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STRUCTURAL COMPONENTS OF CHIKUNGUNYA VIRUS[1]

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SUMMARY Studies were performed on the structural proteins of Chikungunya virus (ChV), some components derived from it and an intracellular component associated with ChV-specific RNA (the "X"-component), by electrophoresis in polyacrylamide gels. The results, supported by morphological and biochemical evidence, showed that ChV contains 2 major structural proteins. One of them is associated with hemagglutinin, and the other with the core of ChV. The molecular weights of these proteins, estimated from their mobilities in polyacrylamide gels containing sodium dodecylsulfate, were 53,000 for the hemagglutinin protein and 30,000 for the core protein.

Pronase treatment of ChV (density 1.24 g/cc) yielded smooth-surfaced particles (density 1.20 g/cc), which seemed to have an intact core surrounded by a membrane containing phospholipid, but which were devoid of hemagglutinin. Further digestion seemed to remove the membrane. On treatment with Tween 80 and ether, small but heavy hemagglutinin (density 1.28 g/cc) was released from ChV. The fundamental structure of the hemagglutinin seemed to be a hollow circular or hexagonal cylinder with a diameter of 3 mμ. Treatment of ChV with Nonidet P40 yielded small, light hemagglutinin (density 1.19 g/cc), which seemed to retain part of the membrane containing phospholipid.

The "X"-component was shown to contain the core protein of ChV as its major protein component, but it was not found to have the phospholipid membrane. The results are compatible with the idea that the "X" component is a nucleoprotein core of ChV accumulating in ChV-infected BHK21 cells.

1. A part of this work was performed during a training course in Microbiology in 1969 held in this Institute by the Government of Japan and arranged by the Overseas Technical Cooperation Agency.
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INTRODUCTION

The presence of an intracellular component associated with Chikungunya virus (ChV)-specific RNA was identified in BHK21 cells infected with ChV. The component was arbitrarily named the " X " (Igarashi and Fukai, 1969). Some of the basic characteristics of the " X " were also reported, supporting the idea that the " X " is a nucleoprotein core of ChV (Igarashi, 1969; Igarashi, Fukuoka and Fukai, 1969). There are several reports on the structural proteins of other group A arboviruses and of their nucleoprotein core found in infected cells, using polyacrylamide gel electrophoresis (Friedman, 1968; Hay, Skehel and Burke, 1968; Sreevalsan and Allen, 1968). To confirm this idea, it was necessary to clarify the structural proteins of ChV and the " X ". This paper reports results of a study on these structural proteins, together with some morphological and biochemical studies on components derived from ChV.

MATERIALS AND METHODS

1. *Virus*

Chikungunya virus (ChV), African strain was obtained from Dr. S. Ahandrik of the Virus Research Institute, Bangkok, Thailand. The stock of seed virus was prepared as described before (Igarashi and Fukai, 1969).

2. *Cells*

The host cell for virus growth was a baby-hamster kidney-cell line (BHK21), clone 13 (MacPherson and Stoker, 1962). The cell line was obtained from Dr. H. Aoki of the Department of Microbiology, Kobe University, and was grown by the method of Karabatsos and Buckley (1967). An established cell line of African green monkey kidney (VERO) was supplied from Dr. A. Oya of the National Institute of Health of Japan, Tokyo, and was grown as described previously (Igarashi and Tuchinda, 1967).

3. *Purification of ChV*

The method was as described before (Igarashi, 1969). Cell growth media were removed from confluent sheets of BHK21 cells. The cell sheets were washed twice with PBS (Dulbecco and Vogt, 1954), and were inoculated with the seed virus at an input multiplicity of 5 PFU/cell. Virus adsorption was carried out at 37 C for 2 hr, spreading virus over cell sheets every 30 min. Then, cultures were added with Eagle's minimal essential medium (MEM, 1959), and incubated at 37 C. When ^{3}H-uridine labeled ChV was necessary, MEM containing 1 μg/ml of Actinomycin S_3 was used as virus phase medium. To obtain ^{3}H-valine, or ^{14}C-valine labeled ChV, the content of amino acids except glutamine was reduced to 1/10 of the ordinary amount and Actinomycin S_3 was added to 1 μg/ml. In both cases labeling was performed from 2 hr after infection, while Actinomycin was added from 0 hr. To prepare ^{14}C-choline labeled ChV, BHK21 cells were preincubated for 2 hr before ChV infection, with ^{14}C-choline in MEM containing 1/10 the ordinary amount of vitamine mixture.

At 16 hr after infection, infected fluid (F), about 600 ml volume, was collected from infected BHK21 cells (about 9×10^{8} total cells). The F was centrifuged at $700\times g$ for 10 min. To the supernatant (S_1) was added 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 4,000 $\times g$ for 20 min. The supernatant was removed, and the precipitate was dissolved in $S_1/10$ volume of BS (0.12 M NaCl, 0.05 M borate buffer, pH 9), and the solution (P_2) was centrifuged at $700\times g$ for 10 min. The supernatant (S_3) was centrifuged at $90{,}000\times g$ for 60 min. The supernatant was removed and the pellet was resuspended in $S_3/10$ volume of BS. To the suspension (P_4), $P_4/10$ volume of protamine sulfate (10 mg/ml in BS) was added. The mixture was kept in an ice bath for 15 min, and then centrifuged at $700\times g$ for 10 min. About 6 ml of the supernatant (S_5) was then layered onto 25 ml of a 10–35% sucrose gradient in STE (0.1 M NaCl, 0.02 M Tris-HCl, 0.001 M EDTA, pH 7.6), and was centrifuged in an SW-25.1 rotor of a Beckman model L ultracentrifuge at 25,000 rev/min for 75 min. A bluish-white light scattering band was observed at a little below the middle of the gradient (Fig. 1). The band was sucked into a syringe equipped with a long needle with an L-shaped tip, and was used as the preparation of purified ChV after dialysis against an appropriate buffer.

4. *Preparation of infected cell homogenate*

BHK21 cell sheets were harvested 16 hr after in-

fection with ChV. Cell sheets were scraped into STE to make a suspension of approximately 3×10^7 cells/ml. The suspension was homogenized in a Teflon homogenizer, and was centrifuged at $700 \times g$ for 10 min. The supernatant was used for further analysis.

5. *Extraction of RNA*

Specimens in STE were mixed with an equal volume of 80% phenol containing 0.001 M EDTA and 2% sodium dodecylsulfate (SDS). Extraction was carried out in a 50 C water bath for 3 min. Then the specimens were cooled in an ice bath, and centrifuged at $1,500 \times g$ for 15 min. The water phase was extracted with 80% phenol containing 0.001 M EDTA for 2 min at room temperature. The mixture were recentrifuged and phenol was removed from the second water phase by 3 successive extractions with ether.

6. *Infectivity titration of ChV and its RNA*

The methods were the same as described before (Igarashi and Tuchinda, 1967; Igarashi, Fukai and Tuchinda, 1967). Titers are presented as plaque forming units (PFU) per ml.

7. *Hemagglutination (HA) and hemagglutination-inhibition (HI) test*

The HA activities of ChV and components derived from it and the HI titer of immune sera were assayed by the method of Clarke and Casals (1958), using goose red blood cells suspended in VAD 6.0. The method was slightly modified for use in a microtiter system (Sever, 1962). Antiserum against ChV was prepared in adult mice as described before (Igarashi, 1969).

8. *Sucrose gradient sedimentation*

Specimens of 0.5 ml of ChV, its components or cell homogenates were overlayed onto 4.5 ml of a 10–35% sucrose gradient in STE, and were centrifuged in an SW-39 rotor of a Beckman model L ultracentrifuge at 30,000 rev/min for 60 min. Twelve fractions were collected dropwise from the bottom of each tube.

Specimens of 0.2 ml of RNA were layered onto 4.8 ml of a 5–20% sucrose gradient in STE, and were centrifuged in an SW-65Ti rotor of a Beckman model L2-65B ultracentrifuge at 55,000 rev/min for 80 min at 10 C. Fifteen fractions were collected dropwise from the bottom of each tube.

Specimens to measure acid-insoluble radioactivity were prepared by drying 0.1 ml of each fraction on a 2.5 cm diameter filter paper disk (Toyo Roshi, No. 5C). Disks were then extracted by Bollum's method (1966). Specimens labeled with ^{14}C-choline were not extracted with ethanol and ether. Dried disks were immersed in 10 ml of toluene scintillator fluid (4 g of diphenyloxazol in one liter of toluene). The total radioactivity of each fraction was measured by adding 0.1 ml of specimen to 10 ml of Bray's solution (1960). Counts were recorded in a Beckman model LSC-150B liquid scintillation spectrometer. With specimens doubly labeled with ^{3}H and ^{14}C, automatic quenching control (AQC) was put on in the machine with an external standard of γ-ray irradiation.

9. *Polyacrylamide gel electrophoresis*

The method of Summers et al. (1965) and Strauss et al. (1968) was followed with slight modifications. The gel mixture contained final concentrations of 7.5% acrylamide, 0.5% *N, N'* methylene-bisacrylamide, 0.1% SDS, 0.1 M sodium phosphate buffer, pH 7.0, 0.034% *N, N, N', N'* tetramethylethylenediamine and 0.07% ammonium persulfate. The mixture was deareated and poured into 5×80 mm glass tubes, held vertically and stoppered in one end, to make 5×50 mm gel columns. The mixture was carefully overlayed to a height of 3 mm with distilled water to give a flat surface to the gel. The gel were allowed to polymerize for 3 hr at room temperature. Gels were prerun with an electrophoresis buffer (0.1 M sodium phosphate, pH 7.0, containing 0.1% SDS), at 5 mamp/gel for 2 hr at room temperature.

Specimens for electrophoresis were dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, in the cold, and then solubilized by incubation with 1% SDS and 1% 2-mercaptoethanol at 37 C for 1 hr. The solubilized specimens were then dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% 2-mercaptoethanol at room temperature overnight. One-fifth volume of 60% sucrose containing bromophenol blue was mixed with the dialyzed specimen, and 0.1 ml of the mixture was applied on the polyacrylamide gel column in the glass tube.

Electrophoresis was performed at room temperature for 4 hr at 5 mamp/gel. After the run, gels were removed from the glass tubes, fixed in 20% sulfosalicylic acid, and stained with Coomassie brilliant blue R 250 and then destained in 7% acetic

acid according to the method of Shapiro, Viñuela and Maizel (1967). When radioactivity was measured, the gel columns were fractionated and counted following the description of Strauss et al. (1968), with the slight modification that gel was cut into fractions of 1.7 mm thickness.

10. *CsCl density gradient centrifugation*

Specimens were mixed with stock solution of saturated CsCl to make an appropriate initial density and density gradient. The mixture in 5 ml volume was buffered with Tris-HCl, pH 7.6, at a final concentration of 0.01 M. Centrifugation was performed in an SW-50L rotor of a Beckman model L2 ultracentrifuge at 35,000 rev/min for 20 hr at 4 C. After the run, fractions of 10 drops were collected from the bottom of the tube. Densities were estimated by measuring the refractive index of each fraction using the equation of Ifft, Voet and Vinograd (1961). Radioactivities were measured as described in the section on sucrose gradient sedimentation.

11. *Electron microscopy*

Test specimens were dialyzed against an aqueous solution of 1% ammonium acetate, pH 7.2, overnight in the cold. One drop of the dialyzed specimen was mixed with an equal volume of saturated uranyl acetate in distilled water. The mixture was applied on a carbon-coated electron microscope microgrid. Excess fluid was removed with filter paper, and the specimen was air dried and observed under a Hitachi model HU-11DS electron microscope at an acceleration voltage of 75 kv and an instrument magnification of 53,000–78,000.

12. *Reagents*

Uridine-5-^{3}H (5 c/mmole), DL-valine-^{3}H (250 mc/mmole) and L-valine-^{14}C (150 mc/mmole) were purchased from Daiichi Pure Chemicals Co. Tokyo. Methyl-^{14}C-choline chloride (60 mc/mmole) was obtained from the Radiochemical Centre, Amersham, England. Actinomycin S_3 was generously supplied by Prof. J. Kawamata of this Institute. Chemical studies on Actinomycin S_3 showed that it corresponded to Actinomycin X_2 (Furukawa et al., 1968). Biochemical studies on Actinomycin S_3 revealed that its mode of action was similar to that of Actinomycin D (Prof. J. Kawamata, personal communication). Pronase E (70,000 p. u. k./g, Kaken-kagaku Co. Ltd., Tokyo) was supplied by Dr. M. Takahashi of this Institute. The nonionic detergent Nonidet P40 was kindly given by Dr. Y. Hosaka of this Department.

RESULTS

1. *Protein components of intact ChV*

A purified preparation of ChV was obtained from the visible band in a sucrose gradient column (Fig. 1). It was disintegrated and analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. Two bands of polypeptide were visible on staining with Coomassie blue (Fig. 2B, 5A, 7A and 12B). The slower moving band was denser than the faster moving one.

FIGURE 1. *Visible band of purified ChV in a sucrose gradient column.*

2. *Protein component associated with the membrane or hemagglutinin of ChV*

1) Hemagglutinin liberated by Tween 80 and ether.

A purified specimen of ChV labeled with ^{3}H-valine in BS was treated with Tween 80 and ether as described by Mussgay and Rott (1964), and the water phase was analyzed by CsCl density gradient centrifugation. HA activity was located with the acid-inosoluble ^{3}H-count at a density of 1.28 g/cc (Fig. 3B). Intact ChV gave main peaks of HA, PFU and ^{3}H-counts at a density of 1.24 g/cc (Fig. 3A). The fraction containing the HA peak of Tween 80-ether treated ChV was analyzed by polyacrylamide gel electrophoresis. A single band was visible on staining with Coomassie blue, corresponding to the slower moving band of intact ChV (Fig. 2A). The Tween 80-ether treated ChV specimens was analyzed by sucrose gradient sedimentation. The HA and ^{3}H-valine count remained near the top of the gradient (Fig. 4B) while those of intact ChV sedimented to fraction 5 (Fig. 4A). Thus Tween 80 ether treatment liberated a hemagglutinating component with a higher density in CsCl than inctact ChV but with a slower sedimentation velocity in a sucrose gradient and so a smaller size than intact ChV. This hemagglutinin contained one of the 2 major structural proteins of ChV. The antigenicity of the hemagglutinin was checked by the HI test using mouse immune sera against ChV.

2) Hemagglutinin liberated by Nonidet P40 (NP40).

Purified ChV labeled with ^{3}H-valine was incubated with 0.1% NP40 at room tem-

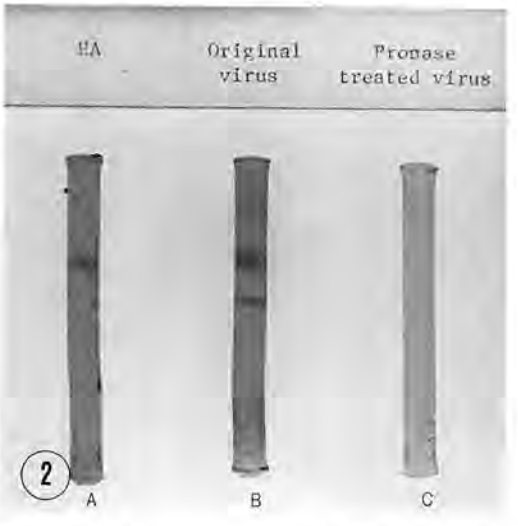


FIGURE 2. *Protein components of ChV after polyacrylamide gel electrophoresis and staining (1).*
(A) HA peak of Tween 80 and ether treated ChV on CsCl density gradient centrifugation
(B) Intact purified ChV
(C) Fraction 7 of pronase treated ChV in a sucrose gradient

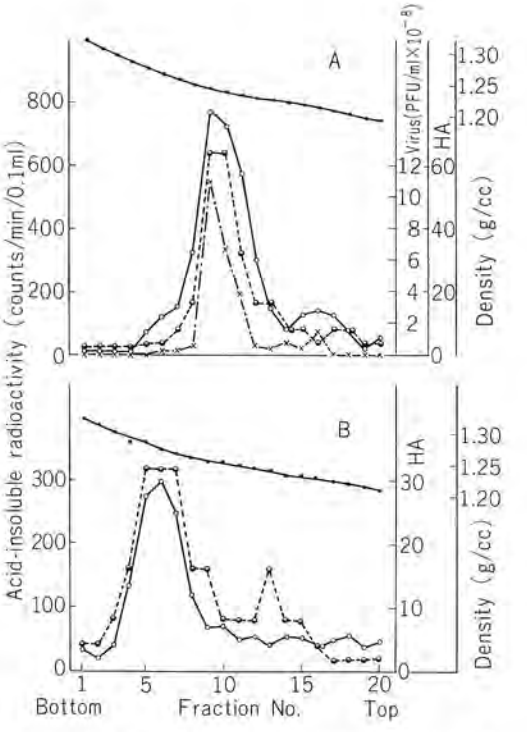


FIGURE 3. *CsCl density gradient centrifugation of (A) ^{3}H-valine labeled intact ChV and (B) Tween 80-ether treated ChV.*
○——○ *acid-insoluble radioactivity*
●------● *HA*
×——× *PFU*
·—·—· *density*

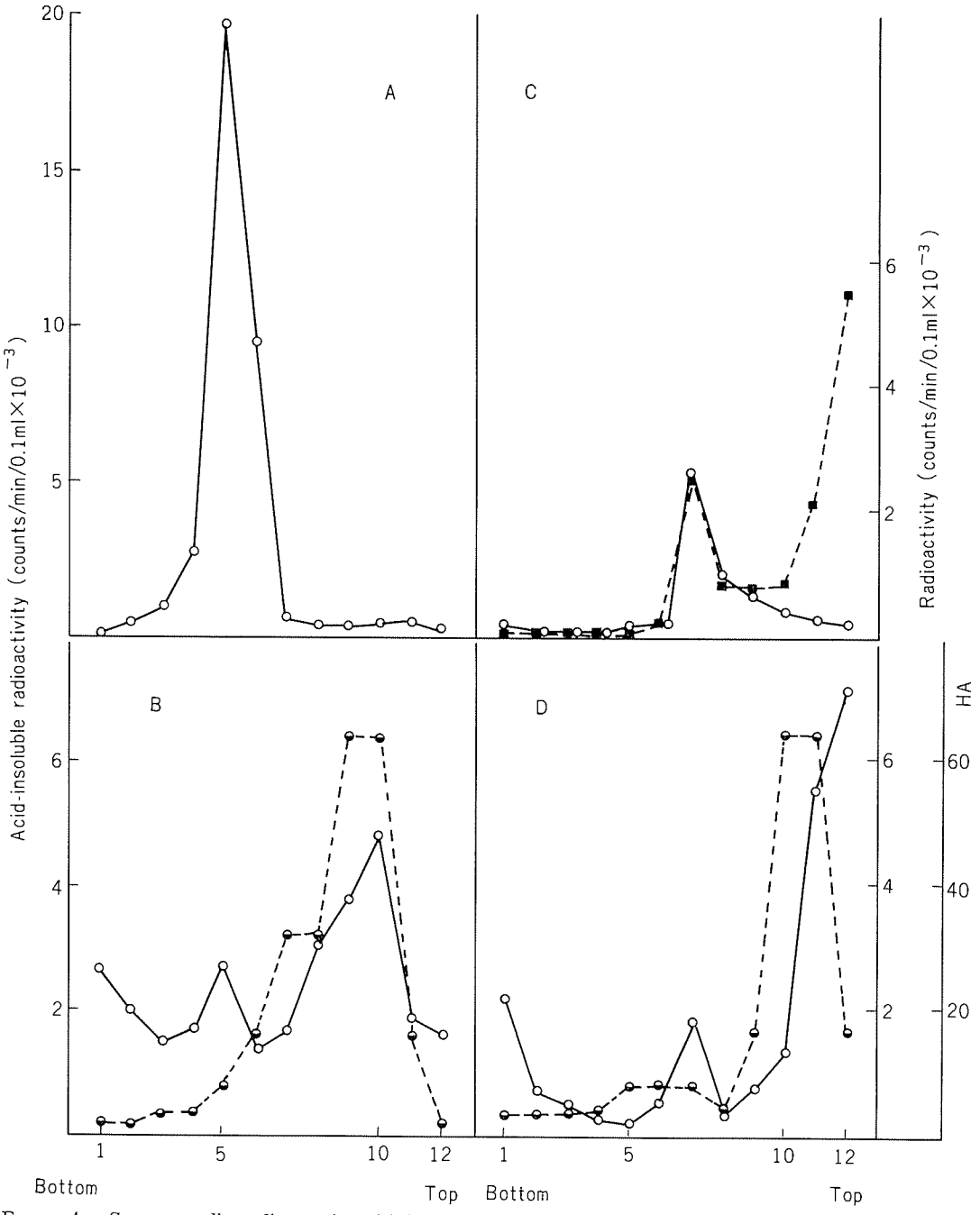


FIGURE 4. *Sucrose gradiet sedimentation of (A) ^{3}H-valine labeled intact ChV, (B) Tween 80-ether treated ChV, (C) pronase treated ChV and (D) NP40 treated ChV.*

○———○ *acid-insoluble radioactivity*

■------■ *total radioactivity*

◒·····◒ *HA*

perature for 10 min and then analyzed by sucrose gradient sedimentation. Two peaks of acid-insoluble ^{3}H-counts were observed in fraction 7 and at the top of the gradient, and the HA was located near the top of the gradient (Fig. 4D). Analysis of fraction 11 of Fig. 4D by polyacrylamide gel electrophoresis gave a single band corresponding to the slower moving band of intact ChV (Fig. 5C). Therefore, a hemagglutinating component seemed to be separated from intact ChV by NP40 treatment, remaining near the top of the sucrose gradient. However, this hemagglutinin differed from that liberated by Tween 80-ether in that it had a lower density of 1.19 g/cc in a CsCl density gradient (Fig. 6). When a ChV preparation labeled with ^{14}C-choline was analyzed under similar conditions, the ^{14}C-count remained at the top of the sucrose gradient centrifugation (Fig. 8C), and were associated with the HA peak on CsCl density gradient centrifigation (data not shown). So that

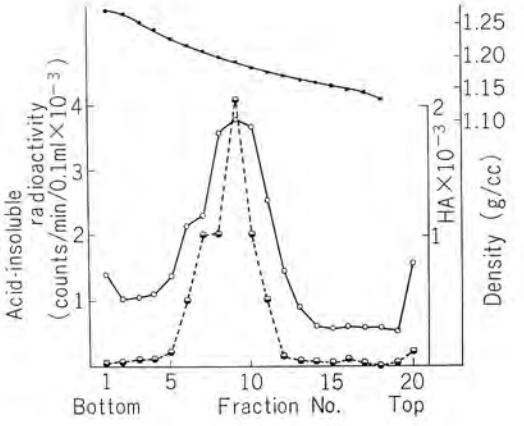


FIGURE 6. *CsCl density gradient centrifugation of fraction 11 of NP40 treated ChV on sucrose gradient sedimentation.*

○——○ *acid-insoluble radioactivity*

◒------◒ *HA*

·-·-·-· *density*

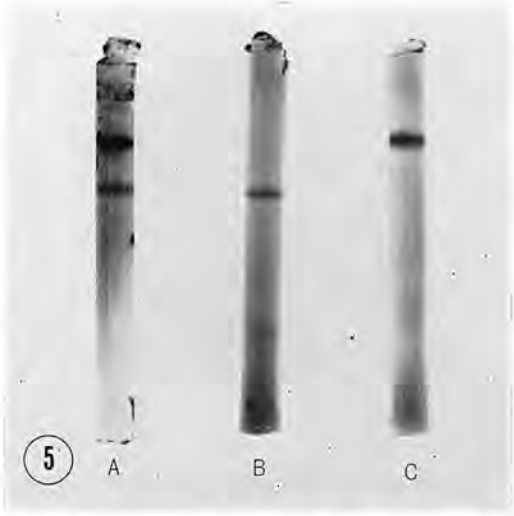


FIGURE 5. *Protein components of ChV observed by polyacrylamide gel electrophoresis and staining (2).*

(A) Purified ChV

(B) Fraction 7 of NP40 treated ChV on sucrose gradient sedimentation

(C) Fraction 11 of NP40 treated ChV on sucrose gradient sedimentation

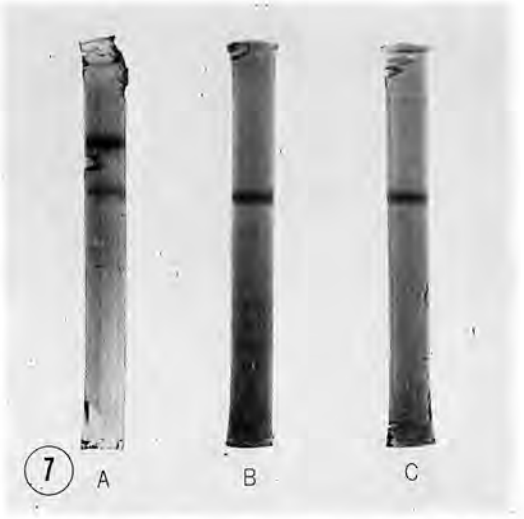


FIGURE 7. *Protein components of ChV and the "X" observed by polyacrylamide gel electrophoresis and staining (1).*

(A) Intact ChV

(B) Fraction 7 of pronase-treated ChV on sucrose gradient sedimentation

(C) Fraction 7 on sucrose gradient sedimentation of the NP40 treated supernatant of a homogenate of ChV-infected BHK21 cells

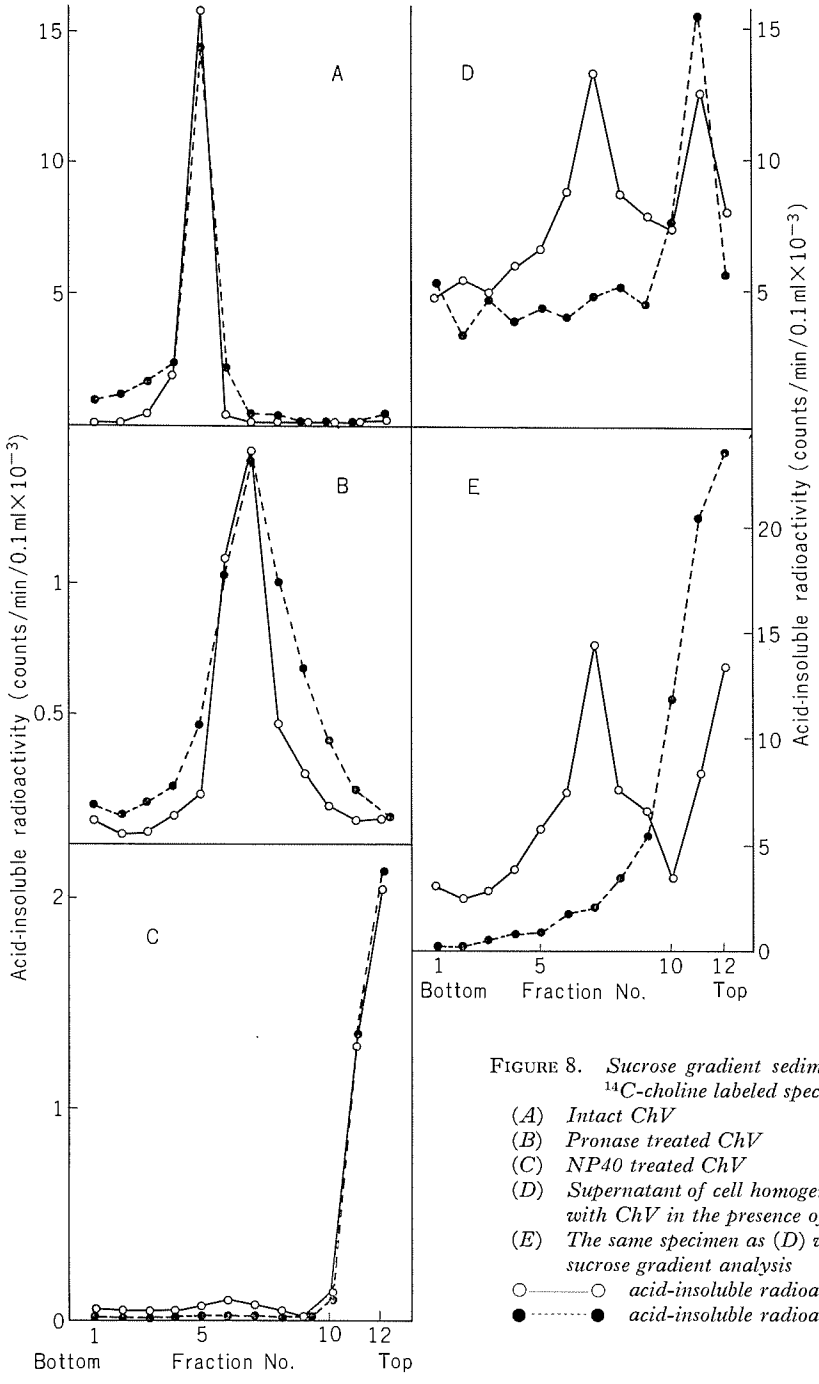


FIGURE 8. *Sucrose gradient sedimentation of ^{3}H-uridine and ^{14}C-choline labeled specimens.*

(*A*) *Intact ChV*
(*B*) *Pronase treated ChV*
(*C*) *NP40 treated ChV*
(*D*) *Supernatant of cell homogenate of BHK21 cells infected with ChV in the presence of Actinomycin S_3*
(*E*) *The same specimen as (D) was treated with NP40 before sucrose gradient analysis*

○——○ *acid-insoluble radioactivity of ^{3}H*
●·····● *acid-insoluble radioactivity of ^{14}C*

on NP40 treatment, another hemagglutinin was liberated, which was small and light and retained part of the membrane containing phospholipid. The hemagglutinins liberated by both Tween 80-ether and NP40 contained only the slower moving component of 2 major structural proteins of ChV.

3. *Protein component associated with the core of ChV*

1) Pronase treated ChV.

Pronase is known to "shave off" the outer projections of Semlike Forest virus in media of low ionic strength (Osterrieth, 1965; Calberg-Bacq and Osterrieth, 1966). Purified ChV labeled with ^{3}H-valine was dialyzed against 0.01 M sodium phosphate, pH 7.0, and the resulting specimen was incubated with 100 μg/ml of pronase at 37C for 60 min. The reaction was stopped by adding 1/10 volume of 2 M NaCl and the specimen was analyzed by sucrose gradient sedimentation. Acid-insoluble radioactivity sedimented to fraction 7, while acid-soluble radioactivity (difference between total and acid-insoluble counts) remained at the top of the gradient (Fig. 4C). Fraction 7 was analyzed by polyacrylamide gel electrophoresis. A single band was obtained corresponding to the faster moving band of intact ChV (Fig. 2C and 7B). When a purified ChV specimen labeled with ^{3}H-uridine and ^{14}C-choline was treated with pronase as above and analyzed by sucrose gradient sedimentation, both the acid-insoluble ^{3}H- and ^{14}C-counts sedimented to fraction 7 as a single peak (Fig. 8B). With intact ChV, both sedimented to fraction 5 (Fig. 8A). When the same pronase-treated ChV specimen was analyzed by CsCl density gradient centrifugation, both the ^{3}H- and ^{14}C-counts were found as a single peak at a density of 1.20 g/cc (Fig. 9A). Thus the protein component responsible for HA, present on the outside of the ChV particle, was digested by pronase, leaving a core particle retaining the membrane containing phospholipid. This conclusion was supported by the results on the RNA extracted from pronase-

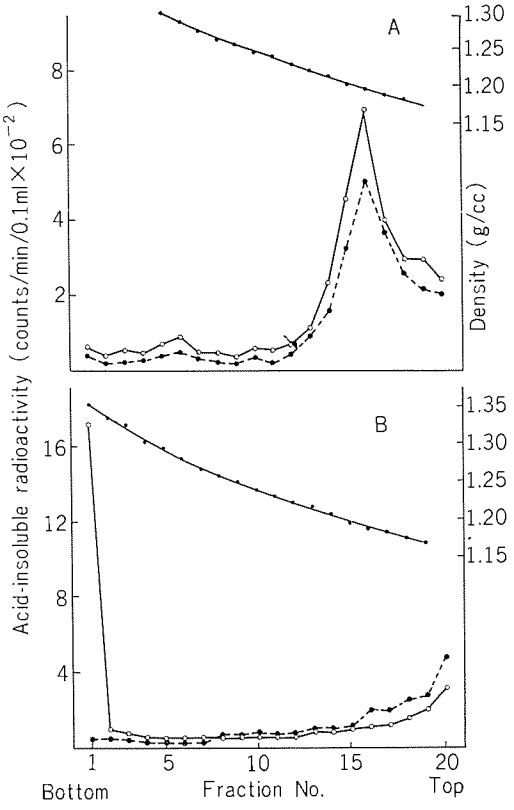


FIGURE 9. *CsCl density gradient centrifugation of ^{3}H-uridine and ^{14}C-choline labeled specimens.*
(A) Pronase treated ChV
(B) Fraction 7 on sucrose gradient sedimentation of the supernatant of a cell homogenate from BHK21 cells infected with ChV in the presence of Actinomycin S_3
○——○ *acid-insoluble radioactivity of ^{3}H*
●------● *acid-insoluble radioactivity of ^{14}C*
·-·-·-· *density*

treated ChV. Although the ChV-PFU and HA were greatly decreased by pronase treatment, the PFU of extracted RNA remained fairly high (Table 1). On sucrose gradient analysis of RNA extracted from ^{3}H-uridine labeled and pronase-treated ChV, a single peak of 45 S was obtained, the value being the same as that of intact ChV-RNA (Fig. 10).

2) Core released by NP40.

As already described, when ^{3}H-valine labeled ChV was treated with NP40 and then analyzed

TABLE 1. *HA and PFU of ChV and PFU of RNA extracted from it, before and after treatment with pronase*

	HA of ChV	PFU of ChV	PFU of extracted RNA
Original ChV	640	5.0×10^9	1.1×10^5
Pronase-treated ChV[a]	<20	1.5×10^5	7.0×10^4

[a] Pronase 100 μg/ml in 0.01 M phosphate buffer, pH 7.0, at 37 C for 60 min.

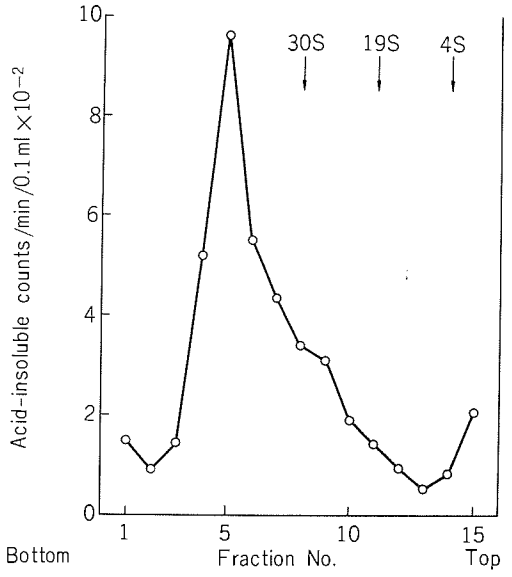


FIGURE 10. *Sucrose gradient sedimentation of RNA from pronase treated ChV labeled with ^{3}H-uridine.*
○——○ *acid-insoluble radioactivity*
Vertical arrows: position of OD_{260} peak of mouse brain RNA added as a marker

by sucrose gradient sedimentation, another peak of ^{3}H-counts appeared in fraction 7 besides that near the top (Fig. 4D). Fraction 7 was removed to analyze its protein component by polyacrylamide gel electrophoresis. The result showed that fraction 7 contained one polypeptide with the same mobility as the faster moving component of intact ChV proteins (Fig. 5B). However, after NP40 treatment of ChV labeled with ^{3}H-uridine and ^{14}C-choline, both the ^{3}H- and ^{14}C-counts remained at the top of the sucrose gradient on centrifugation (Fig. 8C). Thus the core particle released by NP40 treatment seemed to have no phospholipid membrane, and probably no viral RNA, although it contained the core protein of ChV.

From the above results it seems that ChV has 2 major structural proteins. One is localized at the membrane and responsible for HA, and has a slower mobility in SDS-polyacrylamide gel. The other seems to be associated with the core structure of ChV and has a faster mobility. The mobilities were compared with those of bovine serum albumin and cytochrome c, and the relationship between the molecular weight and the mobility of proteins in SDS-polyacrylamide gels was applied (Shapiro et al., 1967). The molecular weight of the membrane or HA protein of ChV was estimated as 53,000 and that of the core protein as 30,000.

4. *Protein component of the "X"*

In a ChV-BHK system, a possible intracellular nucleoid component of ChV, arbitrarily named the "X", was shown to accumulate in the late stage of infection. One purpose of this work was to identify the protein component associated with the "X". The "X"-component was prepared from ChV-infected BHK21 cells. Cells were labeled with ^{3}H-valine from 2 hr after infection in the presence of 1 μg/ml of Actinomycin S_3. A cell homogenate was prpared 16 hr after infection. The homogenate was centrifuged and the supernatant was analyzed by sucrose gradient sedimentation. In addition to the count near the top of the gradient, there was a peak of acid-insoluble count in fraction 7, corresponding to the position of the "X" (Fig. 11A). The distribution of radioactivity was not affected by treatment of the supernatant from the cell homogenate with 0.1% NP40 at room temperature for 10 min before sucrose gradient analysis (Fig. 11B). The peak fraction of the "X" was pooled, diluted 4 times with 0.01 M phosphate buffer, pH 7, and then cencentrated

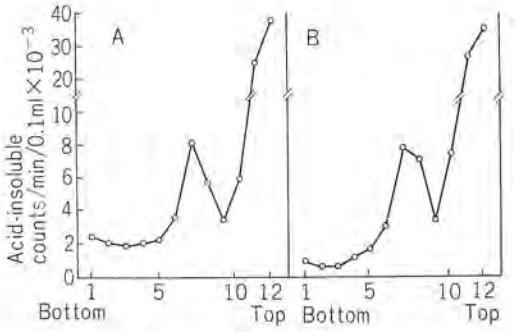


FIGURE 11. *Sucrose gradient sedimentation of the supernatant of a cell homogenate of BHK21 cells infected with ChV and labeled with ^{3}H-valine in the presence of Actinomycin S_3, (A) before and (B) after treatment with NP40.*
○——○ *acid-insoluble radioactivity*

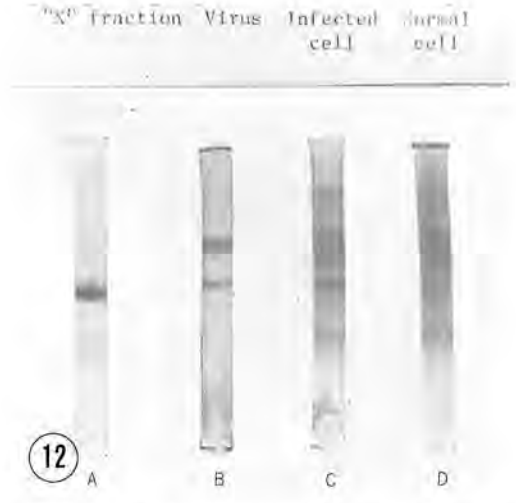


FIGURE 12. *Protein component of ChV and the "X" after polyacrylamide gel electrophoresis* (2).
(*A*) *Concentrated "X" component*
(*B*) *Purified ChV*
(*C*) *BHK21 cells infected with ChV*
(*D*) *Normal BHK21 cells*

by ultracentrifugation (90,000×*g*, 120 min). The pellet was resuspended in 0.01 M phosphate buffer and its protein component was analyzed. Polyacrylamide gel electrophoresis showed that the major protein of the "X"-component corresponded to the core protein of ChV (Fig. 12A). This band was not demonstrated in a preparation of normal BHK21 cells although it was visible in a preparation of ChV-infected cells (Figs. 12C and D). Fraction 7 from a sucrose gradient of a NP40 treated homogenate of ChV-infected BHK21 cells (Fig. 11B) was shown to contain a protein corresponding to the core protein of ChV (Fig. 7C). The "X"-protein was identified with the core protein of ChV by measuring the radioactivity in each fraction from a polyacrylamide gel on electrophoresis of a mixture of ^{3}H-valine labeled ChV and ^{14}C-valine labeled "X" (Fig. 13). There were several minor, additional bands in the specimen of "X", possibly due to contamination of the specimen with cellular proteins, because there were many bands of proteins in specimens of whole cells, some of which corresponded to the minor bands in the specimen of the "X" (Fig. 12).

BHK21 cells were infected with ChV and labeled with ^{14}C-choline and ^{3}H-uridine in the presence of Actinomycin S_3. Then a cell homogenate was prepared and the supernatant of this was subjected to sucrose gradient analysis. There was a prominent peak of ^{3}H-counts corresponding to the position of the "X" (fraction 7 in Fig. 8D), but the ^{14}C-counts, located near the top, were not associated with the "X". After treatment of the specimen with NP40, before centrifugation, the ^{3}H-counts appeared in almost the same position but the ^{14}C-counts near the bottom to the middle of the gradient decreased, while those at the top increased (Fig. 8E). Fraction 7 in Fig. 8D was analyzed by CsCl density gradient centrifugation. The result (Fig. 9B) shows that the ^{3}H-counts precipitated to the bottom, while the ^{14}C-counts were located at the top.

Thus it seems that the "X"-component contains one major protein component with the same mobility as the core protein of ChV and this protein was found only in ChV-infected cells. Moreover, the "X" probably does not contain phospholipid membrane as an integral part. These results support the idea that the "X" is a nucleoprotein core of ChV accumu-

lating in the infected BHK21 cells.

5. *Morphological observations on the components derived from ChV*

The biochemical studies described above indicated a mechanism of degradation of ChV.

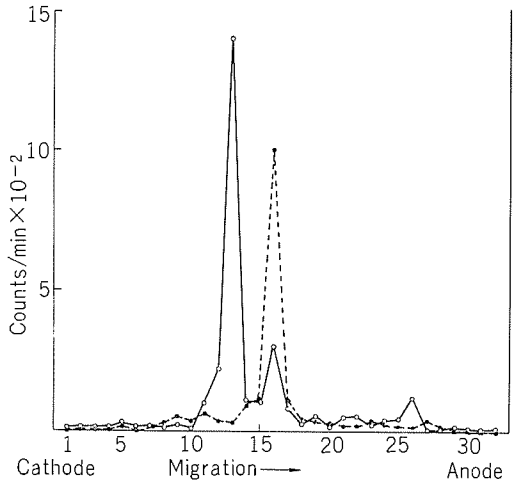


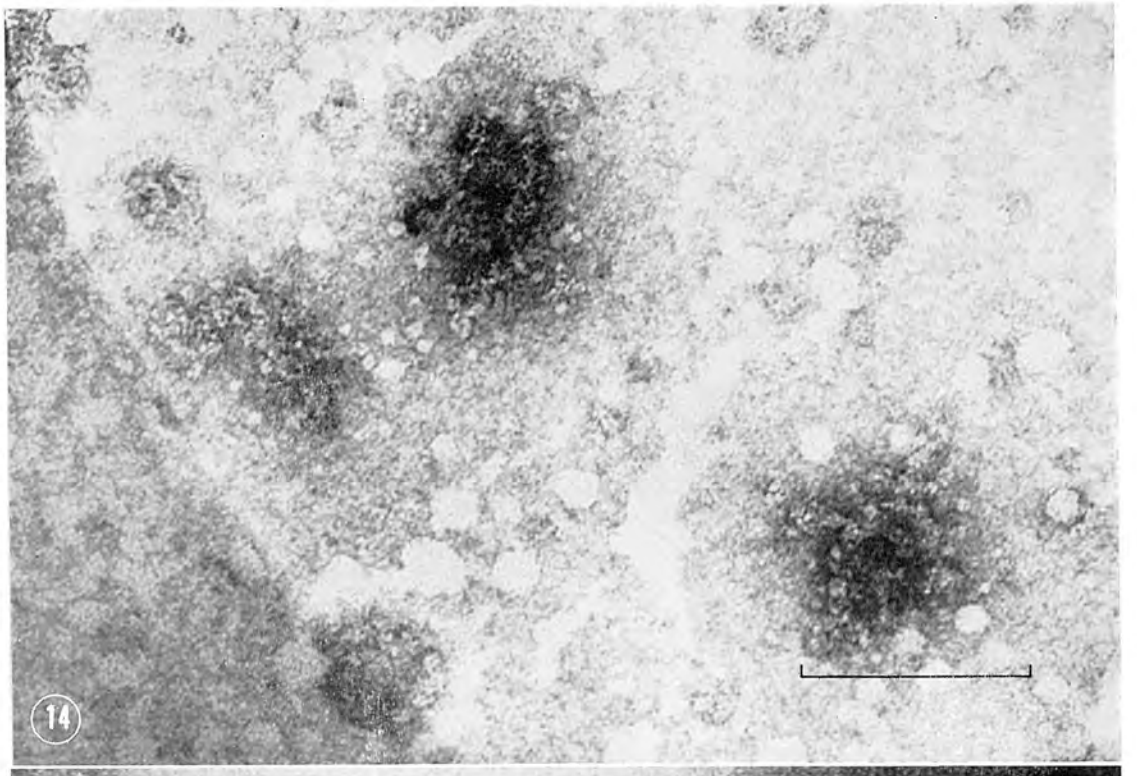
FIGURE 13. *Polyacrylamide gel electrophoresis of ^{3}H-valine labeled ChV and ^{14}C-valine labeled "X".*
○——○ *radioactivity of ^{3}H*
●------● *radioactivity of ^{14}C*

To verify this, morphological studies were made by electron microscopy. ChV was treated with Tween 80-ether and subjected to CsCl density gradient centrifugation. The HA peak obtained was then examined by electron microscope. Fine particulate or granular structures of variable size were seen with a small hollow circular or hexagonal cylinder of 3mμ diameter as their fundamental unit (Fig. 14). The same kind of fundamental structures were sometimes observed on the outside of ChV particles (Fig. 15).

When ChV was treated with pronase (100 μg/ml in 0.01 M phosphate buffer, pH 7, at 37C for 60 min), this outer part of the envelope seemed to be removed, leaving a smooth-surfaced particle (Fig. 16). These particles still seemed to retain their membrane containing phospholipid, because, when the action of pronase was not stopped by adding NaCl, several particles were seen in an apparently more advanced stages of digestion. That is, the phospholipid membrane seemed to be separated from the core, or partly removed or completely removed leaving a naked core. (Figs. 17A and B). The appearance of this core seems very much like that of the "X"-component reported before (Igarashi et al., 1969).

▶
FIGURE 14. *Electron micrograph of hemagglutinin liberated by Tween 80 and ether treatment of ChV.*
Magnification: 300,000
Scale: 100 mμ

▶
FIGURE 15. *Electron micrograph of purified ChV.*
Magnification: 300,000
Scale: 100 mμ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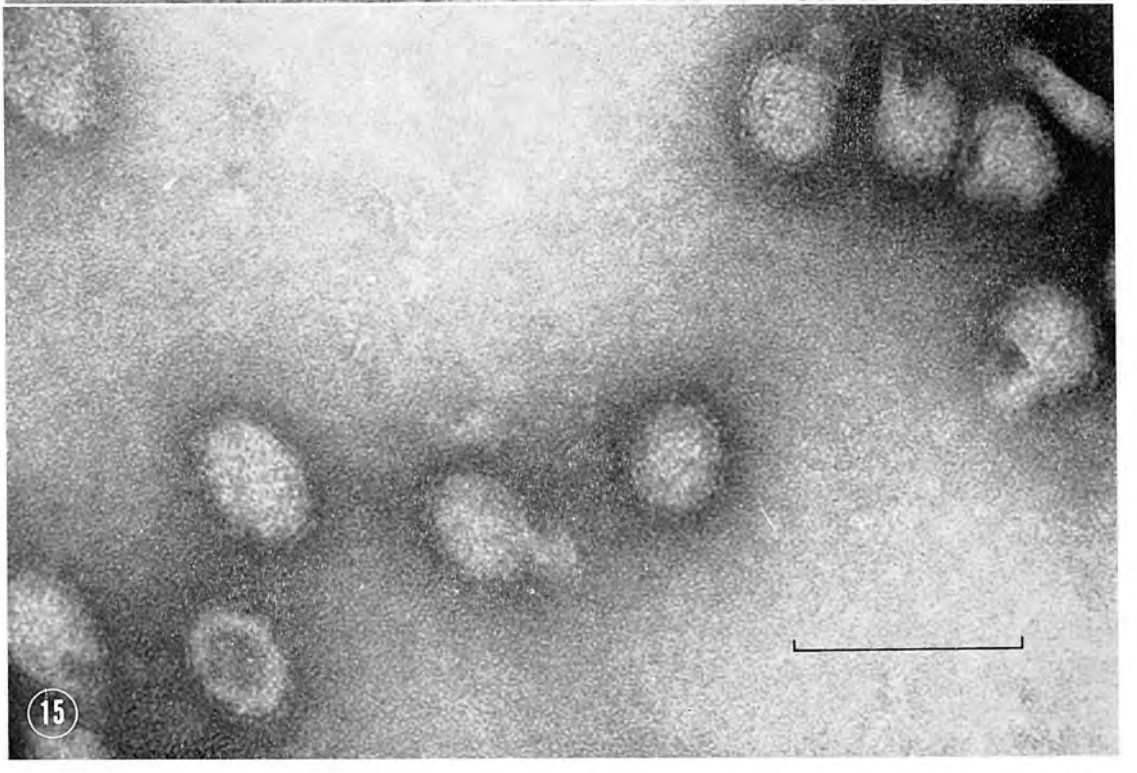
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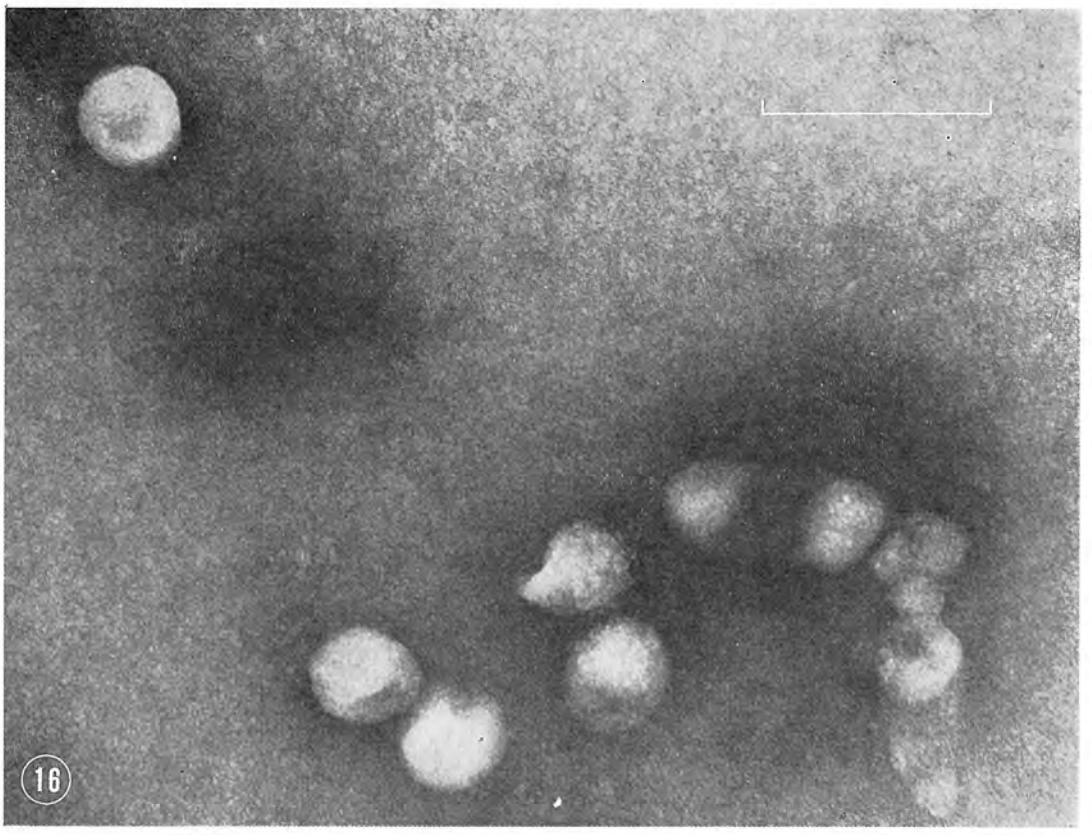
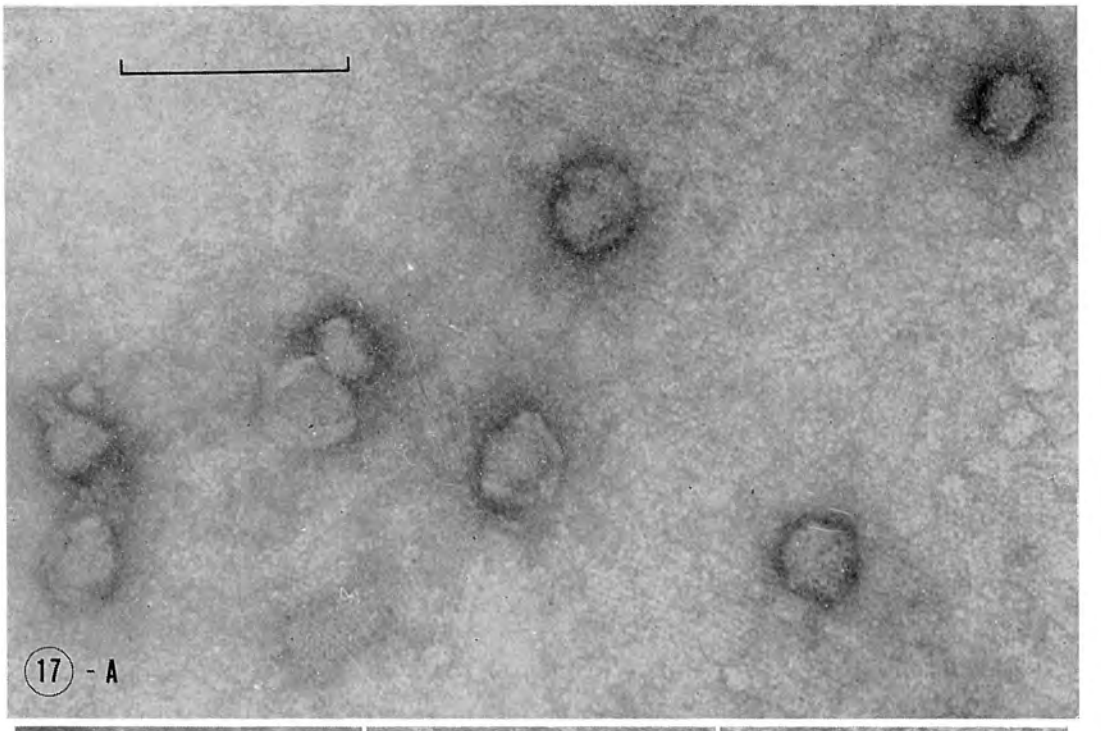
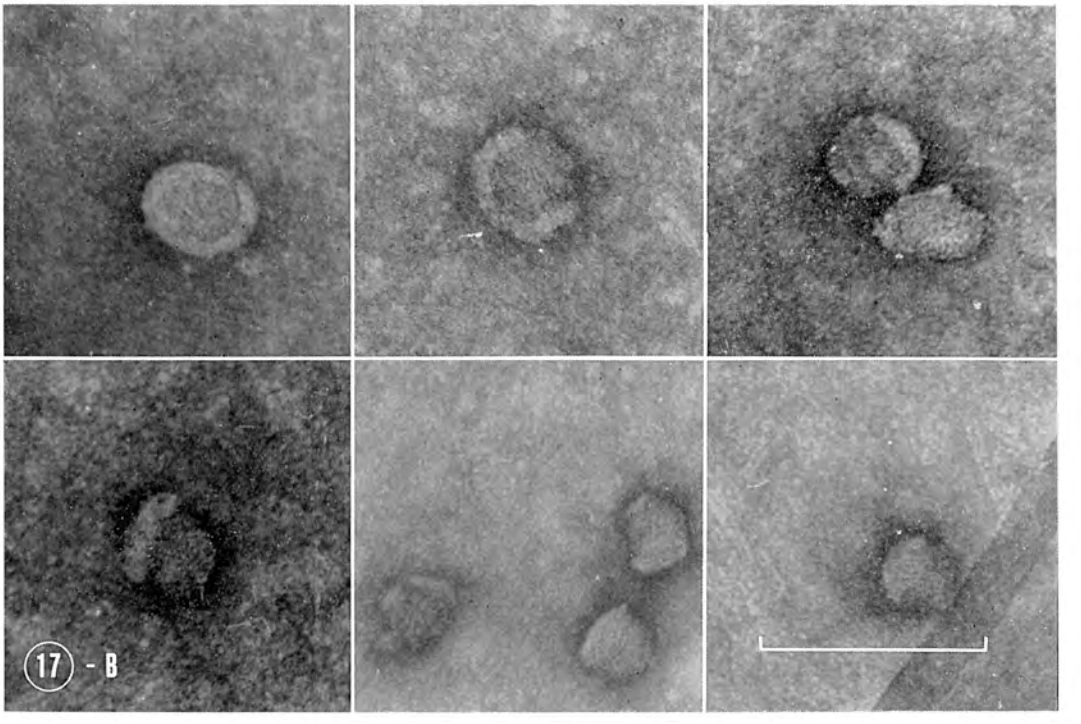


FIGURE 16. *Electron micrograph of pronase treated ChV.*
Magnification : 300,000
Scale : 100 mμ

▶
FIGURE 17. *Electron micrographs of ChV treated with pronase, showing advanced stages of digestion.*
Magnification : 300,000
Scale : 100 mμ


17 - A


17 - B

DISCUSSION

There are several reports on the structural proteins of group A arboviruses. Strauss et al., (1968; 1969) observed 2 structural proteins in the Sindbis virus, a membrane and a core. Yin and Lockart obtained the same result, but Horzinek and Mussgay (1969) reported the presence of a third minor core protein. Friedman (1968) found 3 structural proteins in the Semliki Forest virus, a membrane, a major core and a minor core, although Hay et al. (1968) identified one membrane and one core protein. Sreevalsan and Allen (1968) reported that there were 2 structural proteins in western equine encephalomyelitis virus. Our results on the structural proteins of ChV showed that there are 2 major components, one associated with the membrane and responsible for HA, and the other associated with the core. The intracellular structure, formerly named the "X", possessed the core protein. We did not demonstrate the presence of a third minor core protein, although it is possible that it exists. The molecular weights estimated for these 2 structural proteins of ChV were in fairly good agreement with the values reported for Sindbis virus proteins (Strauss et al., 1969).

From biochemical and morphological studies on the structures of materials derived from ChV by various treatments, we postulate a model of the structure of ChV and the modes of action of several agents on it (Fig. 18). Hemagglutinin of high density (1.28 g/cc) is liberated by treating intact ChV (density 1.24 g/cc) with Tween 80 and ether followed by CsCl density gradient centrifugation. The hemagglutinin has one of the 2 structural proteins of ChV. The molecular weight of the protein is estimated as 53,000. On pronase treatment, this protein is digested out, leaving a smooth-surfaced core as in the case of Sem-like Forest virus (Osterrieth, 1965; Calberg-Bacq and Osterrieth, 1966). This particle (density 1.20 g/cc) has another protein of ChV, i.e. core protein with a molecular weight of 30,000 together with ChV-RNA and phospholipid membrane. On further digestion, this smooth-membrane may gradually be removed leaving a naked core, with an appearance closely resembling that of the "X"-component. On NP40 treatment ChV was separated into a core and hemagglutinin of low density (1.19 g/cc) which seemed to possess

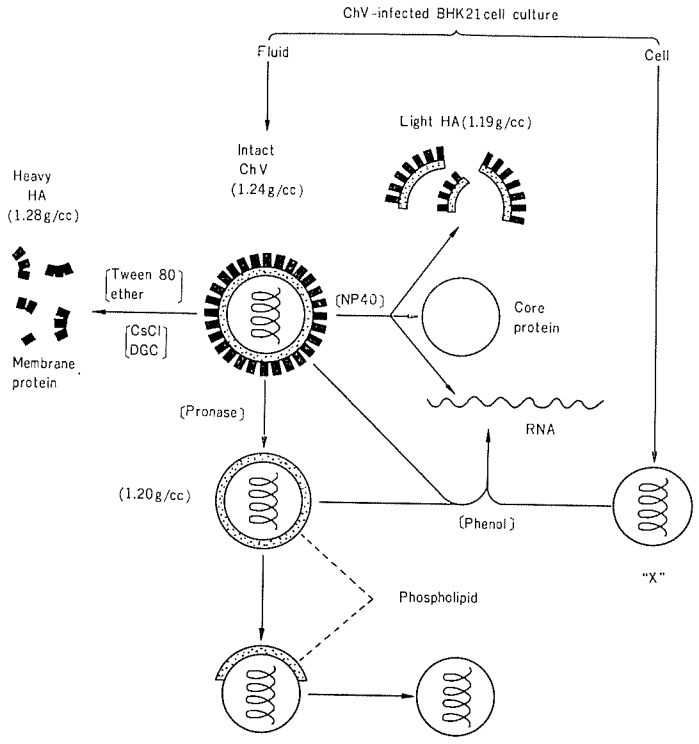


FIGURE 18. *A model of the structure of ChV and the effects of various degradation procedures.*

part of the phospholipid membrane together with HA protein. ChV-RNA also seems to be released by NP40, remaining at the top of the sucrose gradient. Treatment of the "X" with NP40 did not seem to release its RNA. The state of ChV-RNA inside the core may differ when the nucleoprotein core is enclosed in a phospholipid membrane.

There are several reports on multiple hemagglutinins of group A arboviruses, having different densities on CsCl density gradient centrifugation. Mussgay and Rott (1964) reported that on Tween 80 and ether treatment of Sindbis virus, small hemagglutinin of high density (1.27 g/cc) was obtained. They also showed that the density of incomplete virus was 1.19 g/cc, while that of complete virus was 1.24 g/cc. Our data are compatible with these values if the hemagglutinin released by NP40 is considered as part of the envelope with HA protein and phospholipid membrane. Faulkner and McGee-Russel (1968) found 2 hemagglutinins in Semliki Forest virus preparations. One of them (density 1.24 g/cc) was complete virus, while the other (density 1.205 g/cc) consisted of empty particles or fragments of envelopes. Using the same virus, Hay et al. (1968) reported that a component of 1.25 g/cc incorportated both uridine and valine and contained 2 kinds of proteins, while a component of 1.21 g/cc incorporated only valine and contained only membrane protein. Faulkner and Dobos (1968) and Aaslestad, Hoffman and Brown(1968) reported 3 kinds of hemagglutinins having different densities in CsCl from Sindbis virus and eastern equine encephalitis virus, respectively. They observed a certain degree of interconversion of reversible or irreversible type between these hemagglutinins, though their interpretation of the phenomenon differed somewhat. In both cases light hemagglutinin (1.18–1.19 g/cc) was considered to be noninfectious, It seems to have the same density as incomplete virus or the light hemagglutinin released by NP40. In interpretation of the interconversion of hemagglutinins of group A arboviruses observed on CsCl density gradient centrifugation, it seems significant that the hemagglutinin itself is heavier than intact virus, although the hemagglutinin attached to a part of phospholipid membrane is lighter. In consideration of these facts, the reason for the appearance of multiple types of hemagglutinin on CsCl density gradient centrifugation might be understood from analysis of the protein components and RNA in each hemagglutinin.

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