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Citation	Biken's journal : journal of the Research Institute for Microbial Diseases. 1961, 4(4), p. 243-254
Version Type	VoR
URL	https://doi.org/10.18910/83046
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The Structure of HVJ* II. The Fine Structure of the Subunits

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(Received for publication, November 14, 1961)*

SUMMARY

It was found that ether disintegration of HVJ, in the presence of Emasol, in the cold is an effective method for the preparation of an RNA containing fraction as well as the hemagglutinin fraction of the virus. The fine structure of the fractions thus obtained was studied by the negative staining method.

The bulk of the viral RNA was present in the fraction of the treated HVJ sedimenting at 12,000 rpm (17,000 g). This fraction had an RNA content of 2.0 - 2.5 per cent, whereas the RNA content of the original virus particles was 0.75-1.0 per cent, and it showed a specific gel diffusion and sedimentation pattern although contaminated with a small amount of hemagglutinin.

In negatively stained preparations the virus particles of HVJ had various shapes and sizes of 1500 - 6000 Å width. The particles had a surface layer, 60 - 90 Å thick with projections of 130 - 150 Å length and 10 - 20 Å width. Inside the surface layer, inner stranded materials were seen.

The hemagglutinin fraction has particles of 450 - 550 Å width and greater length with approximately 500 Å width, with projections similar to those on the surface of the intact virus particles. These particles seemed to form the outer envelope of the intact virus particles by connect with each other.

The RNA containing fraction consisted mainly of double helical structures of 175 - 190 Å width and varying length and seemed to form the inner structure of the intact virus particles. The non-hemagglutinin fraction consisted of many loosened or unwound helical strands, possibly derived from the original helical structure of the RNA-containing fraction, and further of the double helical structures and the inactivated hemagglutinins.

The significance of these findings was discussed.

INTRODUCTION

In a previous report (Hosaka *et al.*, 1960), Emasol-ether disintegrated HVJ were separated into hemagglutinating and non-hemagglutinating fractions by adsorption onto and elution from fowl red cells and the fractions were studied ultracentrifugally, morphologically and immunologically.

Subsequently the authors studied the localization of viral RNA in the fractions of HVJ disintegrated by the Emasol-ether method. The fine structures of the viral subunits thus obtained were identified and studied by the negative staining technique.

* HVJ is a virus of parainfluenza virus I type and so called Sendai virus

The present paper describes these results.

MATERIALS AND METHODS

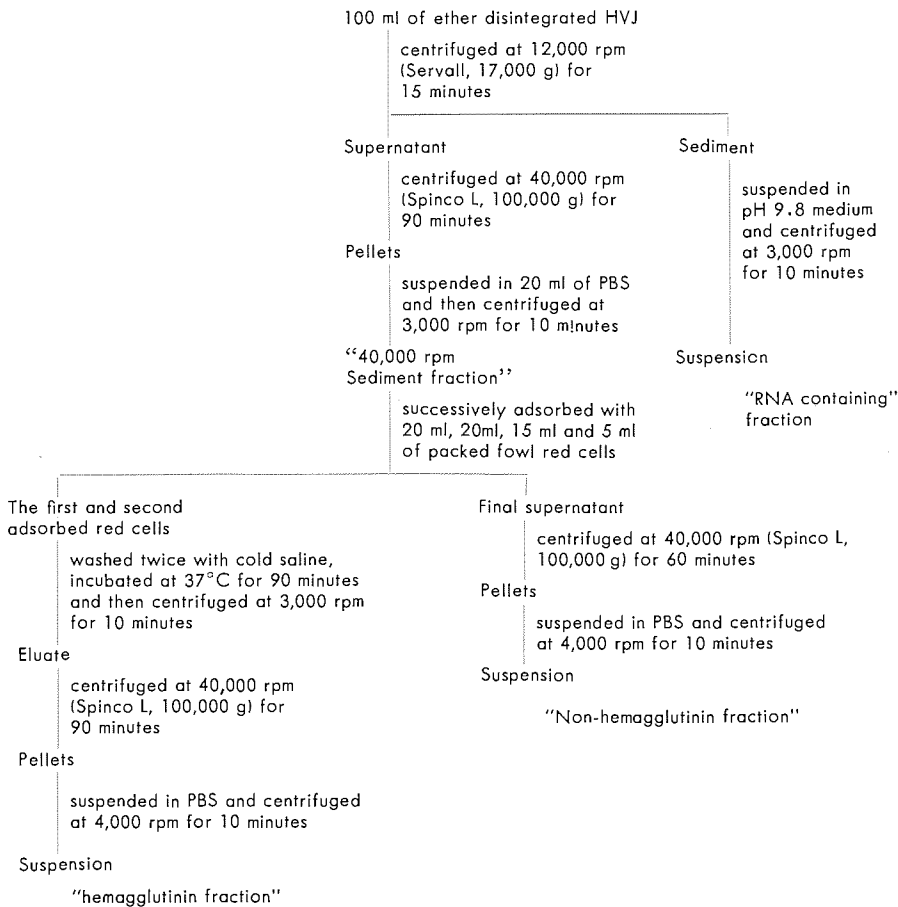
1. *Virus*

The Z strain of HVJ was purified and concentrated from the infected chorioallantoic fluid by two cycles of differential centrifugation. The purified virus was suspended in 0.01 M phosphate-buffered saline (pH 7.2). The virus titer was expressed as the hemagglutinating titer. The hemagglutinating test was described in the previous report (Hosaka *et al.*, 1960).

2. *Preparation of subunits of HVJ*

The principle is similar to that described in the previous report (Hosaka *et al.*, 1960). The disintegrated HVJ was prepared by mixing 100 ml of HVJ suspension (16,000 HAU/ml) with an equal volume of anaesthetic ether in the presence of 160 mg of Emasol in the cold for 10 minutes

Table 1. Fraction of Ether Disintegrated HVJ



and then separating the aqueous layer by centrifugation. The residual ether in the aqueous layer was removed by bubbling the solutions with nitrogen or air. The course of the fractionation is shown in Table 1.

3. *Antisera*

The rabbit antisera against purified HVJ used was the same as that described in the previous report (Hosaka *et al.*, 1960).

4. *Agar Gel diffusion technique*

The double diffusion technique of Ouchterlony (1949) was employed. 0.7 per cent agar solution in isotonic saline containing 0.01 per cent merthiolate was used. 0.15 ml of antigen and two fold dilutions of antisera were placed 7 - 8 mm apart in wells of 6.0 mm diameter and examined after a week incubation at 37°C.

5. *Chemical determinations*

The protein content was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard. The RNA fraction was extracted by Ogur and Rosen's method and the RNA content was estimated by the Orcinol reaction, using yeast RNA as a standard.

6. *Negative staining technique*

A slight modification of Brenner-Horne's method (1959) was used. Samples were mixed with an equal volume of 2 per cent phosphotungstic acid (PTA, pH 7.2) after dialysis against 1 per cent ammonium acetate or distilled water for 20 hours for virus particles, and for 10 hours against distilled water for other samples. They were then mounted on carbon coated collodion grids by the conventional method and examined in a Hitachi, HU 9D electron microscope. Virus particles were also mixed with 2 per cent phosphomolybdic acid (PMA) (Bradley and Kay, 1960) and compared by the same method used for PTA.

The modified technique of negative staining has two advantages; 1) the spontaneously disrupted virus particles are few in preparations by this method, and 2) very small amounts of sample is enough for the preparation of specimens.

RESULTS

1. *Effect of Emasol on ether-disintegration of HVJ*

The previous study (Hosaka *et al.*, 1959, 1960) showed that ether disintegration of HVJ in the cold in the presence of Emasol is an effective method for the preparation of the hemagglutinin of HVJ. Subsequent studies showed that the hemagglutinin fraction had negligible RNA and that the non-hemagglutinin fraction, prepared as shown in Table 1, usually was small and therefore had little RNA. On the other hand RNA-protein of Influenza viruses has been successfully prepared as a non-hemagglutinin fraction, by ether treatment at 37°C for longer periods (Hoyle, 1952; Zillig *et al.*, 1955; Lief and Henle, 1956).

Thus it became necessary to investigate the effects of Emasol on the ether disintegration of HVJ in the cold and at higher temperatures in detail.

To 15 ml aliquots of the purified HVJ suspension were added various concentrations of Emasol in 1 ml of distilled water. The mixtures were shaken with an equal volume of anaesthetic ether for 10 minutes in baths at 10°C and 30°C, re-

spectively. Then the aqueous layers were separated by centrifugation at 2,000 rpm for 5 minutes. Half of each aqueous layer was used directly for the measurement of protein, RNA and the hemagglutinating titer and the other half was recentrifuged at 12,000 rpm (17,000 g) for 20 minutes (Servall centrifuge). The protein content and hemagglutinating titer of the supernatants and the RNA content of the pellets were measured. The values are shown in Fig. 1. The original virus suspension used had 730 $\mu\text{g}/\text{ml}$ of protein, 15.4 $\mu\text{g}/\text{ml}$ of RNA and a hemagglutinating titer of 15,000 HAU/ml.

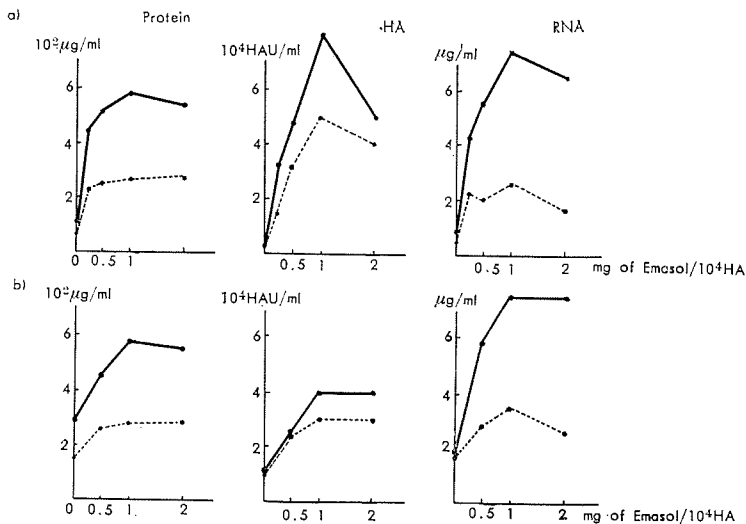


Fig. 1. Effects of Emasol on Ether Disintegration of HVJ at 10° C (a) and at 30° C (b)

The solid lines represent values for the aqueous layer and the broken lines, for the supernatants obtained by centrifugation of the aqueous layer at 12,000 rpm.

The abscissa indicates the amount of Emasol per 10⁴ HA of original virus particles.

The results show that the higher the concentration of Emasol, the more material accumulates in the aqueous layer until a certain maximum; a marked characteristic of the effect of Emasol on the ether disintegration of HVJ is that addition of Emasol to the virus suspension prevents the passage of substances from the aqueous phase into the interphase or the ether phase.

Thus Emasol-ether disintegration is an effective method for the preparation of an RNA-containing fraction as well as the hemagglutinin of HVJ. On treatment at 30°C, the hemagglutinating titer of the aqueous layer was only half of that on treatment in the cold but the distribution of total protein and RNA was similar after the two treatments. Therefore the reduction in the hemagglutinating titer at the higher temperature seems to be due to loss of hemagglutinating

activity. If this is the case, the non-hemagglutinating fraction obtained as shown in Table 1 at 30°C, must contain inactivated hemagglutinin. In fact electron-microscopy showed that the non-hemagglutinin fraction thus obtained, contained the same morphological material as the hemagglutinin fraction, although the non-hemagglutinin fraction obtained in the cold contained a few hemagglutinin-like particles.

After treatment in the cold, most hemagglutinin remains in the supernatant after centrifugation at 12,000 rpm for 20 minutes, while ca. 70 per cent of the viral RNA was present in the pellets. However after treatment at 30°C and centrifugation a little more RNA remains in the supernatant. Therefore the authors used the precipitate formed by centrifugation at 12,000 rpm of the aqueous layer of the treated HVJ at 10°C as the RNA-containing fraction and the resulting supernatant for preparation of the hemagglutinin fraction. The recoveries of protein and RNA, and the RNA content of the fractions of ether disintegrated HVJ at 10°C in the presence of an optimal concentration of Emasol are presented in Table 2.

Table 2. Recoveries of RNA and Protein and the RNA Contents of Fractions of Emasol-ether Disintegrated HVJ

Fraction*	Recoveries		Contents	
	Protein	RNA	RNA/protein	RNA/dry weight
Original virus particles	% 100	% 100	% 1.5-2.0	% 0.75-1.0 (Mean 0.86)
Emasol ether-disintegrated virus	80-90	50-60		
12,000 rpm sediment	15-20	25-30	2.5-3.0	2.0-2.5
40,000 rpm sediment	20-25	5	0.4-0.5	0.3-0.4
hemagglutinin	10-15	<1	<0.1	
non-hemagglutinin	<1	1	4-5	

* Fractions were separated as shown in Table 1

The precipitate on centrifugation of the aqueous layer at 12,000 rpm has a high RNA content (2.0 - 2.5 per cent). The recovery of RNA from the fraction precipitating at the non-hemagglutinin fraction are relatively low, because the RNA content was much reduced when suspensions of the 40,000 rpm pellets and the non-hemagglutinin fraction were clarified by centrifugation at 3,000 rpm for 10 minutes. The RNA containing material in such fractions seems to be insoluble or not readily dispersed at neutral pH. The DNA content of HVJ was negligible.

2. *Some properties of the fraction precipitating at 12,000 rpm*

The fraction, described above, precipitating at 12,000 rpm has much RNA. It was possible that this fraction might only be a mass of partially disintegrated HVJ, since it precipitated on relatively low speed centrifugation.

Thus some properties of the fraction and its sedimentation and gel diffusion patterns were examined. It was difficult to suspend the fraction at neutral pH, because it precipitated spontaneously at neutrality. However it was dissolved in alkaline medium (NaHCO_3 - Na_2CO_3 buffer, 0.02 M, pH 9.8), a soluble suspension was obtained.

The sedimentation pattern of this dispersed suspension showed a single peak with a sedimentation constant of 130 S. (Fig. 2).

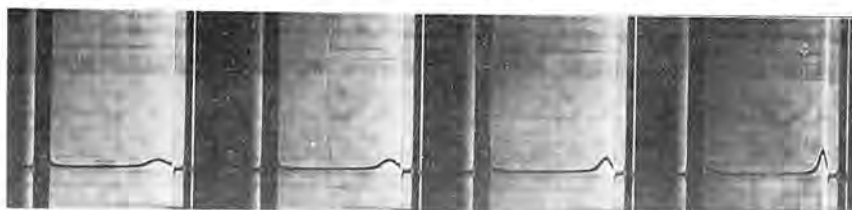


Fig. 2. Sedimentation Pattern of the RNA-containing Fraction

(NaHCO_3 - Na_2CO_3 buffer, 0.02M, pH 9.8)

The figure shows a single peak of 130 S.

Photograph taken at intervals of 4 minutes at 12,590 rpm in a Spinco E ultracentrifuge.

Next the gel diffusion pattern of this fraction was compared with that of the fraction precipitating at 40,000 rpm, as shown in Fig. 3.

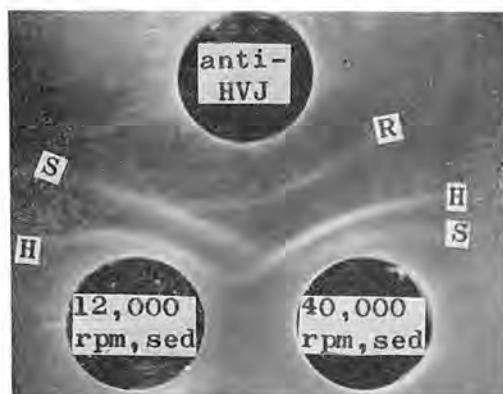


Fig. 3. Gel Diffusion Pattern in Agar Gel of the RNA-containing Fraction and the fraction Precipitating at 40,000 rpm

Both antigens give R, S and H precipitations and H and S by both antigens fuse respectively; a crossing of H and S precipitation lines can be seen.

Fig. 3 shows that the fraction has S, H and R components against anti-HVJ serum (Hosaka *et al.*, 1960) and two precipitation lines, S and H with both antigens were cross: the S line of the 40,000 rpm fraction was nearer to the antigen well and the H line of the fraction precipitating at 12,000 rpm was nearer to the antigen well. These results indicate the fraction precipitating at 12,000 rpm has much more of the S component and much less of the H component, than the fraction precipitating at 40,000 rpm. Thus it is not merely a mass of partially disintegrated HVJ but seems to be a true fraction, of well disintegrated virus, containing much RNA material, which is difficult to be suspended in neutral medium.

This fraction has a hemagglutinating titer of approximately 10 HAU per μ g of protein, which is only half of that of the original virus particles and twenty to thirty times lower than that of the hemagglutinin fraction.

A dissociation of RNA and protein of tobacco mosaic virus under alkaline condition has been reported (Schram *et al.*, 1956), but in our experiments centrifugal studies showed that viral RNA was still associated with the 130 S components in alkaline medium. In these alkaline conditions, intact HVJ virus particles did not disintegrate, but retained their infectivity and other biological properties.

3. *The fine structure of the virus particle and its subunits*

1) *Virus particle*

Photo 1 shows the fine structure of HVJ virus particles, which had been dialysed against distilled water. First, it can be seen that the particles have various shapes and sizes of 1500 - 6000 Å width. Second, the particles are surrounded by a surface layer of 50 - 90 Å thickness with projections 100 - 150 Å long and about 20 Å wide, which are arranged at a center-to-center spacing of 70 - 80 Å and seem to be connected with each other at their tips. Third, within the particles, strands of about 50 Å width, can be seen as tubular structures in relatively loosely packed regions.

When the virus particle was suspended in 1 per cent ammonium acetate and prepared with PTA on grids by the conventional mounting method, the fine structure was not so clear (Photo 2) and the projections were 90 - 140 Å long, and the inner structure of strand-materials appears the same in both preparations.

Virus particles suspended in 0.85 per cent NaCl and prepared with PTA and particles in ammonium acetate and prepared with PMA are shown in Photo 3 and 4, respectively. In Photo 3, the particles of irregular form are relatively few and the inner structure is not so clear as in Photo 1. In Photo 4, the particles are similar in shape and size to those in Photo 1 and 2.

In this modified method, spontaneously disrupted virus particles were seldom seen, which is in contrast to the case with the spray method (Horne and Waterson, 1959). Applying the modified method, the clearest figure was obtained when the sample was suspended in distilled water.

2) *RNA containing fraction (fraction precipitating at 12,000 rpm)*

Photo 5a and 5b show the RNA containing fraction at low and high magnification respectively. In Photo 5, tubular structures, 175 - 190 Å wide, like those of tobacco mosaic virus (Brenner and Horne, 1959) are predominant. The tube has a central hole of 40 - 50 Å diameter and a regular periodicity of 50 - 60 Å along the axis. These structures suggest a helical arrangement of strands, possibly a double helical array, which can be inferred from the distance of the pitches and the angle drawn by the crossing of the periodic strands. In some places hollow rings of the same diameter are seen, which are probably sectional views of the helix. The width of the helical strands was about 50 Å.

The size of these helical structures in alkali ($\text{NaHCO}_3\text{-Na}_2\text{CO}_3$, 0.02M, pH 9.8) was the same as in a neutral medium (Photo 9), although both samples were dialyzed against distilled water before mounting.

3) *Non-hemagglutinin fraction (cf. Table 1)*

Photo 6 and 7 show the non-hemagglutinin fraction. Coiled stranded material of various lengths is predominant and further the same helical structure as seen in the RNA containing fraction and the same morphological structure as the hemagglutinin can be observed. The latter seems to be hemagglutinins, adsorption capacities of which to red cells were inactivated. The coiled stranded materials appear to be loosened or unwound forms of the helical structures observed in the RNA containing fraction, and they still have a coil width of about 200 Å. The loosened or unwound strands have a width of approximately 50 Å, and this suggests an arrangement of subunits, because the strands are not smooth but irregular fibres. These loose structures can scarcely be seen in the fraction precipitated at 40,000 rpm (Photo 9) before fractionation by red cell adsorption and therefore are considered to be the original structures as modified during the separation procedure.

The shadowcast preparations of the non-hemagglutinin fraction, shown in the previous report (Hosaka *et al.*, 1960), seem to contain both the original and the loosened forms of the helical structure.

4) *Hemagglutinin fraction*

Photo 8 shows the hemagglutinin fraction. Various shapes and sizes of the material of 450 - 900 Å can be seen, but a common feature of them can be recognized; projections 150 Å long and 20 - 35 Å wide, similar to those in the surface layer of intact virus particles are arranged on both sides of strand, 50 - 100 Å wide of varying length and sometimes enclosed. Among the various sized particles, relatively uniform and minimum sized particles of approximately 450 - 550 Å can be seen, which seem to be unit hemagglutinin having the capacity of adsorption to and elution from red cells. These findings suggest that the hemagglutinins of HVJ form the outer envelope of intact virus particles by connecting with each other.

DISCUSSION

In the present experiment, the effect of Emasol on ether disintegration of HVJ has been analysed. This effect of Emasol was found to be similar in other myxovirus in subsequent studies.

The RNA-protein of HVJ tended to precipitate readily in neutral media; much RNA was lost when suspensions of various pellets fractions of disintegrated HVJ were clarified by low speed centrifugation. These findings have not yet been reported in the preparation of RNA-protein or S antigen of influenza virus (Hoyle, 1952; Schäfer and Zillig, 1954; Mizutani, 1958; Paucker *et al.*, 1959; Davenport *et al.*, 1960). At present it is unknown whether the finding was due to a peculiar properties of the RNA-protein of HVJ or to an effect caused by Emasol-ether disintegration in the cold. The fact that the inner components of RNA protein released from the envelope of the virus particles of HVJ are very long coils of helical structure in the intact form seems to be at least in part in favour of the first possibility. The fraction containing RNA prepared thus had a relatively low RNA content (2.0 - 2.5 per cent) as an RNA protein, which was considered to be due to contamination by other substances, because a fraction of higher RNA content was recently obtained by chromatographic separation of Emasol-ether disintegrated HVJ, though it is not entirely free from hemagglutinin. The details of its preparation will be described in a forthcoming report.

The helical structures in the RNA containing fraction, which is considered to be RNA-protein, shows the surprising regularity and uniformity of the inner structure of various shapes and sizes of intact virus particles. These helical structures of the RNA containing fraction of HVJ are consistent in size and shape with the helical structures of a preparation of spontaneously disrupted Sendai Virus, observed by Horne and Waterson (1960).

The viral RNA is possibly embedded in the helical structure like tobacco mosaic virus (Franklin *et al.*, 1959; Hoyle *et al.*, 1961). The observed double helical array does not necessarily mean the presence of a double chain of RNA wound complementarily as in DNA, because the RNA of influenza viruses with an inner structure of a double helical array (Hoyle *et al.*, 1961) has no corresponding relation of base like DNA (Schäfer, 1959).

It is interesting that the double helical inner structure of HVJ was modified into a loosened or unwound state during the separation of hemagglutinin and non-hemagglutinin by fowl red cell adsorption. If this modification was caused by the attachment of the helical structure to a red cell component, the question arises of the relation between the modification and the penetration of the inner structural components of HVJ into the cell.

From the figures of the hemagglutinin of HVJ showing that the projections are arranged on strands of various lengths or in some cases on enclosed strands,

the composition of the envelope of the intact virus particle of HVJ could be considered in several ways; the envelope consists of either longitudinal, latitudinal, or randomly arranged strands with projections. The first possibility is not consistent with the occurrence of various length of enclosed strands. If the strands are arranged at random, the densities of the projections in the surface layer varies from place to place. Thus the second possibility is the most probable, but other possibilities can not be excluded at present. The variability and transformability of the morphology of the virus particles of HVJ seems to be best explained by the second possibility. The projections of the hemagglutinin were observed to protrude on both sides of the strands which seems to be caused by disruption of the envelopes of the intact virus particles, which have projections protruding from only one outer side of the surface layer. On comparison of the hemagglutinin of other myxovirus with that of HVJ, those of Influenza (Hoyle *et al.*, 1961) and fowl plaque virus (Waterson and Schäfer, 1961) are a little different; they are more uniform and smaller in size and have projections arranged radially.

When the virus particles of HVJ were suspended in distilled water, mixed with 2% PTA and then dried on the grids, irregular shaped and various sized particles were often seen. This fact confirms the fact that the morphology of such virus particles as HVJ and NDV can vary, depending upon the medium condition (Fukai *et al.*, 1955; Bang, 1948), although even Influenza virus particles have various forms when observed by the mounting method with PTA after dialysis against distilled water. The surface layer of the particles was a little thicker than that described by Horne *et al.* (1960). This may be due to a difference in the method used for preparation of the specimen.

The authors are now studying the relation between the fine structural components and the antigenical components detected by the gel diffusion technique.

ACKNOWLEDGEMENT

The authors wish to thank Miss K. Kawai for her excellent technical assistance.

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EXPLANATION OF PHOTOGRAPHS

Photo 1. Virus Particles of HVJ Dialyzed against Distilled Water and Embedded in PTA.

Various shapes and sizes of particles and a well-defined surface layer and projections can be seen. Mag. $\times 118,000$

Photo 2. Virus Particles of HVJ Dialyzed against 1 per cent Ammonium Acetate and Embedded in PTA.

Mag. $\times 118,000$

Photo 3. Virus Particles of HVJ Suspended in 0.85 per cent NaCl and Embedded in PTA

Mag. $\times 118,000$

Photo 4. Virus Particles of HVJ Dialyzed against 1 per cent Ammonium Acetate and Embedded in PMA.

The figure is essentially the same as the preparations in PTA Mag. $\times 118,000$

Photo 5. The RNA Containing Fraction of HVJ Dialyzed against Distilled Water and Embedded in PTA.

Many double helical structures can be seen. A is interpreted as being a helix viewed along the axis. Mag. $\times 195,000$ (a), $\times 354,000$ (b)

Photo 6,7. The Non-hemagglutinin Fraction of HVJ Dialyzed against Water and Embedded in PTA.

Unwound or loosened helical structures are seen. In the lower left of Photo 7, a clear unwound single strand can be seen. Mag. $\times 195,000$

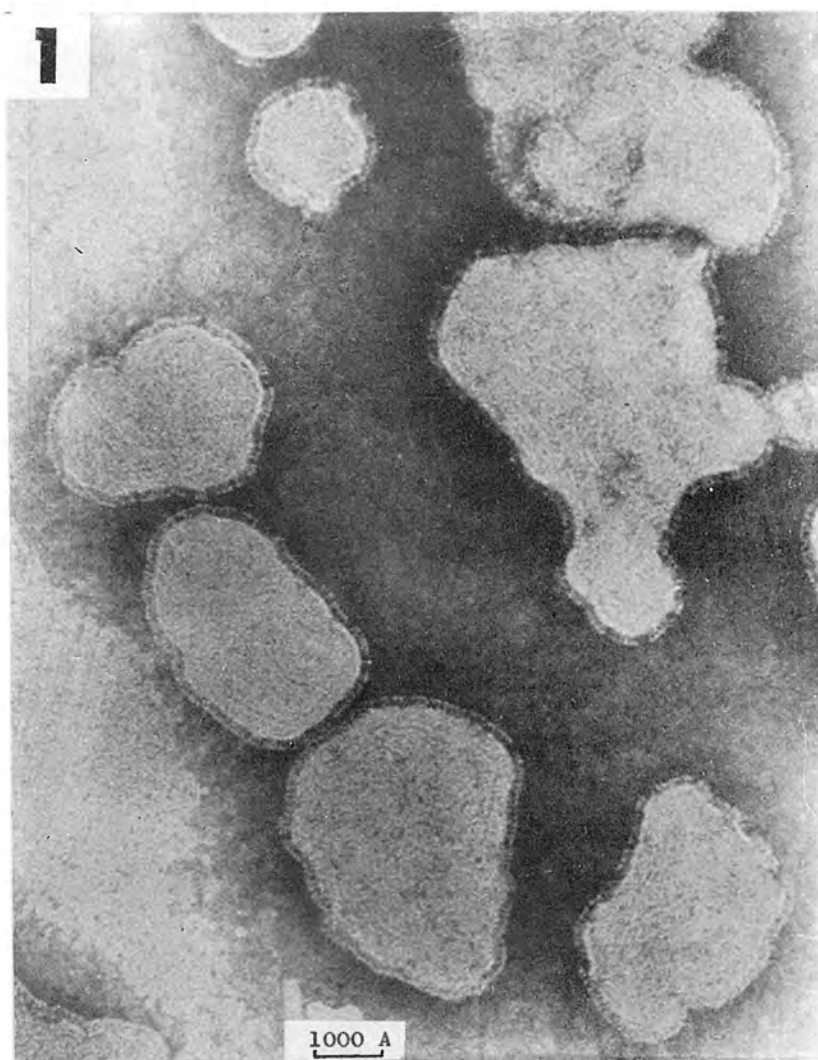
Photo 8. The Hemagglutinin Fraction of HVJ Dialyzed against Distilled Water and Embedded in PTA.

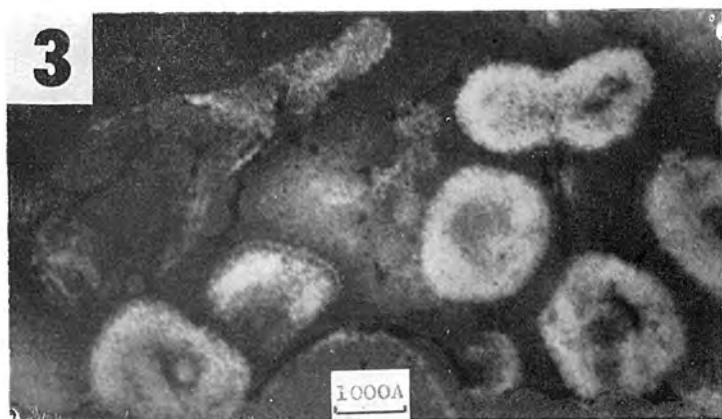
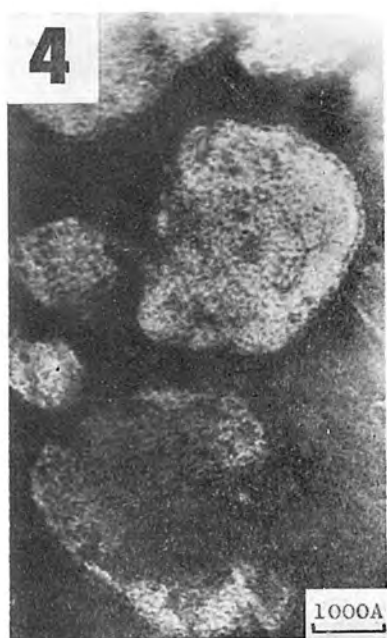
Mag. $\times 19,500$

Photo 9. The 40,000 rpm Sediment Fraction of HVJ Dialyzed against Distilled Water and Embedded in PTA.

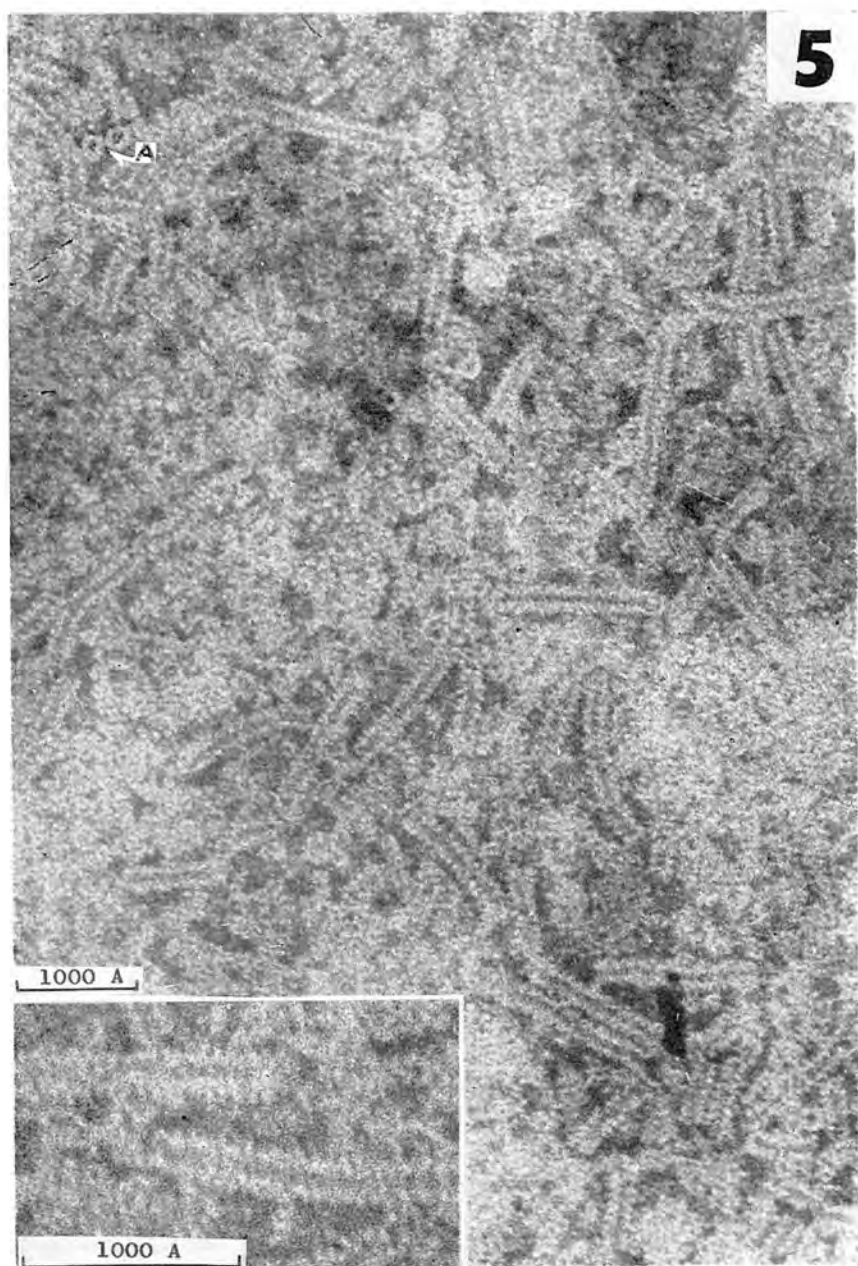
Both hemagglutinins and helical structures are seen. A shows a part of intact envelope.

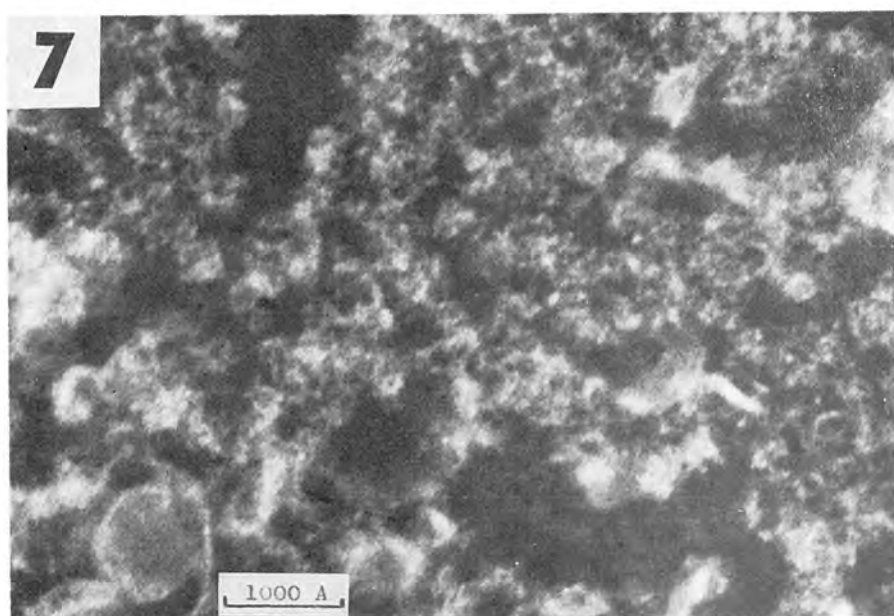
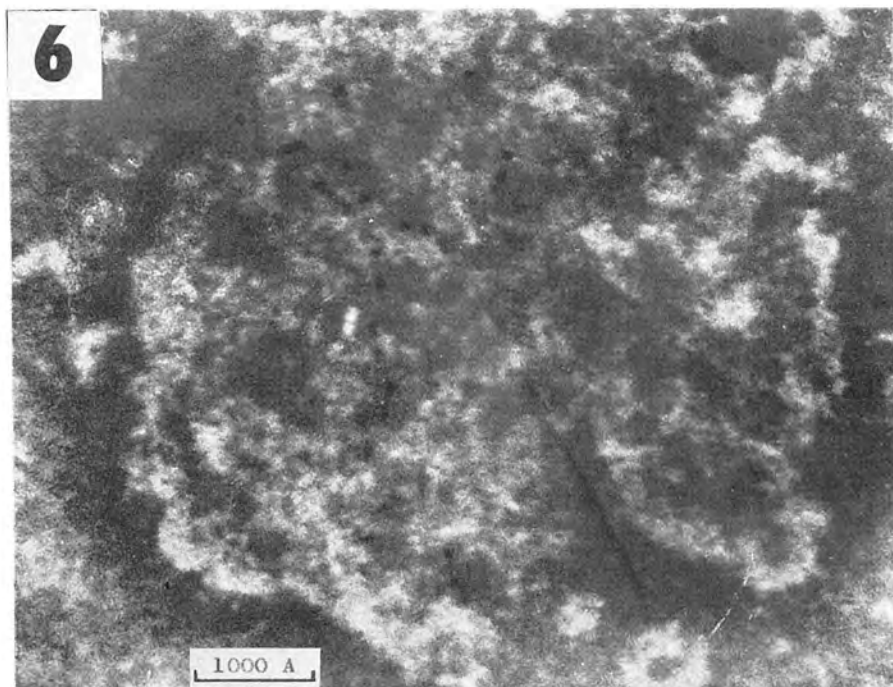
Mag. $\times 19,500$





5





8





All photographs were printed in a reduced scale (4 : 5) of the magnifications described in "Explanation of Photographs".