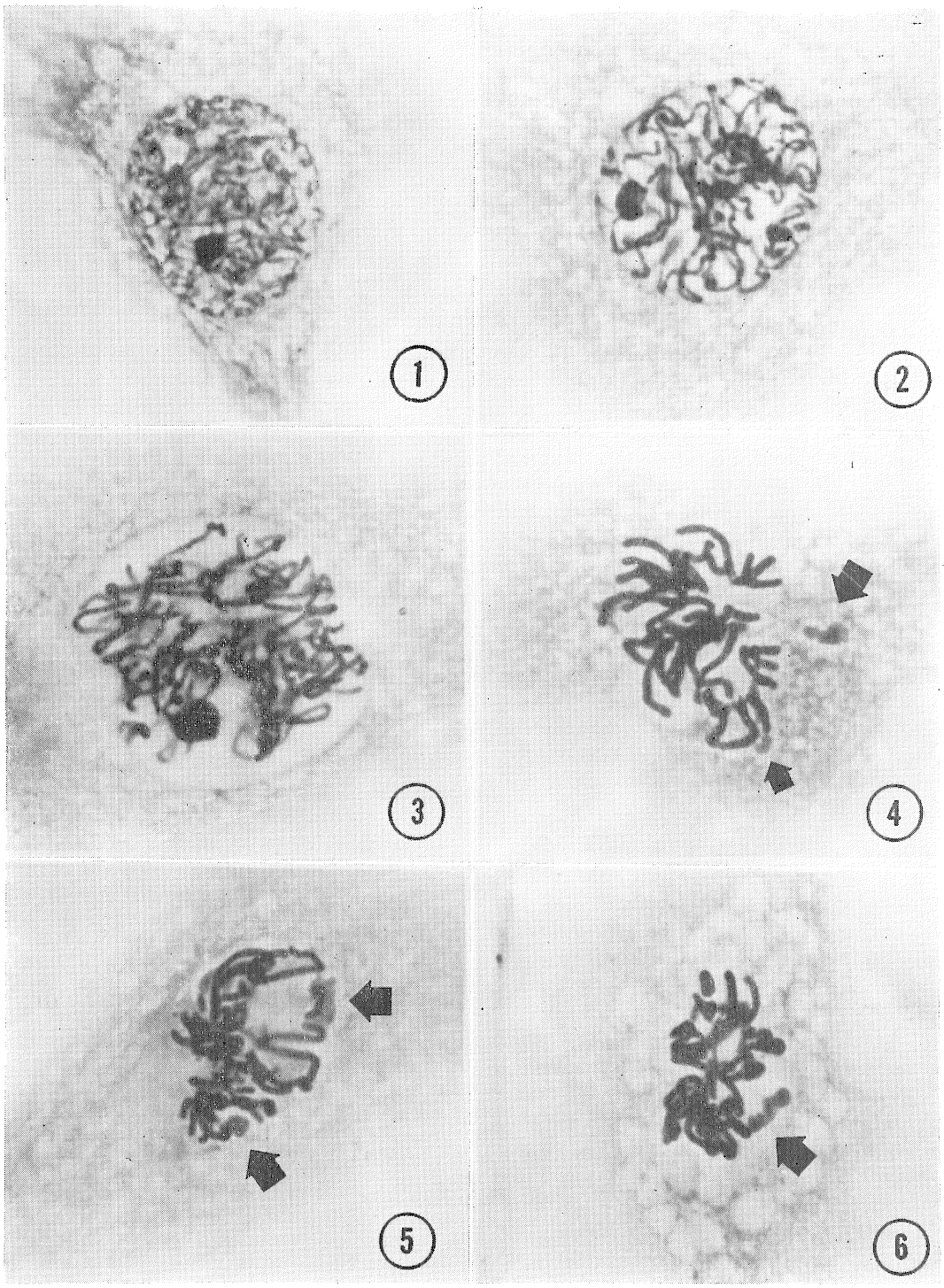
Discussion

Nucleoli are the most obvious and distinctive structures seen within interphase nuclei. However they frequently are inconspicuous by appearance of chromatin after routine nucleolar stainings. In the morphological study of nucleolus of tumor cells, Hori (1957) has pointed out that the real feature of the nucleolus can not be well understood unless any possible close relationship between the nucleolus and its associated chromatin is analysed. Therefore it is primarily necessary to obtain a technique for selective stain of nucleolus from chromatin for demonstration and characterization of interphase nucleoli, which could make possible to detect cytologically nucleoli at mitotic phases.

Among routine stainings attempted in this study, May-Grünwald-Giemsa method was most successfully used. This result was possibly caused by the modification of Munoz (1969) method. Fixed cells were exposed thoroughly to phosphate buffer solution (1/30 m $NaHPO_4$), not exposed to another environment or chemicals, while staining and washing processes. The result suggests that nucleoli can be stained better in the alkaline side by following Munoz's principle, which is, to apply alkaline buffer first and then the dye solution. This buffer solution was also applied to azure B stain which resulted better demonstration of nucleoli than the original method.

Although any staining methods without extraction procedures resulted successful demonstration of nucleoli of cultured cells. Nucleoli at the different stages of mitosis could be appropriately indicated by a certain staining method : for instance, the nucleolus at interphase will be clearly shown by the Feulgen and light-green method, while the extraction procedure for RNA in combination with such double staining as May-Grünwald-Giemsa or azure II-methylene blue method can rise the possibility of visualization of persistent nucleoli.

Observations of nucleoli which persisted through mitosis were made (Table 3), although the result was not conclusive. Above mentioned method, in cooperated with a moderate digestion method for RNA, has shown nucleolar material more frequently in cells of active growing cultures such as HeLa, L, and FKS. The selection of material that has more proliferative ability might be important for the study of nucleoli at mitotic phases. The similar result has been obtained by Heneen and Nichols (1966), and they suspected that the occurrence of persistent nucleoli might result from the presence of increased amount of nucleolar material. However, it still remains



Figures 1–6. Cells from the frog kidney cell strain (FKS) of 9 passages, demonstrating persistence of nucleoli at prophase and metaphase. May-Grünwald & Giemsa stain. ×1600. Figures 1 to 3, prophase cell which has deeply stained nucleolus. Figures 4 to 6, early metaphase and metaphse cells with persistent nucleoli, nucleolar substances are detectable in the cytoplasm (*arrow*).

to be determined what the nature is of the persistent nucleoli.

Investigations on the fate of the nucleolus as well as nucleolar behavior during mitosis have been actively undertaken in cultured mammalian cells (Hsu *et al.* 1965, Heneen and Nichols 1966, Noel *et al.* 1971, Phillips 1972, and others). Hsu *et al.* (1965) have surveyed 18 mammalian cell strains in culture to determine whether the phenomenon of persistent nucleoli in mitotic divisions is limited to the certain cell strain, and it was realized that nucleolar material was always present in mitosis, either in the form of distinct roundish bodies or broken up into shapeless masses surrounding the chromosomes. In the present observation, amphibian cell nucleoli were also found to persist through prophase to metaphase when selected staining methods were employed. And persistent nucleoli were noticed to a much greater extent in the strain cells (FKS) than in the primary culture cells (NCL). Heneen and Nichols (1966) also reported that the amount of persistent nucleolar material was more pronounced in long term cultured cells compared to short term cultured cells. These results will support the suspection that the occurrence of persistent nucleoli might result from the presence of increased amounts of nucleolar material in the lively cell.

Summary

Cytochemical methods proposed for the demonstration of nucleoli were comparatively studied in order to obtain more specific visualization of nucleoli with a light microscope.

The amphibian cell culture derived from frog kidney tissue was successfully used in stain preparations of mitotic cells, and some modifications of staining methods resulted effective manifestation of nucleoli at mitotic phases.

Moderate extraction of RNA from fixed cells with perchloric acid or RNase was recognized more effective to the subsequent nucleolar stainings for the detection of persistent nucleoli in any kind of cultured cells.

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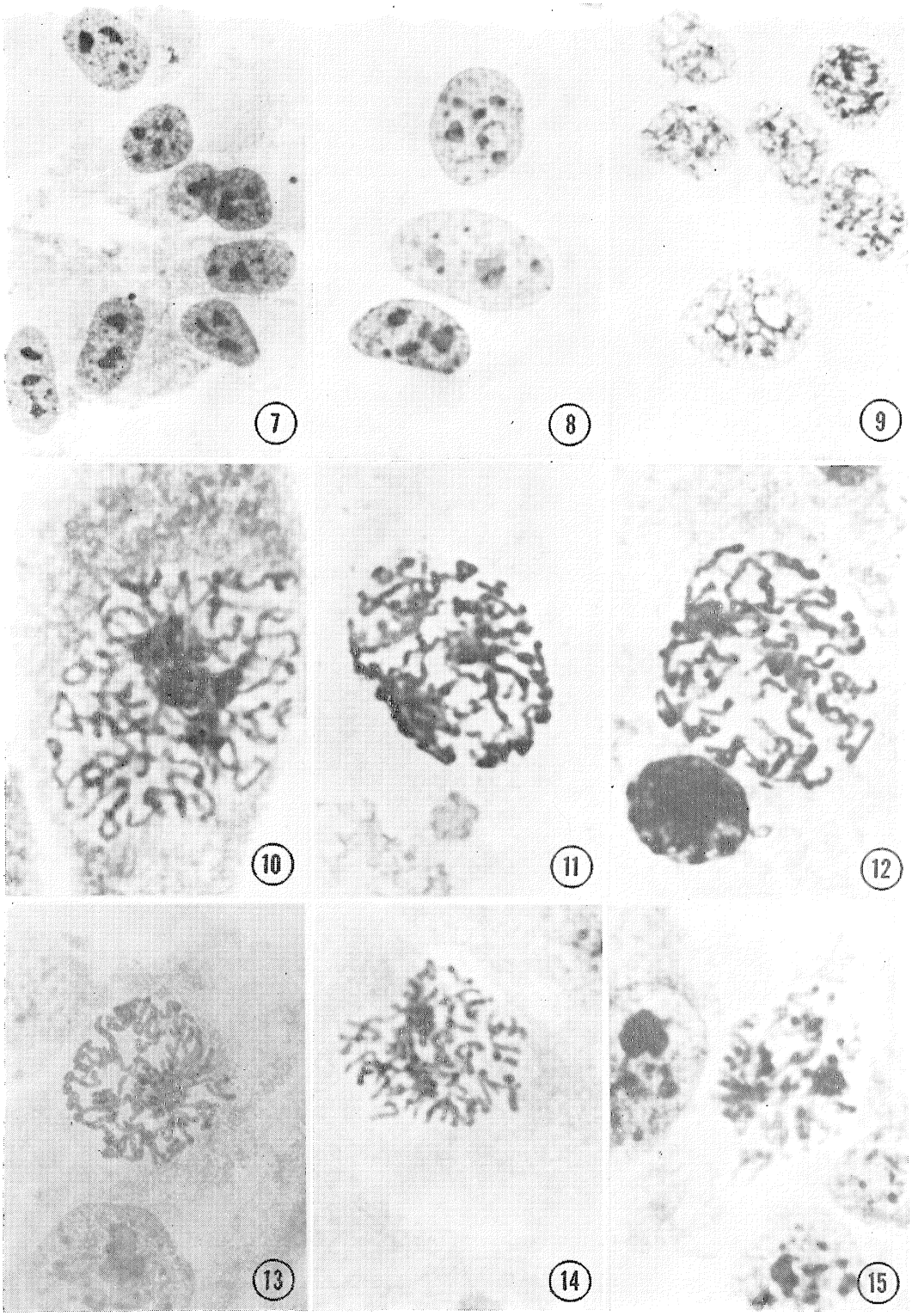
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Figures 7–9. Variable numbers of nucleoli are seen at interphase of L cells, azure II and methylene blue stain.

Figure 7, nucleoli are the most obvious at distinctive structre. Figure 8, contrast was obtained of nucleolus against chromatin after 10% $HClO_4$ treatment for 3 minutes. Figure 9, nucleoli are seemed to be empty spaces surrounded by nucleolus associated chromatin when RNA is removed with fully treatment of RNase.

Figures 10–15. Mitotic cells from HeLa cell line except Fig. 14 of L cell, exsistence of nucleoli is visualized by the extraction procedures. Figure 10, 24 hours of DNase treatment before May-Grünwald & Giemsa stain. Figure 11, late prophase, azure B stain after RNase treatment, nucleoli are detectable against the background of cytoplasm and stained chromosomes. Figure 12, late prophase, a slight treatment of 5% $HClO_4$ before May-Grünwald & Giemsa stain. Figure 13, early metaphase, methyl-green & pyronin stain after RNase treatment. Figure 14, metaphase of L cell, toluidine blue stain after 10% $HClO_4$ treatment, nucleoli are seen in the chromosome group against faint stained background. Figure 15, metaphase cell treated with DNase before May-Grünwald & Giemsa stain, nucleolus is deeply stained aginst chromosomes.

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