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Structural Components of Newcastle Disease Virus I. Differentiation of the Subunits by Physical and Chemical Techniques

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SUMMARY

Newcastle Disease Virus (NDV) was disintegrated by ether treatment in the presence of a non-ionic surface active agent, Emasol 1130, and studied by ultracentrifugation, zone-electrophoresis, and immunologically by agar gel diffusion method.

On ultracentrifugation, ether-disintegrated NDV had two components with $S_{20,w}$ values of 160S and 47S, in a neutral suspending medium and three components, 104S, 15S and 12S, in a suspending medium of pH 8.6.

On agar gel diffusion analysis, ether-disintegrated NDV showed three precipitation lines against anti-NDV serum, which were tentatively disignated as R, S and H component (R; rapid diffusible, S; s-antigen and H; hemagglutinating unit). The N component, present in normal chorioallantoic fluid, was also detected by the gel precipitation reaction. Treatment at pH 8.6 of ether-disintegrated NDV did not affect the gel precipitation pattern.

Trypsinization of ether-disintegrated NDV, caused a 5S component to appear and most of the other components which had been present previously to disappear.

Trypsinization however, had no effect on the whole gel precipitation pattern and this 5S component was identified as R component by the gel diffusion technique.

Ether-disintegrated NDV was separated into hemagglutinating (migrating to the anode) and non-hemagglutinating (migrating to the cathode) fractions by zone-electrophoresis at pH 8.35. From the gel precipitation test, it was found that the former contained R and H components, and the latter contained R and S components.

At least 6 hemagglutinin particles were estimated to be present in a particle of NDV.

INTRODUCTION

NDV has been one of the most extensively investigated animal viruses but little is known about its structural components in contrast to other myxoviruses, such as the influenza virus and the fowl plague virus. This appears to be due mainly to inactivation of its viral hemagglutinating activity during ether treatment, which method has been successfully applied for preparation of subunits of the influenza virus and fowl plague virus (Hoyle, 1952; Schäfer and Zillig, 1954; Lief and Henle, 1956; Mizutani, 1958; Davenport *et al.*, 1959; Paucker *et al.*, 1959).

An improved method devised by Hosaka *et al.* (1959) overcame this difficulty. In this method the myxovirus was treated with ether in the presence of the non-ionic surface active agent, Emasol 1130. This permitted the successful preparation

of subunits of the ether sensitive myxoviruses, HVJ and NDV, and the influenza virus. Thus the authors have been able to study the structural components of these myxoviruses and reported already on those of HVJ. (Hosaka *et al.*, 1960a).

The present paper describes the differentiation and some analysis of the structural components of NDV using ultracentrifugation, agar gel precipitation technique and zone-electrophoresis.

MATERIALS AND METHODS

1. *Virus*

Newcastle Disease Virus (NDV), Osaka strain, was used throughout the experiments. The chorioallantoic fluid of 10 or 11 day old developing eggs infected with NDV was collected after 72 hours incubation. It was centrifuged at 3,000 rpm for 30 minutes and the supernatant was centrifuged at 23,000 rpm for 30 minutes, in a Spinco L ultracentrifuge using a No. 30 rotor. The resulting pellets were suspended in isotonic saline and recentrifuged at 3,000 rpm for 20 minutes. The supernatant was used for experiments. The virus content was expressed as the hemagglutinating titer per ml. The hemagglutinating titer was determined by Salk's pattern method, as described in the previous report (Hosaka *et al.*, 1960a).

2. *Preparation of ether-disintegrated NDV*

0.8 ml of Emasol solution (50mg/ml) was added to 40 ml of purified NDV suspension (20,000 HAU/ml) and then 40 ml of anesthetic ether was added. After shaking for 15 minutes in an ice bath the material was treated as shown in Table 1 (Hosaka *et al.*, 1959).

Table 1. Preparation of ether-disintegrated NDV

Purified NDV suspension
- Emasol 1130 added at concentration of 1 mg/20,000 HA.
- Equal volume of anesthetic ether added.
- Mixture shaken for 15 minutes in the cold.

Mixture
- Centrifuged at 3,000rpm for 5 minutes

Aqueous layer
- Diluted 3 times with isotonic saline and centrifuged at 12,000 rpm for 15 minutes

Supernatant
- Centrifuged at 40,000 rpm for 120 minutes

Pellets
- Suspended in isotonic saline and centrifuged at 10,000 rpm for 15 minutes

Supernatant

"ether-disintegrated NDV"

3. *Preparation of normal protein from the chorioallantoic fluid of uninfected eggs*

The chorioallantoic fluid of uninfected 13 or 14 day old eggs was collected and after centrifugation at 3,000 rpm for 30 minutes the supernatant was recentrifuged at 30,000 rpm for 60 minutes in a Spinco L ultracentrifuge fitted with a No. 30 rotor. The resulting pellet was suspended in isotonic saline at 100 times concentration of the original suspension and was stored in the cold for 2–3 days. It was then centrifuged at 10,000 rpm for 15 minutes,

and the resulting supernatant was designated as normal CAF protein and used for experiments.

4. *Antisera*

Rabbit and guinea pig antisera were obtained as described in the previous paper (Hosaka *et al.*, 1960a). Fowl antisera were obtained from fowls infected with NDV, Osaka strain.

5. *Zone electrophoresis*

Zone electrophoresis was carried out on a starch column (30 × 3 × 1cm) in borate-phosphate buffer (pH 8.35, μ=0.025). After zone electrophoresis at 4°C for 14 hours using a 6 mA current at 550 volts, the starch column was cut into a number of segments of 1cm length and each was eluted with 2.5 ml of isotonic saline. The protein contents were measured with phenol reagent (Lowry *et al.*, 1951).

6. *Agar gel diffusion technique*

The double diffusion technique of Ouchterlony (1949) was employed. 0.6 per cent agar (Difco) and 0.01 per cent methiolate in isotonic saline were used. 0.15 ml of antigen and four times diluted anti-serum were placed 6–7 mm apart in wells of 6.0 mm diameter.

7. *Sedimentation analysis*

A Spinco Model E ultracentrifuge fitted with an analytical rotor, type A was used.

8. *Reagent*

Emasol 1130 like Tween 20 is a non-ionic surface active agent (poly-oxyethylene sorbitan monolaurate) and was kindly supplied by Kao Soap Co., Ltd., Osaka.

RESULTS

1. *Ultracentrifugal and immunological properties of ether-disintegrated NDV*

Five ml of ether-disintegrated NDV (160,000 HAU/ml) was prepared from 40 ml of purified NDV suspension (20,000 HAU/ml). Its sedimentation pattern is shown in Fig. la.

The two peaks seen in Fig. la have sedimentation constants, $S_{20,w}$ of 160S and 47S respectively, as calculated by extrapolation of the values measured at various concentration to zero. (Fig. 2)

The agar gel precipitation pattern of ether-disintegrated NDV with anti-NDV rabbit serum is shown in Fig. 3. Four precipitation lines, tentatively designated R, S (2 lines) and H, are observed.

The S precipitation line was sometimes separated into 2 lines though often only one appeared and the R precipitation line also some times dissociates into two lines. (Fig. 8). In some cases the H precipitation line appeared diffuse (Fig. 5 and 8).

The same gel precipitation pattern was observed with ether-disintegrated NDV and anti-NDV fowl or guinea pig serum. As anti-NDV serum from infected fowl is considered to contain only specific anti-bodies for inherent components of NDV, the gel precipitation pattern seen in Fig. 3 possibly shows the specific lines for the structural components of NDV.

To study the antigenical relationship between ether-disintegrated NDV and

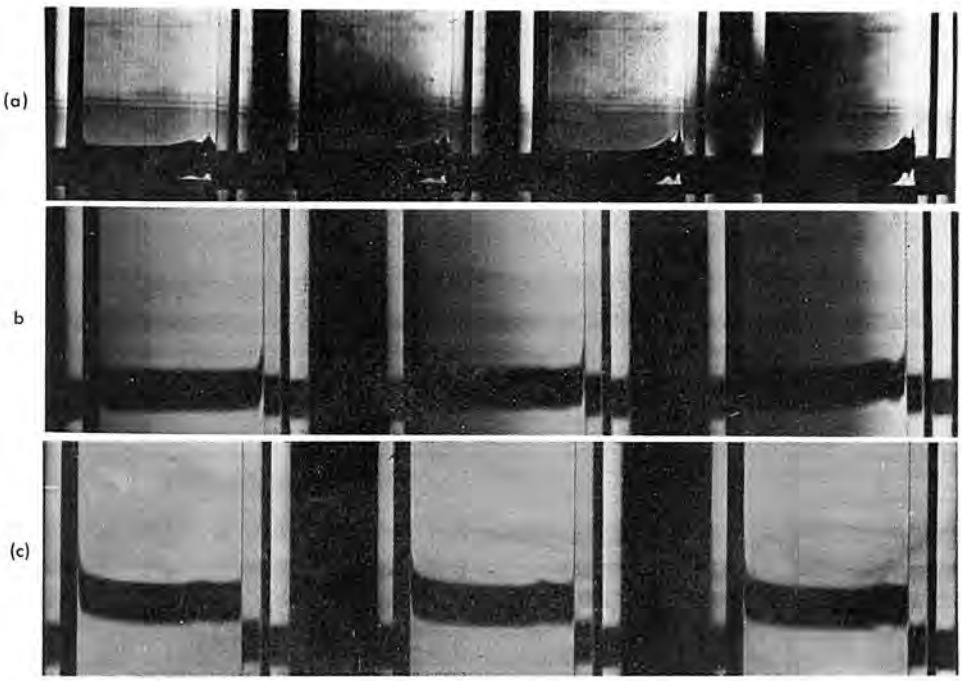

Fig. 1a. Ultracentrifugal pattern of ether-disintegrated NDV (160,000 HA/ml), photographed at 20,410 rpm at intervals of 120 seconds.

1b. Ultracentrifugal pattern of trypsinized ether-disintegrated NDV, photographed at 20,410 rpm at intervals 120 seconds.

1c. Ultracentrifugal pattern of the same sample, photographed at 59,780 rpm at intervals of 480 seconds.

other myxoviruses, and normal CAF protein, these antigens were diffused against anti-NDV rabbit serum. The results are shown in Fig. 4. All the antigens formed a common line N, although ether-disintegrated HVJ produced an additional line fusing with the N precipitation line and forming a spur. These samples, however, did not form the N precipitation line against anti-NDV fowl serum, possibly because the antiserum obtained from the infected fowl did not contain antibodies against normal CAF protein.

2. *The number of hemagglutinins liberated from a virus particle by Emasol-ether treatment*

An attempt was made to determine how many hemagglutinins were liberated from a virus particle of NDV by Emasol-ether treatment by comparing the protein content per HAU of the original virus particles and of the ether-disintegrated particles.

Table 2 shows the results of three experiments. On the same assumptions as used to calculate the hemagglutinin number of a virus particle of HVJ (Hosaka *et al.*, 1960a), about six hemagglutinins were calculated to be liberated from a virus particle. Thus at least six hemagglutinins are considered to constitute a virus particle of NDV.

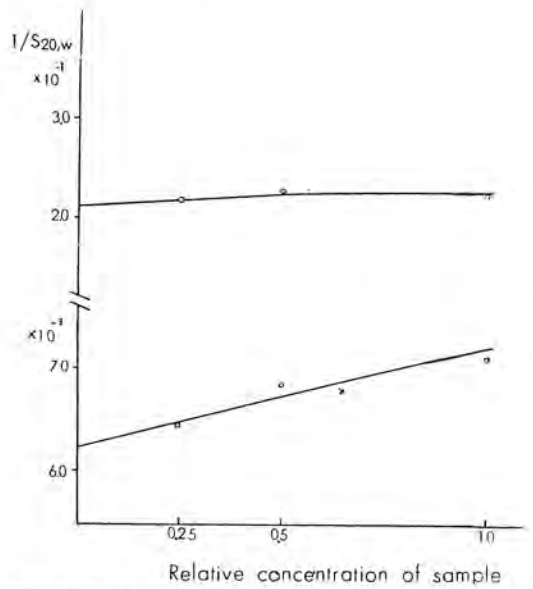

Fig. 2. Relation between the sedimentation coefficient and concentration of ether-disintegrated NDV.

Fig. 3. Gel precipitation pattern of ether-disintegrated NDV (E.D) (160,000 HA/ml) with anti-NDV rabbit serum.

3. *Separation of the 5S component by trypsinization*

While studying the effect of enzymes on the ether-disintegrated virus, the 5S component was found to appear after trypsinization.

0.2 ml of 0.25 per cent trypsin was added to 1.0 ml of ether-disintegrated NDV (160,000 HAU/ml) and the mixture incubated at 37°C for 60 minutes using 0.01 M phosphate buffered saline at pH 7.7 as the reaction medium.

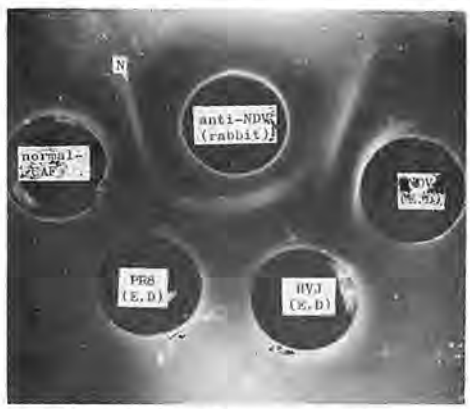

Fig. 4. Antigenical relationships between ether-disintegrated myxovirus and normal protein in uninfected chorioallantoic fluids.

Table 2. Comparison of protein content per HAU of virus particle and ether-disintegrated NDV

No. exp.	virus particle (V.P.)	ether-disintegrated preparation (E.D.)	V.P./E.D.
1	$11\times10^{-2}\mu g$/HA	$1.5\times10^{-2}\mu g$/HA	7.3
2	9.0 〃	1.5 〃	6.0
3	6.0 〃	1.2 〃	5.0

Virus particles were purified by one cycle of differential centrifugation as described in MATERIALS. The ether-disintegrated preparation used was the supernatant of the centrifugation at 12,000 rpm for 15 minutes, described in Table 1.

As shown in Fig. 1b, the sedimentation pattern of the trypsinized preparation at 20,410 rpm differs from that shown in Fig. 1a. The 160S and 47S components are scarcely recognizable but instead one component with a faster sedimention rate (ca. 1,000S), which is probably an aggregate, is observed. Another very slowly sedimenting component in contact with the meniscus is also seen. The latter component did not appear until a much higher speed was attained. Fig. 1c shows the sedimentation pattern at 59,780 rpm of the trypsinized preparation. The sedimentation constant of the latter peak was calculated to be 5.4S.

The immunological identity of the 5S component was tested by the gel precipitation reaction. The trypsinized preparation was centrifuged at 40,000 rpm for 60 minutes and the supernatant and the sedimented fractions, suspended in the original volume of saline, were diffused against anti-NDV rabbit serum, placing the untreated sample on the same plate as the control (Fig. 5).

The supernatant, containing only the 5S component, gave only a R precipitation line and the sediment produced H, S and narrow R lines. It is unknown

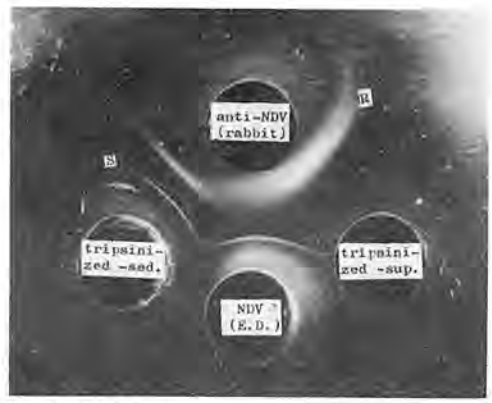

Fig. 5. Antigenical relationship between trypsinized and untreated ether-disintegrated NDV. The trypsinized sample was separated into a supernatant and sedimented fraction by centrifugation at 40,000 rpm for 60 minutes.

whether the R component in the sediment fraction is undigested and still combined or contaminated with free R component. From the experiment, however, it is certain that the 5S component found in sedimentation analysis of the trypsinized sample is immunologically identical with the R component in the agar gel precipitation test.

4. *Fractionation of ether-disintegrated NDV by zone electrophoresis*

Two ml of ether-disintegrated NDV (100,000 HAU/ml) were fractionated by zone electrophoresis under the conditions described in the previous section. The distributions of protein content and hemagglutinating activity are shown in Fig. 6.

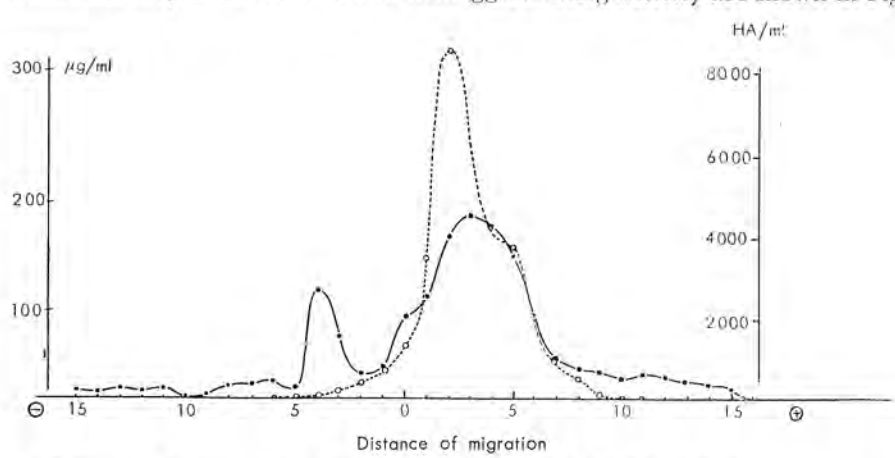

Fig. 6. Zone electrophoresis of ether-disintegrated NDV (160,000 HA/ml). Electrophoresis was carried out at 2 mA/cm² and 20 volts/cm for 14 hours at 4°C, in a vessel (30×3×1 cm).
Solid line; protein content, Broken line; hemagglufinating activity.

The results indicate a separation of hemagglutinating fraction (to the anode side) and non-hemagglutinating fraction (to the cathode fraction).

The behaviour of these fractions against anti-NDV rabbit serum was compared with that of an unfractionated sample by agar gel precipitation analysis. (Fig. 7)

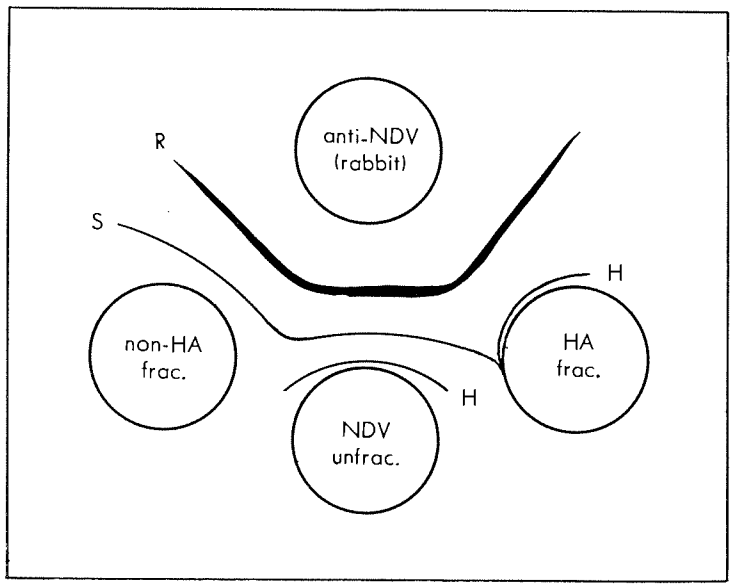

Fig. 7. Antigenical relationship between fractions separoted by zone electrophoresis and the unfractionated sample.

The hemagglutinating fraction formed R and H precipitation lines though often H was scarcely recognizable and the non-hemagglutinating fraction, R and S lines. As R line is shown in both fractions, it appears that line H represents a hemagglutinating unit and S, an s-anigen. Thus the precipitation lines have been tentatively designated as H (hemagglutinating unit), S (s-antigen) and R (rapid diffusible respectively, as shown in Fig. 7. At present it seems advisable not to define the biological identities of the antigenic components conclusively because the role of the R component and the significance of the coexistence of the R component with both the H and S components are still unknown.

5. *Antigenic relationship between ether-disintegrated NDV and the original virus particles*

As reported in the previous paper (Hosaka *et al.*, 1960a), the antigenic relationship between ether-disintegrated HVJ and the original virus particles was recognized and useful for the identification of antigenic components. With NDV, the same type of investigation was planned. Ether-disintegrated NDV (160,000 HAU/ml) and original virus particles (32,000 HAU/ml) were diffused against anti-NDV serum.

Fig. 8. Antigenical relationship between ether-disintegrated NDV (160,000 HA/ml) and original virus particles (20,000 HA/ml).

As shown in Fig. 8, the original virus particles contained a little of R component and gave a precipitation near the antigen well. The latter was situated so close to the well, that it was impossible to see the antigenic relationship between the precipitation and the S or H lines clearly, although in the Fig., it appears to fuse partially with line S. Its precipitation seems to be due to virus particles themselves and the difficulty in its identification seemed to be due to the low rate of diffusion of the particles.

6. *The effect of alkali treatment on ether-disintegrated NDV*

It is difficult to establish a direct relationship between the components found on sedimentation analysis and the components found in agar gel diffusion experiments except for the 5S component which was easily identified as the R component. Therefore an investigation was made of the effect of alteration in the sedimentation pattern under certain conditions on the gel precipitation pattern.

It has already been stated that trypsinization of ether-disintegrated NDV has no effect on the whole precipitation pattern though it had a great effect on the sedimentation pattern, causing the appearance of the 5S component and the disappearance of most of the components present before the trypsinization.

Then the effect of alkali treatment was studied. Disintegrated NDV was suspended in borate-phosphate buffer (pH 8.6, $\mu=0.02$) and dialysed against the same buffer for 24 hours at low temperature.

The sedimentation pattern of the treated sample is presented in Fig. 9a (at 12,590 rpm) and 9b (at 39,460 rpm).

Unlike the pattern of the untreated sample (cf. Fig. 1a) the treated shows 104S, 15S and 12S components. While the sedimentation pattern showed such a large alteration, the agar diffusion pattern of the treated sample was on the whole the same as that of the untreated.

When the treated sample was fractionated by centrifugation at 32,000 rpm for 60 minutes, both the supernatant and the sediment fractions showed similar precipitation patterns in agar gel diffusion experiments, although there was a quantitative difference of their antigen contents.

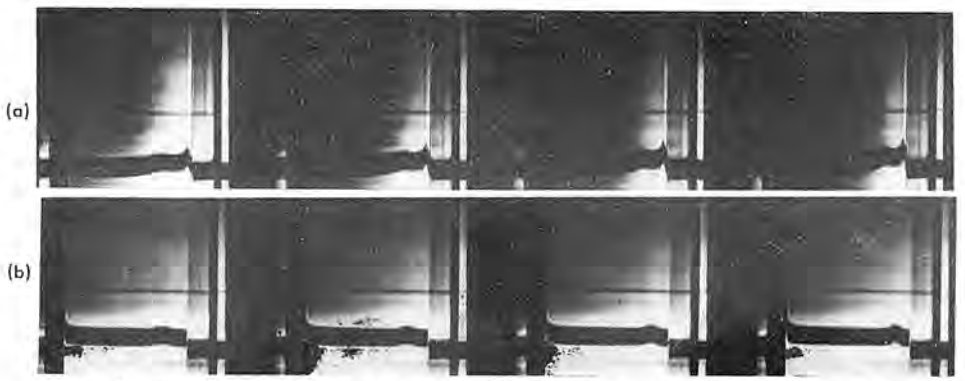

Fig. 9a. Ultracentrifugal pattern of ether-disintegrated material treated with alkali at pH 8.6, photographed at 20,410 rpm at intervals of 240 seconds.

9b. Ultracentrifugal pattern of the same sample at 39,460 rpm at intervals of 240 seconds.

The results suggest that particles containing the same antigenical components vary in size with the conditions employed.

DISCUSSION

In preparation of ether-disintegrated NDV, it was difficult to obtain as high hemagglutinating actitity as in the preparation of ether-disintegrated HVJ. The hemagglutinating titer of ether-disintegrated NDV was the same or a little higher than that of the original virus suspension. This might be due, at least in part, to the fact that about 6 hemagglutinins are liberated from a virus particle of NDV, while about 10 hemagglutinins are liberated from a particle of HVJ (Hosaka *et al.*, 1960a). The hemagglutinins of NDV may also be more sensitive to ether treatment.

On ultracentrifugal analysis, disintegrated NDV showed two components with $S_{20,w}$ values of 160S and 47S. After trypsinization, the sedimentation patterns changed greatly and a new component of 5S appeared. Trypsinzation did not affect the gel precipitation pattern of the sample and the 5S component was identified immunologically as the R component. However the situation and role of the R component is still uncertain. When ether-disintegrated material was separated into hemagglntinating and non-hemagglutinating fractions by zone electrophoresis, the R component was found in both fractions. Further, the occurrence of various sizes of particles, containing the same antigenical components was suggested by alkali treatment experiments. To establish the relationship between the sedimenting and antigenical components and their biological activity, further work is required.

The effect of trypsinization on ether-disintegrated other myxoviruses differed from in the case of NDV. With HVJ there was loss of antigenicity and a change in the sedimentation pattern in the influenzavirus PR8 strain (Hosaka *et at.*, 1960b), the sedimentation pattern was altered, but no smaller component war liberated in both cases.

Further, as with other myxoviruses, the disintegrated sample and the virus particle of NDV contain a fourth antigenic component which was identified antigenically as the CAF protein of normal chorioallantoic fluid in developing eggs. These results are consistent with the finding of Knight (1946) that influenza virus grown in the chorioallantoic fluid is antigenically related to normal protein.

The alteration in the sedimentation pattern of ether-disintegrated NDV at pH 8.6 may be related to a morphological change in the "Untereinheiten des NDV" on alkali treatment as described by Schäfer and Rott (1959).

Valentine and Isaacs (1957) reported that in NDV ribonucleic acid has a ring-like structure and is surrounded by a trypsin sensitive shell. It would be interesting to find out how the trypsin-sensitive shell is related to R component.

Recently Horn *et al.* (1960) worked on the structure and composition of NDV using the negative staining method. It requires further studies to establish the relationship of the subunits described here and the ultrastructure of the virus particles they described.

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