

# A STUDY OF NUCLEOLAR VACUOLES IN CULTURED TOBACCO CELLS USING RADIOAUTOGRAPHY, ACTINOMYCIN D, AND ELECTRON MICROSCOPY

J. MORRIS JOHNSON

From the Whitman Laboratory, The University of Chicago, Chicago, Illinois 60637. Dr. Johnson's present address is Department of Science and Mathematics, Oregon College of Education, Monmouth, Oregon 97361

## ABSTRACT

Previously it has been found that in tobacco callus cells nucleolar vacuoles repeatedly form and contract. In this study, nucleolar vacuoles were investigated by using radioautography, actinomycin D, and electron microscopy. It was found, from grain counts of nucleoli labeled with uridine-<sup>3</sup>H, that nucleoli containing vacuoles had more than three times as many grains/ $\mu^2$  of nucleolar substance as did nucleoli without vacuoles. Treatment of tobacco callus cells with various concentrations of actinomycin D caused the percentage of cells containing nucleolar vacuoles to decrease; with the highest concentration the percentage of these cells dropped from the normal level of about 70% to less than 10%. However, after removal of actinomycin D the cells regained nucleolar vacuoles up to the control level. When radioautography was used with actinomycin D, it was found that the actinomycin D inhibited the uptake of uridine-<sup>3</sup>H, i.e. inhibited RNA synthesis, in those nucleoli which lost their nucleolar vacuoles. In addition, after removal of the cells from actinomycin D, it was found that as the cells regained nucleolar vacuoles the nucleoli also began to incorporate uridine-<sup>3</sup>H. Electron micrographs showed the nucleoli to be composed of a compact, finely fibrous central portion surrounded by a layer of dense particles 100-150 A in diameter. Nucleolar vacuoles occurred in the fibrous central portion. Dense particles similar to those in the outer layer of the nucleoli were found scattered throughout the vacuoles and in a dense layer at their outer edge. These data suggest that in cultured tobacco callus cells the formation and contraction of nucleolar vacuoles is closely related to RNA synthesis in the nucleolus.

## INTRODUCTION

Many reports occur in the literature which mention the presence of nucleolar vacuoles in plant and animal cells. The major papers which discuss nucleolar vacuoles from a morphological viewpoint are those by Montgomery (1898), Dermen (1933), Lafontaine (1958), Lafontaine and Chouinard (1963), Soudek (1960), Soudek and Stránská

(1958) and Raven (1958). Little evidence, however, has been cited to indicate any relationship between nucleolar vacuoles and nucleolar physiology although Höpker (1953) described nucleolar vacuoles and considered the vacuoles to be physiological products of the nucleolus. Recently Johnson (1964) and Johnson and Jones (1967)

have shown that nucleolar vacuoles form and contract repeatedly in cultured tobacco cells. Those studies indicated that nucleolar vacuoles are a normal component of growing cultured tobacco cells and that their presence appears to be related to the physiological condition of individual cells. This report presents additional observations on nucleolar vacuoles and proposes a possible relationship between nucleolar vacuole formation, vacuole contraction, and RNA metabolism of the nucleolus. A preliminary report of this work has been presented by Johnson (1966).

#### MATERIALS AND METHODS

Cultured callus cells from hybrid tobacco (*Nicotiana tabacum* × *N. glutinosa*) established as single cell clones H-196 and H-239 by Hildebrandt (1958) were used in this study. Cells used for experimental purposes were routinely cultured in liquid *D* and *C* tobacco medium. Liquid *D* medium consists of the mineral salts necessary for plant growth, as formulated by Hildebrandt et al. (1946), supplemented with coconut milk (150 ml/l), calcium pantothenate (2.5 mg/l), naphthaleneacetic acid (0.1 mg/l), and 2,4-dichlorophenoxyacetic acid (6.0 mg/l). *C* medium is the same as above except that the 2,4-dichlorophenoxyacetic acid is omitted.

#### Radioautography

Rapidly growing cells were incubated in liquid medium containing uridine-<sup>3</sup>H, 5 μc/ml (specific activity 2.0 c/mnole)<sup>1</sup> for 30 min. After the incorporation period, the cells were fixed for 30 min with cold (4°C) 3% glutaraldehyde in phosphate buffer (pH 6.6). After initial fixation, all operations up to the final steps of embedding were carried out at approximately 4°C. Cells designated as untreated were first rinsed in cold buffer solution for removing traces of glutaraldehyde and then treated with cold 5% trichloroacetic acid for 5 min. After these operations, the cells were rinsed with water, dehydrated with alcohol, and embedded in Epon 812 (Luft, 1961). Control cells, used to check the specific incorporation of uridine-<sup>3</sup>H into RNA, were fixed and rinsed with phosphate buffer as described above, then transferred to one of the following treatments: (a) RNase<sup>2</sup> 1 mg/ml (pH 6.5) at 37°C for 4 hr followed by 5-min rinse in cold 5% trichloroacetic acid; (b) 5% trichloroacetic acid at 90°C for 20 min. After the above treatments, the cells were rinsed in cold water,

<sup>1</sup> Obtained from Schwarz BioResearch, Inc., Orangeburg, New York.

<sup>2</sup> Obtained from Worthington Biochemical, Freehold, New Jersey.

dehydrated, and embedded as described previously. Sections were cut at approximately 1 μ with glass knives on a Porter-Blum MT-1 ultramicrotome and mounted on microscope slides. Since the cells were sectioned at 1 μ, the problem of determining whether silver grains were actually over the nucleolus or in the nucleoplasm was avoided. The nucleoli in this material varied in size from approximately 2 to 10 μ and, since in most cases serial sections were available for examination, it was assumed that silver grains seen over the nucleolus were due to uridine incorporation into RNA in the nucleolus. The slides were processed for radioautography according to the procedure suggested by Kopriva and Leblond (1961). They were coated with Kodak NTB-2 liquid emulsion and developed after 9 days of exposure at 4°C. For facilitating observation, the sectioned material was stained for 1 hr at room temperature with (0.25%) azure B solution at pH 4. Silver grains were counted and photographed with a Zeiss apochromatic 40× oil-phase objective.

For each nucleolus observed, the number of silver grains over it were counted and the diameter of the nucleolus was measured with an ocular micrometer. This process was repeated for each serial section in which the nucleolus occurred. After these data were assembled, the average grains per μ<sup>2</sup> of nucleolar material for each nucleolus was computed. It is this average number which is shown on the histograms (Fig. 1). When grains per μ<sup>2</sup> in nucleoli containing vacuoles were computed, the area occupied by the vacuoles was included.

#### Actinomycin D Treatment of Living Cells

Actinomycin D used in these experiments was supplied by Merck, Sharp, and Dohme (West Point, Pa.) as number 3298 Lyovac Cosmegen, a lyophilized powder (0.5 mg of actinomycin D with 20.0 mg of mannitol). This powder was dissolved in sterile distilled water to make 500 μg of actinomycin D per ml. The concentration used for experimental purposes was made by mixing the above solution with sterile culture medium. Observations on the effects of actinomycin D on the nucleolus and nucleolar vacuoles of living cells were conducted under aseptic conditions with the use of a Sykes-Moore tissue culture chamber.<sup>3</sup> Addition and removal of medium and/or actinomycin D was accomplished by inserting a sterile syringe needle through the rubber O-ring of the culture chamber. A microscope equipped with Zeiss dark-phase optics was used for observations. For each set of observations, 50 cells were randomly selected. These same cells were observed throughout the experiment. Actino-

<sup>3</sup> Obtained from Bellco Glass Inc., Vineland, New Jersey.

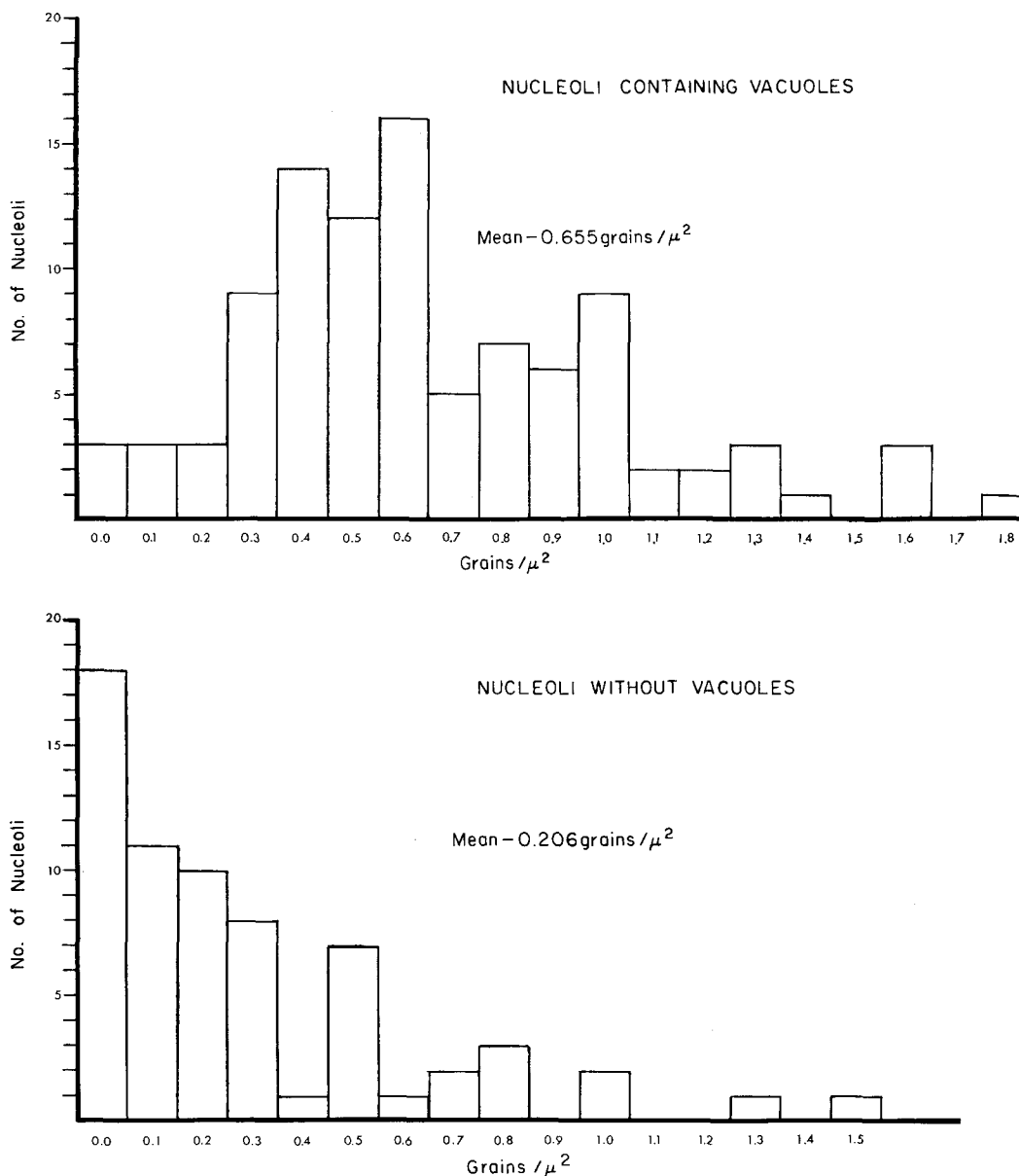


FIGURE 1 Results of grain counts made in a population of growing tobacco callus cells labeled for 30 min with uridine- $^3\text{H}$  ( $5 \mu\text{c}/\text{ml}$ ). The grains/ $\mu^2$  of nucleolar material represents the average of counts made on several serial sections counted for each nucleolus.

mycin D concentrations of 6, 10, 50, and 100  $\mu\text{g}/\text{ml}$  were used. Observations were made at 30 min-1 hr intervals.

#### *Actinomycin D and Radioautography*

Cultures of tobacco callus cells growing under aseptic conditions were treated with 10  $\mu\text{g}/\text{ml}$  actinomycin D for intervals varying from 1 to 3.5 hr.

After 4 hr, the cells were removed from the medium containing actinomycin D, rinsed, and placed in fresh culture medium for the remainder of the experiment. Samples were taken from these remaining cells at intervals of 1, 4, and 9 hr after transfer to fresh medium. Samples of cells from the above treatments were incubated for 30 min with 5  $\mu\text{c}/\text{ml}$  uridine- $^3\text{H}$  so as to check for incorporation into RNA and to

discover whether RNA synthesis was being inhibited by the actinomycin D.

After the above incubation period, the cells were fixed in 3% glutaraldehyde at 4°C for 30 min and processed for radioautography as described above.

Controls for this experiment were an identical culture of cells which were untreated throughout the experiment. Samples were taken from this culture at the beginning of the experiment, at 1 hr after removal of actinomycin D from the experimental culture, and at 9 hr after the end of the experiment. These cells were then incubated in uridine-<sup>3</sup>H and processed in a manner identical with that for the experimental cells.

### Electron Microscopy

H-196 tobacco callus cells were fixed for 12 hr at 4°C in 6% glutaraldehyde in phosphate buffer (pH 6.6) followed by 1% OsO<sub>4</sub> (pH 6.6) for 2 hr. Cells were dehydrated in cold alcohol and embedded in Epon 812 (Luft, 1961). Sections were cut with glass knives on a Porter-Blum MT-1 ultramicrotome and were stained for 1-2 hr with 3% uranyl acetate solution followed by lead citrate (Reynolds, 1963). Grids were examined on an RCA EMU 3C electron microscope.

## RESULTS

### Radioautography of Vacuolated and Unvacuolated Nucleoli

A comparison of the two histograms (Fig. 1) shows that there is a greater incorporation of uridine-<sup>3</sup>H into the RNA of nucleoli containing vacuoles than into that of nucleoli which lack vacuoles. Only nine nucleoli which contained vacuoles had 0-0.2 grains/ $\mu^2$ , whereas 30 of the nucleoli lacking vacuoles had 0-0.2 grains/ $\mu^2$ . A few of the nucleoli which apparently lacked vacuoles were labeled quite heavily. These nucleoli, however, were small in comparison to others measured. These data tend to indicate that some small nucleoli are actively metabolizing even though they lack detectable vacuoles. Figs. 3-5 show examples of the labeled nucleoli used for obtaining the data in this experiment. Figs. 3 and 4 show peripheral incorporation in the matrix material around the nucleolar vacuole. Fig. 5 shows only slight label in a nucleolus with only very small vacuoles. The silver grains seen throughout the nucleus are believed to represent RNA which has been labeled in association with the chromatin. These grains are removed by ribonuclease treatment. There exists the possibility that these grains could be RNA from the nucleolus

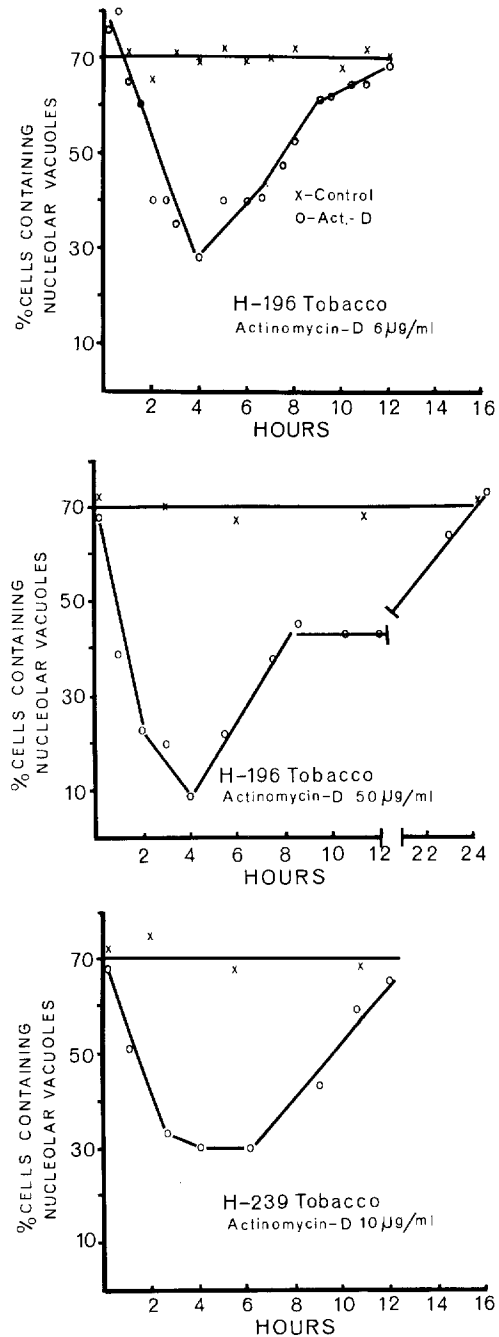


FIGURE 2 The effect of actinomycin D on nucleolar vacuoles in living tobacco cells. The cells were removed from the actinomycin D after a 4-hr treatment and allowed to recover from its effects. Percentages were based on observations of a total of 50 cells for H-196 tobacco cells and a total of 100 cells for the H-239 tobacco cells.

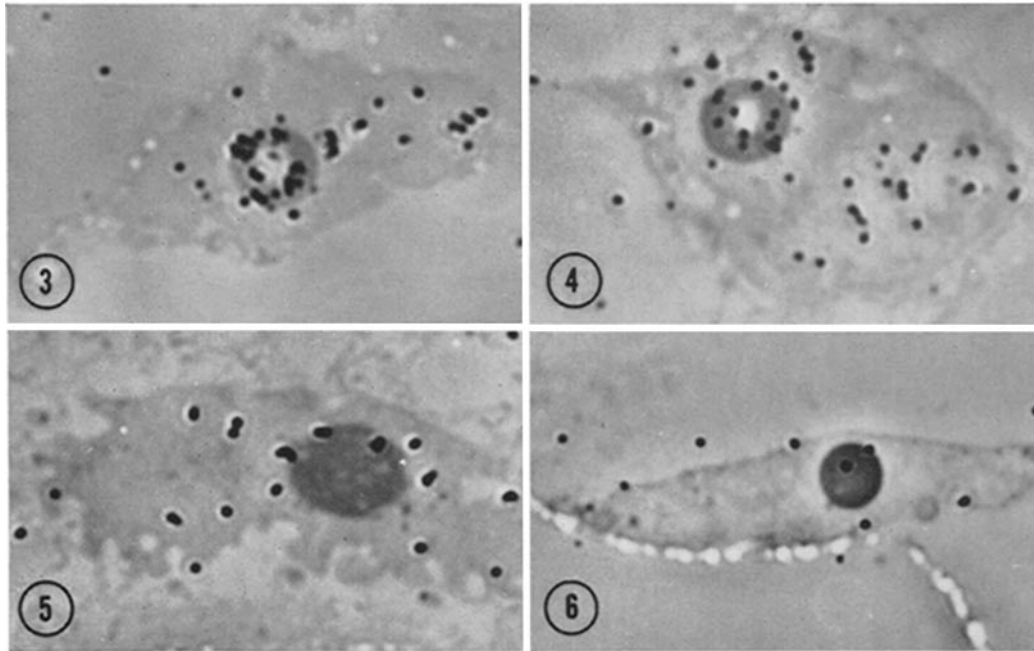


FIGURE 3 Nucleus and nucleolus of a normal, untreated tobacco callus cell labeled for 30 min with uridine-<sup>3</sup>H. Note clusters of silver grains at periphery of nucleolar vacuole. Scattered grains in nucleus are probably due to RNA synthesis associated with the chromatin or to RNA which is on its way from nucleolus to the nucleoplasm.  $\times 1200$ .

FIGURE 4 Same as Fig. 5. Normal, untreated cell with scattered label over vacuolated nucleolus.  $\times 1200$ .

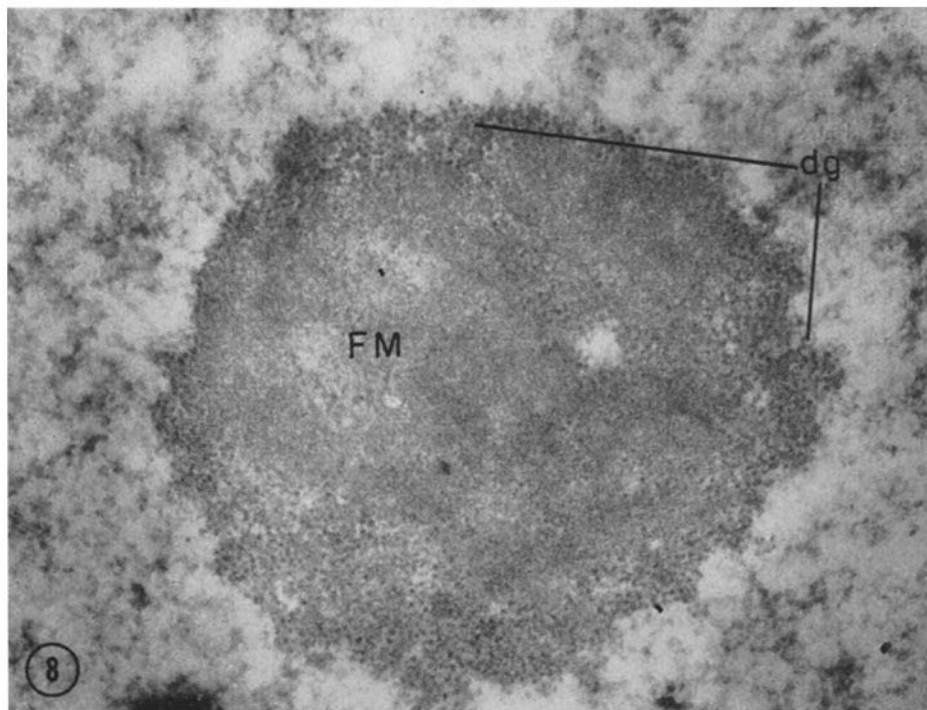
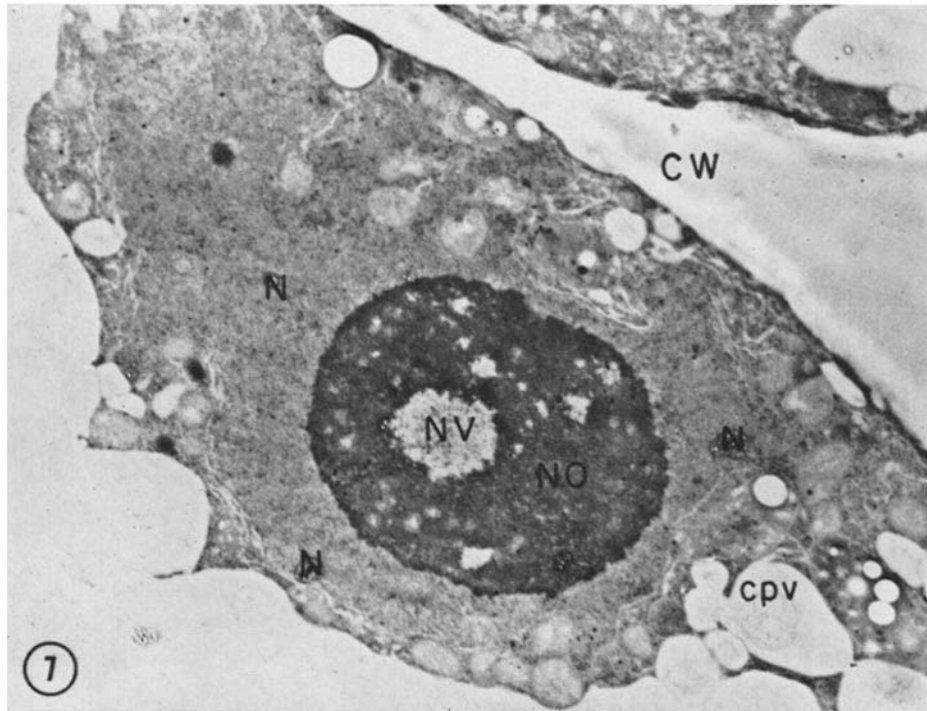
FIGURE 5 Untreated cell, but nucleolus contains only very small nucleolar vacuoles—only a very light label.  $\times 1200$ .

FIGURE 6 Nucleus and nucleolus in a cell which was treated for 3.5 hr with actinomycin D (10  $\mu\text{g}/\text{ml}$ ). Note the homogeneous texture and the very light labeling of the nucleolus and nucleus.  $\times 1200$ .

TABLE I  
*Uptake of Uridine-<sup>3</sup>H into Nucleoli of Actinomycin D-Treated H-196 Tobacco Callus Cells*

	Control cells	Actinomycin D-treated cells					
		Hours of actinomycin D treatment			Hours after cells were transferred to fresh medium		
		1 hr	2.5 hr	3.5 hr	1 hr	4 hr	9 hr
% nucleoli containing vacuoles	58	36	24	12	26	30	54
Mean* No. of silver grains per nucleolus	6.2 $\pm 1.3$	3.7 $\pm 1.5$	3.0 $\pm 1.4$	3.0 $\pm 1.1$	3.9 $\pm 1.4$	3.5 $\pm 2.0$	5.0 $\pm 1.5$
% nucleoli without vacuoles	42	64	76	88	74	70	46
Mean* No. of silver grains per nucleolus	0.55 $\pm 0.32$	0.31 $\pm 0.25$	0.29 $\pm 0.17$	0.40 $\pm 0.22$	0.35 $\pm 0.21$	0.22 $\pm 0.19$	0.48 $\pm 0.34$

\* (All means are expressed at the 5% level of significance).



**FIGURE 7** Nucleus (*N*), nucleolus (*NO*), nucleolar vacuole (*NV*), cell wall (*CW*), and small cytoplasmic vacuoles (*cpv*) in a normal cultured tobacco callus cell. This view of the nucleus and nucleolus is typical for cells from a rapidly growing culture.  $\times 5300$ .

**FIGURE 8** A nucleolus lacking large vacuoles. The nucleolus typically has a central portion composed of finely fibrous material (*FM*) surrounded by a rim of dense particles (*dg*).  $\times 38,000$ .

which is on its way to the nucleoplasm. However, since the time of exposure to label was only 30 min, this possibility would represent a rather rapid movement of RNA out from the nucleolus.

#### *Effect of Actinomycin D on Nucleolar Vacuoles*

A study was undertaken for determining the effect of actinomycin D on nucleolar vacuoles in tobacco callus cells. As has been previously described in this material, nucleolar vacuoles normally occur in about 70% of the nucleoli of growing tobacco callus cells (Johnson and Jones, 1967). In addition, it has been shown that these structures periodically contract and reform. In these experiments, small groups of cells, to be used for experimentation and controls, were taken from liquid cultures and were placed aseptically into Sykes-Moore chambers. The most obvious effect of actinomycin D on the nucleolus at all concentrations was the nearly immediate decrease in the number of nucleoli which contained vacuoles. The decrease in the percentage of cells which contained vacuoles, as seen in Fig.

2, seems to be related to the concentration of actinomycin D. With concentrations of 50 and 100  $\mu\text{g}/\text{ml}$  the percentage of cells containing nucleolar vacuoles dropped to 10%. The other striking phenomenon was the recovery of the cells from the effect of the treatment. When the actinomycin D was removed after 4-hr treatment, new nucleolar vacuoles began to form within 30 min-1 hr. The time required for recovery of nucleolar vacuoles to the control level depended upon the concentration of inhibitor used (see Fig. 2). This recovery was apparently complete since cells from cultures that were observed for several days after treatment were seen to undergo repeated mitosis and cell division.

The only major cytological effect of the actinomycin D noted at low concentrations was the loss of the nucleolar vacuoles and the rounding up of the nucleolus. Fig. 6 shows a light micrograph of an actinomycin D-treated nucleolus. Treatment of cells with concentrations as high as 100  $\mu\text{g}/\text{ml}$  for periods longer than 4 hr did kill some of the cells. No cell death was observed, however, at concentrations of 6, 10, and 50  $\mu\text{g}/\text{ml}$ .

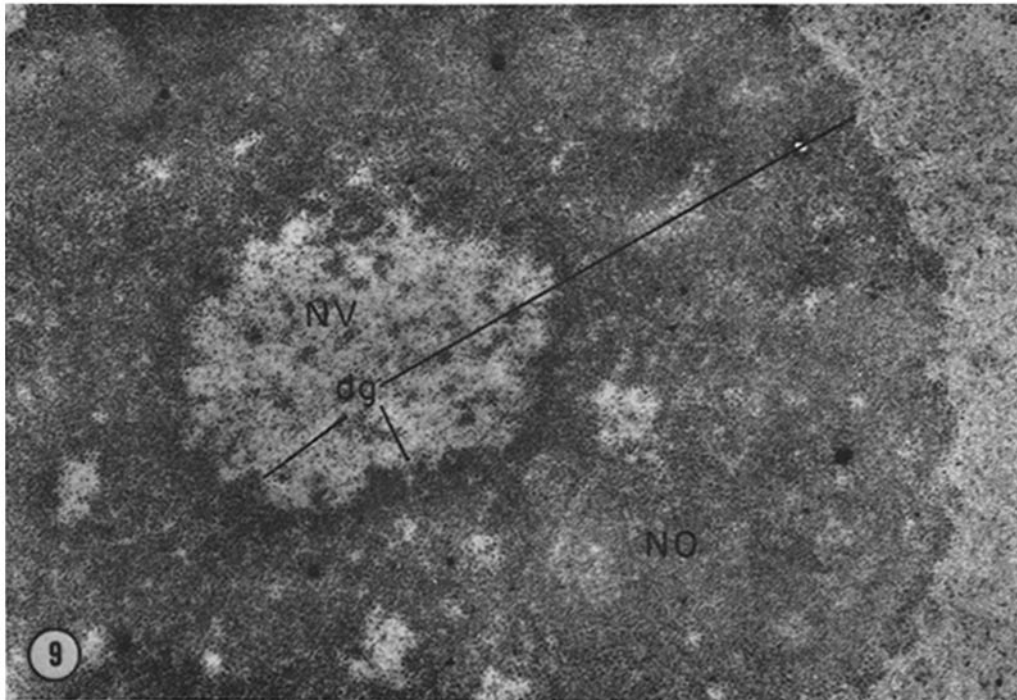


FIGURE 9 A nucleolus with a large central vacuole (NV). Lining the vacuole is a well defined layer of dense particles (dg) which are similar in size and appearance to those at the periphery of the nucleolus (NO).  $\times 14,000$ .

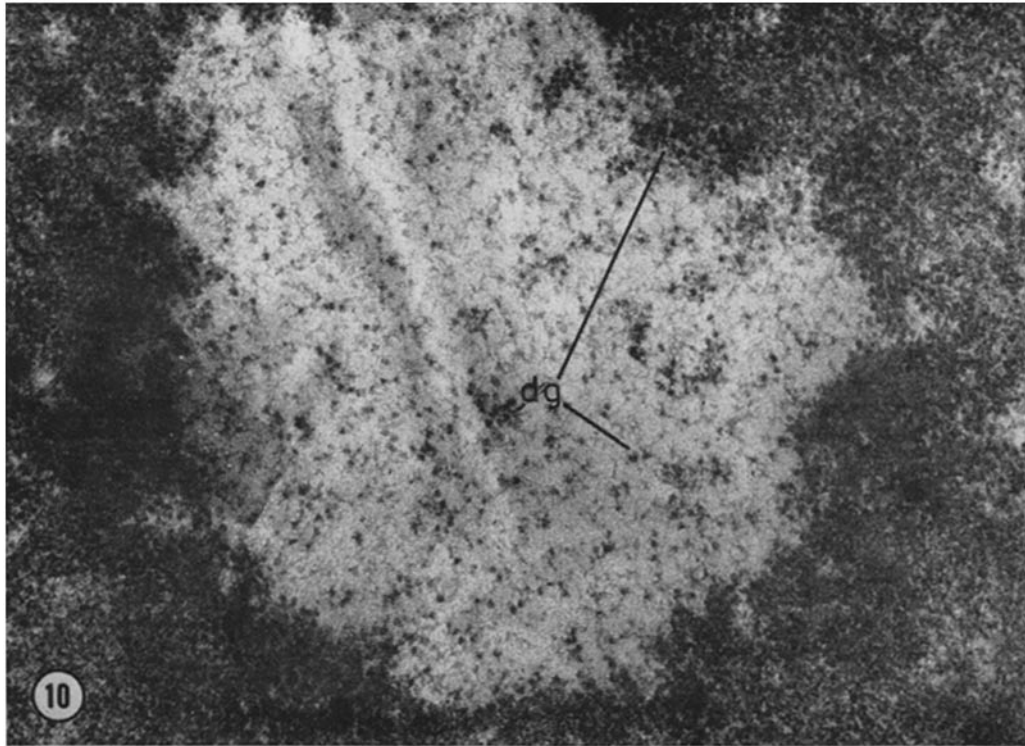


FIGURE 10 A high-resolution micrograph of a nucleolar vacuole showing the dense particles (*dg*) associated with fine filamentous structures scattered throughout the vacuole.  $\times 61,000$ .

#### *Uridine Labeling and Actinomycin D*

This portion of the study was undertaken so as to determine the relationship between the loss of nucleolar vacuoles and the ability of the nucleolus to incorporate uridine- $^3\text{H}$ . The data shown in Table I were obtained by scoring as to presence or absence of nucleolar vacuoles for the first 10 nucleoli encountered on each of five different slides. The grains over each nucleolus were then counted, and the average number of grains for each class of nucleolus was computed. A significant difference exists, in all cases, between the means of nucleoli containing vacuoles and those of nucleoli lacking vacuoles. The results in Table I substantiate the hypothesis that the nucleoli show vacuoles are the ones with higher grain counts and that thus they are presumably the most actively engaged in RNA synthesis. This is particularly evident from the increase in the number of grains found over the nucleoli which are recovering from actinomycin D treatment. The actinomycin D, however, did not completely suppress all incorporation into all the nucleoli,

but merely reduced it to a very low level after a 3.5-hr treatment.

#### *Electron Microscopy*

Nucleoli lacking vacuoles were found to consist of a fibrous compact matrix, as shown in Fig. 8, surrounded by a rim of dense particles 100–150 Å in diameter. Nucleoli containing vacuoles appeared as in Fig. 7. The vacuoles generally were found in the finely fibrous central portion of the nucleolus. Dense particles, similar to those at the periphery of the nucleolus, were scattered throughout the vacuole and in a dense layer at its periphery (Fig. 9). In higher magnification micrographs (Fig. 10), the dense granules occurring throughout the vacuole and at its periphery appeared to be associated with fine filamentous structures about one-half to one-fourth the diameter of the granules.

#### DISCUSSION

In this study, it has been found that vacuolated nucleoli of tobacco callus cells incorporated uridine- $^3\text{H}$  into RNA much more readily than did



nucleoli lacking vacuoles. These data support the hypothesis, suggested by Soudek and Stránská (1958) and Soudek (1960) from their studies on the fungus *Basidiobolus ranarum*, that nucleolar vacuoles are important in its growth, and the suggestion made by Höpker (1953) that nucleolar vacuoles may contain physiological products of the nucleolus. In addition, these data also support our previous suggestion that nucleolar vacuoles may be related to the RNA metabolism of the nucleolus. Actinomycin D has been shown to suppress DNA-directed RNA synthesis (Reich, 1963, 1964) and, in this regard, also to block labeling of the nucleolus with tritiated nucleotides (Perry, 1963; DeMan and Noorduyn, 1967). In addition, other studies have shown that actinomycin D also causes certain morphological changes in the nucleolus (Stevens, 1964; Schoeffl, 1964; DeMan and Noorduyn, 1967). The observations that actinomycin D causes the nucleolar vacuoles to contract and at the same time to inhibit RNA synthesis further suggest a relationship between RNA synthesis and nucleolar vacuole formation. In addition, the return of RNA synthesis and nucleolar vacuoles following removal of actinomycin D also support this hypothesis. Electron micrographs have shown a particulate component also associated with the nucleolar vacuoles. This par-

ticulate component is similar to that described in nucleoli by Stevens (1964, 1965), Lafontaine (1958), Lafontaine and Chouinard (1963), Chouinard (1966), and Hyde (1966). It is also similar to the particulate component described by DeMan and Noorduyn (1967) for hepatic cell nucleoli. Those workers also cite some evidence that it is this portion of the nucleolus which labels with tritiated RNA precursors. In the present study, no such evidence was obtained. However, Fig. 3 shows label associated with the periphery of the nucleolar vacuole, and it is in this area that a dense rim of granules forms as is shown by Fig. 9. It is, therefore, suggested that, at least in tobacco callus tissue, nucleolar vacuoles are closely related to the RNA synthetic activity of the nucleolus.

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