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Separation of Hemagglutinating and Complement Fixing Components from Japanese Encephalitis Virus and Its Biological and Morphological Properties

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Abstract : The morphological and biological properties of hemagglutinating (HA) and complement fixing (CF) components of Japanese encephalitis (JE) virus were examined. When JE virus disrupted with Tween 80-ether was treated with concanavalin A, the effective separation of HA and CF components was made and each purified component was obtained by the centrifugation in density gradient of cesium chloride. It was also shown that the HA component induced neutralizing antibody whereas the CF component only iduced CF antibody. By the electron microscopic observation, it was detected that the HA component contained the dispersed and aggregated projections and the CF components appeared to be aggregations of membraneous and cord-like structures. Aggregation of projections forming small particles was produced by the treatment of JE virion with α-chymotrypsin. It was suggested that small particles of hemagglutinin might be constructed with aggregation of several or more projections.

Many workers have reported that JE virus particles have a spherical shape with an envelope (40 to 50 nm in diameter) (Kitaoka *et al.*, 1963; Fukai, 1970 ; Oka, 1971; Hayashi, 1972 ; Kitano *et al.*, 1974, Ueda, 1976 ; Akashi, 1976). Kitaoka *et al.*, (1963) and Fukai, (1970) demonstrated that the hemagglutinins were associated with both the virion and other non-infectious components. In addition, small particles having hemagglutinating activity could be observed frequently in fresh and old preparations of JE virus. By the electron microscopic study, Fukai (1970) demonstrated that these non-infectious hemagglutinins (named Ht components) revealed as spherical shape measuring 16 to 18 nm in diameter and they were composed of seven or nine subunits. However, there is still little knowledge about the production of these particles. The hemagglutinating (HA) and complement fixing (CF) components separated from sindbis virus and other arboviruses has been characterized by some workers (Mussgay *et al.*, 1964 ; Horzinek *et al.*, 1969 ; Bose *et al.*, 1970). In this paper, the effective separation of HA and CF components of JE virus (a group B arbovirus) by the

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treatment with concanavalin A and by centrifugation of supernatant fluid and precipitate preparation in density gradient of cecium chloride is described. By the electron microscopic observation, the presence of membraneous structure of JE virion is suggested and the construction of small particles having hemagglutinating activity with aggregation of several or more projections is presented.

MATERIALS AND METHODS

JE virus. A twenty percent emulsion of suckling mouse brain infected with Nakayama NIH strain of JE virus suspended in Tris-saline pH 8.0 was centrifuged at 10,000 rpm (refrigerated centrifuge, Tomy Seiko Co., Model RS-18-P2 Rotor No. 3N) for 30 minutes. The supernatant fluid was added protamine sulfate solution (1mg/ml) drop by drop at a final concentration of 4 mg per ml while being shaken for 30 minutes in an ice bath. The mixture was centrifuged again at 10,000 rpm for 30 minutes and the treatment of the supernatant fluid with protamine sulfate was applied four times. Carbon powder (charcoal activated, (CHR-30, Lot V4 N7599), special purified reagent for colum chromatography, Nakarai Chemicals LTD., Japan) was added to the supernatant fluid at a final concentration of 3 percent while being shaken for 30 minutes in an ice bath. The mixture was filtered with a Millipore filter (0.45μ). The filtrate was centrifuged at 110,000g for 4 hours (Spinco Model L2-65B Rotor SW 41 Ti), and the pellet was resuspended with Tris-saline pH 8.0 in a proportion of one tenth volume of original suspension and it was stored at -75°C before use.

Purified JE virus vaccine was also kindly supplied by the Chemo-Sero-Therapeutic Research Institute, Inc. Kumamoto Prefecture, Japan. This purified JE vaccine was treated with protamine sulfate and pelleted by centrifugation at 118,000 g for 4 hours and it was resuspended in Tris-saline pH 8.0 before experiments in our laboratory.

Treatment with Tween 80 and ether. The procedure was modified from the technique described by Mussgay (1964) and Horzinek and Munz (1969). The purified virus suspension was added an equal volume of 2% Tween 80 dissolved in 0.01 M Tris-saline pH 8.0, and the mixture was shaken at 4°C for 20 minutes. An equal volume of ether was added while being shaken at 4°C for 10 minutes. The ether was separated by centrifugation at 1,500 rpm for 15 minutes. The residual ether in the preparation was removed by carefully streaming nitrogen gas over the aqueous phase.

Treatment of disrupted virus with concanavalin A. The virus disrupted with Tween 80 and ether was mixed with one fourth volume of concanavalin A dissolved at a suitable concentration in PBS. After incubation at room temperature for one hour, the mixture was centrifuged at 2,500 rpm at 4°C for 15 minutes. The supernatant fluid was kept at -75°C until required. The precipitate was washed twice with PBS by centrifugation at 4°C and dissociate with 0.5M α-methylmannoside in PBS at a proportion of the original volume. The residual insoluble material was removed by centrifugation at 3,000 rpm at 4°C for 15 minutes. The solubilized precipitate was kept at -75°C until used.

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, 0.8 ml of $\rho=1.350$ and $\rho=1.210$ solutions of CsCl dissolved in 0.01 M Tris buffer pH 8.0 containing 0.001 M EDTA. On the top of gradient, 0.9 ml of the disrupted virus preparation, supernatant or solubilized precipitate was overlaid. The centrifugation was performed in SW 50.1 rotor with Beckman L2-65B at 39,000 rpm for 180 minutes. The fractions were collected by drops from bottom of tubes and dialyzed against Tris-saline pH 8.0 at 4°C overnight.

Preparation of immune ascitic fluid of mice. Adult mice were inoculated intraperitoneally with 0.5 ml of purified vaccine, HA and CF components respectively. The injection of the antigens was made three times at intervals of a week. After two weeks from the last injection of the antigen, mice were inoculated with ascitic tumour cells into the peritoneal sac. Two weeks later, the ascitic fluid was harvested and centrifuged to remove the cellular components.

Hemagglutination test. The microtiter method modified from the technique of Clarke and Casals (1958) was used.

Complement fixation test. The microtiter method modified from the technique of Lennette and Schmidt (1969) was applied.

Treatment with α-chymotrypsin. The purified virus suspension was mixed with equal volume of α-chymotrypsin solution (1 mg per ml). The mixture was put on microgrids at certain intervals and dried in air. The specimens on microgrids were washed with drops of water and negatively stained prior to observation in the electron microscope (JEM Model 100B).

Electron microscopic observation. Carbon coated microgrids were placed on a drop of the specimens. After drying in air, the grids were washed 2 or 3 times with cacodylate buffer pH 7.4. The fixation of the specimens on grids was made with 4% paraformaldehyde solution pH 7.3 and washed 2 or 3 times with 1% ammonium bicarbonate buffer pH 7.3. Negative staining was carried out with 2% phosphotungstic acid (PTA) solution pH 7.4 or 1% uranyl acetate (UA) solution. The PTA-UA double staining method described by Hayashi *et al.* (1972) was also applied.

RESULTS

Separation of HA and CF components

The good yield of HA and CF components from the disrupting JE virus with Tween 80 and ether were obtained by treatment of 60 μg or 120 μg of concanavalin A per ml of phosphate buffered saline pH 7.2 (Table 1) and by dissociation of the precipitate in the adequate volume of 0.5 mol or 1.0 mol solution of α-methylmannoside (α-MM) (Table 2). The HA and CF components in the supernatant fluid and the precipitate preparation dissolved in 0.5 mol of α-MM were effectively separated as shown in Fig. 1. The fractions having HA or CF activity separated from the precipitate preparation or the supernatant fluid by centrifugation in CsCl density gradient were combined respectively (Fig. 1).

Table 1. Hemagglutination and complement fixation activity of the precipitate and supernatant fluid 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 2. 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 treated with 120μg of concanavalin A per ml and separated into the precipitate and supernatant fluid.
(2) The precipitate was resuspended with the original volume of phosphate buffered saline containing various concentrations of α-methylmannoside.

Properties of antibody induced by HA and CF components

The immune ascitic fluids were prepared from mice inoculated intraperitoneally with both HA and CF components as antigens and ascitic tumor cells. It was demonstrated that HA component was able to induce the neutralization antibody as well as hemagglutination inhibition (HI) antibody, but no CF antibody. In contrast, CF components induced CF antibody but no neutralization or HI antibody (Table 3).

Electron microscopic observation of purified vaccine, HA and CF components

The complete and disrupted particules of JE virus in the purified vaccine were revealed as homogeneous pictures when the preparation was stained with 2% phosphotungustic acid solution (PTA) (Fig. 2A). In contrast, when the double staining with 1% PTA and 1% uranyl acetate solution (UA) was applied, the images of complete, imcomplete and disrupted virions were presented as clear and fine features (Fig. 2B).

The dispersed and aggregated projections were observed in the fractions of No. 5 and 6 (Fig. 1) obtained by fractionation of the precipitate in CsCl density gradient as seen in

Table 3. Induction of antibodies in mouse ascitic fluid by immunizing with fractionated components

Antigens / Test	Antibody titer					
	NT		HI		CF	
MAF and serum	V	HA	V	HA	CF	V
MAF immunized with HA component	64	128	64	<2	<2	2
MAF immunized with CF component	<4	<2	<2	<2	8	16
Hyperimmune rabbit serum against JE virus	>256	640	1280	<2	16	64
Non immune MAF	<4	<2	<2	<2	<2	<2

Remarks: Antigens were used as follows :
V ····· complete virion, HA ····· hemagglutinating component,
CF ····· complement fixing component.
MAF mean the mouse ascitic fluid.

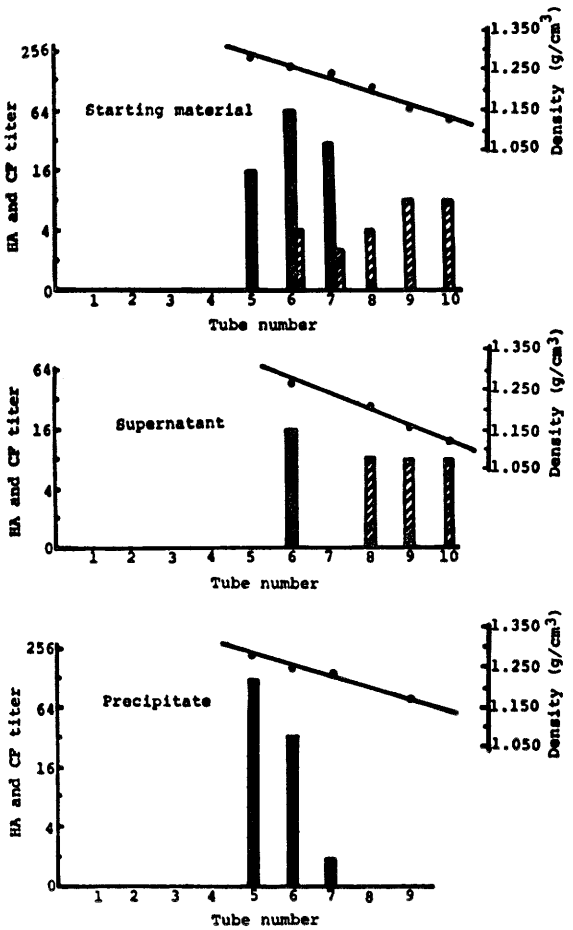

Fig. 1. Sedimentation pattern of the disrupted JE virus, the supernatant and the precipitate in discontinuous density gradient of CsCl centrifugation.

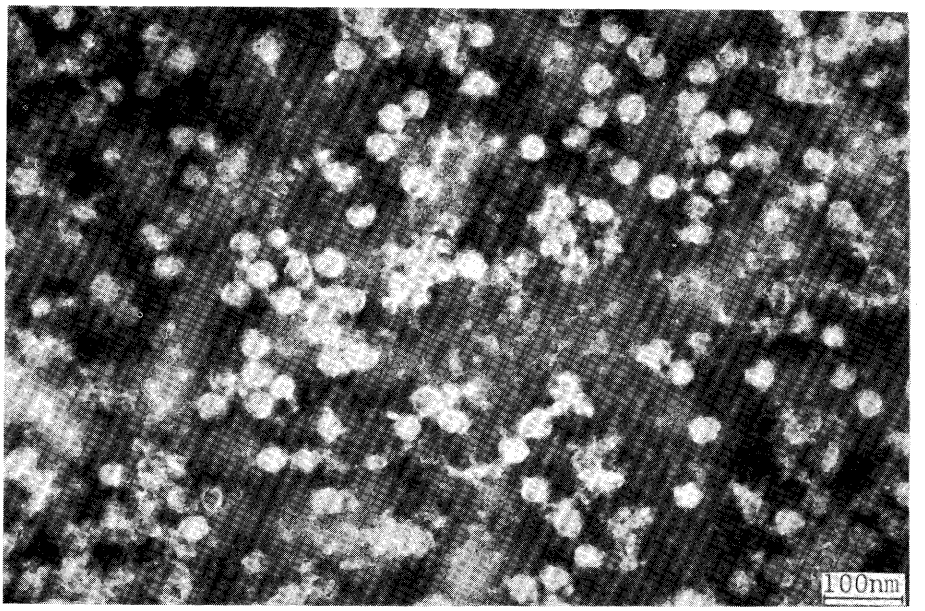

Fig. 2A. Single staining of the purified vaccine of JE virus with 2% PTA. Magnification 1:100,000.

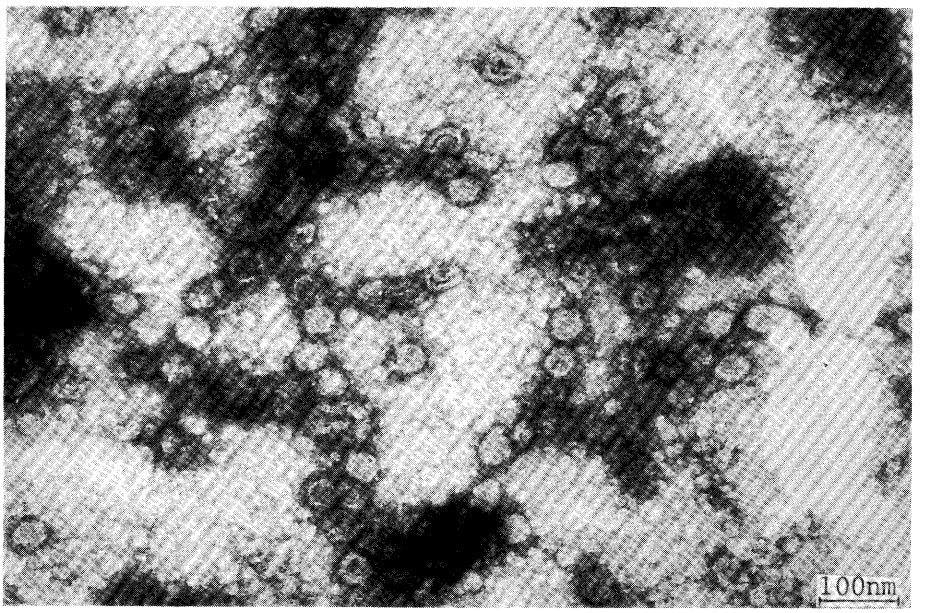

Fig. 2B. Double staining of the purified vaccine of JE virus with 1% PTA and 1% UA. Magnification 1:100,000.

Fig. 3. In contrast, as shown in Fig. 4, the aggregation of cord-like and membraneous pictures were revealed in the fractions of No. 8, 9 and 10 (Fig. 1) obtained by fractionation of CF component in CsCl density gradient. The membraneous forms as shown in Fig. 5A and 5B were also detected in the fractions of No. 9 and 10 separated from CF components. As seen in Fig. 6, the membraneous picture was also observed inside the envelope shape devoide projections, when the purified JE virus preparations were kept at 4°C for over a mounth. It was usually observed that the considerable membraneous picture of imcomplete virion appeared in the purified vaccine of JE virus as shown in Fig. 7. The thin sections of JE virions were suggested the presence of membraneous structure as seen in Fig. 8.

Small particles of hemagglutinating components

Figure 9 shows the presence of small particles apparently released from the virions in the purified virus preparation. These small particles having HA activity appeared to form spherical particles of diameter 16–18nm. The purified virus preparation was treated with α-chymotrypsin as described in Materials and Methods. It was demonstrated that the small particles could be obtained in good yield by this treatment (Fig. 10), and these particles might be consisted of several or more projections as seen in Fig. 11.

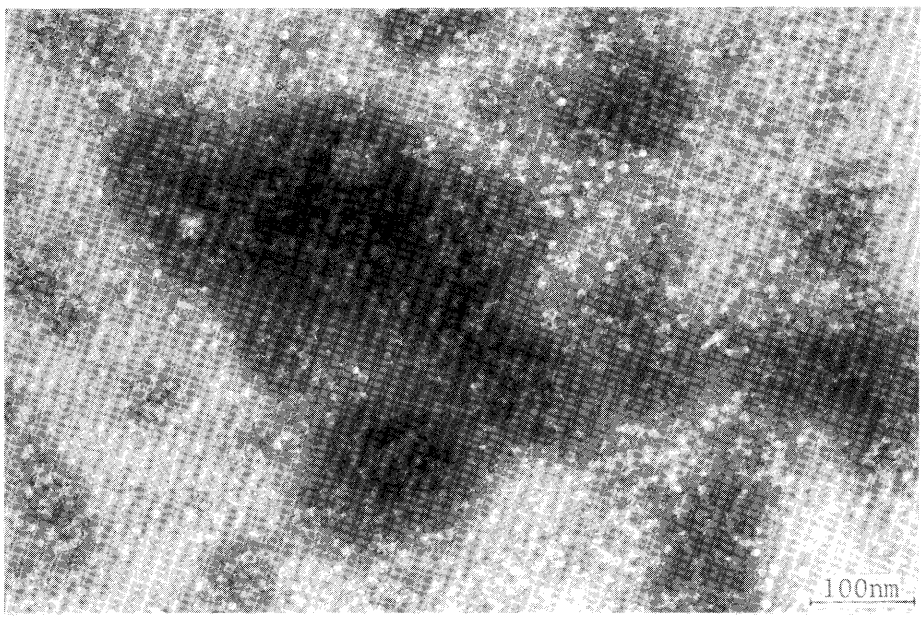

Fig. 3. Dispersed and aggregated projections in hemagglutinating component separated by centrifugation in CsCl density gradient. Magnification 1:140,000.

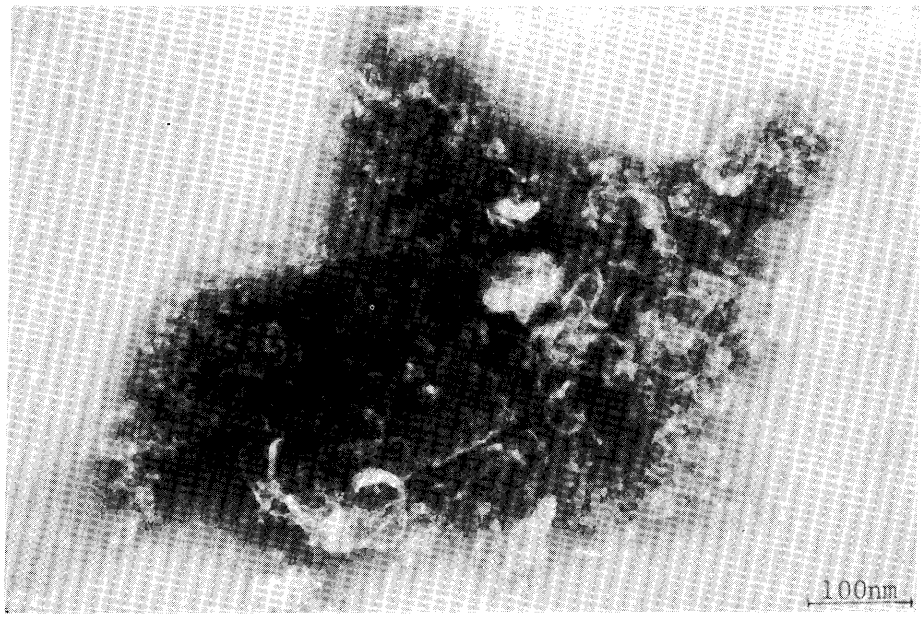

Fig. 4. Aggregation of cord-like and membraneous pictures in complement fixing components separated by centrifugation in CsCl density gradient. Magnification 1:140,000.

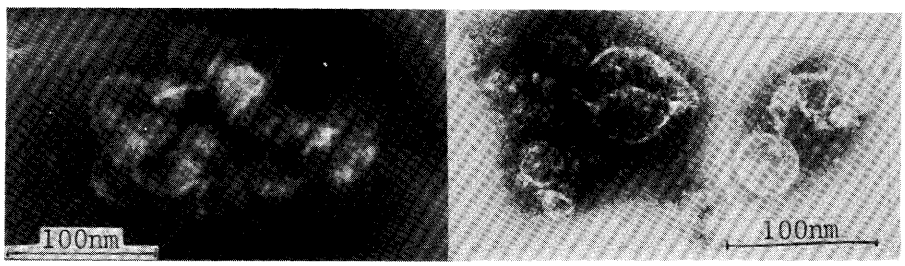

Fig. 5A and 5B. Membraneous pictures observed in the fraction of the complement fixing components separated by centrifugation in CsCl density gradient. Fig. 5A aud 5B were obtained by staining with 2% PTA and 1% UA respectively. Magnification 1:200,000.

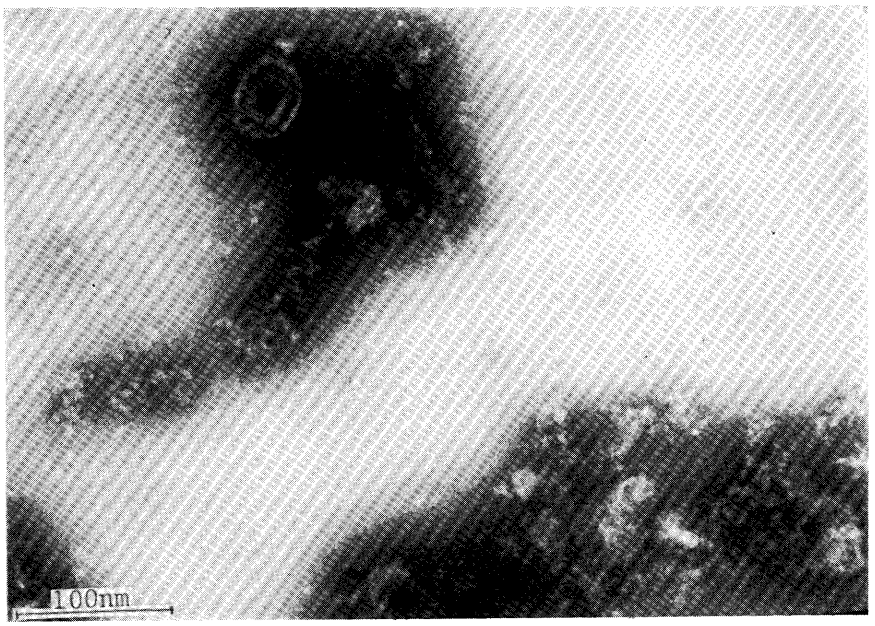

Fig. 6. Double layer form of virion devoid of projections. Magnification 1:210,000.

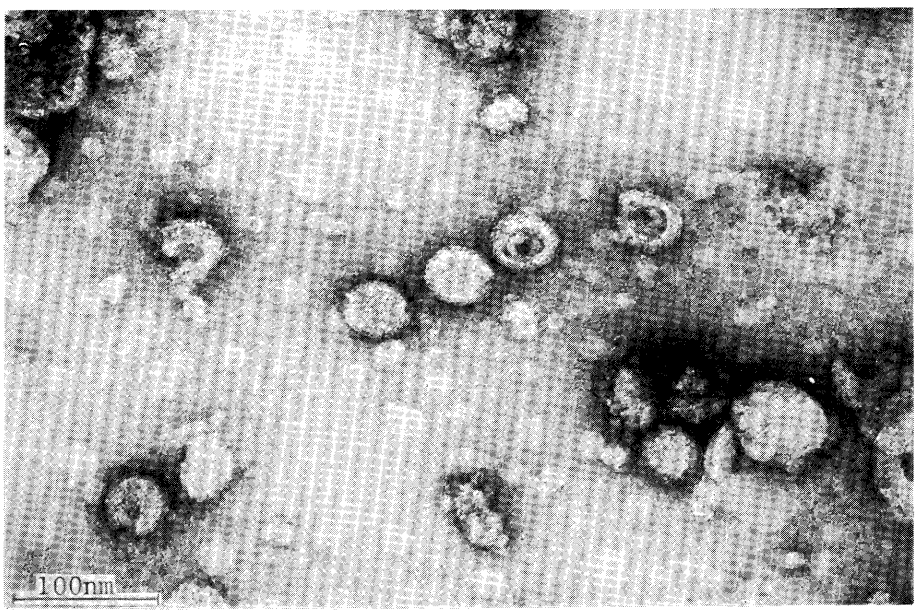

Fig. 7. Complete and disrupting JE virions. Magnification 1:200,000.

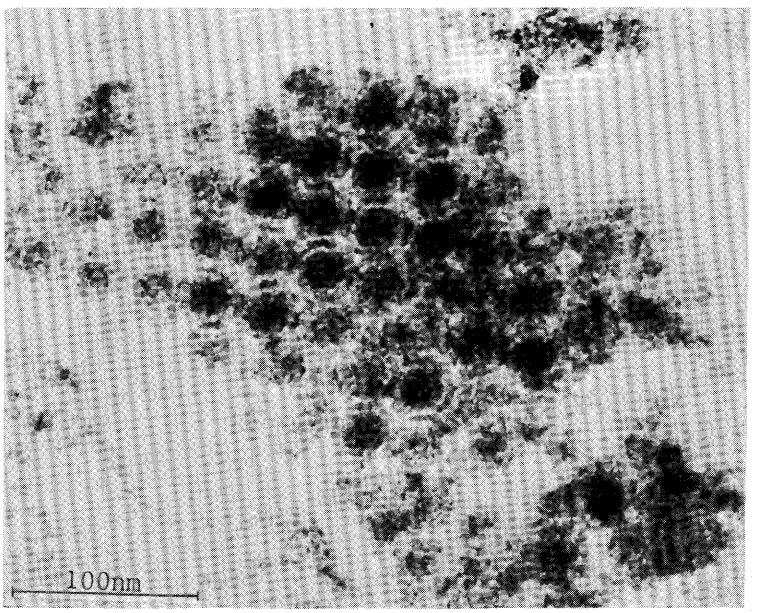

Fig. 8. Ultrathin section of JE virions. Magnification 1:250,000.

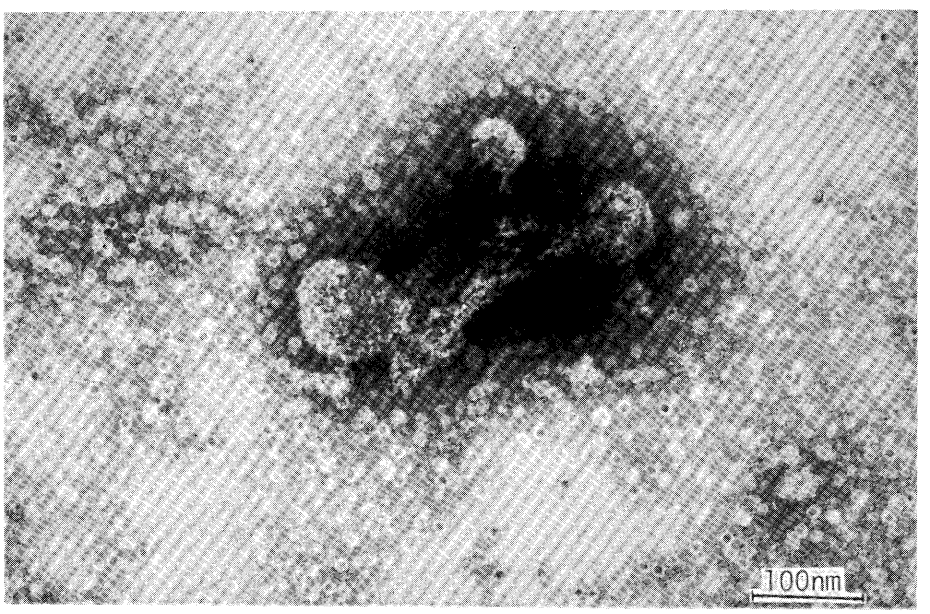

Fig. 9. Small particles of hemagglutinating component in an old sample of purified JE virus preparation Magnification 1:150,000.

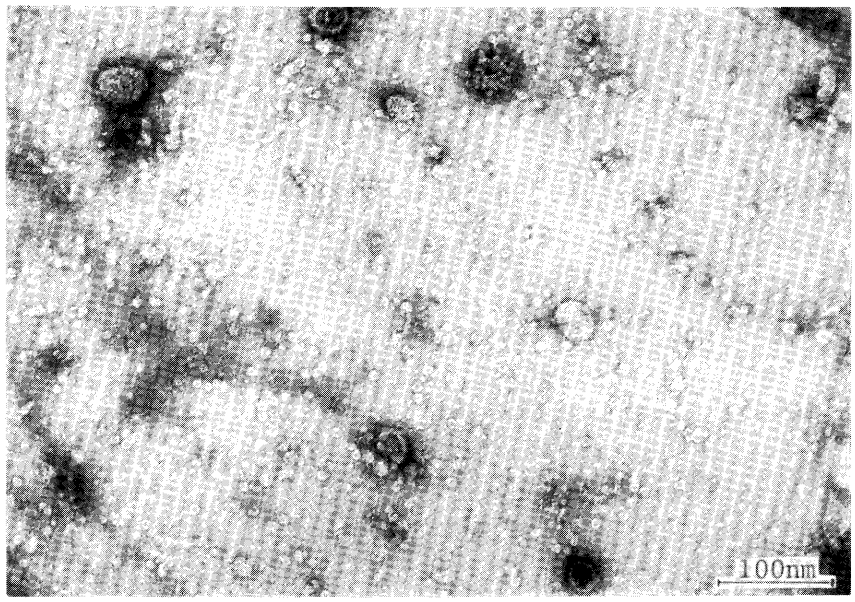

Fig. 10. Aggregation of projections produced by treatment of purified JE virus vaccine with α-chymotrypsin. Magnification 1:150,000.

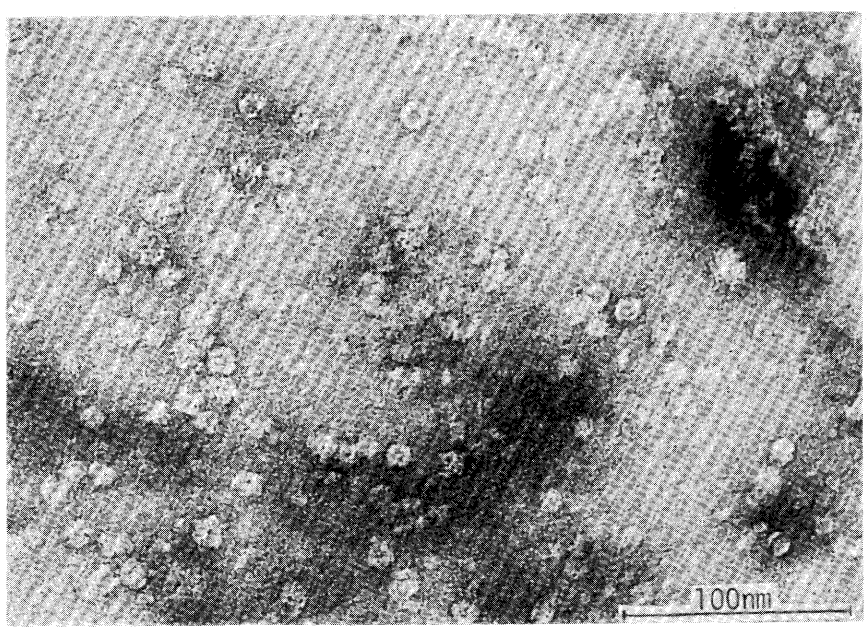

Fig. 11. Aggregation of projections produced by treatment of purified JE virus vaccine by α-chymotrypsin. Magnification 1:300,000.

DISCUSSION

Many workers have reported that togaviruses have a spherical shape surrounded by net-like structures. Horzinek (1973) and Kitano *et al.* (1974) pointed out that the projections may appear a diffuse halo of filamentous or rod shaped structures according to the different staining procedures. In this study, it has been shown that JE virus have a spherical shape with filamentous halo and a net-like structure particularly when the specimens were stained with the double staining method appling 1% PTA and 1% UA.

Many workers have also attempted to isolate the envelope having HA activity and the core components having CF activity from the virions of sindbis virus (Mussgay *et al.*, 1964 ; Bose *et al.*, 1970). So far as JE virus was concerned, the separation of both purified components from the virus preparation has not been performed effectively before. It has been shown that structural glycoprotein components of enveloped viruses can react with concanavalin A (Calaft *et al.*, 1972 ; Stewart *et al.*, 1973), and that 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, HA and CF components could be separated effectively from JE virus preparation by disruption with Tween 80 and ether followed by agglutination of hemagglutinin with concanavalin A. The purified HA and CF components were obtained from the supernatant fluid and the precipitate preparation by centrifugation in discontinuous density gradient of CsCl respectively. It was noteworthy evidence that HA component induced the neutralization antibody as well as HI antibody, whereas CF components induced CF antibody but no neutralization nor HI antibody. By the electron microscopic observations, the purified HA component was revealed as dispersed and aggregated projections (Fig. 3). On the other hand, the purified CF components contained the aggregation of cord-like and membraneous structures (Fig. 4). The membraneous structures was also clearly detected in the top fraction of the purified CF components separated by centrifugation in CsCl density gradient (Fig. 5A and 5B). The appearance of transparent imagination of membraneous structure might be due to the treatment of JE virion with Tween 80 and ether. The presence of membraneous structure could be presumed from the images of incomplete virions appeared in the purified virus preparation particularly in the JE virus vaccine (Fig. 6 and 7). On the basis of these findings, it was considered that the core of JE virion might be surrounded with membraneous structure. The small particles measuring 16 to 18 nm in diameter having HA activity could be observed frequently in the fresh or old preparation of purified virus. These particles were also recognized by Nishimura *et al.* (1963), and the term Ht components was proposed by Fukai (1970). It was also reported that these Ht components may be constructed with seven or nine subunits (Fukai, 1970). The treatment of purified JE virus with α-chymotrypsin resulted apparently in the aggregation of several or more projections forming small particles in this study.

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日本脳炎ウイルスの血球凝集性及び補体結合性要素の分離とその生物学的，形態学的性状

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日本脳炎ウイルスを Tween 80 及びエーテルで破壊し，Concanavalin A で処理すると血球凝集素（HA）を含む沈澱と補体結合（CF）成分とを含む上清とに分けられる．沈澱は 0.5M α-Methylmannoside 溶液に融解する．HA 及び CF 成分は更らに CsCl 不連続密度勾配で超遠心して精製画分とすることが出来る．そして HA 画分は中和抗体及び血球凝集抑制（HI）抗体を産生するが CF 抗体は産生しない．また CF 画分は CF 抗体のみを産生し，中和抗体及び HI 抗体は産生しない．電顕的に HA 画分は projection の集合体として観察された．CF 画分は膜様構造物と索状構造物の集合として観察された．また，CF 画分の中には電子密度の低い径 30～40mm の膜様構造物を認めた．一方，4°Cに長期間保存した精製ウイルス試料やワクチン製剤にもしばしば 2重膜構造の粒子を散見する．こうした一連の所見を考え合せると日本脳炎ウイルス粒子の内部には core を包み込んでいる膜様構造があることを推察することが出来る．また，精製ウイルス試料やワクチン製剤にしばしば小型粒子がみられ，このものは血球凝集能をもっている．日本脳炎ウイルス粒子を α-chymotrypsin で処理すると電顕的には projection の単離及び集合がみられ，血球凝集性小型粒子に類似のものが多数認められた．このような所見から小型粒子は数個またそれ以上の数の projection が集合して作られるものであろうと推定した．

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