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AN INTRACELLULAR COMPONENT ASSOCIATED WITH CHIKUNGUNYA VIRUS-SPECIFIC RNA

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SUMMARY A component associated with Chikungunya virus (ChV) specific RNA was found in a homogenate of BHK-21 cells infected with ChV. The component, arbitrarily named " X ", yields infective RNA after extraction of RNA, but on assay of ChV infectivity it shows low infectivity. " X " sediments slower in sucrose gradient columns and is more labile on freezing than mature ChV. The RNA associated with " X " is rather labile and seems to be readily accessible from outside. This component tends to accumulate inside the cells at a late stage of infection. Clear-cut data showing that " X " is a precursor of ChV, have not been obtained yet.

INTRODUCTION

In our previous paper on Chikungunya virus (ChV) from samples of infected suckling mouse brain (Igarashi, Fukai and Tuchinda, 1967), we reported that some components, other than mature virus were found by sucrose gradient sedimentation analysis, from which infective RNA could be obtained by phenol extraction. These components were not found in ChV samples from infected tissue culture fluid. In the latter, only a single component with the same sedimentation rate as mature virus was found to yield infective RNA (Igarashi, 1968).

Therefore, it seemed interesting to look for components yielding infective RNA, like those found in mouse brain, in tissue culture cells infected with ChV. This analysis, together with radioisotopic studies, was necessary to obtain information on these components and on the process of intracellular multiplication of ChV.

MATERIALS AND METHODS

1. *Cells*

The host cells used for virus multiplication were the BHK-21 cell line, clone 13 (MacPherson and Stoker, 1962). This cell line was kindly supplied by Dr. H. Aoki, Department of Microbiology (chief, Prof. S. Hotta), Kobe University, and it was cultivated by the technique of Karabatsos and Buckley (1967). A specimen of the established cell line of African green monkey kidney (VERO) was kindly sent by Dr. A. Oya, the National Insitute of Health of Japan, Tokyo, and was used for infectivity titration of virus and RNA.

2. *Virus*

Chikungunya virus (ChV) African strain at the 175th passage level in suckling mouse brain was

kindly supplied by Dr. S. Ahandrik of the Virus Research Institute, Bangkok, Thailand. The virus suspension made from stock infected suckling mouse brains was adapted to BHK-21 cells by 3 successive inoculations onto monolayers in fluid medium composed of 0.02% gelatin in Eagle's minimal essential medium (MEM, 1959), followed by 5 successive plaque purifications by selection of large plaques. The plaque isolate was further passaged 3 times successively on BHK-21 cell monolayers in fluid media, and then inoculated into a number of BHK-21 cell monolayers in fluid medium. After 16 hr incubation at 37 C, infected culture fluid was collected and centrifuged at 700 *g* for 10 min. The supernatant (S_1) was mixed with an equal volume of saturated ammonium sulfate, pH 7.2. The mixture was kept in an ice bath for 15 min and then centrifuged at 2,500 *g* for 15 min. The supernatant was removed, and the precipitate was dissolved in 1/5 volume of S_1 of BS (0.05 M borate buffer, 0.12 M NaCl, pH 9.0), and centrifuged at 700 *g* for 10 min. From the supernatant (S_3), virus was collected by ultracentrifugation at 90,000 *g* for 60 min. The supernatant was removed and the precipitate was resuspended in 1/10 volume of S_3 of BS. To this suspension, protamine sulfate was added at a final concentration of 1mg/ml. The mixture was kept in an ice bath for 15 min and then clarified by centrifugation at 700 *g* for 10 min. The final supernatant (S_5) was diluted 10 fold with virus diluent (0.2% gelatin in MEM), distributed into a number of tubes and stored at −70 C as virus stock. All the experiments reported in this paper were carried out using this stock.

3. *Virus inoculation and preparation of a cell homogenate*

Growth medium was drained from monolayers of BHK-21 cells and they were inoculated with stock virus. Adsorption was carried out at 37 C for 2 hr, by spreading virus over cell sheets every 30 min. Then the sheets were washed twice with PBS (Dulbecco and Vogt, 1954), MEM was added to them and they were incubated at 37 C. This point was taken as the zero time of infection. After a certain period of incubation, culture fluid was removed, and the cell sheets were scraped into STE (0.02 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA, pH 7.6). The cell suspension was homogenized in a Waring blendor and centrifuged at 700 *g* for 10 min. The supernatant was used for sucrose gradient analysis.

4. *Sucrose gradient sedimentation*

Samples of 0.5 ml were layered onto 4.5 ml of 10–35% (w/w) sucrose gradient in STE, and centrifuged with an SW-39 or SW-50 rotor in a Beckman model L ultracentrifuge at 30,000 rev/min for 60 min. Fractions were collected dropwise from the bottom of the centrifuge tubes.

The radioactivity of the acid-insoluble fraction was measured by drying 0.1 ml samples on 2.5 cm diameter filter paper disks (Toyo Roshi, No. 5 C), which were then extracted by the method of Bollum (1966). Radioactivity was counted in a Beckman liquid scintillation spectrometer, model LS-200 B, with scintillator consisting of 4 g of diphenyloxazol in one liter of toluene.

5. *Extraction of RNA*

RNA was extracted from fractions obtained by sucrose gradient analysis after dilution of these fractions to 2 ml with STE. Each sample was mixed with an equal volume of water-saturated phenol containing 0.001 M EDTA and 1% of sodium dodecyl sulfate (SDS), which had been kept at 60 C. Extraction was carried out at 60 C for 5 min and then samples were cooled in an ice bath and centrifuged at 1,500 *g* for 15 min. The aqueous phase was reextracted with 1 ml of water-saturated phenol and recentrifuged. Phenol was removed from the second aqueous phase by 3 successive extractions with ether.

6. *Infectivity titration of ChV and its RNA*

The method used was essentially as described previously (Igarashi and Tuchinda, 1967; Igarashi et al., 1967). The titer was presented as plaque forming units (PFU) per ml.

7. *Reagents*

Uridine-5-H^3 (5 c/mmole) was purchased from Daiichi Pure Chemicals Co. Ltd. Tokyo. Actinomycin S_3 was generously supplied by Prof. J. Kawamata of this Institute. Chemical studies on Actinomycin S_3 showed that it corresponds to Actinomycin X_2 (Furukawa et al., 1968). Biochemical studies of Actinomycin S_3 demonstrated that its mechanism of action is similar to that of Actinomycin D (Prof. Kawamata, personal communication). Crystalline pancreatic ribonuclease (RNase) was a product of Worthington Biochemicals Co. Sodium deoxycholate of Difco Laboratories and protamine sulfate of Nutritional Biochemicals Co. were used.

RESULTS

1. *Presence of an intracellular component associated with ChV-specific RNA*

In the presence of 1 μg/ml of Actinomycin S_3, over 95% of cellular RNA synthesis measured by H^3-uridine incorporation was inhibited in non-infected BHK-21 cells as in the case of FL cells (Mantani et al., 1967), and no prominent radioactive peak of acid-insoluble material was detected from cell homogenate by sucrose gradient analysis. While the yield of ChV was not affected at this concentration of the drug. So that the acid-insoluble count incorporated was considered as ChV-specific, when ChV-infected cells were labelled with H^3-uridine in the presence of 1 μg/ml of Actinomycin S_3.

ChV-infected BHK-21 cells were labelled with H^3-uridine in the presence of Actinomycin S_3, and the cell homogenate was analyzed by sucrose gradient sedimentation. The main peak of radioactivity was found in about the middle of the gradient column, while ChV-PFU sedimented far toward the bottom of the gradient, so that ChV-specific, acid-insoluble radioactivity separated from ChV-PFU (Fig. 1A). The component associated with ChV-specific radioactivity in fraction 7 in Fig. 1A was arbitrarily named the " X "-component.

On the other hand, when the superatant of the culture fluid was analyzed under the same condition, the main radioactive peak was close to the ChV-PFU peak, sedimenting faster than " X " (Fig. 1B). The faster sedimentation of ChV-PFU in the experiment shown in Fig. 1A than in Fig. 1B may be attributed to the association of ChV infectivity with some cellular components.

When a cell homogenate prepared from infected BHK-21 cells, which had been kept frozen at —20 C for 2 days was used, the radioactive peak around fraction 7 was 1/8–1/10 of that of fresh material (Fig. 1A).

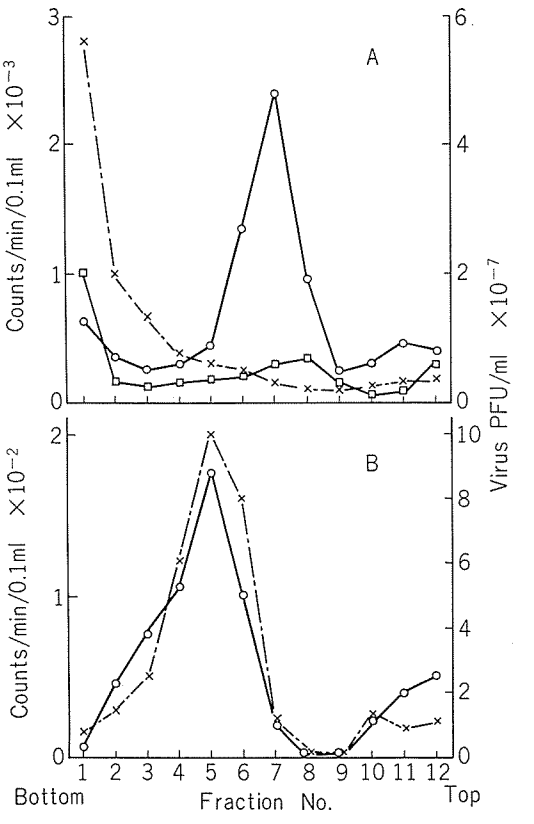


FIGURE 1. *Sucrose gradient analysis of ChV-infectivity and radioactivity from BHK-21 cells infected with ChV and labelled with H^3-uridine in the presence of Actinomycin S_3.*

BHK-21 cells (10^9) were infected with ChV at an input multiplicity of 5. After adsorption, 120 ml of MEM containing 1 μg/ml of Actinomycin S_3 were added, and incubated at 37 C for 2 hr. Then H^3-uridine was added (1 μc/ml, final concentration), and incubation was continued another 14 hr. The infected culture fluid was centrifuged at 700 g for 10 min, and the supernatant (S_1) was used for sucrose gradient analysis (B) to determine ChV-PFU (×—·—·—×) and the acid-insoluble count (○———○). Infected cells were scraped into 12 ml of STE, and the suspension was divided into two parts. One part was homogenized in a Waring blendor and then centrifuged at 700 g for 10 min. The supernatant was analyzed as above (A). The other part was kept at —20 C for 2 days and then treated as described above to measure the acid-insoluble count (□———□) in A.

2. *Appearance of the " X "-component during infection*

To study the nature of the " X "-component, its appearance during the course of infection was examined. Replicate cultures of BHK-21 cells were infected with ChV and pulse labelled for 2 hr at 2 hr intervals after infection. Cell homogenates were prepared after each labelling period. A sample of each homogenate was used for measuring ChV-PFU and radioactivity. Results are shown in Fig. 2 and Table 1. The virus titer increased from 2 hr to 12 hr after infection. The incorporation of H^3-uridine into the acid-insoluble fraction within 2 hr labelling period increased with time after infection until 10 hr after infection and decreased slightly 10–12 hr after infection. The PFU released during each of the 2 hr periods increased with time until 10 hr after infection and then decreased slightly in the period 10–12 hr after infection. The ratio of ChV-PFU released during the 2 hr period to the H^3-count incorporated was found to increase as infection proceeded, except for a slight drop in the last period. At present there are no data on the rate of incorporation of H^3-count into mature virus particles, but it seems from the data in Table 1, that ChV is produced at most in the 8–10 hr period, followed by the 6–8 hr period after infection.

The cell homogenates prepared in the above experiments were analyzed by sucrose gradient sedimentation. The results are shown in Fig. 3A-E. The peak of " X " was detectable from 4–6 hr after infection (Fig. 3B) and subsequently became more prominent (Fig. 3C-E). There was another peak of acid-insoluble radioactivity at fraction 10, and this peak was prominent from an early stage of infection. When H^3-uridine labelled RNA extracted from purified ChV was centrifuged under the same conditions, the H^3-peak was found in fraction 10, so that the radioactivity around fraction 10 in Fig. 3 might be attributable to the free ChV-specific RNA complex.

The radioactive counts corresponding to " X " and to the RNA-complex are given in Table 2, showing the approximate amounts of these compounds labelled within the 2 hr periods. There was a steady increase both in the absolute amount of " X " and in its ratio to the total count incorporated, as infection

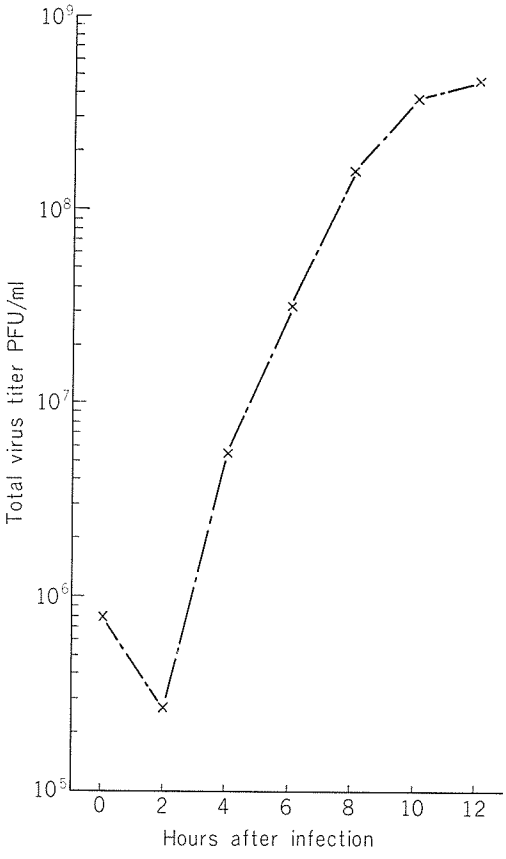
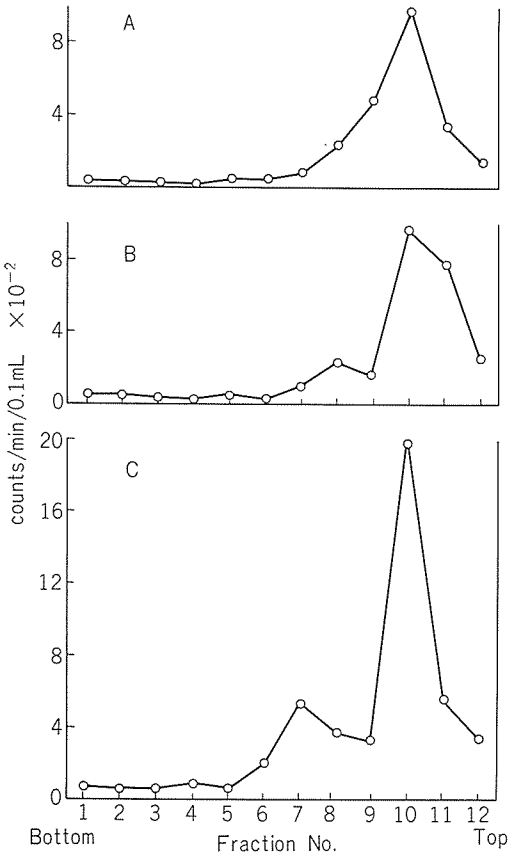


FIGURE 2. *Growth of ChV in BHK-21 cells.*
Fourteen replicate cultures of BHK–21 cells, each containing 7×10^6 cells, were infected with ChV at an input multiplicity of 20. After adsorption, cell sheets were washed twice with PBS and then MEM containing 1 μg/ml of Actinomycin S_3 (3 ml/culture) was added and cell cultures were incubated at 37 C. Every 2 hr after infection, 2 replicate cultures were labelled with H^3-uridine (60 μc/culture) for 2 hr, and then the cells were homogenized. Part of the homogenate was used for assay of ChV-PFU (×—·—·—×) and radioisotope count (in Table 1). The rest was used for the experiment in Fig 3.

TABLE 1. *Incorporation of H^3-uridine and ChV-PFU released during successive 2 hour periods after infection*

Labelling period (hr after infection)	H^3-count incorporated[a] (count/min/ 0.1 ml)	ChV-PFU released[a] (PFU/ml)	PFU released per H^3-count incorporated
2— 4	4,650	5.23×10^6	1.13×10^2
4— 6	6,651	2.55×10^7	3.84×10^2
6— 8	12,056	1.29×10^8	1.07×10^3
8—10	17,144	2.25×18^8	1.31×10^3
10—12	12,817	9.00×10^7	7.03×10^2

a: values in 6 ml of homogenate.



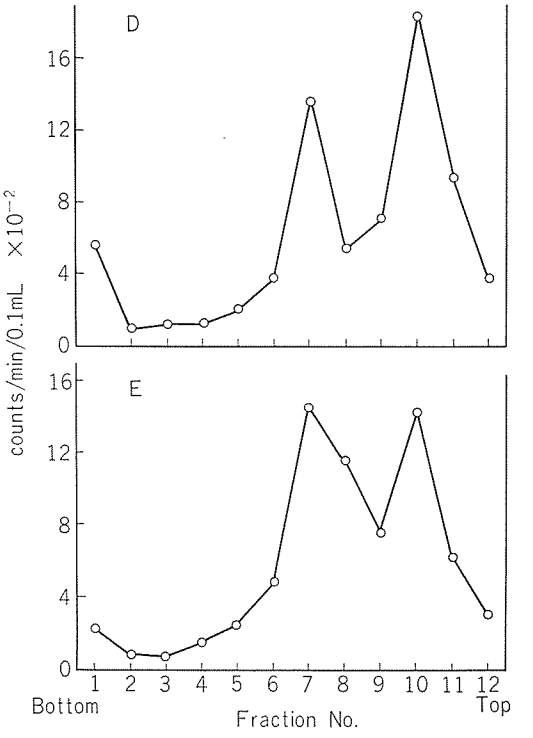


FIGURE 3. *Efficiency of labelling the "X" -component during ChV infection.*

The cell homogenates prepared in the experiment of Fig. 2 were centrifuged at 700 g for 10 min. The supernatants were analyzed by sucrose gradient sedimentation and the acid-insoluble count of each fraction was measured. The labelling periods were A; 2–4 hr, B; 4–6 hr, C; 6–8 hr D; 8–10 hr, and E; 10–12 hr after infection.

proceeded. Meanwhile the highest ratio of radioactivity in the RNA-complex to total count was in the 2–4 hr period, and the lowest in the 8–10 hr followed by the 10–12 hr period.

If the " X "-component was the precursor or immature particles of ChV, and if there was no inefficiency in the step of conversion of " X " to ChV, there should be some correlation between the amount of " X " and the efficiency of production of mature ChV in infected cells. To test this, the ratio of ChV-PFU released to the amount of the " X " at each labelling period was measured. These values increased from the 2–4 hr period until the 6–8 hr period and then declined (Table 2). This may imply that in the late stage of infection, there is some overproduction of " X " or inefficiency in the conversion to mature ChV. Another explanation may be that " X " is not a precursor of ChV, but some kind of by-product which accumulates inside ChV-infected cells. The following experiment was to clarify this point.

3. *Pulse and chase experiment*

In the presence of Actinomycin S_3 ChV-infected BHK-21 cells were pulse labelled in the early (3–4 hr) and late (8–9 hr) stages of infection with H^3-uridine. Then the radioactive medium was removed and H^3-uridine was cased out by MEM containing cold uridine and Actinomycin S_3, for 30 min and 60 min. Cell homogenates were prepared and analyzed by sucrose gradient sedimentation. The results in Fig. 4. indicate that the H^3-count in " X " could not be chased out efficiently either during the early or the late stage of infection. The count in the RNA complex in the late stage of infection was chased out, while that in the early stage was not. These results suggest that the H^3-count accumulates in " X " in the late stage of infection. Chasing out of radioactivity from " X " could not be demonstrated using longer chasing periods of up to 4 hr.

4. *Association of the " X "-component with infective ChV-RNA*

The above experiment did not directly demonstrate a relationship between " X " and ChV. Hence the next approach was to see whether the " X "-component could yield infective ChV-RNA. A cell homogenate prepared from fresh BHK-21 cells 16 hr after infection, was analyzed by sucrose gradient sedimentation. Each fraction was assayed for its ChV-PFU and, after RNA extraction, for PFU of infective RNA. The results (Fig. 5A) show that infective RNA was recovered from fraction 7 with a high titer, while most of the ChV-PFU sedimented to the bottom. A cell homogenate

TABLE 2. *Incorporation of H^3-uridine into " X " and the RNA-complex during successive 2 hour periods after infection*

Labelling period (hr after infection)	H^3-count in " X "[a]		PFU released per H^3-count in " X "	H^3-count in RNA[a]	
	count/min/ 0.1 ml	ratio to total H^3-count[b]		count/min/ 0.1 ml	ratio to total H^3-count[b]
2— 4	89	0.0191	5.88×10^4	992	0.214
4— 6	203	0.0305	1.26×10^5	950	0.143
6— 8	520	0.0431	2.40×10^5	1976	0.164
8—10	1350	0.0788	1.67×10^5	1836	0.107
10—12	1460	0.114	6.16×10^4	1428	0.111

a : values from Fig. 3.
b : not corrected for change in volume during sucrose gradient analysis.

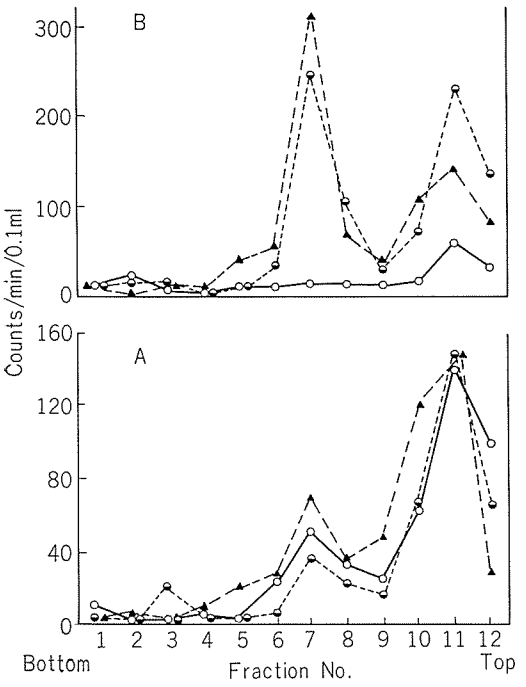


FIGURE 4. *Pulse and chase with H^3-uridine of BHK-21 cells infected with ChV.*

Replicate cultures of BHK-21 cells were infected with ChV as for Fig. 2, and MEM containing 1 μg/ml of Actinomycin S_3 was added. Cultures were labelled with H^3-uridine (A) 100 μc/culture, 3–4 hr and (B) 50 μc/culture, 7–8 hr after infection. Then they were chased with MEM containing 300 μM of non-radioactive uridine for 30 min (◒------◒) and 60 min (▲———▲). Then cell homogenates were prepared as described in Materials and Methods, and fractionated in sucrose gradients to measure acid-insoluble counts. Zero time of chase (○———○).

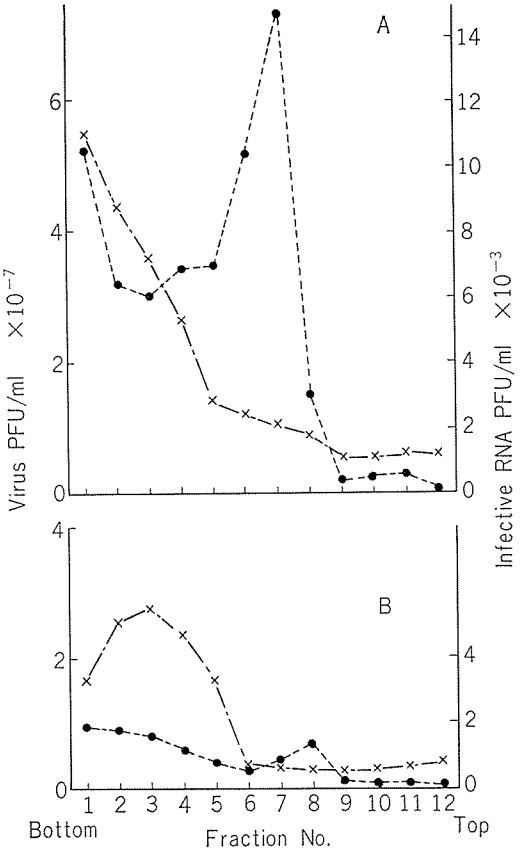


FIGURE 5. *Association of the "X"-component with ChV-infective RNA.*

(A) Cell homogenate was prepared in 6 ml of STE from 6×10^8 of BHK-21 cells 16 hr after infection, and fractionated by sucrose gradient sedimentation. A portion of each fraction was used for assaying ChV-PFU (×—·—·—×) and RNA was extracted from the rest of the fraction for assaying infective RNA (●------●).

(B) Cell homogenate was prepared from similar BHK-21 cells to (A) but kept at −20 C for 2 days after harvest before homogenization. Symbols as in (A).

prepared from the same kind of BHK-21 cells after storage at −20 C for 2 days, yielded less (about 1/8) infective RNA (Fig. 5B), than fresh cells. The result in Fig. 5 is comparable with that in Fig. 1A, where a high count was found in fraction 7 of fresh specimen and less in a specimen from frozen cells.

The identity of "X" with the component yielding infective RNA was confirmed using a cell homogenate of BHK-21 cells infected with ChV and labelled with H^3-uridine in the presence of Actinomycin S_3. Each fraction from a sucrose gradient was assayed for its ChV-PFU, acid-insoluble count, and, after extraction, for RNA-PFU (Fig. 6A). There was a correspondence between the H^3-count

and the titer of infective RNA around fraction 7, although another H^3-peak around fraction 9–10 was visible. The peak of ChV-PFU was found near the bottom. Part of fraction 7 was recentrifuged and analyzed under the same conditions as before. The result (Fig. 6B) showed that the " X- "-component was a distinct entity carrying infectinve RNA. The ChV-PFU in fractions 6 and 7 was much lower than that expected from the titer of RNA extracted. The reason for the appearance of a small radioactive peak in fraction 11 in Fig. 6B is not clear, but it might represent a portion of RNA in " X " which is easily broken off.

Plaque formation by RNA in fraction 7 of Fig. 6B was completely inhibited, when 10^{-2} dilution of anti-ChV rabbit serum was present in the agar overlay medium. Moreover the PFU of RNA in fraction 7 was completely destroyed by RNase (1 μg/ml, 15 C, 5 min).

In contrast to the cell homogenate, when purified ChV (S_5) was analyzed in a sucrose gradient and each fraction was tested as in Fig. 5, the PFU of extracted RNA was distributed like that of mature ChV (Fig. 7). In this case only one component carrying infective RNA may be mature ChV, and the ratio of RNA-PFU to ChV-PFU was 10^{-4}–10^{-5}, which was far less than in fraction 7 of Fig. 6B.

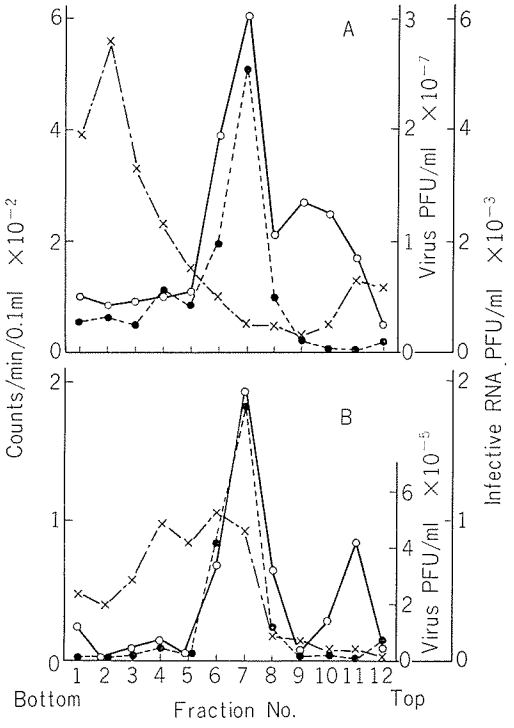


FIGURE 6. *Identification of " X " as an entity yielding ChV-infective RNA.*

Cell homogenate similar to that in Fig. 1A, but labelled with less radioisotope (0.4 μc/ml), was analyzed by sucrose gradient sedimentation. (A) The ChV-PFU (×—·—·—×) and acid insoluble count (○———○) of each fraction was assayed and RNA was extracted for assay of infective RNA-PFU (●------●). (B) Fraction 7 in Fig. 6A was pooled and diluted with STE and analyzed under the same conditions as above.

5. *Other properties of " X "*

Further studies were made on the nature of " X ". As shown in Fig. 8, when a cell homo-

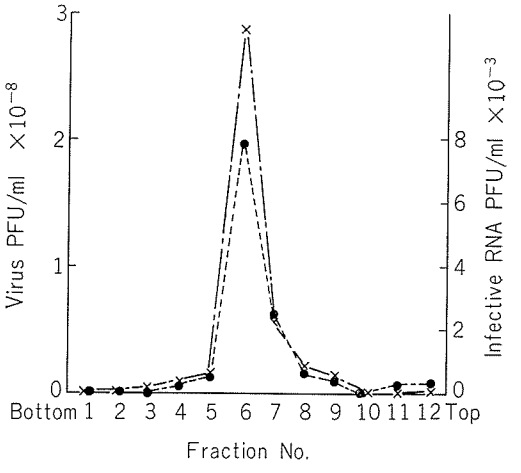


FIGURE 7. *Sucrose gradient analysis of partially purified ChV (S_5) prepared from infected BHK-21 cell culture fluid.*

The ChV-PFU (×—·—·—×) of each fraction was measured and RNA was extracted for assay of infective RNA-PFU (●------●).

genate was treated with RNase before sucrose gradient analysis, the peak of radioactivity corresponding to " X " decreased. At the same time, the H^3-count in the RNA complex also decreased. The effect of deoxycholate on " X " was different. The H^3-peak corresponding to " X " disappeared, while the count in the RNA-complex increased. This suggests that RNA was released from " X " by brief treatment with deoxycholate. When a cell homogenate was treated with protamine sulfate, and clarified by centrifugation, scarcely any radioactive peak was obtained from the supernatant on sucrose gradient analysis. This is contrary to the result for mature ChV, where the PFU remaines almost entirely in the supernatant.

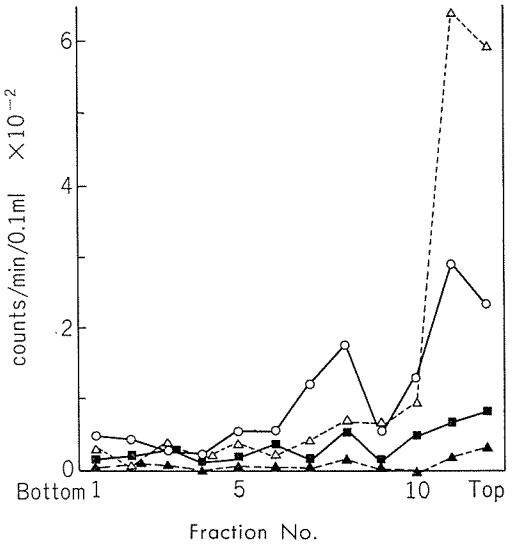


FIGURE 8. *Properties of the " X "-component.*
The cell homogenate used in Fig. 3D was diluted and fractionated by sucrose gradient sedimentation for assay of the acid-insoluble count (○——○). The same specimen was treated with 10 μg/ml of RNase at 37 C for 30 min (■——■) or with 0.2% sodium deoxycholate at 15 C for 5 min (△------△) before centrifugation. Part of the same cell homogenate was treated with protamine sulfate (1 mg/ml, 4C, 15 min) and clarified by centrifugation (700 g, 10 min) before sucrose gradient analysis (▲———▲).

DISCUSSION

An intracellular component, arbitrarily designated as " X ", was found and shown by ultracentrifugation to be a distinct entity associated with ChV-spefific RNA. It sedimented slower in a sucrose gradient than mature ChV. Using Japanese encephalitis virus, as a reference of 200 S (Fukai, Kitano and Fukunaga, 1966), the sedimentation coefficient of mature ChV was estimated as 230 S, and that of the " X " as 170 S. On the other hand, " X " sedimented more rapidly than ChV in potassium tartrate gradients. This may imply that " X ", despite its smaller size has a higher density than mature ChV particles. Using partially purified ChV samples from suckling mouse brain, Igarashi et al. (1967) found some components other than mature ChV, yielding infective RNA. When purified ChV from infected tissue culture fluid was used, only mature ChV yielded infective RNA. This discrepancy can be interpreted by the intracellular presence of " X " as a carrier of infective RNA. In the cytoplasm of chick embryo cells infected with Semliki Forest virus, Friedman and Berezesky (1967) found a possible precursor particle of 140 S, which was labelled with a 1 hr pulse, was RNase-resistant and contained 42 S viral RNA. This particle of 34–40 mμ diameter, which lacked an outer envelope, was not infectious under the conditions used for assaying mature virus. Similar cytoplasmic structure were also found in the cases of Sindbis virus (Ben-Ishai, Goldblum and Becker, 1968) and western equine encephalitis virus (Sreevalsan and Allen, 1968).

The " X "-component yield infective ChV-RNA when extracted with hot SDS-phenol, although " X " itself has low infectivity as virus, if any. By the cold phenol method, a lower amount of RNA-PFU was extracted from " X ", also " X " seems to exist primarily intracellular. These results are different from the case of eastern equine encephalitis virus (Wecker and Richter, 1962; Richter and Wecker, 1963), in which infective RNA was

extracted efficiently by the cold phenol method from immature particles, and these particles were also found in infected culture fluid.

At present we have no direct evidence that " X " is the precursor or an immature form of ChV. Pulse and chase experiments did not show that radioactivity in " X " was incorporated into mature ChV. On the other hand the H^3-count in the RNA complex was chased out in the late stage of infection, but not in the early stage. This might be interpreted as showing that in the early stage of infection there was a multiplication of ChV-RNA template as an RNA-replication mechanism increasing the size of the pool of RNA-complex, while in the late stage the size of this pool did not increase. This hypothesis could also explain why the efficiency of virus liberation relative to the total count was low in the early stage of infection but increased later.

It seems interesting to see that " X " tended to accumulate in the late stage of infection, but the efficiency of virus release relative to the amount of the " X " labelled during the 2 hr periods decreased from 8 hr after infection. These results could be explained in two ways: One explanation is that " X " is not a direct precursor of ChV but only a by-product, the production of which is not related to the release of ChV, but increases steadily as infection proceeds. By electron microscopy Mussgay and Weibel (1962) observed a nucleoid structure of 25 mμ diameter in the cytoplasm of KB cells infected with Venezuelan equine encephalitis virus, but they are uncertain whether this structure is a precursor particle. The other explanation is that although " X " is a precursor of ChV, the maturation step of " X " into ChV may be blocked or inhibited. In electron microscopic stdies on a western equine encephalitis virus-chick embryo cell system, Morgan, Howe and Rose (1961) observed precursor particles of 22 mμ diameter, which were absent in an advanced stage of infection. Higashi et al. (1967) reported similar particles seen on electron microscopic examination of VERO cells in a later period of infection with ChV. McGee-Russel and Gosztonyi (1967) described a virus core-like structure in suckling mouse brain infected with Semliki Forest virus. Acheson and Tamm (1967) studied the Semliki Forest virus-chick embryo cell system by electron microscopy and reported the presence of the same structure, and they also noted that accumulation of nucleoid was observed in the late stage but not in the early stage of infection. Evidently the possibility that " X " is a precursor cannot be ruled out even from the negative results of pulse and chase experiments, because it may be difficult to chase out when the pool size is large and is increasing, and when " X " is treated as a mass population, in contrast to the case in electron microscopic studies.

In the case of group B arboviruses, Yasuzumi et al. (1964) and Yasuzumi and Tsubo (1965) reported the presence of dense particles of 20–25 mμ diameter in mouse brains infected with Japanese encephalitis virus. Using a Kemerobo virus-chick embryo cell system, Shestopalova et al. (1964) observed particles of 26—27 mμ in diameter in the nuclei and cytoplasm. No clear-cut relationship was established between these particles and mature virus particles. Igarashi, Kitano and Fukai (1963) reported briefly on a slow-sedimenting infectivity of Japanese encephalitis virus differing from that of mature virus by sucrose gradient analysis and LD_{50} titration in mice. However, this was not confirmed by our subsequent work using a titration in tissue culture system and virus preparations from frozen brains (Igarashi, Fukunaga and Fukai, 1964). This contradiction may be reconciled by assumption of the presence of a component like " X " also in Japanese encephalitis virus system.

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