BIKEN JOURNAL Vol. 17, 39-49, 1974

# GROWTH INHIBITION OF JAPANESE ENCEPHALITIS VIRUS BY PROLINE DEFICIENCY IN SINGH'S *AEDES ALBOPICTUS* CELLS[1]

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(Received January 7, 1974)

SUMMARY Growth of Japanese encephalitis virus (JEV) was inhibited by proline deficiency in Singh's *Aedes albopictus* (SA) cells. This deficiency inhibited accumulation of JEV antigen as well as synthesis of JEV-specific RNA. However, it did not inhibit growth of Sindbis virus or synthesis of Sindbis virus-specific RNA. Proline deficiency did not appreciably reduce the synthesis of cellular RNA or affect the adsorption and penetration of infecting JEV. The yield of JEV was reduced by proline deficiency, even when SA cells were infected with infective JEV-RNA. These results suggest that certain steps in the synthesis of JEV-specific RNA in SA cells require proline.

## INTRODUCTION

We reported before that proline was required for the maximal growth of JEV in SA cells (Igarashi, Sasao and Fukai, 1973b). There are many reports of amino acid requirements for growth of animal viruses in mammalian cells (Rouse, Bonifas and Schlesinger, 1963; Tankersley, 1964; Goldblum, Ravid and Becker, 1968; Loh and Oie, 1969; Ito et al., 1969; Levine, Buthala and Hamilton, 1971; Winters and Consigli, 1971; Iinuma, Maeno and Matsumoto, 1973), but there seem to be no other reports of proline requirement, especially in arbovirus and mosquito cell systems. Thus the mechanism of proline deficiency and growth inhibition of JEV in SA cells in a previous paper (Igarashi, Sasao and Fukai, 1974) was examined in detail.

1 Part of this work was presented at the 21st Annual Meeting of the Society of Japanese Virologists.

## MATERIALS AND METHODS

### 1. *Virus*

Japanese encephalitis virus (JEV), JaOH-0566 strain which had been passaged in Monkey kidney (MK) and BHK21 cells was mainly used. The strains used for comparison were Nakayama and JaGAr-01 with or without passages in BHK21 cells, and the JaOH-0566 strain of the original suckling mouse brain (SMB) virus and that passaged in MK cells. The origins and histories of these JEV strains and of Sindbis virus were described before (Igarashi et al., 1973d; Igarashi et al., 1974). Virus infectivity was measured by plaque titration on BHK21 cells and recorded as plaque forming units (PFU) per ml. Hemagglutination (HA) and complement-fixation (CF) reactions were performed by the methods of Clarke and Casals (1958) and Hammon

and Work (1964), respectively using microtiter systems (Sever, 1962). Hyperimmune mouse serum against the JaOH-0566 strain was prepared as described previously (Igarashi et al., 1973a) for use in the CF test.

2. *Cells*

The origins of Singh's *Aedes albopictus* (SA) cells and BHK21 cells were reported before (Igarashi et al., 1973d). SA cells have been adapted to grow in Eagle's minimal essential medium (MEM) (Eagle, 1959) supplemented with 10% calf serum and 0.2 mM concentrations of all non-essential amino acids (Igarashi et al., 1973b) at 28 C. BHK21 cells were grown with 10% calf serum in MEM at 37 C.

3. *Predeplection of proline and inoculation of SA cells with virus*

The growth medium was removed from SA cell cultures in 2 oz bottles ($5\times10^6$ cells/bottle). Cell sheets were washed twice with PBS and overlayed with proline-free medium (2% calf serum in MEM supplemented with 0.1 mM concentrations of all non-essential amino acids except proline). Cultures were incubated at 28 C for 2 days. Then the fluid medium was removed and the SA cells were inoculated with 0.2 ml of virus specimen (about 10 PFU/cell). During the adsorption period of 2 hr virus was spread every 30 min. Then unadsorbed virus was removed by washing the cells twice with PBS and cultures were incubated at 28 C in medium without proline or complete medium (2% calf serum in MEM supplemented with 0.1 mM concentrations of all non-essential amino acids).

4. *Extraction and assay of infective RNA*

Suspensions of infected cells or concentrated JEV in STE (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6) were extracted by phenol method at room temperature as described previously (Igarashi, Sasao and Fukai, 1973c). Then specimens were diluted with 2 M $MgSO_4$ in 0.05 M Tris-HCl, pH 7.6, and 0.2 ml of the diluted specimen was inoculated onto monolayer cultures of BHK21 cells which had been washed twice with PBS. After adsorption of the specimen at room temperature for 20 min, cells were washed twice with PBS and overlayed to allow plaque formation. In using SA cells, RNA was diluted with 100 $\mu$g/ml of DEAE dextran (molecular weight, ca. $2\times10^6$, Pharmacia, Uppsala, Sweden) in PBS, before inoculation onto SA cells. After adsorption for 20 min at room temperature, cells were washed twice with PBS and incubated at 28 C in medium without proline or in complete medium.

5. *Measurement of RNA synthesis*

Duplicate cultures of SA cells in 2 oz bottles were labeled with 0.2 $\mu$Ci/ml of $^3$H-uridine (5 Ci/mmole, Daiichi Pure Chemicals Co., Tokyo) for 11 hr at 28 C. Then the $^3$H-uridine medium was discarded and the cell sheets were washed three times with PBS and scraped off into PBS. Part of the cell suspension was used to measure the protein content by the method of Lowry et al. (1951). The rest was mixed with an equal volume of 10% trichloroacetic acid (TCA) and kept for 45 min at 4 C. The mixture was centrifuged at $1,500\times g$, 15 min, and the supernatant was discarded. The precipitate was washed twice with cold 5% TCA by resuspension and centrifugation. The final precipitate was resuspended in 1 ml of 5% TCA and heated at 80 C for 10 min to extract the RNA. After centrifugation, the supernatant was mixed with 8 ml of scintillator fluid, consisting of 4 g of diphenyloxazol in one liter of a 1:2 mixture of Triton X-100 and toluene (Patterson and Greene, 1965). Radioactivity was recorded in a Beckman model LS-150B liquid scintillation spectrometer at a counting efficiency of 30% for $^3$H.

In experiments on virus-specific RNA synthesis, 1 $\mu$g/ml of actinomycin $S_3$ (Furukawa et al., 1968) was added to the medium 1 hr before labeling.

6. *Sucrose gradient sedimentation of RNA*

Labeled cells were scraped into STE containing 1% sodium dodecyl sulfate (SDS) and lysed at room temperature by pipetting them through a hypodermic needle. The lysed specimen was placed on a 5–20% sucrose gradient in STE containing 0.5% SDS (Becker and Joklik, 1964) and centrifuged in the SW 50.1 rotor of a Beckman ultracentrifuge, model L2-65B at 45,000 rev/min for 90 min at 15 C. Fractions were collected dropwise from the bottom of the tube. Samples of 0.1 ml of each fraction were dried on 2.5 cm diam filter paper disks. The disks were then washed three times with 5% trichloroacetic acid (Bollum, 1966), dried and placed in a counting vial containing scintillator fluid, consisting of 4 g of diphenyloxazol in one liter of toluene. Counts were recorded as described above. The rest of each fraction was diluted with water and its

absorption at 260 mμ was measured in a Carl Zeiss spectrophotometer, type PMQ III.

7. *Preparation of $^3$H-uridine labeled JEV*

BHK21 cells in roller bottles (11 × 28 cm) were infected with JEV and labeled with 10 μCi/ml of $^3$H-uridine in the presence of actinomycin $S_3$ (1 μg/ml in 2% calf serum in MEM). Virus was collected and purified from the culture fluid 20 hr after inoculation, as described before (Igarashi et al., 1973a). The specific activity of the purified JEV was $4 \times 10^6$ counts/min/$6 \times 10^8$ PFU.

8. *Fluorescent antibody staining*

SA cells were grown in 20 × 45 mm flat bottles containing 11 × 40 mm cover slips. Cells were infected and incubated at 28 C as described above. The medium was removed after appropriate times of inoculation. The cells were rinsed twice with PBS and dried for 30 min and then fixed with acetone for 5 min at room temperature. Then 0.1 ml of fluorescen isothiocyanate-conjugated hyperimmune rabbit serum was placed on the cover slip and the specimen was incubated at 37 C for 40 min. Then it was rinsed three times in PBS and mounted on glass slides with glycerol. Specimens were observed under a Leitz Panphoto microscope, equipped with an ultraviolet light illuminating system (Philips CS150 high pressure mercury lamp and 4 mm UG1 transmission UV filter). Objective lenses were equipped with K430 specimen filters. Photographs were taken with an Leitz Orthomat automatic camera using Kodak Tri-X Pan film.

Unlabeled specimens of anti-JaOH-0566 strain hyperimmune rabbit serum, and those labeled with fluorescent dye were kindly supplied by Dr. M. Takagi, Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University Kanonji, Kagawa. The sera were prepared by repreated injection of infected SMB suspension with Freund's complete adjuvant, and had a neutralizing antibody titer of 4.3 log units estimated by the 50% plaque reduction method.

## RESULTS

### 1. *Proline deficiency and inhibition of JEV antigen formation in SA cells*

As the first step in studies on the stage in JEV replication requiring proline the direct fluorescent antibody staining technique was applied to detect the appearance of viral antigen using medium for virus growth with or without proline. Replicate cultures of proline deficient SA cells were inoculated with JEV. In the control series, incubated in complete medium, specific fluorescence began to appear one day after infection, starting from a certain perinuclear point and spreading to encircle the whole perinculear region and diffusing into the cytoplasm. On the second day, almost all the cells showed specific fluorescence around the nucleus, diffusing into the cytoplasm (Fig. 1A). When infected cells were incubated in medium without proline, both the number of

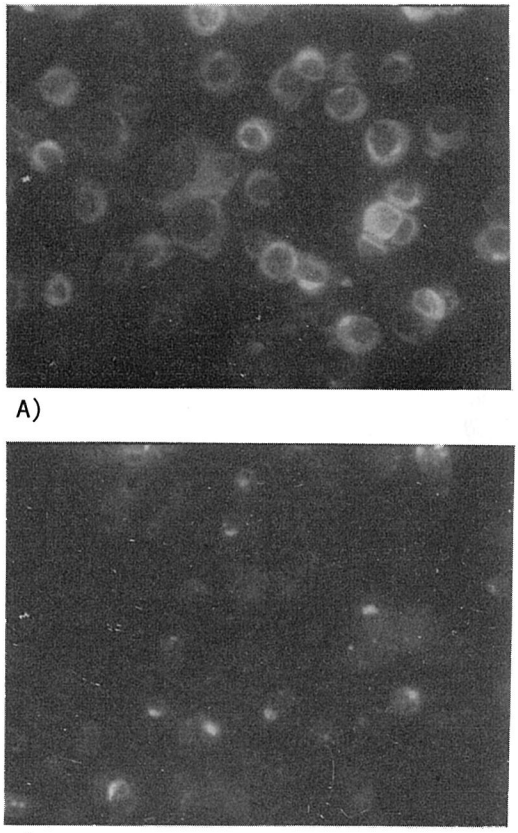


FIGURE 1. *Photomicrographs of SA cells infected with JEV 2 days after incubation in complete medium (A), or proline-free medium (B). Stained by the direct fluorescent antibody method. Magnification:* × 800

fluorescent cells and the amount of antigen reacting with fluorescent antibody were less, the latter sometimes being confined to part of the perinculear region only (Fig. 1B). There were only about 10% as many fluorescent-positive cells as in control cultures in complete medium.

Thus it seemed that growth of JEV in SA cells was reduced by proline deficiency at an early stage before viral antigen synthesis. The HA titer released into infected culture media was also reduced by proline deficiency to less than 10% of that in control cultures in complete medium, which excluded the production of non-infectious hemagglutinin, and also the yield of infective virus (Igarashi et al., 1973b).

2. *Effect of proline deficiency on adsorption, penetration and release of RNA from infecting JEV particles*

Thus we examined whether some early synthetic events in infection of SA cells by JEV were impaired by proline deficiency. Two series of SA cells were prepared. One was incubated for 2 days before virus inoculation in medium without proline to deplete the cells of the amino acid. The other was incubated in complete medium. Each series was further divided into 2 groups. One was inoculated with purified $^{3}$H-uridine labeled JEV diluted with proline-free medium, and the other group with JEV diluted with complete medium. After adsorption at 28 C for 2 hr, cells were washed three times with PBS, detached and suspended in PBS. Part of each suspension was used for protein determination and for counting cells in a hemocytometer. The rest of the suspension was washed with hyperimmune rabbit serum diluted 10 fold with PBS. Then it was washed with PBS and assayed for infective centers by diluting it with complete medium and measuring plaque formation on BHK21 cells under overlay of complete medium to see the extent of virus penetration. The radioactivity in the RNA fraction of the cell suspension was also determined to measure the amount of JEV adsorbed to SA cells. Table 1 shows that neither the infective centers or the radioactivity bound to the cells were reduced when SA cells were depleted of proline or when proline was omitted from the medium used for virus inoculation.

Thus it seems that neither adsorption nor penetration of infective JEV was affected by proline deficiency.

To examine the effect of proline deficiency on the release of infecting virion RNA, SA cells were inoculated with infective JEV-RNA extracted from purified JEV. After adsorption and washing with PBS, cells were overlayed with either proline-free medium or complete medium. JEV-inoculated SA cells were incubated in parallel as controls. Specimens were taken to measure infective JEV released into the culture media. The PFU of infective RNA extracted from the cells and CF antigen bound to the cells were also measured on the 4th day after inoculation. Table 2 shows that production of infective JEV in SA

TABLE 1. *Effect of proline deficiency on the adsorption and penetration of JEV into SA cells*[a]

| Proline in medium for | | Activity of JEV bound to SA cells | |
|---|---|---|---|
| Preincubation of SA cells | Inoculation of JEV | Radioactivity (count/min/cell) | Infective centers (PFU/cell) |
| − | − | 1.59±0.92 | 0.65±0.15 |
| − | + | 1.39±0.22 | 0.72±0.10 |
| + | − | 1.27±0.08 | 0.81±0.17 |
| + | + | 1.16±0.41 | 0.54±0.14 |

[a] Average of values in 3 experiments ± S.E..

TABLE 2. *Effect of proline on the growth of JEV in SA cells infected with JEV or infective RNA of JEV*

| Time of sampling (days after infection) | Titer of | Inoculated with | | | |
|---|---|---|---|---|---|
| | | Infective JEV-RNA Incubation medium | | JEV Incubation medium | |
| | | Proline-free | Complete | Proline-free | Complete |
| 2 | JEV-PFU[a] | $3.0\times10^{2}$ | $2.5\times10^{3}$ | $4.0\times10^{7}$ | $3.1\times10^{8}$ |
| 4 | JEV-PFU | $1.2\times10^{4}$ | $1.6\times10^{7}$ | $5.6\times10^{7}$ | $4.3\times10^{8}$ |
| | RNA-PFU[b] | $<50$ | $1.8\times10^{2}$ | $8.5\times10$ | $1.2\times10^{3}$ |
| | CF[c] | nt[d] | nt | $<4$ | 32 |

[a] Infectivity of JEV released into the medium.
[b] Infectivity of RNA extracted from cells.
[c] CF antigen titer extracted from cells by homogenization.
[d] Not tested.

cells on the second day after inoculation was reduced by proline deficiency in the medium, even when cells were inoculated with infective RNA. The difference in the yields of infective JEV became more on the 4th day, possibly due to the multiple steps involved in virus growth. Table 2 also shows that production of infective RNA and CF antigen were reduced in proline deficient SA cells. These results suggest that the growth of JEV in SA cells was inhibited by proline deficiency at some point after release of infective virion RNA.

### 3. *Proline deficiency and inhibition of JEV-specific RNA synthesis in SA cells*

Based on the above results JEV-specific RNA synthesis was next emamined. SA cells in 2 oz bottles were inoculated with JEV and incubated at 28 C in complete medium. At 12 hr-intervals after infection, JEV-specific RNA synthesis was measured in duplicate cultures as incorporation of $^{3}$H-uridine in the presence of actinomycin $S_3$. Table 3 shows that actinomycin-resistant RNA synthesis was maximal 49–60 hr after infection. Actinomycin reduced the incorporation of $^{3}$H-uridine in uninfected cultures to 4% of that in control cultures without actinomycin (Table 4). Therefore JEV-specific RNA synthesis was examined 48–60 hr after infection.

Proline deficient SA cells were inoculated with JEV and overlayed with either proline-free medium or complete medium. Sindbis virus-infected cultures and mock-infected cultures were used as controls. At 48 hr after infection, 1 $\mu$g/ml of actinomycin $S_3$ was added. Two other series of mock-infected SA cells were incubated in proline-free medium and in complete medium as controls without actinomycin treatment. At 49 hr after infection,

TABLE 3. *Synthesis of JEV-specific RNA in SA cells*

| Labeling time (hr after infection[a]) | $^{3}$H-uridine incorporated (counts/min/mg protein) |
|---|---|
| 1–12 | 274.1 |
| 13–24 | 576.1 |
| 25–36 | 1937.5 |
| 37–48 | 3535.8 |
| 49–60 | 4646.3 |
| 61–72 | 3405.7 |
| 73–84 | 1895.7 |
| 85–96 | 2443.9 |
| 97–108 | 1640.1 |

[a] One hr before labeing, 1 $\mu$g/ml of actinomycin $S_3$ was added.

0.2 $\mu$Ci/ml of $^3$H-uridine was added and incubation was continued for 11 hr. Then specimens were prepared to measure the specific radioactivity incorporated into the RNA fraction. Table 4 indicates that actinomycin-resistant RNA synthesis in JEV-infected SA cells was reduced by proline deficiency to almost the background level of unifected and actinomycin-treated cells. However, the syntheses of Sindbis virus-specific RNA and of mock-infected cellular RNA were not affected appreciably by proline deficiency. Moreover, the production of infective Sindbis virus was not reduced by proline deficiency.

Sucrose gradient sedimentation analysis of the RNA synthesized in mock-infected cells revealed three peaks of $^3$H-uridine incorporation corresponding to the peaks of UV absorption (28 S, 18 S and 4 S), and this pattern was not changed greatly by proline deficiency in the medium, but was lost almost completely by actinomycin treatment, except

TABLE 4. *Proline deficiency and incorporation of $^3$H-uridine into SA cells*

| Virus | AM[a] | Incubation medium | | | |
|---|---|---|---|---|---|
| | | Proline-free | | Complete | |
| | | PFU/ml[b] | Count/min[c] | PFU/ml | Count/min |
| JE | + | $7.3\times10^5$ | 932.3 | $1.5\times10^7$ | 2426.6 |
| Sindbis | + | $2.6\times10^7$ | 27422.2 | $2.1\times10^7$ | 19923.1 |
| ,, | + | — | 1151.8 | — | 1037.5 |
| ,, | − | — | 31358.3 | — | 34833.5 |

[a] 1 $\mu$g/ml of actinomycin $S_3$ was added 48 hr after infection.
[b] Virus titer in medium 48 hr after infection.
[c] Incorporation per mg of protein after labeling with 0.2 $\mu$Ci/ml of $^3$H-uridine 49–60 hr after infection.

TABLE 5. *Effect of proline on the growth of JEV and synthesis of virus-specific RNA in SA cells infected with various strains of JEV*

| Strain of JEV | Passage history[a] | Incubation medium | | | |
|---|---|---|---|---|---|
| | | Proline-free medium | | Complete medium | |
| | | PFU/ml[b] | Count/min[c] | PFU/ml | Count/min |
| Nakayama | SMB | $7.8\times10^4$ | 446.2 | $4.7\times10^6$ | 3071.4 |
| | SMB-BHK | $1.1\times10^6$ | 1427.1 | $2.2\times10^7$ | 9686.0 |
| JaGAr-01 | SMB | $5.2\times10^4$ | 780.1 | $1.7\times10^6$ | 12670.0 |
| | SMB-BHK | $6.9\times10^5$ | 674.4 | $1.1\times10^7$ | 9961.0 |
| JaOH-0566 | SMB | $2.2\times10^4$ | 1002.8 | $1.2\times10^6$ | 3424.2 |
| | SMB-BHK | $1.0\times10^5$ | 741.7 | $4.2\times10^6$ | 4958.0 |
| | SMB-MK | $1.8\times10^5$ | 582.9 | $3.2\times10^6$ | 3096.9 |
| | SMB-MK-BHK | $2.8\times10^6$ | 584.2 | $5.5\times10^7$ | 3366.0 |

[a] SMB: Suckling mouse brain; BHK: BHK21 cells; MK: Monkey kidney cells.
Details of the passage histories were reported before (Igarashi et al., 1973d).
[b] Virus titer in the medium 48 hr after infection.
[c] Incorporation per mg of protein, on labeling 49–60 hr after infection with 0.2 $\mu$Ci/ml of $^3$H-uridine in the presence of 1 $\mu$g/ml of actinomycin $S_3$, 48–60 hr after infection.

for a small peak of 4S RNA (Fig. 2A). The pattern of actinomycin-resistant, JEV-specific RNA synthesized in infected SA cells revealed that $^3$H-uridine was incorporated into 42 S virion RNA as well as into 20S, which is presumably the replicative form or replicative intermediate form of RNA, and 4S RNA. The latter might reflect the small 4S RNA peak present in mock-infected and actinomycin-treated cells. However, incorporations into 42 S and 20 S JEV-specific RNA's were reduced when proline was omitted from the medium for virus growth (Fig. 2B).

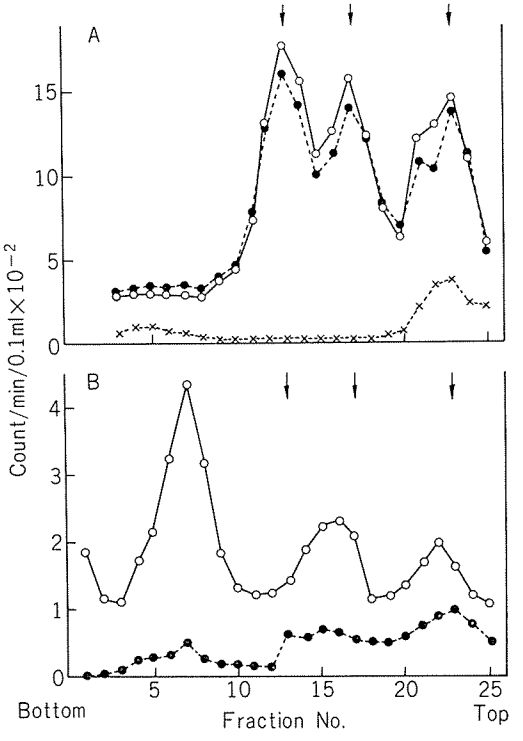


FIGURE 2. *Sucrose gradient sedimentation of RNA's synthesized in SA cells. A Mock-infected SA cells were incubated in complete medium* (○——○), *or proline free medium* (●----●). *Cells were labeled with 5 μCi/ml of $^3$H-uridine 49–60 hr after infection. In one series 1 μg/ml of actinomycin $S_3$ was added 48 hr after infection, before labeling* (×----×). *B JEV-infected SA cells were incubated in complete medium* (○——○), *or proline-free medium* (●----●). *1 μg/ml of actinomycin $S_3$ was added 48 hr after infection and cells were labeled 49–60 hr after infection with 10 μCi/ml of $^3$H-uridine. Arrows indicate the position of UV absorption peaks.*

The replication of JEV in SA cells seems to involve some proline dependent steps and these steps seem to occur after exposure of infective virion RNA and before progeny viral RNA synthesis.

JEV, JaOH-0566 strain, passaged in MK and BHK21 cells was used to obtain the results mentioned above. Depression of virus growth and virus-specific RNA synthesis in the absence of proline were also observed with other strains of JEV, (i.e. Nakayama and JaGAr-01, with or without passage histories in BHK21 cells, and JaOH-0566 strain passaged only in SMB or MK cells). Cultures of proline deficient SA cells were inoculated with various strains of JEV. After incubation at 28 C for 48 hr in proline-free medium or complete medium, 1 μg/ml of actinomycin was added. Synthesis of virus-specific RNA was measured by incorporation of $^3$H-uridine 49–60 hr after infection. The virus yield 48 hr after infection was also measured. Table 5 shows that growth and synthesis of specific RNA by all the JEV strains tested depended on the presence of proline in the medium.

4. *Reversibility of proline deficiency*

Deficiency of proline might damage SA cells so that they cannot support the maximal growth of JEV. The yield of JEV from JEV-infected SA cells was less in proline-free medium, than in complete medium. However, when 0.1 mM proline was added to proline-free medium the yield of JEV began to increase after a lag period of about 12 hr, and within 48 hr reached the same level as that in complete medium (Fig. 3). Thus reduction of the JEV yield in proline-deficient SA cells was not due to irreversible damage to the cells. The observation of a lag period before increase in the virus titer after addition of proline also supports our conclusion that the inhibition occurred at an early stage, before progeny virus RNA synthesis.

JEV-infected SA cells were incubated in

complete medium. At 60 hr after infection, when viral RNA synthesis had reached a maximum, the medium was discarded and the cell sheets were washed with PBS and incubated in proline-free medium or complete medium. Synthesis of JEV-specific RNA was measured at intervals after changing the medium as incorporation of $^3$H-uridine in the presence of actinomycin $S_3$. Table 6 shows that incorporation in proline-free medium decreased relative to that in complete medium with time after changing the medium. There are two explanations of this. One is that the proline dependent steps in JEV-RNA synthesis are not transient, that is, the products of the steps are not stable, but decay after removed of proline from the medium. The second is that the presence of proline is required for activity of the site of JEV-RNA synthesis, as a result of recycling of progeny RNA to support the continuous synthesis of viral RNA in infected SA cells.

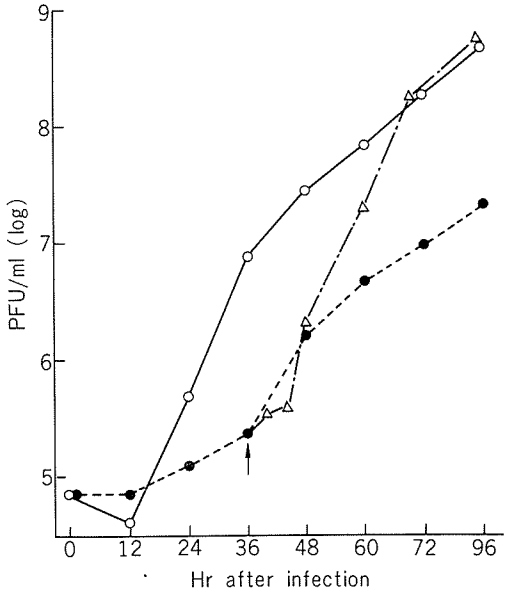


FIGURE 3. *Growth of JEV in SA cells with or without proline replicate cultures of proline deficient SA cells were infected with JEV and incubated in complete medium* (○——○) *or proline-free medium* (●----●). *At 36 hr after infection (indicated by an arrow) some of the latter series were supplemented with 0.1 mM proline* (△·····△). *Portions of the infected culture fluids from each series were titrated for infective JEV.*

TABLE 6. *Effect of proline-withdrawal on further synthesis of actinomycin-resistant RNA in SA cells*[a]

| Labeling time (hr after infection) | $^3$H-uridine incorporated[b] (count/min/mg protein) | |
|---|---|---|
| | Proline-free medium | Complete medium |
| 61–72 | 2873.8 | 3838.1 |
| 73–84 | 2498.4 | 2878.4 |
| 85–96 | 1870.1 | 3493.0 |
| 97–108 | 829.0 | 3851.9 |

[a] JEV-infected SA cells were incubated in complete medium for 60 hr after infection. Then the medium was changed.

[b] 1 $\mu$g/ml of actinomycin $S_3$ was added 1 hr before labeling with 0.2 $\mu$Ci/ml of $^3$H-uridine.

## DISCUSSION

Certain steps in the replication of JEV in SA cells seem to have a high requirement for proline, and the SA cells cannot supply adequate proline to support maximal growth of JEV. SA cells themselves require proline for maximal growth, but the requirement of proline for growth of JEV is even more pronounced (Igarashi et al., 1973b). The steps in JEV replication requiring proline seem to be after exposure of infecting virion RNA and before progeny viral RNA synthesis for the following reasons: (1) The earlier steps of adsorption and penetration of JEV into SA cells seem to proceed well in proline deficiency, (2) JEV growth was inhibited by proline deficiency even when infective RNA was inoculated into SA cells, (3) synthesis of viral RNA and accumulation of viral antigen were reduced by proline deficiency. A lag period was observed before increase of virus production on addition of proline to proline-free medium and this also indicates that early steps are blocked by protein deficiency. These early steps may not be transient, that is, their

products may not be stable, because when proline was removed from infected SA cells at the time of maximal viral RNA synthesis, this synthesis decreased. This may also indicate that the activity of the site of JEV-RNA synthesis as the result of recycling of progeny RNA was inhibited by proline deficiency. The latter possibility is compatible with results obtained by fluorescent antibody staining, showing that the fluorescent-positive site was confined to a part of the perinuclear region in cells under proline-free conditions but not in cells in complete medium.

In complete medium almost all SA cells infected with JEV, JaOH-0566, showed fluorescence on the second day after infection. This seems to be peculiar to our system, because the percentages of fluorescent-positive cells are reported to be low in arbovirus-infected arthropod cells (Rehacek, 1972; Singh, 1972).

Many animal virus-cell systems require amino acids and it has been found that in these systems late steps of virus maturation are blocked by amino acid deficiencies. For example the syntheses of adenovirus DNA and virus-specified antigens occurred in the absence of arginine (Rouse and Schlesinger, 1967). Moreover, early functions of herpesvirus replication (Inglis, 1968) including the syntheses of viral DNA (Becker, Olshevsky and Levitt, (1967) and early antigens (Roizman, Spring and Roave, 1967) were not affected by arginine deprivation, while synthesis of late antigens (Roizman et al., 1967) or their compartmentalization (Courtney, McCombs and Benyesh-Melnick, 1970) were inhibited. Furthermore, synthesis of T-antigen of SV40 virus took place in the absence of arginine (Goldblum et al., 1968). Arginine deficiency seems to inhibit the encapsidation of polyoma viral DNA (Winters and Consigli, 1971). Arginine deprivation also blocked the maturation of Newcastle disease virus (Iinuma et al., 1973). In a serine-dependent subline of HeLa cells this virus required serine for production of hemagglutinin and infectious virus (Ito et al., 1969). However, in both cases, the syntheses of viral RNA and certain viral antigens occurred. Lysine deficiency caused some inhibition of the assembly of reovirus, without affecting the synthesis of viral structural proteins or virus RNA (Loh and Oie, 1969). Phenylalanine deficiency prevented the synthesis of envelope antigen but allowed the synthesis of viral nucleocapsid in respiratory syncytial virus (Levine et al., 1971). However, arginine deprivation inhibited both early and late stages in the replication of vaccinia virus (Archard and Williamson, 1971). In this case marked reductions in the syntheses of DNA, RNA and protein were observed both in infected and control cultures. This is in contrast to our system. Proline deficiency did not reduce RNA synthesis in uninfected SA cells appreciably. Moreover it did not reduce synthesis of Sindbis virus-specific RNA or infective Sindbis virus production. Thus, inhibition of JEV-RNA synthesis by proline deficiency did not reflect non-specific inhibition of RNA synthesis in SA cells, or non-specific inhibition of arboviral RNA synthesis, but it was observed with all the JEV strains tested. Both in uninfected cells and in cells infected with JEV protein synthesis, measured as incorporation of $^{3}$H-valine in proline-free medium, was reduced slightly, to 85% of the level in cultures in complete medium.

The steps in the replication of JEV in SA cells requiring proline may be the formation of viral RNA synthetase, or some cofactors involved in the synthesis of viral RNA. It would be interesting to find that some proline-rich polypeptides were synthsized in JEV-infected cells, and to measure the activity of viral RNA synthetase in infected SA cells incubated in media with and without proline.

ACKNOWLEDGEMENTS

We thank Prof. J. Kawamata of this Institute for a gift of actinomycin $S_3$, and Dr. M. Takagi, Kanonji Institute, The Research Foundation for Microbial Diseases of Osaka University, Kanonji, Kagawa, for a

generous supply of hyperimmune rabbit sera against JaOH-0566 strain, and a specimen of fluorescent antibody.

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