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Synthesis of Japanese Encephalitis Virus in Porcine Kidney Stable Cells Observed by Fluorescent Antibody Technique and Autoradiography*

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SUMMARY

The site of the synthesis of Japanese encephalitis virus (JEV) in the actinomycin-treated and infecter PS Y15' cells (a porcine kidney stable cell line) was observed by the immunofluorescent antibody technique, acridine orange staining, and the autoradiographic analysis.

In the parallel studies by immunofluorescent technique and acridine orange staining in the infected cells, viral protein (as an antigen) and viral RNA were detected at the same site of cytoplasm.

In the autoradiographic analysis, the cytoplasmic labeling of ^{3}H-uridine was due to the synthesis of JEV-RNA, while the nucleolus and nucleus were not involved. In the autoradiographic studies on the secton of infected cells, the ^{3}H-uridine was frequently incorporated around the cytoplasmic vacuoles. This localization of labeling agreed with the site of acridine orange positive granules.

The results suggest that the syntheses of the viral RNA and viral protein occurred in the similar site of cytoplasm of the infected cells, and also the virus particles seem to be assembled in the sites of the viral RNA and protein syntheses.

INTRODUCTION

The sites of the viral RNA synthesis in the infected cells have been extensively studied by a number of workers. While it has been shown in the several recent studies that the RNA of Mengovirus (Franklin and Rosner, 1962), poliovirus (Penman et al., 1964; Holland and Basset, 1964), Mouse-Elberfeld (ME) virus (Scholtissek et al., 1962), and encephalo-myocarditis (EMC) virus (Dalgarno and Martin, 1965) has been synthesized in the cytoplasm of the infected cells, there is only few reports concerning the site of the synthesis of Japanese encephalitis virus (JEV), the representative of the members of arbovirus B group, in the infected cells. There is a suggestion from the electron microscopic observations that the JEV particles develope by budding from the membrane of the cytoplasmic vacuole but in the present state of the matters few paper has been presented on the site of the synthesis of JEV or JEV-RNA.

In addition to the previous paper (Lee, 1966),

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the author studied on the site of the synthesis of JEV in this report, especially its RNA was analysed in infected PS Y15' cells by the fluorescent antibody technique, acridine orange staining, and the autoradiography. To suppress the cellular RNA synthesis actinomycin D and S_{2+3} were applied to the cells throughout the work. The actinomycin S_{2+3}, a new antitumor agent, which has been derived from Streptomyces, has a function similar to the actinomycin D (Fujita, 1959; Kawamata, et al., 1965).

MATERIALS AND METHODS

Cell culture

The cells of porcine kidney stable cell line (PS Y15'), kindly supplied by Dr. Y.K. Inoue, Institute for Virus Research, Kyoto University, have been cultivated in TC medium 199 (Difco Laboratories, U.S.A.) supplemented with 5 per cent of inhibitor-free calf serum. The monosheets of the cells for the viral plaque assay and the virus multiplication were obtained by seeding 10^6 cells in 5 ml of the same medium per prescription bottle (2 oz.) and incubating for three days.

A cytological observation and immunofluorescent antibody staining (FA) as well as ^{3}H-uridine labeling were performed during the exponentially growing phase of the cells. The cells were those that had proliferated in the same medium described above on a cover slip (11×45 mm^2) in Leighton tube after at least 3 or 4 divisions from the original cells of 5×10^4 per tube.

Virus

The Nakayama strain of Japanese encephalitis virus (JEV), clonized from the largest plaque of PS adapted virus, was used throughout the experiments. The infected PS monolayer cells were mechaniqally scraped and homogenized by VirTis 45 homogenizer (Virtis Co., Gardiner, N.Y., U.S.A.) at the maximum speed for 2 min. at 4°C and centrifuged to remove the cell debris. The supernatant was used as the virus sample.

Plaque assay for virus

The assay was carried out by a modified method of Porterfield (1959).

Fluorescein conjugated immune globulin

The immune sera were obtained from the rabitts whch were immunized by highly purified JEV antigen (Lee, 1966). The immune globulin fraction was purified repeatedly by salting-out with saturated ammonium sulfate and desalting through the column of Sephadex G25 according to the method of Kawamura (1964).

The immune globulin fraction (adjusted protein content to 0.5 per cent) was conjugated with fluorescein isothiocyanate (B.B.L.) of 1/200 of total weight of the protein in carbonate-bicarbonate buffer solution with pH 9.4 for 10 to 12 hours at 4°C. An excess dye was eliminated by passing through the column of Sephadex G25 and of DEAE-cellulose.

Nonspecific fraction of the conjugate was adsorbed with acetone-dried liver powder of the normal mouse or the normal cells of fresh porcine kidney.

Nonspecific reactivity of the conjugate was checked on the frozen sections of the normal mouse brain and PS Y15' cell monosheets to verify its purity. The conjugate of F:P ratio of 2-4 was employed for all the preparations.

Immunofluorescent antibody staining

The cells on the coverslips were washed several times with cold phosphate buffer saline, and fixed in acetone for 5 min. and treated with Daifron (commercial name; same to Genetraon 226; Daikin Co., Japan) for 5 min. at room temperature. They were reacted directly with anti-JEV globulin conjugate in a moist chamber for one hour at 37°C, and for overnight at 4°C to complete the reaction. Then the cells were washed in five changes of phosphate buffer-saline on a vibrator to remove the excess of the conjugate. As controls, normal cells were treated with the conjugated globulin, and infected cells were treated first with unlabeled blocking antiserum and then with the conjugate. Immunofluorescence of the cells was observed under the dark-field illumination and also under the UV light source of Nikon fluorescent microscope (Nippon Kogaku Co., Tokyo, Ja-

pan). The FA cells were photographed using Fuji color film R-100 and the black and white Fuji SSS film (Fuji Photo Film Co., Tokyo, Japan).

^{3}H-uridine incorporation into the cells

The cells on the coverslips were exposed to the amount varying 2 to 10μc/ml ^{3}H-uridine (specific activity 100 mC/mMole, The Radiochemical Center, Amersham, England), and 5 μg/ml of unlabeled thymidine in Eagle's essential medium containing 3 per cent of calf serum. Following the varied times of exposure, between 5 and 60 min., the cells were immediately fixed in a large amount of cold acetic alcohol (1:3) for 10 min., and then washed with 70 per cent ethanol.

Treatment with nucleases

RNase treatment: One-third of the coverslips in each group was treated with 0.2 mg/ml RNase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) in phosphate buffered saline with pH 7.2 for 60 min. at 37°C.

DNase treatment: Another one-third of the coverslips was treated with 0.25 mg/ml DNase (Worthington Biochemical Corp.) in phosphate buffered saline with pH 7.2 containing 0.005 M Mg^{++} for 60 min. at 37°C.

The remaining one-third was not treated with the nucleases.

Autoradiography

The coverslips were treated with 2 per cent perchloric acid for 40 min. at 4°C to remove an acid-soluble materials and washed extensively with running water at room temperature before the emulsion coating. Coating was made with NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y., U.S.A.) diluted with double distilled water to 1:1 proportion, or with NR-M 1 emulsion (Konishiroku Photo Industry Co., Tokyo, Japan). The exposure time in both cases in the dark was between 7 to 20 days at 4°C. The coated coverslips were developed in D-19 solution or in Konidol X (Konishiroku) 5 min. for each case and fixed with Fuji fixer FF-H (Fuji Photo Film Co.). Those coverslips were then stained with 3 per cent Giemsa's staining solution in M/30 phosphate buffer with pH 6.2.

The specially made ocular micrometer of fine square section type was employed for accurate grain counting and for measuring of cell area.

In topographical experiment, the cells after the exposure to ^{3}H-uridine(4 μc/ml) for 30 min., were fixed with 5 per cent neutral formalin at °4C, and extracted with 2 per cent perchloric acid at 4°C for 40 min., then washed extensively with runningwater, and refixed with Palade's osmium tetra-oxide fixative for 10 min. at 4°C. After the dehydration through ethanol series (50 to 100 per cent) the fixed cells were removed from the glass wall of the bottle and embedded in Epon 812 following the method of Luft (1961). Sections of 0.2 to 1 u in thickness were made in LKB ultratome (LKB Produkter AB., Stockholm 12, Sweden) with glass knives and autoradiography was performed on those sections.

Acridine orange staining

Cultures on the coverslips were placed immediately without air-drying into acetic alcohol (1:2) for 10 min., rinsed in each of three bottles of citric acid phosphate buffer (McIlvaine) pH 3.8 for 1 min. and after the staining with 0.01 per cent acridine orange solution for 4 min. the excessive dye was removed by washing in each of the three bottles of citric acid phosphate buffer for 1 min. Those cells were examined under the same optics and light for FA observation.

Phase contrast optics

For the morphological observations of the cells the Nikon phase contrast optics of dark-medium contrast (Nippon Kogaku Co., Tokyo) were applied.

Actinomycins

As mentioned in the preceded paragraph, actinomycin S_{2+3} shows a similar effect to that of actinomycin D in suppressing the cellular RNA synthesis, but this drug is about four times more powerful than actinomycin D. Both D and S_{2+3}

were used in these experiments. In another series of experiments, a comparison of the effect of actinomycin S_{2+3} with actinomycin D was made (Lee, 1966) and in the subsequent experiment, when actinomycin S_{2+3} was employed, the equivalent concentration as regards the effect on the cells were used.

The drugs were handled under the safety lamp, FL 20 RF type (Matsushita Electric Co., Osaka, Japan) and kept in the dark at -20°C.

Actinomycin D and S_{2+3} were kindly supplied by Prof. Junichi Kawamata, Department of Chemotherapy of the Institute for Microbial Diseases, Osaka University.

RESULTS

To establish the optimal conditions for the analyses, several preliminary checks were made before the detailed works were undertaken.

i) Growth of JEV in PS Y15' cells

The Nakayama strain of JEV grows well in PS Y15' cells. The one-step growth curve of JEV in the monolayer cultures of the cells is shown in Fig. 1. The start of infection is taken as the zero time when the cells are first exposed to the virus.

The growth of total plaque forming unit (PFU) of the virus was logarithmic from 8th to 12th hours post-infection, and it was easily reproducible in the repeated experiments. The appearance of PFU in the tissue culture fluid was delayed about 2 hours compared with the total PFU, including the cell-associated virus, of the system.

ii) Effect of actinomycin D or S_{2+3} on PS Y15' cells

To suppress the cellular RNA synthesis, either actinomycin D or S_{2+3} were employed in the subsequent experiments. The cells grown on the coverslips were treated with actinomycin D or S_{2+3} for appropriate times, then they were incubated in a ^{3}H-uridine medium and the incorporation was estimated by the autoradiographic procedures to determine the effect of inhibition on cellular RNA synthesis by the drugs

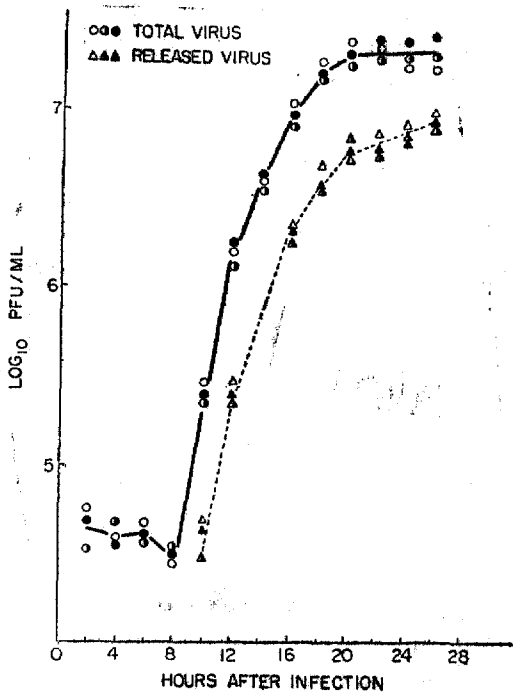

Fig. 1. Growth of JEV in monolayer cultures of PS Y15' cells
The start of infection was taken as the zero time when the cells were first exposed to the virus. The virus was adsorbed to the cells for 2 hours, and then the number of infective particles was measured by plaque forming unit at the intervals of 2 hours. Closed circles (●—●) : total virus. Triangles (▲···▲) : virus released from the cells.

(cf. Table 1).

The total number of grains on the nucleolus (n) and nucleus (N) per 100 μ^2 are shown in Table 1. The silver grains on the nucleolus were decreased more rapidly than those on the nucleus after treating with actinomycins. At the concentration of 0.5μg/ml of actinomycin S_{2+3}, the suppression of the cellular RNA synthesis was almost equivalent to that of 2 μg/ml of actinomycin D. The studies in detail on the suppression of the cellular RNA synthesis of both drug will be reported elsewhere.

iii) Effect of actinomycin S_{2+3} on JEV synthesis

As actinomycins have extensive effect on the cells, the effect of the drugs on the total yield of

Table 1. Inhibition of Cellular RNA Synthesis on the Nucleoli and Nuclei at Different Exposure Times of Actinomycin D or S_{2+3}

Duration of Iuncbation in The Drug	Total Nnumber of grains on the area①($100\mu^2$) of Cell Compartments							
	Amd②				Ams③			
	n④		N⑤		n		N	
	No.	%	No.	%	No.	%	No.	%
0	20,085.4	100	5,260.5	100				
0.5	1,561.0	7.5	1,766.3	33.6	1,060.0	5.3	1,866.9	35.5
1	525.2	2.6	895.4	17.0	—		—	
1.5	166.8	0.8	559.6	10.6	130.7	0.7	613.3	11.6
2	7.4	0.04	312.0	5.9	0		357.6	6.8
2.5	0		46.7	0.9	0		16.9	0.3
3	0		0		0		0	

The cells were exposed to the concentration of 2 μg/ml actnomycin D or 0.5μg/ml actinomycin S_{2+3} during appropriate times, then they were incubated in the medium containing 5μc/ml ^{3}H-uridine and 5μg/ml unlabeled thymidine for 30 min. Immediately afterward, the cells were fixed in ice-cold acetic alcohol and extracted with 2 per cent perchloric acid, and then the autoradiographic procedure was performed. The grain count was made on the cell compartments of 100 randomly selected cells. The total number of silver grains of each cell compartment was eliminated the grains of emulsional back ground by unit area. ① : estimated from 100 cells. ② : actinomycin D, 2 μg/ml. ③ : actinomycin S_{2+3}, 0.5μg/ml. ④ : nucleolus. ⑤ : nucleus.

JEV infective particles was tested in four series of PS Y15' cells: two of them (A and B) were previously treated with 2μg/ml actinomycin S_{2+3} for two hours before the virus inoculation. The first (A) was treated with the drug after the infection. In the second series (B), actinomycin was removed after the infection. The third (C) was treated with the drug after the infection without the pretreatment before the virus inoculation. The last one (D), untreated before and after the inoculation was regarded as the control.

The results are presented in Table 2 which shows that the virus synthesis took place regardless of the addition of actinomycin, before or after the infection. The levels of the yield of infective JEV particles are essentially same in the presence or absence of this actinomycin S_{2+3} concentration, although the cytopathological changes of the cells due to the drug were more severe than those of the control.

Development of JEV antigen in cells infected

The presence of viral protein in the cells was determined by staining with fluorescein conjugated antibody globulin.

Table 2. Effect of Actinomycin S_{2+3} on JEV Multiplication in PS Y15' cells

Name of The Series	Addition of Ams_{2+3} (2μg/ml)		Total Vvirus Yields of post infection pfu/ml	
	2hrs Before Infection	After Infection	12hrs	22hrs
A	+	+	0.2×10^7	1.4×10^7
B	+	—	1.0×10^7	2.6×10^7
C	—	+	1.2×10^7	2.5×10^7
D	—	—	1.1×10^7	1.7×10^7

+ : indicate the addition of actinomycin S_{2+3}.
— : indicate no addition of actinomycin S_{2+3}.

In the cells infected with freshly harvested virus, a specific small bright fluorescent mass was observed in the perinuclear zone where the viral antigen was supposed to be localized in 6 or 8 hours after the infection (Fig. 2 and 3). The viral antigen showed two ways of spreading into cytoplasm as the infection proceeded. The one way which seemed relatively rapid, was such that the small bright mass of immunofluorescence spread around the nucleus and further diffused into the entire cytoplasm (Fig. 3 and 4). In the

other way, the mass developed into a compact type forming like a cluster in the cytoplasm containing many small bright fluorescent masses (Fig. 2). This cluster further diffused into the entire cytoplasm. A considerable number of cells presented many small bright masses over the diffuse-type immunofluorescent cytoplasm (Fig. 4).

Under the phase contrast optics, the compact cluster of masses or the small bright fluorescent masses were observed like protrusion from the cytoplasm.

The development of the viral antigen in actinomycin-treated cells

To demonstrate the effect of actinomycin on production of JEV protein (as antigen), the study dealt with the synthesis of viral antigen in the cells in which all the cellular RNA synthesis has been considered to be inhibited by actinomycin.

Fig. 5 shows the rate of immunofluorescent cells with small bright perinuclear fluorescent mass and diffuse fluorescence over the cytoplasm in the actinomycin-pretreated cells as the infection proceeded.

The viral antigen in actinomycin-pretreated and infected cells was developed rapidly in the entire cytoplasm and the intensity of the fluorescence was much greater than in the actinomycin-untreated and infected cells; the number of the immunofluorescent cells was greater throughout the infection process than that of control.

These results strongly suggest that the synthesis of viral protein has no relation to the cellular metabolic processes of the host cells, and also it indicates that the synthesis of viral protein is enhanced more readily in the actinomycin-treated cells than in the control cells.

Analysis by acridine orange staining

The cells, grown on the coverslips and treated with actinomycin and infected with JEV, were studied as the infected series. And the cells, treated with actinomycin and uninfected, were regarded as the control.

By the staining with acridine orange, the control cells were stained in such a way as to make

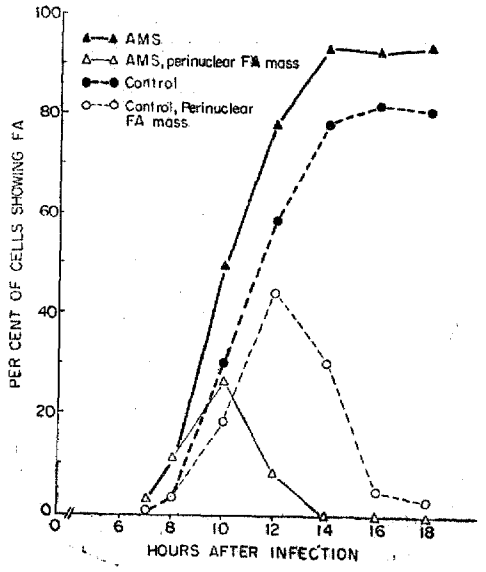

Fig. 5. Production of JEV Antigen in Actinomycin-treated PS Y15' Cells. The cells were treated with 0.25μg/ml actinomycin S_{2+3}, being added 5 hours before virus inoculation and again after virus infection. The immunofluorescent cells were counted at intervals of 1 hour at the beginning of the observation and 2 hours at the end of it.
Closed triangles(▲—▲) : rate of immunofluorescent cells in actinomycin-treated and infected cells. Open triangles(△—△) : rate of the cells showing small bright perinuclear cytoplasmic immunofluorescent masses. Closed circles (●—●) : rate of immunofluorescent cells in actinomycin-untereated and infected cells. Open circles(○—○) : rate of the cells (untreated, infected) showing perinuclear cytoplasmic immunofluorescent masses.

the DNA in the nucleus to appear in light green, and the cytoplasm, in reddish color. In infected cells, the brilliant-red granules appeared in the perinuclear zone (Fig. 6), and spread over the cytoplasm as the infection proceeded. Simultaneously, the cytoplasm of the infected cells showed a brilliant-red color. These observations suggested that viral RNA was supposed to be localized around the perinuclear zone, indicating where the sites of the RNA-positive granules and RNA-positive cytoplasm are located.

The development of RNA-positive substances

detected by AO staining in the perinuclear zone occurred coincidentally with the appearance of the immunofluorescent antigenic substance of the virus at the same sites. In comporatory studies as above the brighter immunofluorescent mass of granules or packings RNA-positive masses in the acridine orange stained cells could be detected in numbers at the same sites. The observation supports the suggestion that the JEV-protein (antigen) was produced at identical sites where the RNA of JEV was formed.

^{3}H-uridine incorporation into JEV-infected cells

The uninfected and JEV-infected cells were labeled with 10μc/ml of ^{3}H-uridine by pulse labeling technique for 30 minutes; both of them were treated with 0.5μg/ml of actinomycin D for 5 hours before and after the virus inoculation and continuous drug exposure, except 2 hours of virus adsorption. Under this condition the uninfected cells observed were essentially free of the label (silver grains), indicating a strong inhibition of the cellular RNA synthesis as shown in Fig. 7. The silver grains (labels) in the infected cells were found mainly over the cytoplasm and they appeared particulary as dense masses covering the deeply stained parts of cytoplasm in Giemsa stained preparates (Fig. 8).

When the infeceted cells into which ^{3}H-uridine had been incorporated were treated with both RNase and DNase as described above before the perchloric acid treatment, the cytoplasmic labels (silver grains) which densely covered the deeply stained parts or loosely scattered as described above were completely removed by RNase, but the labels were not affected by DNase.

Kinetics of ^{3}H-uridine incorporation into the cellular RNA and JEV-RNA

To examine whether the cytoplasmic labels are viral origin, the kinetics of ^{3}H-uridine incorporation into JEV-RNA was studied in the infected cells, both treated and not treated with actinomycin. The uninfected cells of those were set up as control. The cell groups were arranged into four: (1) actinomycin-untreated and uninfected; (2) actinomycin-untreated and infected; (3) actinomycin-treated and infected; and (4) actinomycin-treated and uninfected. The incorporation studies of all the groups were conducted in parallel applying 2μC/ml of ^{3}H-uridine (Perry et al., 1961).

The general patterns of ^{3}H-uridine incorporation into cellular RNA of the nucleolus (n), nucleus (N), and cytoplasm (C) are presented in Fig. 9. Fig. 9A shows the incorporation into each cell compartments of the group (1) and the incorporation in group (4) is shown in Fig. 9B.

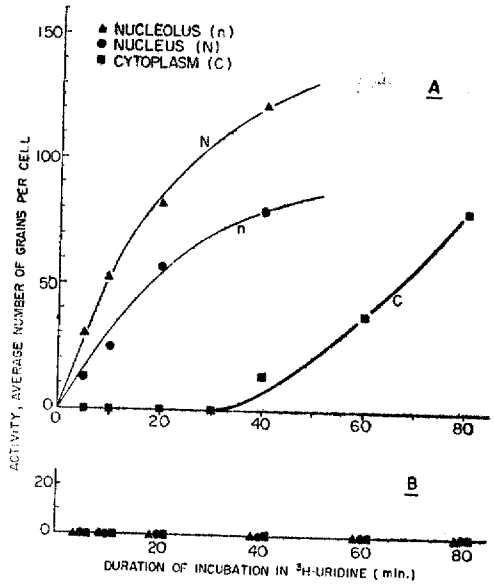

Fig. 9-A. Incorporation of ^{3}H-uridine into RNA in PS Y15' cells(top).

Fig. 9-B. Incorporation of ^{3}H-uridine into RNA in actinomycin treated cells(bottom). The cells were divided into 2 cell groups: Group 1 cells were not treated with actinomycin; Group 4 cells were pretreated with 4μg/ml actinomycin D. Both cell groups were exposed to 2μc/ml ^{3}H-uridine. At the termination of a period of exposure the cells were fixed in cold acetic alcohol(1 : 3), washed in 70 per cent ethanol, treated with 2 per cent perchloric alcohol at 4°C for 40 minutes, and washed for about 2 hours in running water and autoradiographic process was performed. Triangles(▲—▲) : the number of grains on the nucleolus; Circles(●—●) : the number of grains on the nucleus; Quadrangles(■—■): the number of grains on the cytoplasm.

As shown in Fig. 9A, the incorporation into cytoplasm (C) was appeared after 30 min. in ^{3}H-uridine and the rate of the incorporation was slowly increased, whereas those of nucleolus (n) and nucleus (N) were enhanced within a short incubation periods showing the steep initial constant slopes during first 30 min.

The most of cytoplasmic labelings in the infected cells (group (2)) were observed around the cytoplasmic blebs which was supposed to be increased by the infection

The observation indicates that the nucleolar or nuclear RNA has been translocated into cytoplasm after 30 min. since those RNA had been synthesized in the case of non-infected cells.

In the cells of group (4), actinomycin-treated and uninfected, the incorporation activity was not found in any cell compartments as shown in Fig. 9B.

Immediately after the virus adsorption (2 hours after the virus addition to the cells) the kinetic patterns of the cells, belonging group (1) and (2), were similar to Fig. 9A, while that of the cells, belonging group (3) and (4) were similar to Fig. 9B. No viral RNA synthesis was detectable at this stage.

After 8 hours post-infection, the kinetics of ^{3}H-uridine incorporation into RNA of all the cell groups (1)-(4) expressed the curves as shown in Figs. 10A and 10B.

The incorporation activity of ^{3}H-uridine was strikingly different between the cytoplasm of infected and uninfected cell groups. As shown in those figures, regarding of early incorporation in both infected cell groups (2) and (3), the kinetics of the C-curves rise along an acute slope in a short incubation time; Fig. 10A, $C_{(-)}$ and Fig. 10B, $C_{(+)}$; whereas one of the uninfected control group (1) manifested a delayed slope, while another, group (4), indicated free from uridine incorporation.

In contrast, the kinetic curve of the actinomycin-untreated and infected nucleolus [group (2) in Fig. 10A, $n_{(-)}$] shows an apparently more gentle slope than that of uninfected cell [group (1) in Fig. 10A, $n_{(-)}$]. And that of N of the infected cell [group (2) in Fig. 10A, $N_{(-)}$] rises almost same as the control [group (1) in Fig. 10A, $N_{(-)}$]. The fact indicates a definite modification in the metabolic process between the infected and uninfected cells, but it is clear that the infected nucleolar or nuclear RNA synthesis was not replaced by viral RNA synthesis, since no significant incorporation activity of ^{3}H-uridine was shown in the nucleus and nucleolus of actinomycin-treated and infected cells [group (3) in Fig. 10B, $n_{(+)}$ and $N_{(+)}$]

During 10 hours post-infection, the same analyses were undertaken and the kinetic patterns were obtained as shown in Fig. 11A and B. In this experiment, all the kinetics of each cell compartment showed almost similar to those of 8 hours post-infection time as shown in Figs. 10A and B. The C-curves of both infected cell groups [group (2) and (3)] indicated acute slopes, while those of N and n of the infected and actinomycin treated cells [group (2)] had kept continuously more gentle slopes [Fig. 11A, $n_{(-)}$ and $N_{(-)}$] than that of the control [Fig. 11A, group (1) $n_{(-)}$ and $N_{(-)}$].

These results suggest that the phenomenon of the cytoplasmic labeling is due to the synthesis of JEV-RNA, while the nucleolus and nucleus are not involved.

Autoradiography on the sections of the infected cells

To confirm the topography of the viral RNA synthesis in the cytoplasm, the autoradiography was applied to sections of the infected cells. The infected cells, cultures in bottles, were exposed for 30 min. to ^{3}H-uridine (4 μc/ml) at 7th and 14th hour of the infection. Uninfected cells were similarly labeled as the controls.

The cells, infected and uninfected controls, were treated for 3 hours with 5 μg/ml actinomycin D before the exposure to hot uridine. The autoradiographic procedures were performed as described in materials and methods.

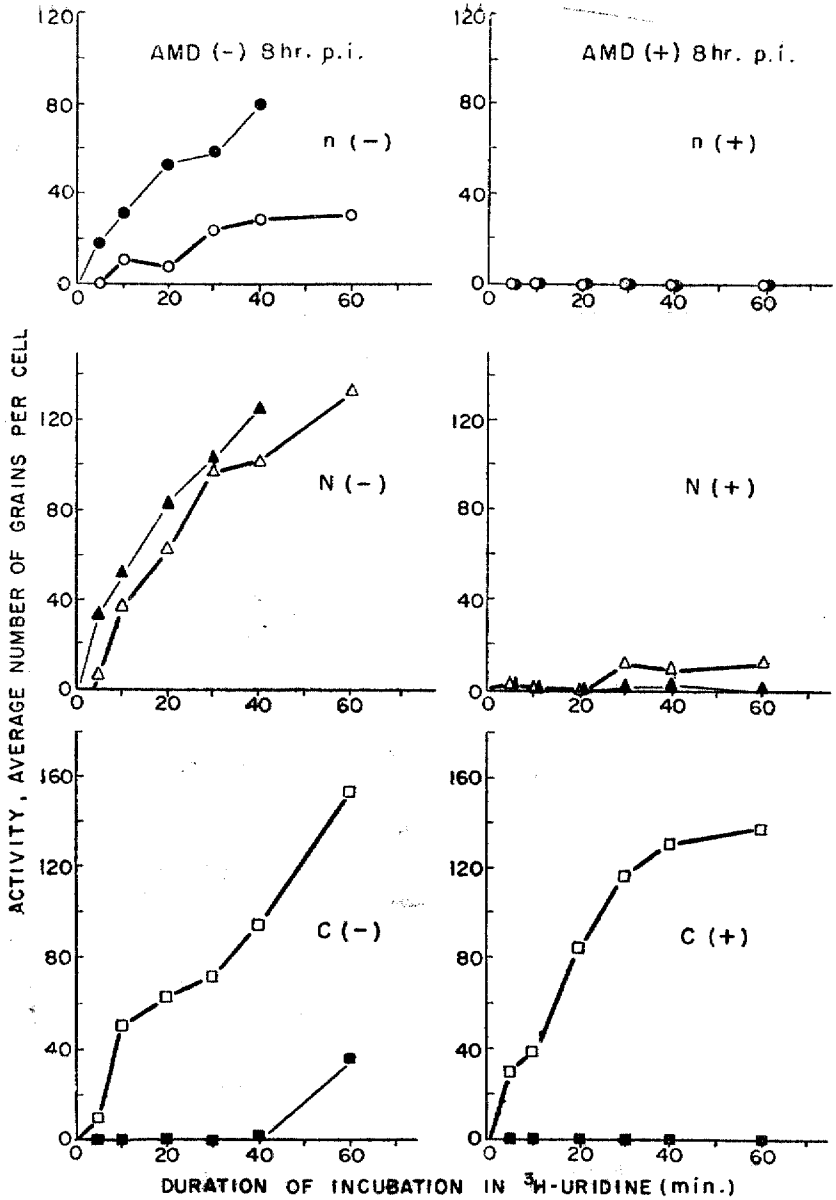

Fig. 10A. Incorporation of ^{3}H-uridine into RNA of various parts of infected and uninfected PS cells (left side set).

Fig. 10B. Incoporation of ^{3}H-uridine into RNA of various parts of actinomycin D(AMD)-treated-infected and AMD-treated-uninfected PS cells (right side set).

All groups of the cells were exposed to 2μc/ml ^{3}H-uridine at 8th hour post-infection (8hr. p. i.) For the grain count, randomly taken ca. 200 cells were used per point.

n : nulceolus; N : nucleus; C : cytoplasm

●—● ▲—▲ ■—■ : uninfected

○—○ △—△ □—□ : infected

Group 1 : AMD untreated-uninfected	AMD(−)
Group 2 : AMD untreated-infected	AMD(−)
Group 3 : AMD treated-infected	AMD(+)*
Group 4 : AMD treated-uninfected	AMD(+)*

* Treated with 0.5μg/ml AMD 5 hrs. before inoculation and continuously drugged after the infection except absorption time.

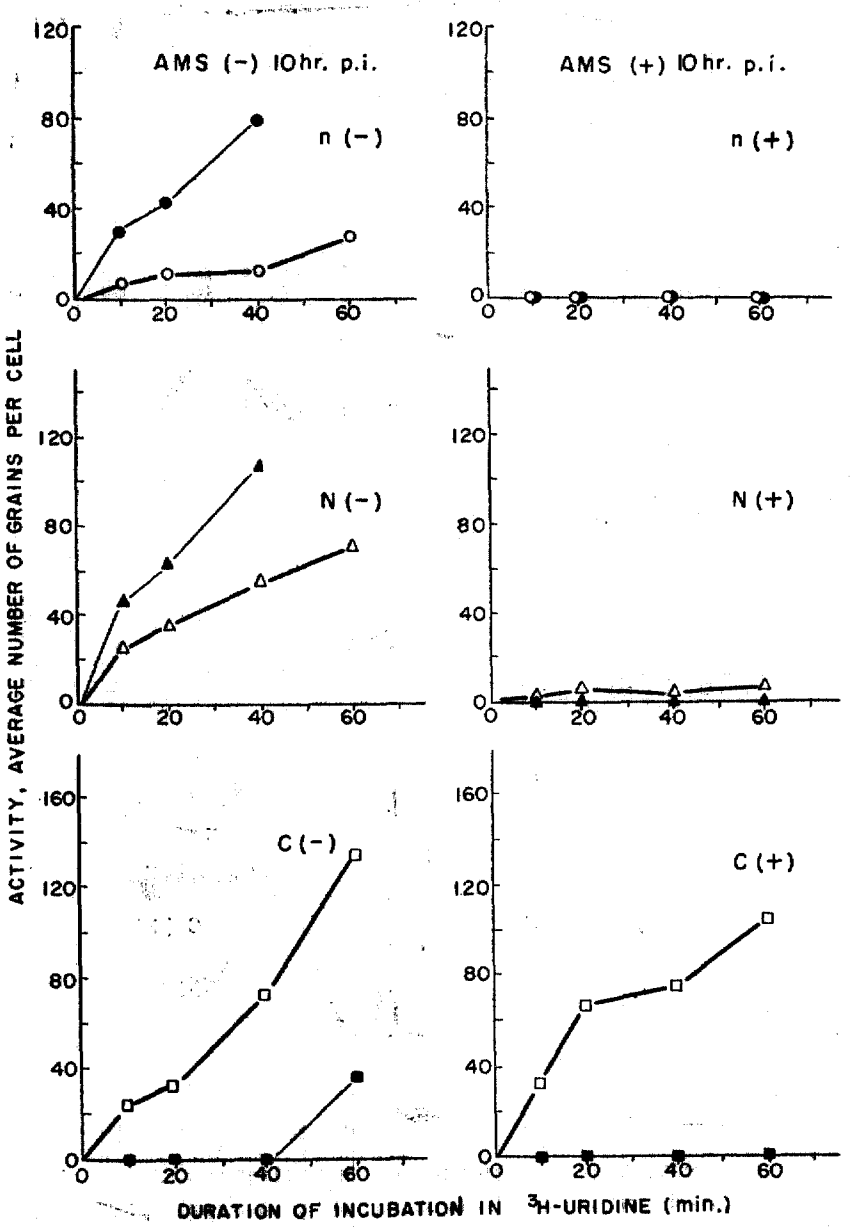

Fig. 11A. Incorporation of ^{3}H-uridine into RNA of various parts of infected and uninfected PS cells (left side set).

Fig. 11B. Incorporation of ^{3}H-uridine into RNA of various parts of actinomycin S(AMS)-treated-infected and AMS-treated-uninfected PS cells (right side set).

At 10th hour after infection all the cell groups were exposed to 2 μc/ml ^{3}H-uridine for a period. (10 hr. p. i.)

Group 1 : AMS-untreated-uninfected	AMS(−)
Group 2 : AMS-untreated-infected	AMS(−)
Group 3 : AMS-treated-infected	AMS(+)*
Group 4 : AMS-treated-uninfected	AMS(+)*

* Treated with 0.5μg/ml actinomycin S_{s+s} for 3 hours before the ^{3}H-uridine incorporation.

Other symbols are same to Fig. 10.

In the most of the cells labeled at 7th hour after infection, the silver grains were seen around the walls of cytoplasmic vacuoles as shown in Fig. 13, but no labeled part of the cytoplasm was seen in the control cells as shown in Fig. 12. And no labeled nucleus and nucleolus were observed. The cells labeled at 14th hour of the infection showed a heavy cytoplasmic labeling even in later stages of infection as shown in Fig. 14.

DISCUSSION

Dulbecco (1962), in discussing the site of RNA synthesis of animal viruses, suggested that the reports on cytoplasmic synthesis could have been the result of an extensively rapid synthesis of viral RNA in the nucleus followed by an equally rapid movement into the cytoplasm.

Takeda et al. (1965) reported that the RNA synthesis by JEV infection under the condition in which cellular RNA synthesis was completely uppressed by chromomycin A_3 was demonstrated autoradiographically to occur in the nucleus. We have surveyed extensively the site of synthesis of JEV-RNA in the actinomycin-treated and JEV-infected cells, and could not find out any evidence for a nucleolar or nuclear involvement in the synthesis of JEV-RNA in the infected cells.

In the analysis of the kinetics of incorporation of ^{3}H-uridine into RNA in the infected cells, no preferential increase of labeling on the nucleolar or nuclear compartments was observed, but the curves of cytoplasmic labeling (uridine incorporation activity) rose even at the shortest incubation in ^{3}H-uridine on both actinomycin-treated and untreated infected cells.

It was demonstrated that the cytoplasmic labeling was due to the synthesis of viral RNA, whereas neither nucleolus nor nucleus was the possible synthetic site, or an initial synthetic site, and there was no rapid translocation of the synthesized products into the cytoplasm.

The site of RNA synthesis of JEV in the infected cells does not differ from any of those of the other small-sized RNA viruses, such as Mengovirus (Franklin and Baltimore, 1962; Franklin and Rosner, 1962) ME-virus (Hausen and Schäfer, 1961), poliovirus (Darnell et al., 1961), EMC virus (Dalgarno and Martin, 1965), and western equine encephalitis (WEE) virus (Wecker et. al., 1962).

In the topographical investigation, the silver grains (^{3}H-uridine labeling) around the cytoplasmic vacuoles were frequently observed. Comparing this uridine incorporation with the electron microscopic observations which suggested that the JEV particles developed by budding from the membrane of the cytoplasmic vacuoles (Higashi, 1963; Ota, 1965), it seems to suppot the idea that the site of the development of JEV particles may be similar to that of viral RNA synthesis.

Furthermore, as the results of the studies of immunofluorescent antibody and acridine orange staining of the infected cells, the viral protein and viral RNA or viral RNA-protein were detected at similar sites of the cytoplasm of actinomycin-treated and infected cells. In addition, the cytoplasmic labeling was found at almost same sites of the acridine orange positive granules showing the existence of RNA.

Thus it seems understandable that the viral protein is synthesized in the site of the cytoplasm of infected cells similarly to that of the viral RNA synthesis, where also the virus particles develope.

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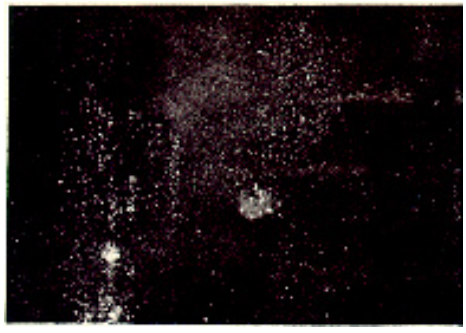


Fig. 2: Perinuclear cytoplasmic immunofluorescent mass and a cluster of compact typed mass.
7 hours post infection.

Fig. 3: Perinuclear cytoplasmic fluorescent mass and immunofluorescence developed surrounding the nucleus.
8 hours post infection.

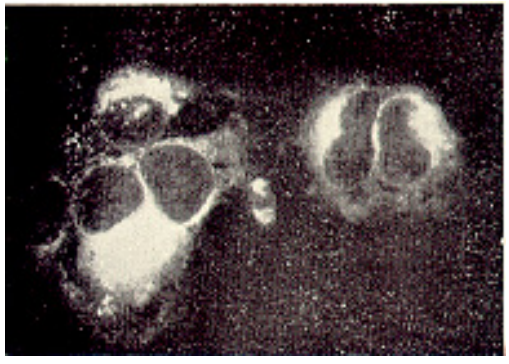


Fig. 4: Immunofluorescence diffused into the entire cytoplasm and many small bright masses contained over the diffused fluorescence.
11 hours post infection.

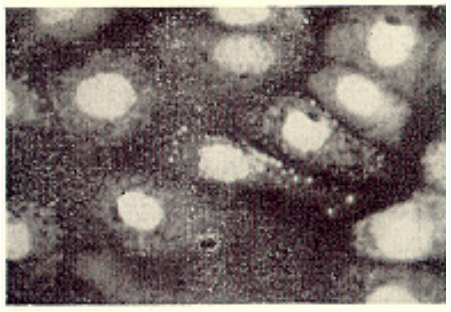


Fig. 6: RNA or RNA protein stained with acridine orange in the perinuclear zone of cytoplasm and developed into cytoplasm
The cells were pretreated with 4μg/ml actinomycin D before virus inoculation

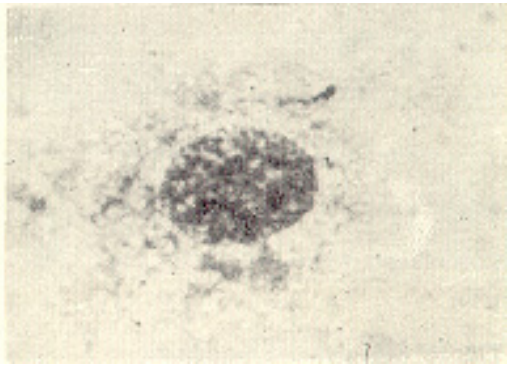


Fig. 7: AMD-treated-uninfected cells labeled with ^{3}H-uridine, 10μC/ml, for 30 minutes. Notice the label-free status

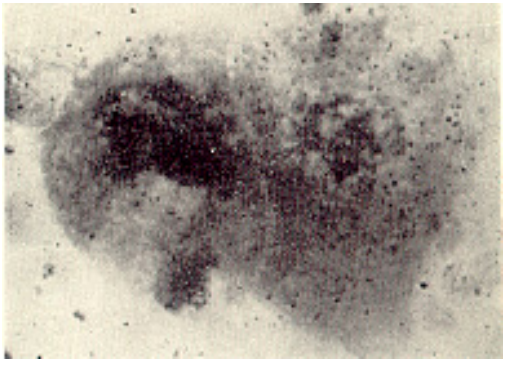
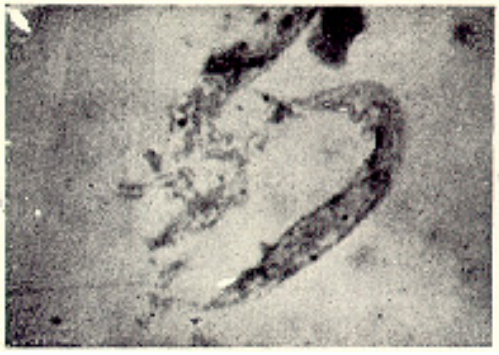
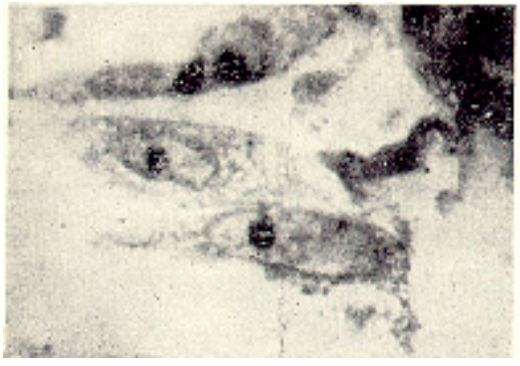


Fig. 8: AMD-treated-infected cells labeled with ^{3}H-uridine, 10μC/ml, for 30 minutes at 8th hour post-infection. Notice the cytoplasmic heavy labelings, especially dense over the deeply stained parts, and no preferential labelings in the nucleus.
The chromatin aggregation in the nucleus was

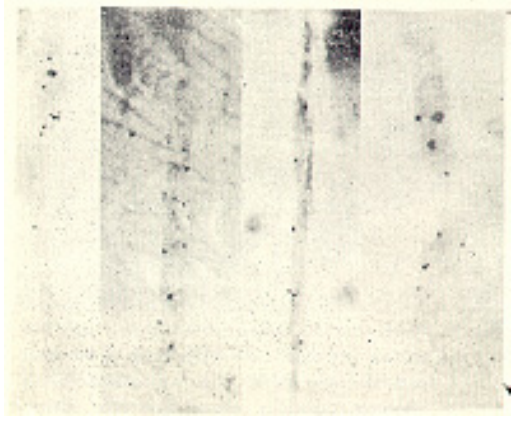


(A)



(B)

Fig. 12: AMD-treated-uninfected cells
Cells were fixed as monolayers after 30 min. exposure to ^{3}H-uridine. After dehydration through ethanol series, the cells were scraped off the bottle wall and embedded into Epon 812. Sections of 0.2 to 1μ in thickness were made and autoradiography was performed on the sections. No labeling is observed.



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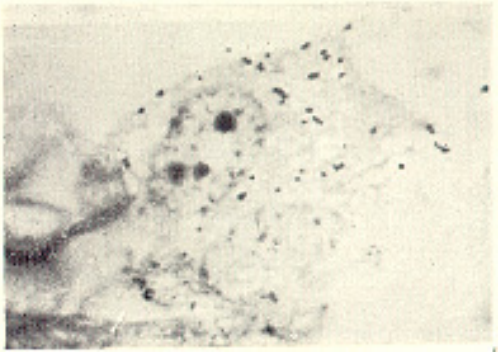

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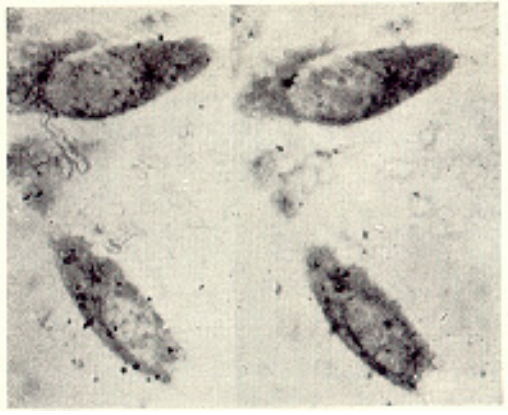
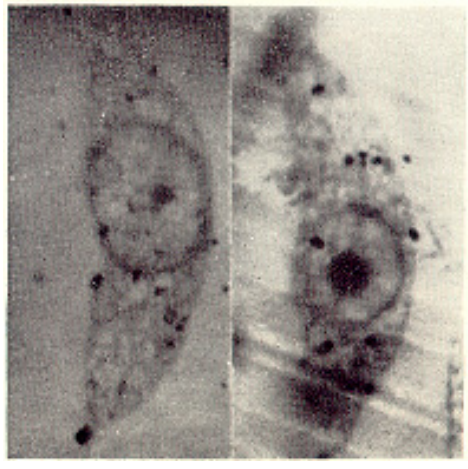


(C)

Fig. 13: Monolayers of AMD-treated-infected cells were fixed after 30 min. exposure to ^{3}H-uridine After dehydration through ethanol series and embedding in Epon 812 the thin sections were made and radiography was applied on the sections. Notice the labelings around the walls of cytoplasmic vacuoles and free of label on the nucleus. 7th hour post-infection. Photo. C shows a pair of successive sections.



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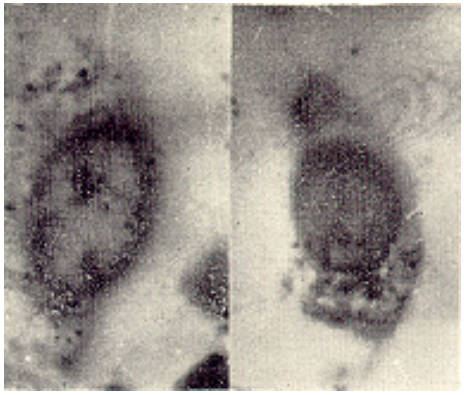

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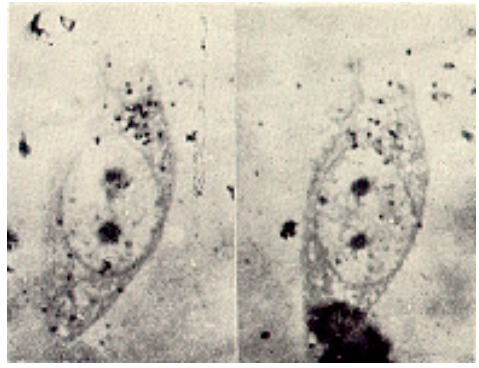

(C)

Fig. 14: Autoradiographs on the sections of AMD-treated-infected cells at 14th hour post-infection. Notice the severe cytoplasmic vacuole formation and the labelings around the walls of the vacuoles, while the nucleolus and nucleus are free of labelings. Photo. C shows pair of two successive sections in a series