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AUTORADIOGRAPHIC STUDIES ON THE INTRACELLULAR DISTRIBUTION OF ^{3}H-ACTINOMYCIN S IN TG CELLS[1]

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SUMMARY The intracellular distribution of actinomycin S_3 randomly labeled with tritium (^{3}H-ACT) was investigated by autoradiography. Monolayer cultures of TG cells were exposed to 10 or 20 μg/ml of ^{3}H-ACT for 15 to 120 minutes at 37°C. By counting the number of silver grains observed on autoradiograms it was found that the ^{3}H-ACT accumulated preferentially in the nuclear region. Treatment with DNase resulted in the disappearance of silver grains, while RNase had no effect. The finding of the accumulation of ^{3}H-ACT in the nuclear region evidently support the idea that the biochemical mechanism of the action of actinomycin is to inhibit DNA dependent cellular RNA by forming a complex with DNA.

INTRODUCTION

Since the discovery of the preferential formation of a complex between actinomycin and DNA (KAWAMATA and IMANISHI, 1959; KAWAMATA and IMANISHI, 1960; RAUEN *et al.*, 1960; KIRK, 1960) there have been reports on the biochemical mechanism of action of actinomycin and, particularly on its inhibition of DNA dependent cellular RNA synthesis (HURWITZ *et al.*, 1962; REICH *et al.*, 1963; LEVINTHAL *et al.*, 1963). At concentrations of actinomycin that do not completely suppress RNA formation, however, differential inhibition of the various classes of RNA is observed (PERRY, 1962; GEORGIEV, 1963; YAMADA *et al.*, 1964; KLENOW and FREDERIKSEN, 1964). From these observations it was assumed that cellular RNAs are synthesized in different parts of the cell. Meanwhile in animal cells, morphological changes of the nucleoli are claimed to be the most characteristic of the effects of actinomycin (GOLDSTEIN *et al.*, 1960; JOURNEY and GOLDSTEIN, 1961; SCHOEFL, 1964).

The demonstration of the actual localisation of actinomycin in the cell, therefore, seems to

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be a good approach to the elucidation of its action and also to the function of the organellae of the cells in reference to cellular RNA synthesis. Autoradiography with tritium labeled actinomycin is considered to be a useful method for this purpose. Only a few reports have appeared on the intracellular distribution of actinomycin (HARBERS *et al.*, 1963 a; HARBERS *et al.*, 1963 b). The results reported in this paper are mainly on the intracellular localisation of tritium labeled actinomycin S as demonstrated by autoradiography. Part of this work has already been published in a preceding paper (KAWAMATA *et al.*, 1964).

MATERIALS AND METHODS

1. *Cell line*

The cells used in these experiments were the established strain TG Cl-33 derived from normal human fallopian tube (BARSKI *et al.*, 1959) which were supplied by Dr. G. BARSKI, Paris.

2. *Methods of cell culture*

The cells were grown on cover slips in Leighton type square tubes containing Eagle HeLa medium (Difco) supplemented with 3.75 per cent calf serum and 3.75 per cent horse serum, and as antibiotics 50 mg/l of dihydrostreptomycin sulfate and 100, 000 μ/l of sodium penicillin G. Unless otherwise stated 1×10^5 cells were inoculated onto each cover slip and incubated at 37°C for 4 days. Each experimental group consisted of three tubes.

3. *^{3}H-actinomycin S_3*

^{3}H-actinomycin S_3 (^{3}H-ACT) was prepared by the gas exchange method with tritium in the laboratory of Dr. T. KOMAI at the National Institute of Health, Tokyo. This preparation of randomly labeled ^{3}H-ACT was purified by aluminum column chromatography and ethyl acetate treatment and finally by precipitation with thymus DNA. The procedure is indicated as follows:

calf thymus DNA (2 mg/ml) + ^{3}H-ACT (200 μg/ml) > mix in equal volumes
↓
stand for 10 minutes
↓
add 1/10 volume of 1 M NaCl and 2 volumes of 95 per cent ethanol
↓
shake gently for 10 minutes
↓
precipitate (^{3}H-ACT-DNA complex)
↓
dissolve in H_2O
↓
stand in boiling water bath for 10 minutes
↓
cool rapidly to room temperature
↓
extract with ethyl acetate
↓
evaporate the ethyl acetate
↓
recrystallize from ethyl acetate

The purity of the purified ^{3}H-ACT preparation was examined by paper chromatography employing a solvent system composed of aqueous solution of 2 per cent β-naphthalene sulphonic acid, *n*-dibutylether and ethyl acetate (2 : 1 : 1). The purified preparation of ^{3}H-ACT showed a single spot of the characteristic orange red color of actinomycins and had a specific activity of 30 μc/m mol. The antibacterial actvity and radioactivity on the paper were only demonstrated in this spot.

4. *^{3}H-actinomycinic S_3 acid*

Actinomycinic acid, which is a biologically less active derivative of actinomycin from which the lactone rings are split off, was obtained by treatment of actinomycin with alkaline alcohol solution. ^{3}H-actinomycinic S_3 acid was prepared by similar treatment of ^{3}H-ACT. ^{3}H-ACT was dissolved in methanol containing 1/10 N NaOH and treated in a water bath at 4°C for 4.5 hours. Then the solution was neutralized with 1 N HCl. The purity of this preparation was also examined by paper chromatography.

5. *Morphological observation*

Cells grown on a cover slip were washed three times with chilled (4°C) phosphate buffered saline (PBS) and fixed in methanol for 2 to 3 minutes. Then they were stained by immersion in Giemsa (pH 5.7) for 30 minutes.

6. *Autoradiography*

After treatment with ^{3}H-compounds, the cover slip was washed 5 to 7 times with cold PBS and then the cells fixed with methanol for 2 to 3 minutes. The slide was immersed in PBS again for 4 to 6 hours at 37°C and then washed twice with PBS. After

drying, the cover slip was dipped in Kodak emulsion NTB2 and then exposed for 2 weeks in a refrigerator. Then the slip was developed, fixed and stained with Giemsa. One hundred cells were selected at random for silver grain counts.

7. *Treatment with nucleases*

After treatment with ^{3}H-ACT, the cells were fixed with methanol and treated with DNase at 37°C for 24 hours or RNase at 40°C for 4 hours. To prepare the DNase solution 0.05 mg of DNase (crystallized, Worthington) was dissolved in 1 ml of Gomori's tris buffer (pH5.7) containing 0.2 M $MgSO_4 \cdot 7H_2O$. RNA solution was prepared by dissolving 1 mg of RNase (crystallized, salt free, Worthington) in 1 ml of distilled water.

RESULTS

1. *Penetration of ^{3}H-ACT into the cells*

The cells were treated with 10 or 20 μg/ml of ^{3}H-ACT for 2 hours at 37°C. At intervals specimens were taken for autoradiography and the number of silver grains was counted. As shown in Table 1 few silver grains were detected either in the nuclear and cytoplasmic regions in 15 or 30 minutes. After 60 minutes incubation, the number of silver grains was greater, particularly in the nuclear region and after 120 minutes the number reached a maximum. In the cytoplasmic regions, however, the number of silver grains did not change through out the observation period. Figs. 1 a and b show the effect of time on the grain counts in the nuclear or cytoplasmic regions, respectively, of the cell. Figs. 2a and b represent similar results on the distribution of silver grains in the nuclear or cytoplasmic regions per 100 μ^2, respectively. Figs. 3a–e show autoradiograms. When cells were incubated at 4°C with 10 and 20 μg/ml of ^{3}H-ACT for 15, 30, 60 and 120 minutes, no silver grains were detected either in the nuclear or the cytoplasmic regions.

TABLE 1 *Distribution of silver grains in TG cells after treatment by ^{3}H-ACT**

	Concentration of ^{3}H-Act (μg/ml)	Time (min.)			
		15	30	60	120
Nucleus	20	4.0	7.9	13.5	22.5
	10	3.5	4.7	12.3	16.1
Cytoplasm	20	2.0	4.7	2.3	5.3
	10	1.8	1.3	1.9	1.6

* Average number of silver grains per 100 cells.

2. *Effects of nucleases*

After treatment of the cells with 20 μg/ml of ^{3}H-ACT for 2 hours, the cells were treated with DNase or RNase and then observed by autoradiography. In the control, which was

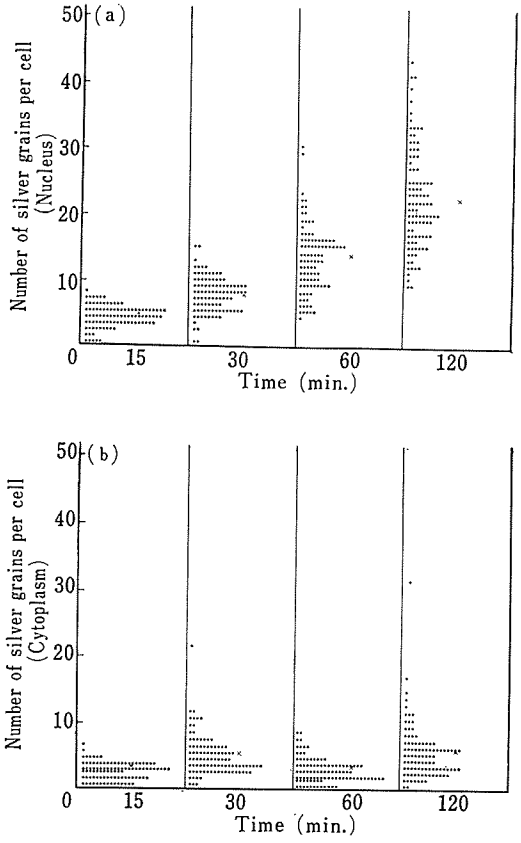

FIGURE 1 Distribution of silver grains in TG cells after treatment by ^{3}H-ACT.
(a) Nucleus (b) Cytoplasm
× Average number of silver grains per 100 cells

a preparation not treated with DNase, a mean number of 30.6 silver grains were found in the nuclear region of 100 cells. However, in the DNase treated specimen, no silver grains were detected in the nuclear or cytoplasmic regions. With RNase, the mean number of silver grains in the nuclear region was 31.4, which was similar to the mean number in the untreated control, which was 32.4. Figs. 4a and b show these results.

3. *Effect of ^{3}H-actinomycinic S_3 acid*

When the cells were treated with 10 μg/ml of ^{3}H-actinomycinic S_3 acid at 37°C for 2, 4, 6, 18 and 24 hours, no silver grains were detected at all in the cells. The treated culture grew as well as the untreated control culture. This means that ^{3}H-actinomycinic S_3 acid at this concentration does not affect the cells, for it does not penetrate them.

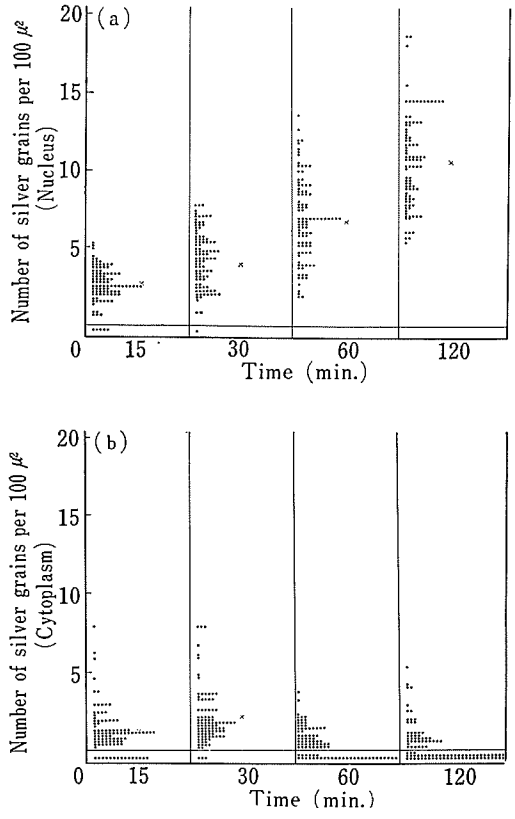

FIGURE 2 Distribution of silver grains per 100 μ^2 of TG cells after treatment by ^{3}H-ACT.
(a) Nucleus (b) Cytoplasm
× Average number of silver grains per 100 μ^2 of cells

DISCUSSION

The accumulation of silver grains particularly in the nuclear region was demonstrated after treatment of TG cells in culture with ^{3}H-ACT. This is in accord with the results of HARBERS *et al.* (1963 a) who found that ^{3}H-actinomycin C_1 accumulated in the nuclei of Ehrlich's ascites tumor cells. To identify the grains found in the nuclear regions the cells were treated with DNase or RNase after the ^{3}H-ACT had been fixed in the cells. Complete elimination of silver grains from the cells was observed only after DNase treatment. This suggests that the ^{3}H-labeled compound is bound to DNA and not to RNA. In another series of experiments the quantitative recovery of actinomycin from actinomycin treated cells was confirmed. These results strongly support the assumption that the grains found in the cells originated from ^{3}H-ACT itself, because only actinomycin forms a complex with highly polymerized DNA and other degradation products of actinomycin do not. Our results with ^{3}H-actinomycinic S_3 acid supports this assumption. Although not mentioned in the results, no accumulation of silver grains in the nucleolar region has so far been observed but a rather dense localisation of grains in the perinucleolar region was noticed. These findings are now under investigations.

The finding of a particular localisation of a drug in the cell, of course, does not necessarily indicate the site of action of the drug. In the case of actinomycin, however, its accumulation in the cell nuclei is a necessary step in the exhibition of its cytological effects. Further studies on the localisation of actinomycin at the organellae and also the relationship of actinomycin to RNA synthesis in the cells are now under progress.

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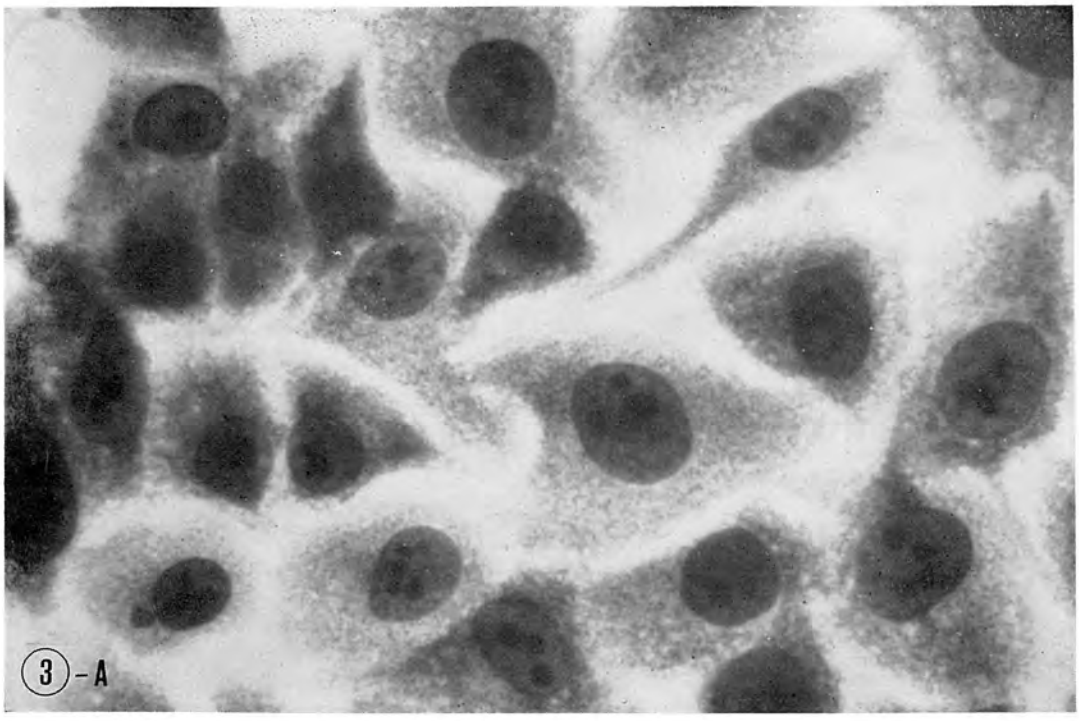


FIGURE 3 Autoradiogram of TG cells treated by 20 μg/ml of ^{3}H-ACT.
A : Control, untreated TG cells

B : Treatment for 15 minutes	C : Treatment for 30 minutes
D : Treatment for 60 minutes	E : Treatment for 120 minutes

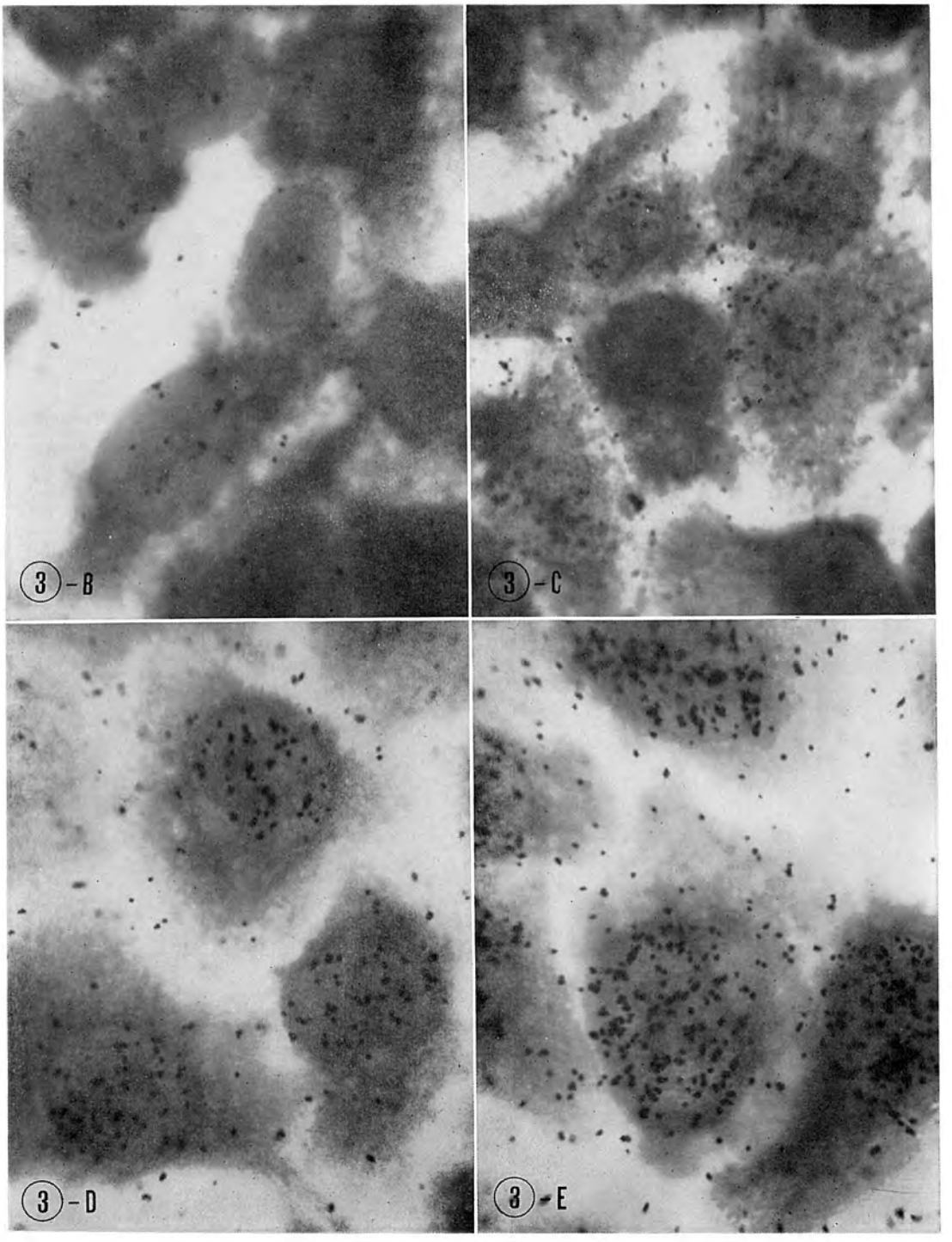
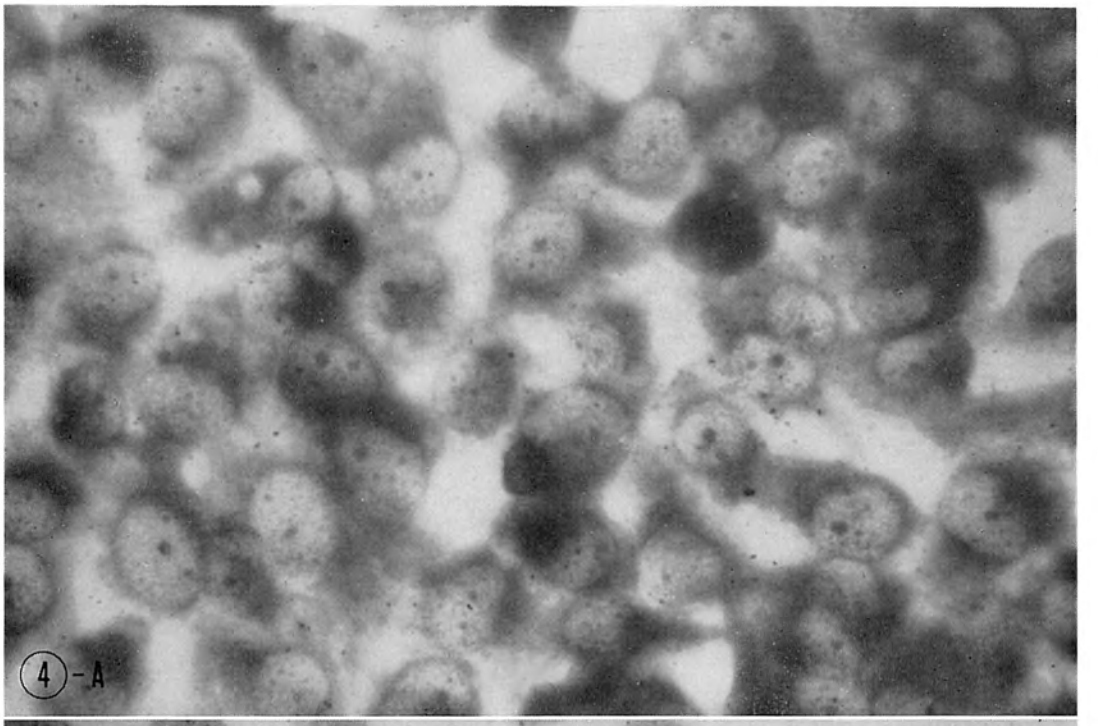

3-B
3-C
3-D
3-E

FIGURE 4 Effects of DNase or RNase on the accumulation of silver grains by ^{3}H-ACT in TG cells.

A : Treatment by DNase

B : Treatment by RNase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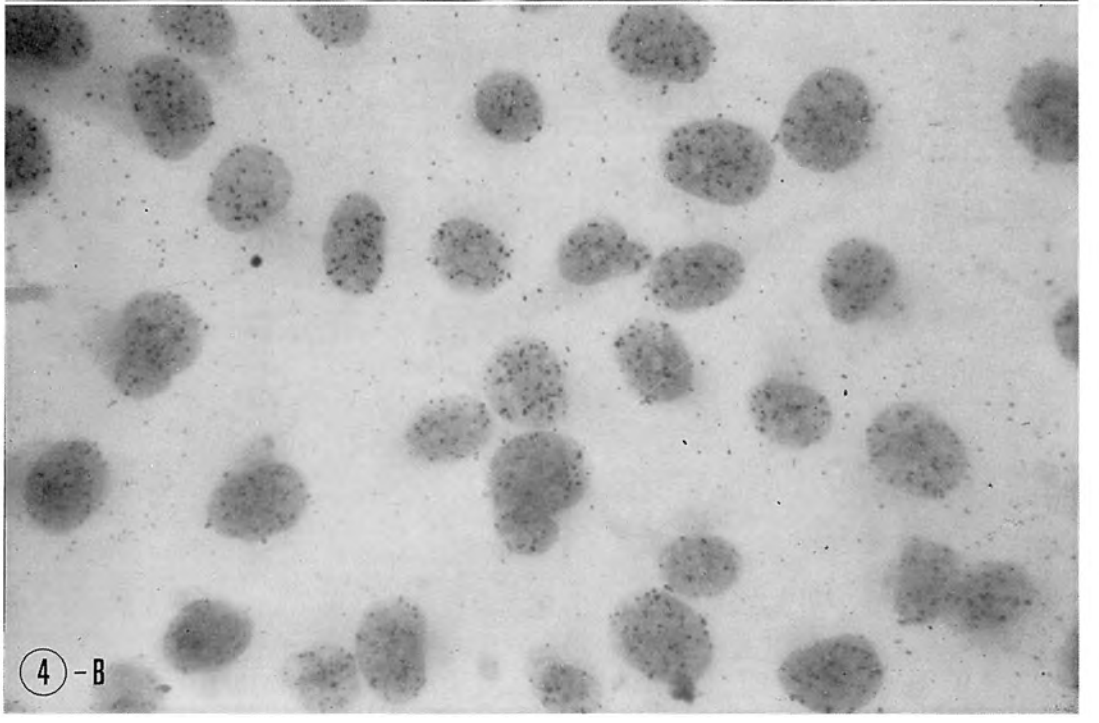
4-B