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Structure of HVJ
I. Two Kinds of Subunits of HVJ

YASUHIRO HOSAKA, YASUSHI HOSOKAWA AND KONOSUKE FUKAI

Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University, Osaka

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SUMMARY

Subunits of HVJ, prepared by ether treatment in the presence of a surface active agent, were studied ultracentrifugally, morphologically and immunologically by the agar gel diffusion method.

The virus particle of HVJ is composed of two kinds of subunits; one is hemagglutinin which is 40–50 mμ and 90 S and consists of at least 3 antigenically different components (H_1, H_2 and R), as judged by agar gel precipitation against anti-HVJ rabbit serum, two of which (H_1 and H_2) seem to be inherent for the hemagglutinin, and the other is a soluble antigen which is 15–20 mμ and 37 S. This reacts with anti-NDV rabbit serum as well as anti-HVJ rabbit serum in the gel precipitation test.

Antigenical relationships are recognized between the virus particle and the hemagglutinin of HVJ in the gel precipitation test; both materials have identical R component and the possibly inherent components (V and H) of both materials fuse forming a spur. No antigenical relationship is recognized between the soluble antigen and the virus particle or the hemagglutinin in the gel precipitation test. It was concluded that a virus particle is probably composed of about ten hemagglutinins arranged in the surface layer and of soluble antigens inside the hemagglutinin layer.

INTRODUCTION

Studies of the subunits of Influenza virus and Fowl plague virus have been reported by Hoyle (1952), Hoyle *et al.* (1953), Lief and Henle (1956), Mizutani (1958) and Davenport *et al.* (1959) and Schäfer and Zillig (1954), Zillig *et al.* (1955) and Schäfer (1957, 1959). From these studies, it was shown that the viruses were composed of two kinds of subunits; one was hemagglutinin of protein with carbohydrate, which was 30–40 mμ in size and was arranged in the surface of the virus particle, the other was "S-antigen" of nucleoprotein which was 10–20 mμ in size and was below the hemagglutinin layer.

The principle used by these workers for preparing subunits of the viruses was to treat the virus particles with ether. However in our experiences, the hemagglutinating titer so obtained was several times lower than the original titer and the method could not applied to the other myxoviruses, HVJ and NDV, since they are too sensitive to ether. Now the improved method described in the previous report (Hosaka *et al.*, 1959) has permitted the successful and simple preparation of subunits of myxoviruses. The method used was the treatment of the virus with ether in the presence of the non-ion surface active agent, Emasol 1130.

The present paper describes results of studies on the ultra-centrifugal, morphological and immunological nature in the agar gel diffusion test, of the subunits of HVJ prepared by the improved method.

MATERIALS AND METHOD

1. *Virus*

HVJ; Z strain, NDV (Newcastle Disease Virus); Osaka strain, Mumps virus; Enders strain and Influenza virus; PR8 strain and NWS strain were used.

The allantoic fluids of 10 day chick embryos infected with HVJ and NDV were collected after 72 and 48 hours incubation respectively and those for Influenza viruses of 11 day chick embryo, collected after 48 hours incubaton. The fluids were centrifuged at 3,000 rpm for 30 minutes and the supernatant centrifuged further at 22,000 rpm for 30 minutes (Spinco L. No. 30). The pellets were suspended in isotonic saline and recentrifuged at 3,000 rpm for 20 minutes. The resulting supernatant was used for experiments. The virus content was expressed as its hemagglutinating titer. The hemagglutinating titer was estimated as follows; 0.5 ml of 0.5 per cent fowl red cells in saline was added to 2 fold serial dilutions of virus. The reciprocal of the highest dilution which agglutinated the red cells was expressed as the hemagglutinating titer (HA).

2. *Antiserum*

1) *Rabbit antiserum*

One ml of purified HVJ and NDV (10,000 HA/ml) was injected into rabbits intravenously and intramuscularly alternately once a week. Six days after the 3rd injection, the blood was withdrawn by cardiac puncture. One ml of Influenza virus, PR8 strain (10,000 HA/ml), was injected intravenously once a week. Six days after the 2nd injection, the rabbits were bled. In later experiments, immune sera were obtained by injection of virus purified twice by differential centrifugation.

2) *Guinea pig antiserum*

Guinea pigs were immunized by the method of Lief *et al.* (1958); guinea pigs were infected with 0.2 ml of purified virus (10,000 HA/ml) by intranasal inhalation under light anesthesia and after 2 weeks, injected intraperitoneally with 1 ml of purified virus. One week later the blood was withdrawn by cardiac puncture.

3) *Fowl antiserum*

One ml of purified virus (10,000 HA/ml) was injected intramuscularly into roosters once a week. Six days after the 2nd injection the blood was withdrawn by cardiac puncture.

Anti-mumps virus serum was obtained by intravenous injections with infected amniotic fluids.

The antisera of these species were heated at 56°C for 30 minutes and stored at −10°C before use. The hemagglutination inhibition titer against HVJ were 640 ±, 160, 320 and 40 for anti-HVJ rabbit, guinea pig and fowl serum and anti-NDV rabbit serum respectively. The hemagglutination inhibition titer was expressed as reciprocal of the highest dilution (2 fold serial dilutions) of 0.5 ml of the antiserum which inhibited agglutination of 0.5 ml of 0.5 per cent fowl red cells with 4 HA of HVJ.

3. *Agar gel diffusion*

The double diffusion technique of Ouchterlony (1949) was employed. 0.6 per cent agar (Difco) and 0.01 per cent merthionate in isotonic saline were used. Often a small volume of phosphate buffer (pH 7.0) was added but the precipitation pattern was not changed. 0.15 ml of antigen and twice diluted antiserum were placed in each 8.0 mm diameter well 10 mm apart. The plates were incubated at 37°C and photographed after a week.

4. *Reagents*

Emasol 1130 (poly-oxyethylen sorbitan monolaurate) is a non-ion surface active agent like Tween 20 and was kindly supplied from KAO Soap Co., Ltd., Osaka.

Anesthetic ether was employed.

RESULTS

1. *Ultracentrifugal analysis of ether-disintegrated HVJ*

Fig. 1. Sedimentation diagram of ether disintegrated HVJ (2,000,000 HA/ml) 24,630 rpm at intervals of 120 seconds, at room temperature (20°C). Arrow shows the sedimentation direction.

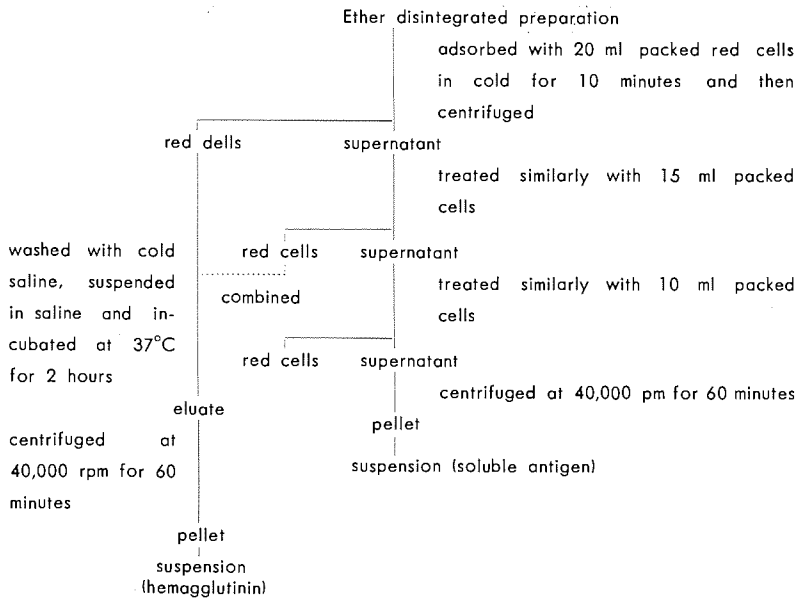
100 mg of Emasol 1130 in 2 ml of distilled water was added to 30 ml of HVJ suspension (30,000 HA/ml). The mixture was shaken for 10 minutes in an ice bath and then centrifuged at 3000 rpm for 5 minutes. The aqueous layer was removed, diluted 3 times with isotonic saline and centrifuged at 15,000 rpm for 15 minutes (Spinco L#30). The supernatant was centrifuged further at 40,000 rpm for 60 minutes and the resulting pellets were resuspended in 3 ml of isotonic saline and recentrifuged at 12,000 rpm for 15 minutes (Kubota KR6-B). The sedimentation pattern of the supernatant was analysed by Ultracentrifuge (Spinco E). The HA titer of the supernatant was 2,000,000 HA/ml. The preparation was designated as ether-disintegrated HVJ.

The sedimentation pattern is presented in Fig. 1. Two peaks were recognized, showing sedimentation coefficients of 90 S and 37 S for the fast and the slow components respectively, uncorrected for the standard condition. There was much less contents of the 37 S component than the 90 S component. These values are much smaller than those of the virus particle of HVJ: 1200 S (Fukumi, 1956 and Tadokoro, 1958). The 90 S component was shown to be hemagglutinin, since the hemagglutinin fraction prepared by adsorption and elution of ether-disintegrated HVJ with fowl red cells was found to have a similar sedimentation pattern. Therefore the 37 S component represents the non-hemagglutinin fraction, that is, the soluble antigen.

2. *Shape and size of the subunits*

The hemagglutinin and the soluble antigen of the ether-disintegrated HVJ were separated by adsorption and elution with fowl red cells

Table 1. Separation of hemagglutinin and soluble antigen of HVJ by adsorption and elution with fowl red cells.



(Table 1) and an appropriate dilution of each fraction was studied by electron microscopy under shadowing.

The hemagglutinin fraction is shown in Fig. 2. Spherical particles of a size of 40–50 mμ are observed. The soluble antigen fraction is shown in Fig. 3a and b. Spherical particles of 15–20 mμ are observed to form chains and rosaries or to be free.

3. *Number of hemagglutinins constituting a virus particle*

An attempt was made to determine how many hemagglutinins constitute a virus particle of HVJ by comparison of the protein contents per HA of the virus particles and the ether-disintegrated particles.

Table 2. Comparison of protein content per HA of virus particle and ether-disintegrated particles

No. Exp.	virus particle (V.P.)	ether-disintegrafted particles (E.D.)	V.P./E.D.
Exp. 1	$4.3\times10^{-2}\mu g/HA$	$0.52\times10^{-2}\mu g/HA$	8.2
Exp. 2	3.8 〃	0.32 〃	11.9
Exp. 3	4.0 〃	0.39 〃	10.3

Virus particles were purified by one cycle of differential centrifugation and ether-disintegrated particles were the supernatant of diluted aqueous layer centrifuged at 15,000 rpm for 15 minutes after ether treatment of the virus particle. The protein content was measured by the method of Lowry et al. (1951).

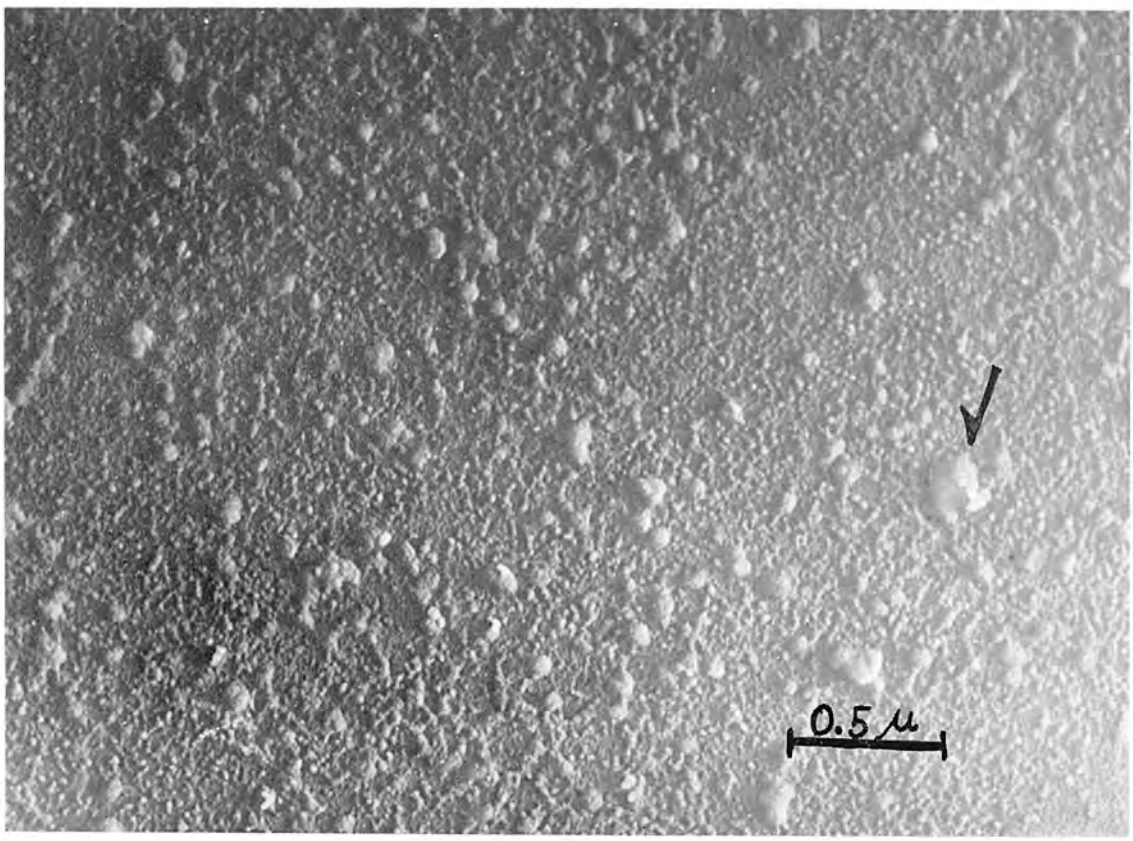
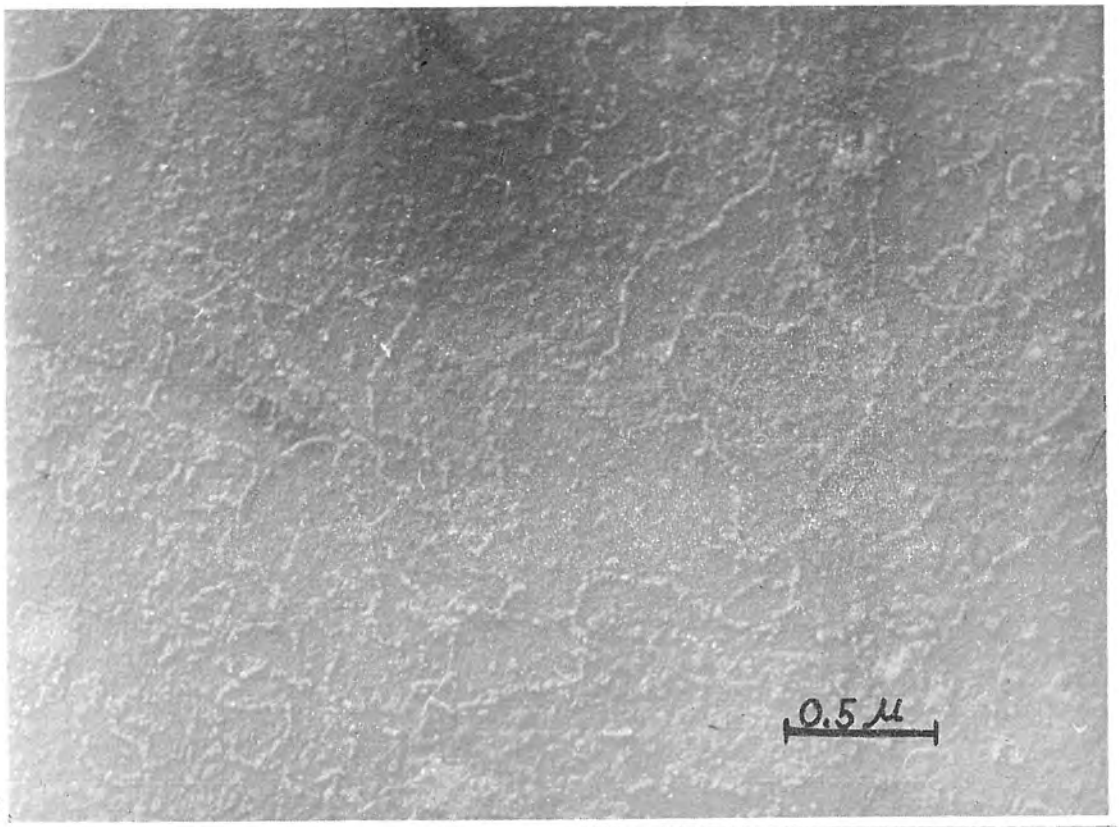
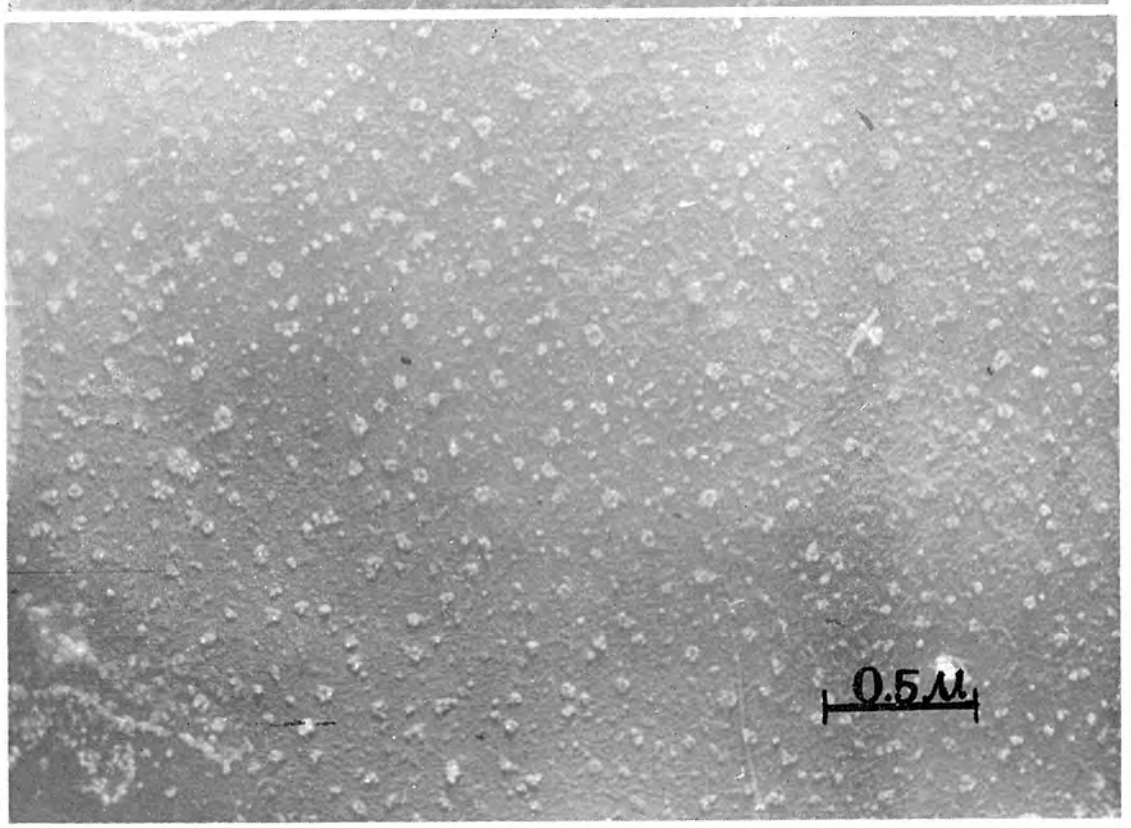


Fig. 2. Hemagglutinin of ether-disintegrated HVJ. (× 40,000) Arrow shows original virus particle.



(a)



(b)

Fig. 3.a and b Soluble antigen of ether-disintegrated HVJ. (× 40,000)

Table 2. presents the results of three experiments. For the samples of ether-disintegrated HVJ, the supernatant was used which was centrifuged at 15,000 rpm for 15 minutes but was not further centrifuged at 40,000 rpm.

Assuming that no protein of HVJ was lost during the ether treatment, the virus paticles not disintegrated by ether, if any, were completely removed by centrifugation at 15,000 rpm and the HA titer for fowl red cells was proportional to particle number having an hemagglutinating capacity irrespective of the particle size, it would be calculated that a virus particle of HVJ consists of about ten hemagglutinins

4. *Immunological study of the subunits by gel diffusion*

The agar gel diffusion technique has proved a very sensitive method for antigenical identification of viral components; Jensen and Francis (1953) have used it for influenza virus, Gipsen (1955) for pox group virus, Boden (1955) and Brown and Crick (1957, 1958) for foot-and-mouth disease virus, Brown and Crick (1957) for vesicular stomatitis virus, Mansi (1957) for myxomatosis virus. Polson *et al.* (1958) and Le Bouvier *et al.* (1957) for poliovirus and Klemperer and Pereira (1959) for adenovirus.

In the present experiments, the antigenical components of the subunits and the virus particles of HVJ were studied by agar gel diffusion.

1) *Hemagglutinin*

The hemagglutinin (800,000 HA/ml) of HVJ prepared according to Table 1 was diffused against homolgous antiserum of rabbit, guinea pig and fowl and heterologous antiserum of fowl (anti-Influenza virus, PR8 strain).

As shown in Fig. 4, two common fused precipitin lines (H_1 and H_2) were

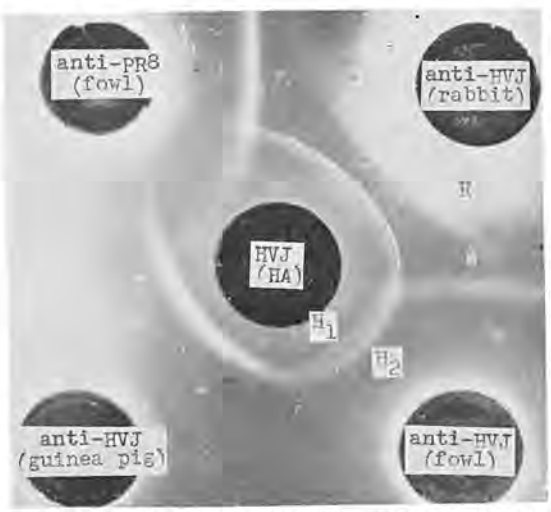


Fig. 4. Precipitation of the hemagglutinin (800,000 HA/ml) of HVJ against the homologous antiserum of rabbit, guinea pig and fowl and the anti-PR8 fowl serum in agar gel. (photographed on 9th day of incubation) HA : hemagglutinin

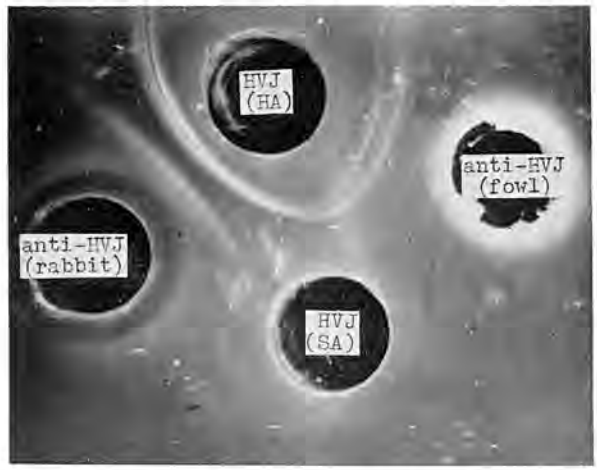


Fig. 5. Precipitation of the hemagglutinin (500,000 HA/ml) of HVJ against anti-HVJ rabbit and fowl serum in agar gel. (photographed on 8th day of incubation) HA: hemagglutinin, SA: soluble antigen SA showed no visible precipitation because of its low concentration.

observed against homologous antiserum of 3 species, though these appeared to overlap with guinea pig antiserum. Sometimes these 2 precipitin lines appeared to overlap even with rabbit and fowl antiserum, particularly when a relatively large amount of antiserum was employed. However they were due to different antigen-antibody reactions, as was indicated by the observation that the two precipitin lines crossed, when hemagglutinin was diffused against the homologous antiserum of rabbit and fowl in appropriate dilutions. (Fig. 5)

In Fig. 4, R precipitations are recognized in front, besides H precipitation with rabbit antiserum. In many repeated experiments, the R components usually formed one precipitation 2–3 days after incubation and thereafter separated or not into 2 precipitin lines. So it requires further study whether R precipitation was due to one or more antigen-antibody reactions. Further in the figure, one more precipitation is observed with rabbit antiserum behind the H precipitation. Often this precipitation appeared to overlap with the H precipitations.

In Fig. 4, unexpectedly, a precipitation was shown between anti-HVJ rabbit and fowl serum, which crossed over viral precipitations. To investigate which antigen-antibody reaction this precipitation was due to, fowl antiserum immunized by other myxoviruses, normal fowl serum and guinea pig antiserum were diffused against the rabbit antiserum of HVJ and Influenza virus.

It is shown in Fig. 6 that the various fowl antisera irrespective of the kinds of antibody react with anti-HVJ and anti-PR8 rabbit antiserum and form a fused precipitation but that the guinea pig antiserum does not react. Normal fowl serum also formed an identical precipitation against the rabbit serum. However, these antigens did not react with rabbit antiserum immunized by the viruses purified by two cycles of differential centrifugation. Therefore, the unexpected precipitation is possibly due to a fowl serum protein-specific antibody reaction in rabbit serum.

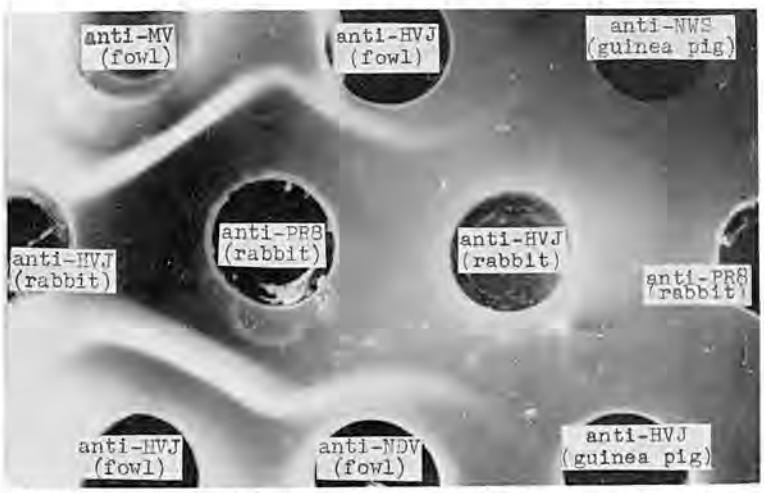


Fig. 6. Precipitation of fowl serum and guinea pig serum containing various antibodies against anti-HVJ and anti-PR8 rabbit serum in agar gel. (photographed on 9th day of incubation) Guinea pig serum showed no precipitation against rabbit antiserum. MV : mumps virus

This antibody seemed to be produced by injection of virus preparation contaminated with chick serum protein, particularly with γ-globulin, which is increased in allantoic fluids of chick embryo infected with virus (Ishida *et al.*, 1959) and removed by two but not one cycle of differential centrifugation. Normal allantoic fluids and the supernatant obtained by centrifugation at 22,000 rpm for 30 minutes of allantoic fluid infected with HVJ did not precipitate with anti-HVJ and anti-PR8 rabbit serum, probably because of the low contents of chick serum protein. This was sufficiently high to produce antibody in rabbits injected with it.

Thus, in the following experiments, rabbit antiserum was used which had been immunized with virus preparation purified by two cycles of differential contrifugation.

2) *Soluble antigen*

Soluble antigen and hemagglutinin was separated according to the method shown in Table 1. To obtain soluble antigen which was sufficiently concentrated to produce precipitation in agar gel, it was necessary to use concentrated ether-disintegrated particles as starting material, otherwise soluble antigen became mixed with the hemagglutinin fraction.

The soluble antigen thus obtained and the hemagglutiuin of HVJ purified by 3 cycles of adsorption-elution with fowl red cells were diffused against anti-HVJ and anti-NDV rabbit serum.

As shown in Fig. 7, the soluble antigen formed a clear precipitation S band near the antigen well with anti-NDV serum and a fainter one with anti-HVJ serum. The precipitations with both anti-sera were due to an identical antigen-antibody reaction, as indicated by the fusion of the precipitations when the antiserum wells were close together. These precipitations showed no antigenical

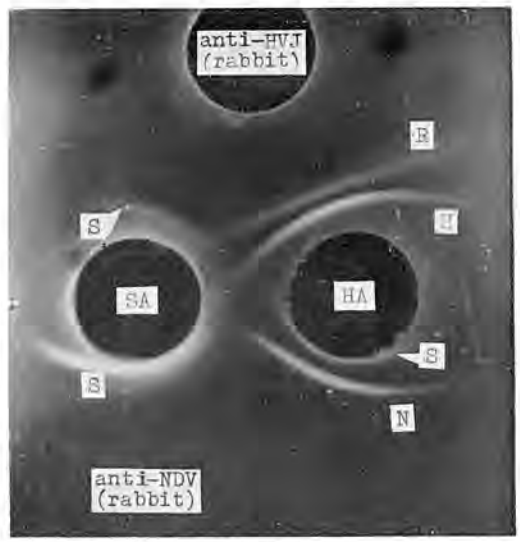


Fig. 7. Antigenic relationship betwen the hemagglutinin and the soluble antigen of HVJ against anti-HVJ and anti-NDV rabbit serum in agar gel. (photographed on 6th day of inucubation) Hemagglutinin purified by 3 cycles of adsorption-elution onto red cells was shown to contain still a little soluble antigen.

relation to that of hemagglutinin, as seen in the figure. Even such extensively purified hemagglutinin showed a precipitation with anti-NDV rabbit serum very close to the antigen well, possibly due to contamination with soluble antigen.

With anti-NDV rabbit serum, hemagglutinin formed a precipitation N band, which is related to the protein in the uninfected chorioallantoic fluids. (Hosaka *et al.*, 1960)

3) *Components in the supernatant obtained by centrifugation at 40,000 rpm of the ether-disintegrated preparation*

In the experiments described above, particles of the ether-disintegrated preparation of HVJ sedimented at 40,000 rpm for 60 minutes were studied immunologically by agar gel diffusion. In the present experiment, the supernatant was studied immunologically by gel diffusion, to determine whether other components constituting the virus particles were present in the supernatant.

To concentrate the supernatant materials, the supernatant was saturated with $(NH_4)_2SO_4$. Then the flocculate floated on the solution and 2–3 days later precipitated. The precipitate was collected, dissolved in phosphate buffer saline (pH 7.0, 0.01 M) and dialysed for 48 hours against the same buffer. The dialysate was thus concentrated about 15–20 times. It was diffused against anti-HVJ and anti-NDV rabbit serum, with particles sedimented at 40,000 rpm.

It is demonstrated in Fig. 8 that the supernatant materials react with both antisera like the sedimented particles. Both antigens form fused precipitations by the hemagglutinin but no precipitation by the soluble antigen was observed

Fig. 8. Antigenic relationship between the supernatant and the sediment of ether-disintegrated HVJ centrifuged at 40,000 rpm against anti-HVJ and anti-NDV rabbit serum.

with the supernatant. Therefore no component other than the hemagglutinin was detectable antigenically in the supernatant, which could not completely sedimented by the centrifugation, under the employed condition.

4) *Antigenical relationship between virus particles and ether-disintegrated particles*

It is interesting to see how the ether-disintegrated particles make up the original form and structure of the virus particle. If there is a component antigenically related to the virus particle, it would be a somatic antigen of the virus particle and be present on the surface. Thus the antigenical relationship between virus particles and ether-disintegrated particles was studied by agar gel diffusion against anti-HVJ and anti-NDV rabbit serum.

As shown in Fig. 9, against anti-HVJ, the R component formed an identical precipitation with both the virus particles and the ether-disintegrated particles and the H component fused with the V component near the antigen well, forming a spur. The V precipitation seemed to be due to the inherent virus particle of HVJ, as judged by its formation of a common fused precipitation against anti-HVJ rabbit, guinea pig and fowl serum, though this is not shown in figure, and its low diffusion rate, which was indicated by the curvature and the location of the precipitation near the antigen well.

Against anti-NDV serum, both antigens formed a fused precipitation N, although the virus particle formed a fainter and diffuser one. The virus particles produced one more precipitation V', possibly due to the virus particles themselves near the antigen well, which can scarcely seen in the photograph. The soluble

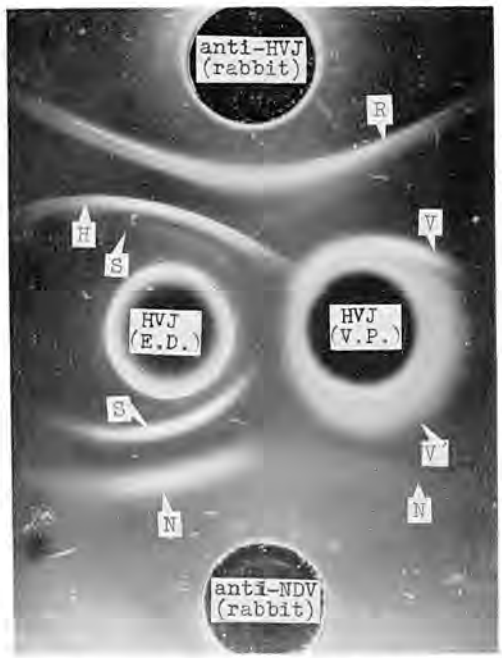


Fig. 9. Antigenic relationship between virus particle (40,000 HA/ml) and ether-disintegrated particles (1,000,000 HA/ml) against anti-HVJ and anti-NDV rabbit serum in precipitin reaction in agar gel. (photographed on 8th day of incubation)
E. D. : ether disintegrated
V. P. : virus particles

antigen was not detectable in the virus particle preparation by agar gel precipitation.

To confirm these relationship between the virus particles and the ether-disintegrated particles, mixtures of both antigens were diffused against anti-HVJ rabbit serum.

The results are shown in Fig. 10. V, H_1, H_2 and R precipitations were observed from the antigen well towards the antibody well. Thus it was confirmed that the R components of both were identical and the H and V components were antigenically related but different. It was concluded from the above results that the hemagglutinin was arranged in the surface layer of virus particle and the soluble antigen, inside the hemagglutinin layer.

5) *R component after purification of HVJ*

Attempts were made to separate the R component from the virus particles of HVJ by its adsorption and elution on red cells and by sonic vibration. But they were unsuccessful. As shown in Fig. 11, the R component was still combined with the virus particle after these procedures. Therefore the R component seemed to be an integral part of the virus particle of HVJ.

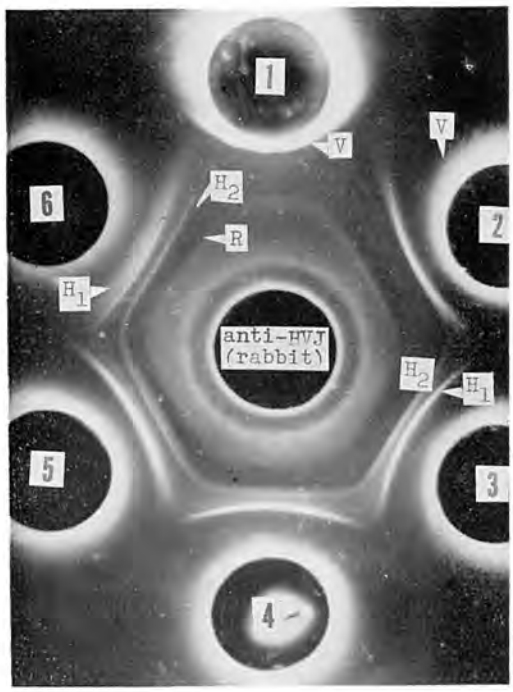


Fig. 10. Precipitation in agar gel by a mixture of the virus particle (40,000 HA/ml) and the ether disintegrated particles (10,000,000 HA/ml) against anti-HVJ rabbit serum.
Antigen 1. virus particle 0.15 ml
+ ether-disintegrated particle 0.0 ml
2. virus particle 0.12 ml
+ ether-disintegrated particle 0.3 ml
3. virus particle 0.09 ml
+ ether-disintegrated particle 0.6 ml
4. virus particle 0.06 ml
+ ether-disintegrated particle 0.9 ml
5. virus particle 0.03 ml
+ ether-disintegrated perticle 1.2 ml
6. virus particle 0.00 ml
+ ether-disintegrated perticle 1.5 ml
V precipitation near antigen well (2) was just recognizable

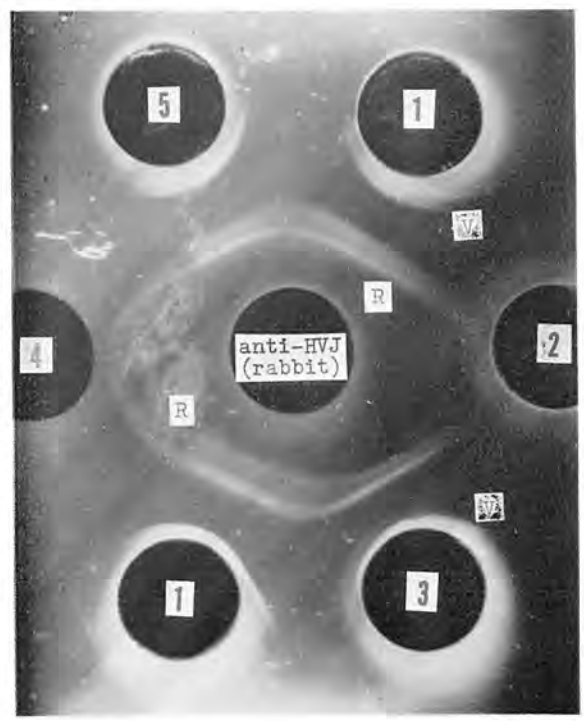


Fig: 11. Precipitation of R component of virus particle purified by adsorption-elution onto red cells or sonic vibration against anti-HVJ rabbit serum.
Antigen. 1. original virus particle (purified by one cycle of differential centrifugation)
2. supernatant centrifuged at 20,000 rpm for 30 minutes after sonic vibration for 15 minutes (in Raytheon sonic oscillator, Model S-102, 9 K. C.)
3. pellets treated as above
4. supernatant not adsorbed with red cells of virus particle
5. virus particles adsorbed and eluted from red cells.

DISCUSSION

Subunits of HVJ could be obtained in large quantity by a recently developed method (Hosaka *et al.*, 1959). The subunits, like those of Infiuenza virus and Fowl plague virus, were shown to consist of two kinds of units. They were also of a similar size and shape to those of these viruses; hemagglutinin units are spherical particles of 30–40 mμ and 100–150 S and "S-antigen" are spherical particles of 10–20 mμ and 30–40 S forming chains and rosaries. (Hoyle, 1952; Hoyle *et al.*, 1953; Davenport *et al.*, 1959 and Schäfer and Zillig, 1954; Schäfer, 1957, 1959)

That there are antigenical relationships between the hemagglutinin and the virus particle of HVJ but not between these antigens and the soluble antigen of HVJ in the agar gel precipitation test is consistent with the immunological findings on the subunits of Influenza and Fowl plague virus, although these viruses were differentiated by the complement-fixing reaction and by quantitative precipitation. (Hoyle, 1952; Lief and Henle, 1956; Mizutani, 1958 and Davenport *et al.*, 1959 and Schäfer and Zillig, 1954; Schäfer, 1957, 1958) Such antigenical relationships suggest that the hemagglutinin units of HVJ are arranged in the surface layer of the virus particle of HVJ and the soluble antigens are inside the hemagglutinin layer. These characteristics seems to be common to myxoviruses.

The hypothesis that a virus particle is probably composed of about ten hemagglutinin units is also supported by the ratio of the size of the hemagglutinin unit to the virus particle of HVJ: that is 110–140 mμ and 1,200 S (Fukai and Suzuki, 1955; Fukumi, 1956 and Tadokoro, 1958). Ten units are more than the six hemagglutinins constituting a virus particle of Fowl plague (Schäfer, 1959).

By agar gel precipitation, the hemagglutinin of HVJ was found to consist of at least 3 antigenically different components (H_1, H_2 and R). The R component reacted with only anti-HVJ rabbit and not with anti-HVJ guinea pig or fowl serum. At present it is still unknown why an antibody against R component was not produced in fowl and guinea pig and a study is in progress to determine whether the R component is a material related to the chorioallantois of chick embryo. On the other hand, H_1 and H_2 appeared to be inherent components of the hemagglutinin of HVJ, since they both reacted with antisera of various species, forming fused precipitates. A slight differences in the molecular configuration and weight of the antibodies in antiserum of various species, having the same determinant group did not prevent the formation of fused precipitation reactions, as shown in Fig. 4. The intersection of H_1 and H_2 indicated that they are due to different antigen-antibody reactions (Fig. 5). It is uncertain on the nature of a precipitation behind the H precipitation with rabbit antiserum, observed in the Fig. 4. Further study is required to define the biological significance of these viral components.

The soluble antigen of HVJ gave a faint precipitation with anti-HVJ guinea pig and fowl serum. This was possibly due to a low titer of antibody against the soluble antigen in the antiserum of these animals. It reacted with anti-NDV rabbit serum as well as anti-HVJ rabbit serum. This indicates that a close relationship exists between HVJ and NDV.

It will be shown in the next paper that the N component of the hemagglutinin of HVJ and the virus particle of HVJ are related to the normal component in the chorioallantoic fluids of chick embryo (Hosaka *et al.*, 1960)

In some experiments, (Fig. 4 and 11) V and R precipitation separated into 2 lines respectively. At present it is unknown whether this was caused by one antigen-antibody reaction or two.

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