Study on the Basic Morphology of Japanese Encephalitis Virus: The isolation of purified hemagglutinin and the detection of core membrane

Mitsunobu AKASHI

Department of Virology, Institute for Tropical Medicine, Nagasaki University

(Chief : Prof. Kaoru HAYASHI)

ABSTRACT : The isolation of hemagglutinating and complement fixing components from the disrupted Japanese encephalitis virus with Tween 80 and ether was performed successfully by the method of the precipitation of hemagglutinins (projections) with concanavalin A. The purified hemagglutinating (HA) and complement fixing (CF) components were obtained by the centrifugation of the precipitate and the supernatant in the discontinuous density gradient of cesium chloride. The purified hemagglutinating component showed a major polypeptide in 10 % acrylamide gel electrophoresis and revealed the aggregation of fine projections by electronmicroscopic observation. It was found that the CF components contained the nucleocapsid and membranous structure (core membrane) by the electronmicroscopic observation. The core membrane was distinguished from the membranous structure at the basis of projections (basal membrane). The CF components showed a major and a minor polypeptides which were considered to correspond to the nucleocapsid and the core membrane respectively. The biological properties of HA and CF components were also examined.

The isolation of hemagglutinating (HA) and complement fixing (CF) components from certain arboviruses was made previously by some workers (Mussgay *et al.* 1964, Horzinek *et al.* 1969, Bose *et al.* 1970). However, the isolation of both components from Japanese encephalitis (JE) virus was not performed sufficiently. It was found that certain RNA viruses were agglutinated with concanavalin A by Calafat *et al.* 1972, Stewart *et al.* 1973, Becht *et al.* 1972, and Oram *et al.* 1971. In this study, an attempt was made to isolate the HA and CF components from JE virions by using concanavalin A. The purified HA and CF components obtained by the centrifugation in discontinuous density gradient of cesium chloride solution were examined morphologically by electronmicroscopy. The fine structure and the biological properties of these componets are presented in this paper.

Contribution No. 757 from the Institute for Tropical Medicine, Nagasaki University
Received for publication, February 23, 1976

MATERIALS AND METHODS

Purification of JE virus: Nakayama NIH strain of JE virus was used throughout the experiment. The history of that strain was unknown, but the strain underwent 49 passages intracerebrally in the suckling mouse brain in this laboratory. The purification of the virus was performed with procedure described in the previous paper (Ueda, 1976).

Disruption of the virus: *Treatment with Nonidet P40 (NP40)*: The disruption of the virus treated with NP40 was carried out according to the procedure described by Appleyard *et al.* (1970) and Kääriäinen *et al.* (1971). NP40 dissolved in 0.01 M tris saline pH 8.4 and the purified virus suspension were mixed at the ratio of 1 : 1 in volume and shaken at 4°C for 30 minutes. The mixture was kept in ice bath until use.

Treatment with Tween 80 and ether: The disruption of the virus particles treated with Tween 80 and ether was performed by the modified procedure described by Mussgay *et al.* (1964), Horzinek *et al.* (1969) and Dold *et al.* (1969). Equivalent volume of Tween 80 dissolved in 0.01 M tris saline pH 8.4 and the purified virus suspension were mixed and shaken at 4°C for 20 minutes. Then, the mixture was added with an equal volume of ether, and shaken again at 4°C for 10 minutes. The ether phase was separated by centrifugation at 1,500 rpm for 5 minutes. The residual ether in aquous phase was removed by carefully streaming nitrogen gas.

Treatment with Tween 20: The disruption of the virus treated with Tween 20 was carried out by the procedure described by Webster *et al.* (1969). Equivalent volume of Tween 20 dissolved in 0.02 M bicarbonate buffer pH 10 and the purified virus suspension were mixed and shaken at 4°C for one hour. The disrupted virus preparation was kept in ice bath until use.

Treatment with Silica gel: The purified virus suspension was added with silica gel No. IV (Nakarai chemicals Co.) at a proportion of one tenth volume and shaken for 30 minutes at 4°C. The mixture was filtered with filter paper. The filtrate was stored at −75°C until use.

Biological assay methods: *Infectivity*: The assay for the infectivity was performed by the procedure of hemadsorption negative (HAD^-) plaque described by Mifune *et al.* (1975). Monolayer cells of BSC−1 grown in 60 mm petri dishes were inoculated with the virus and allowed to adsorb at 37°C for 1 hour in a 5 % CO_2 atmosphere. Then, monolayer cells were covered with 4.5 ml of maintainance medium. After incubation at 37°C for 48 hours, the cells were challenged again with Newcastle disease virus (NDV) at a Moi of more than 10 PFU per cell. The plates were incubated at 37°C for an additional 18 hours. At the end of incubation, the culture medium was discarded and 2 ml of 0.5% sheep red cells suspended with physiological saline was added to examine for the hemadsorption onto the cells. The negative plaques were counted in the background of a confluent layer of sheep red cells.

Neutralization test: The diluted antiserum of rabbit or immune ascitic fluid was mixed with an equivalent volume of an appropriate dilution of JE virus suspension to give

approximately 200 plaques per dish. The mixture was incubated at 37°C for 90 minutes. The surviving viruses after neutralization were assayed in BSC−1 cells by HAD⁻ plaque method previously described by Mifune *et al.* (1975). Neutralization antibody titer was expressed as the reciprocal of antiserum or immune ascitic fluid dilution showed the 50 % reduction of HAD⁻ plaques.

Hemagglutination (HA) and Hemagglutination inhibition (HI) test: The procedure was performed by the microtiter method modified from the technique of Clarke and Casals (1958).

Complement fixation (CF) test: The microtiter method modified from the technique of Lennette *et al.* (1969) was carried out.

Preparation of immune ascitic fluid: Male adult mice were inoculated intraperitoneally with 0.5 ml of the antigens. The antigens were injected 3 times at intervals of 6 days. After 2 weeks from the first injection of the antigen, the mice were inoculated with the ascitic tumor cells into the peritoneal sac. Further, 2 weeks later, ascitic fluid was harvested and centrifuged to remove the tumor cells.

Treatment of the disrupted virus with concanavalin A: The disrupted virus with NP40, Tween 20 or Tween 80 and ether was mixed with one fourth volume of concanavalin A dissolved at an adequate concentration in phosphate buffered saline (PBS). After incubation at 25°C for 1 hour, the mixture was centrifuged at 2,500 rpm at 4°C for 15 minutes. The supernatant was stored at −75°C until use. The precipitate was washed twice with PBS at 4°C by centrifugation and dissociated with 0.5 M α−methylmannoside in PBS at a proportion of original volume. The residual insoluble substance was removed by centrifugation at 2,500 rpm at 4°C for 15 minute. The solubilized precipitate was harvested and kept at −75°C until use.

Cesium chloride (CsCl) density gradient centrifugation: The discontinuous gradient of CsCl was prepared by layering carefully at a proportion of 1.5 ml of $\rho=1.450$ solution, 0.9 ml of $\rho=1.350$ and $\rho=1.210$ solutions of CsCl in 0.01 M tris HCl containing 0.001 M EDTA pH 8.0 respectively. On the top of the gradient, 0.9 ml of the disrupted virus preparation was overlaid. The centrifugation was performed in the SW 50.1 Roter with Beckman L_2−65B, at 39,000 rpm for 180 minutes. The fractions were collected by 15 drops from the bottom of tubes and dialyzed against tris-saline pH 8.0 at 4°C for 18 hours.

SDS-polyacrylamide gel electrophoresis: The purified virus, hemagglutinating and complement fixing components were assayed by 10 % polyacrylamide gels electrophoresis. The procedure was performed by slightly modified method described by Weber *et al.* (1969). The test specimens were added with the equivalent volume of 50 % glycerol, 2 % sodium dodecyl sulfate (SDS) and 4 % 2−mercaptoethanol (2ME) in 0.02 M phosphate buffer pH 7.2. The mixture was boiled at 100°C for 2 minutes and cooled in water, then added with a drop of 0.05 % bromphenol blue (BPB). The treated specimens were layered on the top of 10 % acrylamide gel columns (9 cm high with 0.5 mm diameter). Electrophoresis was performed 3 mA per column for 10 to 14 hours. The running buffer was used 0.1 M sodium phosphate buffer containing 0.1 % SDS. The

gels were stained with 0.01 % coomassie brilliant blue and scanned at 605 nm wave with the microdensitometer. The gels were stored in 20 % methanol 7.5 % acetic acid solution.

Electronmicroscopic observation: A carbon coated microgrid was placed on a drop of the specimen. After drying in air, the grid was washed 2 or 3 times with cacodylate buffer pH 7.4. The fixation was carried out with 4 % paraformaldehyde solution pH 7.3 and washed 2 or 3 times with 1 % ammonium buffer pH 7.3. Negative staining was performed with 3 % phosphotungstic acid (PTA) solution pH 7.4 or 3 % uranyl acetate (UA) solution. The PTA−UA double staining method as described by Hayashi *et al.* (1972) was also made.

RESULTS

Effect of detergents on the hemagglutination (HA) activity

The disruption of the purified virus with NP40, Tween 20 or Tween 80 and ether did not affect on the HA activity. The HA titer of the specimens rised slightly with 0.01% NP40 treatment and much more rising of HA titer was observed in case of Tween 80 and ether treatment (Table 1).

Concentration of concanavalin A for the precipitation of HA components

The disrupted purified virus preparation with Tween 80 and ether was treated with various concentration of concanavalin A as described in materials and methods. An adequate concentration of concanavalin A was obtained at a proportion of 120 μg or 240 μg per ml for the precipitation of HA components. However, the lower HA titer in the supernatant remained. It was considered due to the partial disruption of the virus with Tween 80 and ether treatment (Table 2).

Concentration of α − methylmanoside for the dissociation of HA components fromthe precipitate with concanavalin A

HA components precipitated with concanavalin A were released with varions concentration of α−methylmannoside. The HA activity was recovered sufficiently by addition of 0.5 M or 1 M α−methylmannoside (Table 3).

Characteristics of HA and CF components separated by the treatment of concanavalin A from the disrupted virus preparation

Table 1. Effect of nonionic detergents and ether on the hemagglutination activity of JE virus (Nakayama NIH strain)

Final concentration of detergents (%)	Nonidet P40 (at 4°C/30min.)	Tween 20 (at 4°C/60min.)	Tween 80/Ether (at 4°C/20, 10min.)
0	512	64	512
0.01	1024	—	512
0.1	512	32	1024
1	Hemolysis	Hemolysis	2048
2	Hemolysis	Hemolysis	Hemolysis

The disrupted virus, the supernatant and the precipitate dissolved with 0.5 M α−methylmannoside were centrifuged in discontinuous density gradient of CsCl as described in materials and methods. HA and CF components in the disrupted virus preparation could not be clearly fractionated. However, the both components in the supernatant and the precipitate were successfully separated from each other. Although the supernatant frequently contained the both components, the precipitate showed only HA activity by the careful treatment of concanavalin A (Fig. 1 and Fig. 2). Furthermore, it was indicated that the disrupted virus preparation showed the optimal pH in HA reaction changing from pH 6.4 to pH 6.2 or 6.0 (Table 4).

Electronmicroscopic observation

The purified virus particles were disrupted remarkably by the treatment of Tween 80 and ether. The inner substances (nucleocapsid) and the envelope were aggregated as seen in photos. 1, 2, 3 and 4. The precipitate having HA activity contained the

Table 2. Hemagglutination and complement fixation activity of the precipitate and supernatant separated by the treatment with various concentration of concanavalin A

Final concentration of concanavalin A (μg/ml)	Titer			
	Supernatant		Precipitate	
	HA	CF	HA	CF
0	1024	8	0	0
60	32	8	512	0
120	16	2	512	4
240	4	2	256	4

Remarks: (1) The disrupted JE virus was obtained by the treatment with Tween 80 and ether as described in materials and methods.
(2) The precipitate was resuspended with original volume of phosphate buffered saline containing 0.5M α−methylmannoside.

Table 3. Concentration of α−methylmannoside for the dissociation of the hemagglutinating components precipitated with concanavalin A

Concentration of α−methylmannoside (Mol.)	Titer	
	HA	CF
0	256	32
0.01	16	0
0.05	128	2
0.5	512	4
1.0	512	4

Remarks: (1) The disrupted JE virus with Tween 80 and ether was trated with 120μg of concanavalin A per ml and separated into the precipitate and supernatant.
(2) The precipitate was resuspended with the original volume of phosphate buffered saline containing various concentrations of α−methylmannoside.

Table 4. Effect of pH ranges on hemagglutination activity of the dissociating components from the precipitate obtained by treatment with Concanavalin A

pH	HA titer
6.6	8
6.4	32
6.2	512
6.0	256
5.75	64

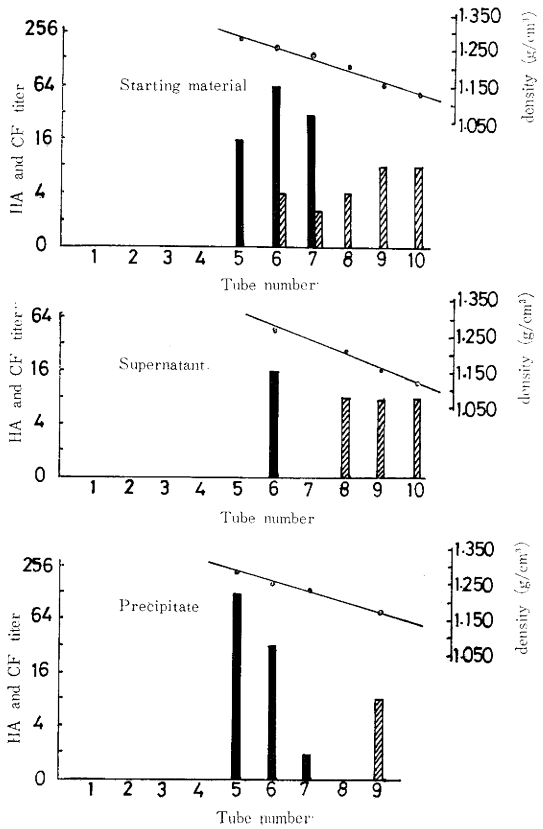


Fig. 1. Sedimentation pattern of the disrupted JE virus, the supernatant and the precipitate in discontinuous density gradient of CsCl (experiment 1).

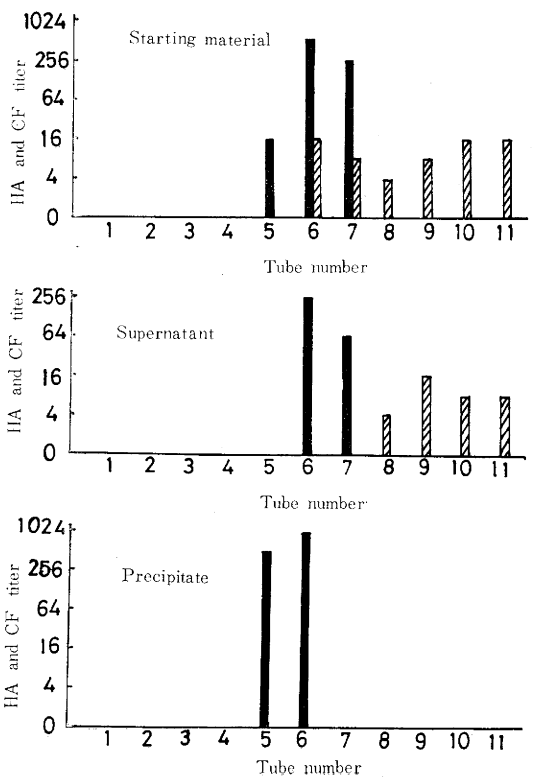


Fig. 2. Sedimentation pattern of the disrupted JE virus, the supernatant and the precipitate in discontinuous density gradient of CsCl (experiment 2).

aggregation of fine projections (fraction No. 5) and free fine projections among aggregated ones (Fraction No. 6) as seen in Photo. 5 and Photo. 6 respectively. Rope-like aggregations were observed in Fraction No. 8 and No. 9 obtained from the fractionation of the supernatant having CF activity. It was considered to be nucleocapsid (Photo. 7 and Photo. 8). It was a surprising and remarkable finding that the aggregation of membranous structure was observed in Fraction No. 9 and No. 10 obtained from the fractionation of the supernatant as seen in Photo. 9, 10, 11, 12 and 13. The similar membranous structure and the aggregation of nucleocapsid could be observed, after the purified virus was treated with silica gel and fractionated by centrifugation in the discontinuous density gradient of CsCl as described in materials and methods (Photo. 14). The membranous structure was observed more clearly in the preparation kept at 4°C for a long time (over six months) and spontaneously released the nucleocapsid as seen in Photo. 15. The double layer form was also observed in the purified virus preparation as seen in Photo. 16.

Analysis of polypeptides of JE virus

The disrupted virion showed four major and a minor polypeptides. The last (rapidly scanned) polypeptide might be contaminated. The middle major polypeptide among three major one was usually eliminated by the purification of the HA and CF components. The HA component showed a major polypeptide (peak No. 3). The CF components showed a major and a minor polypeptides (peak No. 2 and No. 1). From the judgement of sedimentation pattern in discontinuous density gradient of CsCl (Fig. 1 and Fig. 2), it was considered that the peak No. 2 in the CF components might be nucleocapsid and the peak No. 1 might be membranous structure (Fig. 3).

Properties of antibody induced by HA and CF components

The immune ascitic fluid was obtained as described in materials and methods.

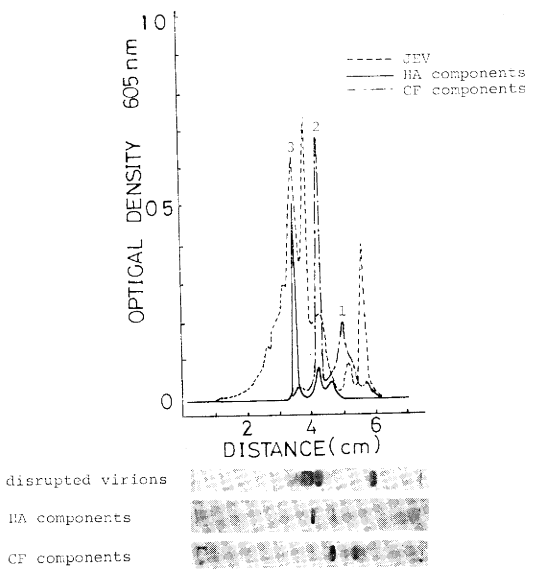


Fig. 3. Polyacrylamide gel electrophoresis of disrupted JE virions, HA and CF components.

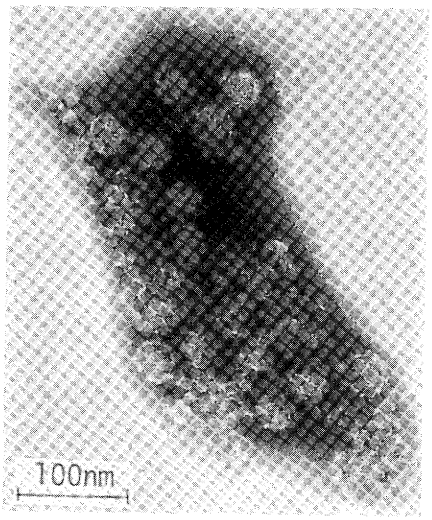


Photo. 1. Disrupted purified JE virus with Tween 80 and ether (magnification 1:140,000).

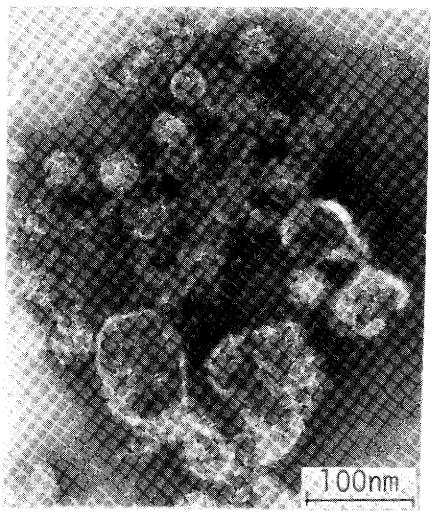


Photo. 2. see Photo. 1.

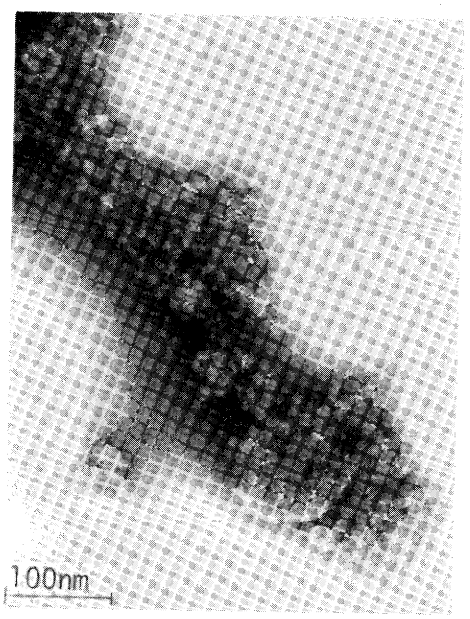


Photo. 3. see Photo. 1.

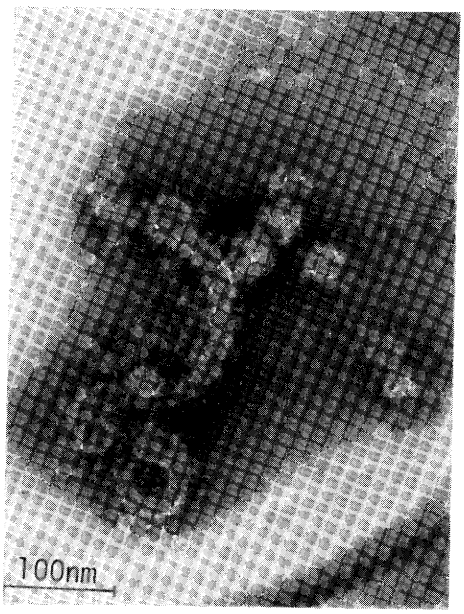


Photo. 4. see Photo. 1.

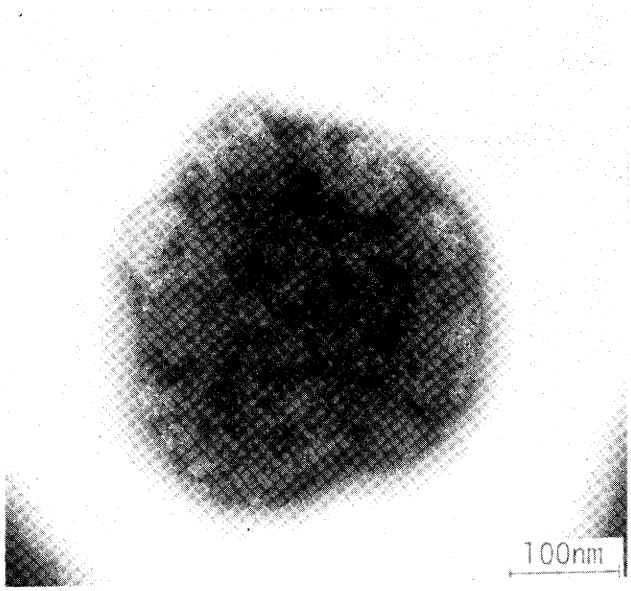


Photo. 5. The aggregation of fine projections observed in Fr. No. 5 of the precipitate (magnification 1:140,000).

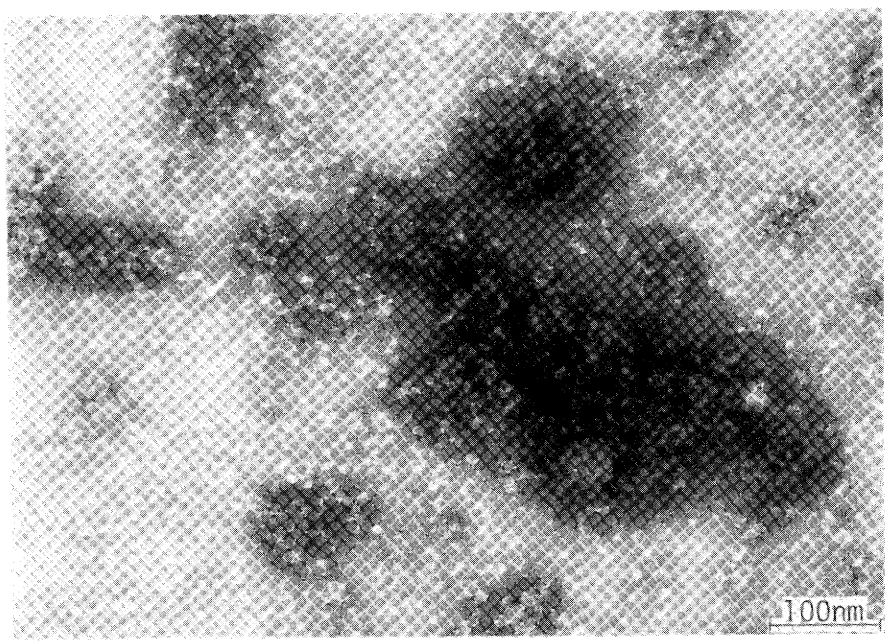


Photo. 6. The free and aggregated projection observed in Fr. No. 6 of the precipitate (magnification 1:140,000).

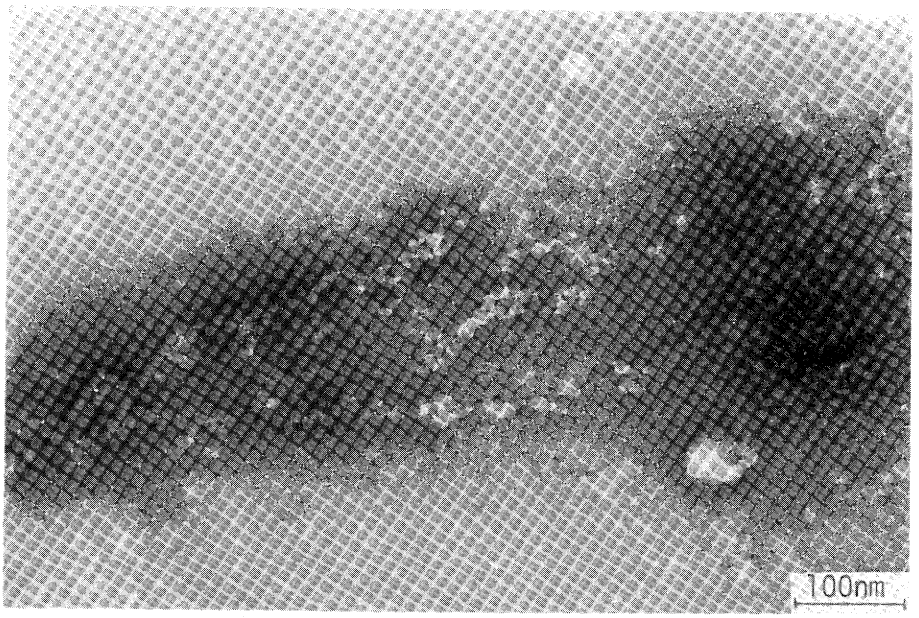


Photo. 7. The rope-like structure of nucleocapsid observed in Fr. No. 8 of the supernatant (magnification 1:140,000).

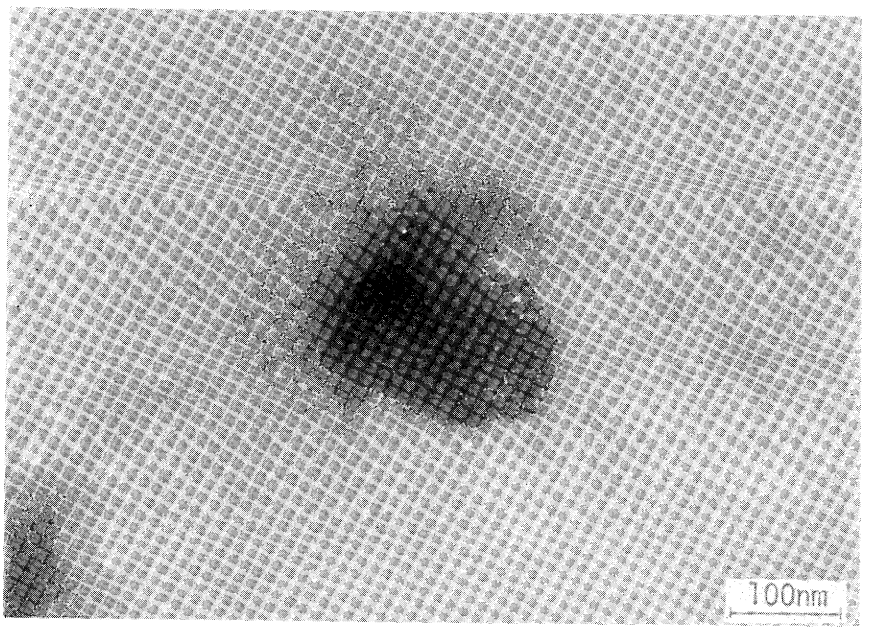
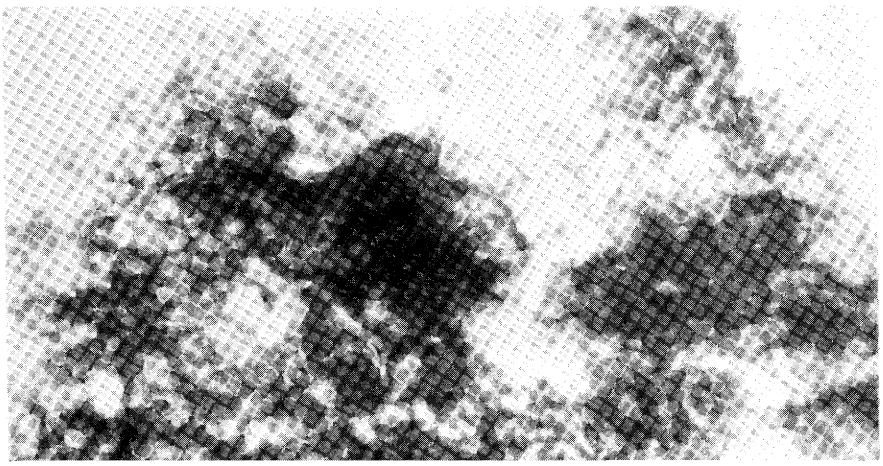
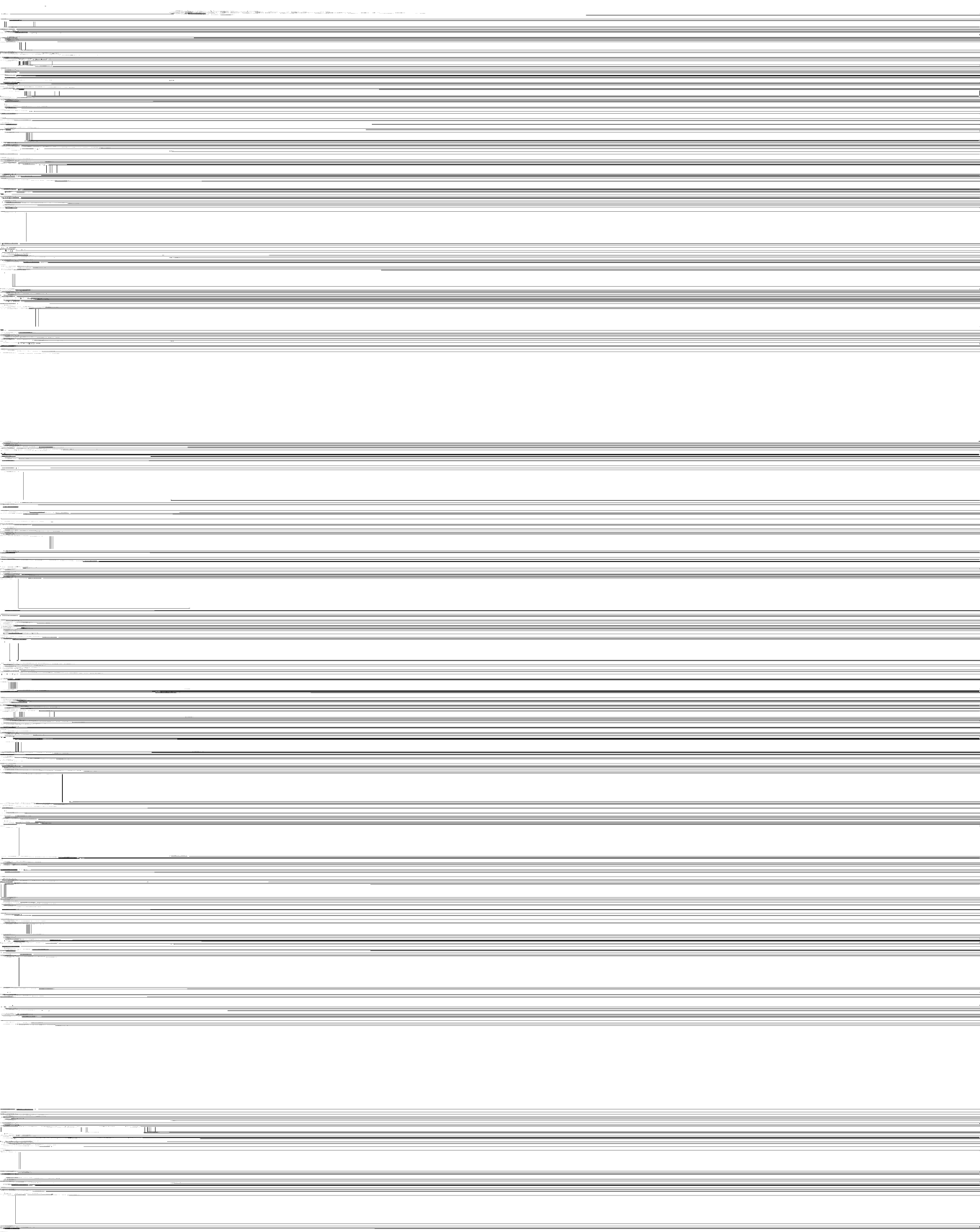


Photo. 8. The aggregation of rope-like structure of nucleocapsid observed in Fr. No.9 of the supernatant (magnification 1:140,000).

Photo. 9. The aggregation of rope-like structure of nucleocapsid and the membranous structures observed in Fr. No. 9 of the supernatant (magnification 1:140,000).





It was interesting to note that HA component was able to induce the neutralization antibody as well as HI antibody, but no CF antibody. In contrast, CF components induced CF antibody but no neutralization nor HI antibody (Table 5).

Table 5. Production of neutralization, hemagglutination inhibition and complement fixation antibodies in immune mouse ascitic fluid against fractionated components

Antibody \ Test / Antigen	antibody titer					
	NT	HI		CF		
	V*	HA	V	HA	CF	V
Immune MAF against HA components	64	128	64	<2	<2	2
Immune MAF against CF components	<4	<2	<2	<2	4	8
Hyperimmune rabbit serum against JE virus	>256	640	1280	<2	16	64
Nonimmune MAF	<4	<2	<2	<2	<2	<2

Remarks: V* ····· complete virions
MAF····· mouse ascitic fluid

DISCUSSION

Many workers attempted to isolate successfully the envelope having HA activity and inner core substances having CF activity from the virions of Sindbis virus (Mussgay, *et al.* 1964, Bose *et al.* 1970), Semliki Forest virus (Kääriäinen *et al.* 1971), and Venezuelan equine encephalitis virus (Horzinek *et al.* 1969). So far as JE virus was concerned, the isolation of the both components from the virion particularly from the amount of the purified virus was performed sufficiently. Recently, it was demonstrated that concanavalin A, a phytagglutinin derived from jack beans, binds specifically to saccharides with the terminal $\alpha-$ D$-$ mannopyranosyl, $\alpha-$ D$-$ glucopyranosyl or $\beta-$ D$-$ fructofuranosyl residues. Glycoprotein representing structural components of enveloped virus reacts with concanavalin A (Carafat *et al.* 1972; Stewart *et al.* 1973). It was also found that the cells infected with myxoviruses (Becht *et al.* 1972), arboviruses (Oram *et al.* 1971) particularly JE virus (Yoshinaka *et al.* 1975) were agglutinable with concanavalin A. In this study, it was attempted to isolate HA and CF components from the virion and the both components could be isolated successfully from the disrupted JE virions with Tween 80 and ether by the agglutination of hemagglutinin with concanavalin A. It was considered that concanavalin A allowed to agglutinate rapidly and selectively the projections in the disrupted virus preparation. The HA component precipitated with concanavalin A showed a major polypeptide

in acrylamide gel electrophoresis, and revealed an aggregation of fine projections by electronmicroscopic observation.

The supernatants having CF activity which have been obtained after the treatment of concanavalin A contained the aggregation of nucleocapsid and membranous structure named as "core membrane" by the author. The core membranes revealed electron dense shells as seen in Photo. 9, 10, 11, 12, 13, and 14. These membranous structures (core membrane) were clearly different from the membranous structure at the basis of projection which was proposed to name "basal membrane" by Ueda (1976). It was considered that these core membranes surrounded closely the nucleocapsid of the virions. This finding might have been similar to the capsidal shell demonstrated in Sindbis virus by Horzinek *et al.* (1969). On the other hand, Igarashi *et al.* (1970) had reported that the core of Chikungunya virus was surrounded by a membrane containing phospholipid devoid of hemagglutinin. It was, however, considered that the core membrane stated by Igarashi *et al.* (1970) might correspond to the membranous structure of JE virion at the basis of projections (basal membrane) as it was observed previously by Ueda (1976). The supernatant obtained after the treatment of concanavalin A showed a major and a minor polypeptides in 10% acrylamide gel electrophoresis as shown in Fig. 3. The major and the minor polypeptides might be nucleocapsid and membranous structure (core membrane) respectively. Kitano *et al.* (1975) reported that, when the disrupted JE virus with NP40 was centrifuged in the equilibrium density gradient by adding solid CsCl to the virus preparation, the top fraction contained amorphous substance (however, there was no presentation of that component) showing the smallest polypeptide in acrylamide gel electrophoresis. He suggested that this smallest polypeptide might be a membrane protein embedded in the envelope. Taking into consideration the smallest polypeptide stated by Kitano *et al.* (1975), it may correspond to the core membrane which was clearly distinguished from the membranous structure (basal membrane) associated with the envelope at the basis of projections demonstrated previously by Ueda.

SUMMARY

The treatment of disrupted JE virion with concanavalin A was effective to isolate the HA and CF components. The aggregation of fine projections was revealed in HA components, whereas the CF components contained the nucleocapsid and core membrane distinguished from the membranous structure at the basis of projections. The biological properties of these both components were also presented.

ACKNOWLEDGEMENT

Author would like to express sincere thanks to Prof. K. Hayashi for his continous encouragements throughout this study.

REFERENCES

1) Appleyard, G., Oram, J. O. & Stanley, J. L. (1970) : Dissociation of Semliki Forest virus into biologically active components. J. gen. Virol., 9, 197−189.

2) Becht, H., Rott, R. & Klenk, H. D. (1972) : Effect of Concanavalin A on cells infected with enveloped RNA viruses. J. gen. Virol. 14, 1−8.

3) Bose, H. R. & Sagik, B. P. (1970) : Immunological activity associated with the nucleocapsid and envelope components of an arbovirus. J. Virol., 5 (3), 410−412.

4) Calafat, J. & Hageman, P. C. (1972): Binding of Concanavalin A to the envelope of two murine RNA tumor viruses. J. gen. Virol., 14, 103−106.

5) Clarke, D. H. & Casals, J. (1958): Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Amer. J. Trop. Med. Hyg., 7, 561−573.

6) Dold, H. J. & Northrop, R. L. (1969): Analysis of the diluents used for Rubella virus hemagglutination and hemagglutination-inhibition test. Appl. Microbiol., 18(2), 221−227.

7) Hayashi, K. & Suematsu, T. (1972): Double negative stain with phosphotungstic acid and uranyl acetate solution for the electronmicroscopic observation of Japanese encephalitis virus particles. Igaku-no-Ayumi, 79(10), 591−592.

8) Horzinek, M. & Mussgay, M. (1969): Studies on the nucleocapsid structure of a group A arbovirus. J. Virol., 4(4), 514−520.

9) Horzinek, M. & Munz, K. (1969): Structural constituents of Venezuelan equine encephalitis virus. Arch. ges. Virusforsch., 27, 94−108.

10) Igarashi, A., Fukuoka, T., Nithiuthai, P., Hsu, L. C. & Fukai, K. (1970): Structural components of Chikungunya virus. Biken J., 13, 93−110.

11) Kääriäinen, L. & Söderlund, H. (1971) : Properties of Semliki Forest virus nucleocapsid. Virology, 43, 291−299.

12) Kitano, T., Suzuki, K. & Yamaguchi, T. (1974) : Morphological, chemical and biological characterization of Japanese encephalitis virus virion and its hemagglutinin. J. Virol. 14(3), 631−639.

13) Mifune, K. & Makino, Y. (1974): The interference by Japanese encephalitis virus with Newcastle disease virus in Vero cells. Intervirology, 4(3), 150−161.

14) Lennette, E. H. & Schmidt, N. J. (1969): Diagnostic procedures for viral and rickettsial disease. 4th ed., 52−58, American Public Health Association, Inc., New York.

15) Mussgay, M. & Rott, R. (1964): Studies on the structure of a hemagglutinatig component of a group A arbovirus (Sindbis). Virology, 23, 573−581.

16) Oram, J. D. Ellwood, D. C., Appleyard, G. & Stanley, J. L. (1971): Agglutination of an arbovirus by concanavalin A. Nature New Biology, 233, 50−51.

17) Stewart, M. L., Summers, D. F., Soeiro, R., Fields, B. N. & Maizel, J. V. (1973): Purification of oncornaviruses by agglutination with concanavalin A. Proc. Nat. Acad. Sci., 70(5), 1308−1312.

18) Ueda, Y. (1976): Study on the basic morphology of Japanese encephalitis virus: The envelope and the origin of the large particles of hemagglutinin. Trop. Med., 18(1), 29−44.

19) Weber, K. & Osborn, M. (1969): The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electropholesis. J. Biol. Chem., 244(16), 4406−4412.

20) Webster, R. G. & Darlington, R. W. (1969): Disruption of myxoviruses with Tween 20 and isolation of biologically active hemagglutinin and neuraminidase subunits. J. Virol., 4(2), 182−

187.

21) Yoshinaka, Y. & Shiomi, T. (1975): Agglutination of Japanese encephalitis virus with concanavalin A. J. Virol., 15(3), 671−674.

日本脳炎ウイルスの基本構造に関する研究: 血球凝集性粒子の分離，純化と芯被膜の確認

明石光伸（長崎大学熱帯医学研究所ウイルス学部門）

Concanavalin A は糖蛋白と特異的に結合するほか， glycoprotein を有する enveloped RNA ウイルスをも特異的に凝集することが知られてきた。この性質を利用して，Tween 80 and ether で破壊した日本脳炎ウイルスから血球凝集性及び補体結合性成分を分離し，更に塩化セシウム密度勾配遠心法で両成分の純化を行った。血球凝集性成分は電顕的に projection の集合であることを確認し，かつ単一の polypeptide から成っていることを認めた。一方，補体結合性成分は nucleocapsid と membrane 構造物の集合であることを電顕的に確認し，かつそれぞれに相当する polypeptide が証明された。membrane 構造を core membrane と呼び，さきに上田が見出した envelope の基底膜 (basal membrane, unit membrane) とは異なるものであることを指摘した。本構造の電顕的証明は最初の所見であり，日本脳炎ウイルスの基本構造に新しい所見を提示したものである。血球凝集性成分でマウスを免疫すると血球凝集抑制抗体は勿論中和抗体もよく産生されたが，補体結合性抗体は検出されなかった。これに反して，補体結合性成分で免疫したマウスでは補体結合性抗体のみが証明され，血球凝集性抗体は勿論中和抗体の産生も認められなかった。この事実は日本脳炎ウイルスの感染防禦にあずかる抗原成分の解析に重要な資料となるものである。

熱帯医学　第18巻　第1号　11−28頁，1976年3月