Some Electron Microscopic Studies on the Satellite Tobacco Necrosis Virus and its IgG-antibody

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(Accepted 12 December 1967)

SUMMARY

Satellite Tobacco Necrosis Virus is the smallest virus known to date. It has a mean diameter of 180 Å with icosahedral symmetry which was determined from shadow-cast and negatively contrasted specimens. Immunological complexes of virus and anti-virus (IgG) were freed from non-specific IgG by particle sieve chromatography. The individual antibody molecules could be detected by electron microscopy in the small complexes formed with slight antigen excess. IgG molecules either bridged adjacent virus particles by their ends or were combined to a single virus particle by one of its ends, thus radiating out from the virus capsid. A few subunits could be discerned on the IgG molecules arranged in different structural configurations.

INTRODUCTION

Satellite Tobacco Necrosis Virus is an extremely small nucleoprotein with a calculated particle weight of 1.97 x 10^6, containing 20% of RNA. The molecular weight of the empty virus-capsid is thus approximately 1.6 x 10^6, which is composed of a number of capsomeres (Reichmann, 1964; Fridborg et al. 1965). Reichmann (1964) suggested that the virus capsid is a regular icosahedron consisting of 42 crystallographic sub-units. TNV satellite is thus an excellent model for electron microscopic studies of virus antibody complexes, as it is the smallest virus known to date and it is remarkably stable (Fridborg et al. 1965).

The IgG-molecules observed on electron microscopic specimens appear heterogeneous in shape, and their dimensions vary considerably. The reported lengths of IgG average between 100 and 250 Å (Hall, Nisonoff & Slayter, 1959; Höglund & Levin, 1965; Feinstein & Rowe, 1965; Höglund, 1967a, b). Chemical studies have established that IgG antibody molecules contain two identical combining sites (Nisonoff, Winkler & Pressman, 1959).

In this paper some electron microscopic investigations of TNV satellite and its IgG antibody complexes are presented. A high-pressure spray gun for preparing specimens for electron microscopy is also described.

METHODS

Virus production. Satellite Tobacco Necrosis Virus was produced and purified according to methods previously described by Fridborg et al. (1965). Dr B. Strandberg kindly supplied purified virus suspensions suspended in 0.01 M-MgSO₄.
Preparation of IgG-antibodies. Antisera against TNV satellite, elicited by immunization of rabbits, were kindly supplied by Dr L. Philipson. The antiserum was dialysed against 0.1 M-veronal buffer at pH 8.6, with 0.02 % NaN₃ as a bacteriostatic agent. About 40 ml. of serum were applied to a 65 cm. x 11 cm. zone electrophoresis column packed with Munktell cellulose powder (Porath, 1964) and equilibrated with the same buffer as was used for dialysis. Electrophoresis was performed for 65 hr with an initial current of 560 mA which gradually rose to 650 mA. The column was eluted with the same buffer used for electrophoresis, and fractions of about 30 ml. were collected. The slower-moving region of the γ-globulin zone was pooled and concentrated by negative-pressure dialysis to 10 ml. of a 1 % solution. This was further purified by chromatography on a 100 cm. x 3 cm. column packed with Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated with 0.2 M-tris + HCl buffer at pH 8.0 containing 0.02 % NaN₃. Twelve ml. fractions were collected (Fig. 1a), and the zones containing IgG were pooled and concentrated to a volume of about 2 ml. (about 2 % concentration). The protein concentration of the solution was estimated by ultraviolet absorption at 280 nm using a value of 14.0 for \( E_{280}^{\%} \) (Porath & Ui, 1964). The virus concentration (about 0.2 %) was determined according to Kassanis & Nixon (1962), who reported a value of 6.5 for \( E_{280}^{\%} \).

Analytical ultracentrifugation. The homogeneity of TNV satellite and the IgG suspensions (Fig. 1b) was investigated with a Spinco model E analytical ultracentrifuge at a temperature of 20°. The sedimentation coefficients previously reported for IgG
and satellite virus, 6·6 S and 49 S (Fridborg et al. 1965) respectively, were used for their identification.

Immuno-electrophoresis. The immunological homogeneity of the IgG suspensions was tested by immuno-electrophoresis according to the micromodification of Scheidegger (1955) using 1·5 % Bacto Nobel agar (Difco) in veronal buffer (I = 0·045), of pH 8·2 at 6 v cm. Electrophoresis was continued for 3 hr at room temperature, and the components were developed against sheep anti-rabbit globulin (Pl. 1, fig. 1) (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.).

Precipitation tests. The precipitability of IgG was analysed by the Ouchterlony technique in 0·3 M-phosphate buffer using 1·5 % agar or by precipitation in capillary tubes; 0·1 % TNV satellite and 0·2 % IgG suspensions were used as stock solutions. IgG was serially diluted to estimate the antibody concentration, just below the equivalence zone (at antigen excess), in which small precipitates were formed to be studied in the electron microscope: 0·1 %TNV satellite precipitated by approximately 0·0125 % IgG-antibody solutions (Pl. 1, fig. 2) was found to be suitable for these investigations.

Purification of the immunological complexes. The TNV satellite-IgG complexes were afterwards separated from non-specific IgG by particle sieve chromatography on a 44 cm. × 1 cm. column packed with 10 % agarose (Hjertén, 1964), which was kindly supplied by Dr S. Hjertén. The buffer used was 0·1 M+tris HCl at pH 8·0 and the chromatography was performed at 4°. Two ml. fractions were collected (Fig. 2), which were examined in the electron microscope.

Electron microscopy. The samples were diluted in 0·1 M-ammonium acetate (pH 6·6) or ammonium bicarbonate (pH 8·0) buffers with protein concentrations between 0·1 and 0·01 %.
**Shadow casting.** The samples were applied to freshly cleaved mica with a high-pressure spray gun (Fig. 3). This was driven by air and was supplied with two air-filters ($F_1, F_2$), an air-valve ($V$), an inner glass tube for the sample ($I$) and an outer tube ($O$) with a $0.1$ mm. wide capillary at the front. About $10 \mu l$ of sample was placed in the inner tube, an air stream was regulated with the air valve, and the sample was sprayed on to a vertically placed piece of mica. Occasionally $880 \AA$ polystyrene latex spheres (Dow Latex) were mixed with the sample to be used as reference particles. The shadow casting was performed in the Siemens evaporating unit as described by Hall (1960), using platinum or a platinum alloy ($85\% \text{Pt} + 15\% \text{Ir}$). Afterwards the specimens were backed with carbon (Bradley, 1954), stripped on to double-distilled water and picked up on platinum apertures with $70 \mu$ holes.

![Fig. 3. A high pressure spray gun for application of particles and molecules on the specimens. This is driven by air filtered by two air filters ($F_1$ and $F_2$) and supplied with an air-valve ($V$), an inner glass tube for the sample ($I$) and an outer tube ($O$) with a $0.1$ mm. wide capillary at the front.](image)

The sizes of the IgG molecules were calculated from prints at a magnification of 160,000 with an ocular micrometer graduated to $0.1$ mm. Measurements were also made on both shadowed and unshadowed $880 \AA$ polystyrene latex spheres in order to estimate the approximate depth of the metal cap on the particles. The widths measured perpendicular to the shadow direction were exaggerated by approximately $50 \AA$ due to the metal cap, and this correction was applied to the widths reported in this communication.

**Negative contrasting.** The viruses were negatively contrasted by two different methods: the sample was mixed with an equal volume of $2\%$ uranyl acetate (UAc) at pH 4.6 dissolved in water or in methanol, and then placed in the spray gun. The mixture was then applied to freshly cleaved mica. This was then backed with carbon, stripped on to water and picked up on platinum apertures. Sometimes the diluted samples were applied instead to carbon-coated platinum discs by a micropipette, and the excess was
drawn off by a piece of filter paper. A drop of 1% UAc, dissolved in water or methanol, was immediately added to the sample on the disc, and the excess liquid was sucked off by a piece of filter paper. Occasionally the viruses were contrasted by 1% uranyl oxalate at pH 6.3, prepared according to Mellema et al. (1967). The mean minimum diameters of negatively contrasted particles were calculated from prints at a magnification of 400,000. The specimens were examined in the Siemens Elmiskop I, the double condenser lens being used at a magnification of \( \times 40,000 \) or \( \times 80,000 \). A 400\( \mu \) condenser aperture and a 50\( \mu \) objective aperture were utilized at an operating voltage of 60kv.

**Fig. 4.** Histograms showing the widths of UAc-contrasted TNV satellite particles at pH 4.6.

**Stroboscopy.** The micrographs of negatively contrasted TNV satellite were enlarged photographically to 4,000,000 times. These photographs of individual virus particles were placed beneath a camera and were then centred and rotated some definite angle, which was repeatedly photographed by the Markham technique (Markham, Frey & Hills, 1963).

**RESULTS**

**Characterization of Satellite Tobacco Necrosis Virus**

The diameter of 527 UAc-contrasted TNV satellite particles was calculated to be between 150 and 200\( \AA \) with a mean diameter of 180\( \AA \) (Fig. 4). Most of the particles were hexagonal, indicating a threefold or sixfold symmetry. Occasionally some virus particles with twofold or fivefold symmetry also appeared (Pl. 2, fig. 1, 2). The stroboscopy technique was used on some prints of negatively contrasted virus particles in order to evaluate possible symmetry of the 2-dimensional projections. Plate 1, fig. 3, shows two-, three- and fivefold symmetry in different TNV satellite particles with the aid of stroboscopy by rotation 180°-, 120°- and 72°-angle respectively.
The shape of the shadows which were formed from shadow-cast TNV satellite particles was also studied (Pl. 2, fig. 3) and compared with the shadows which were formed from different polyhedral paper models, which were illuminated with a spotlight at the same angle as the shadow casting was performed. Structural similarities in the shape of the shadows of the electron micrographs and from icosahedral models could be discerned. Pointed or obtuse shadows were then observed, but the exact geometrical shape of the shadow on highly magnified electron micrographs was not easily determined because of a disturbing granular background.

Occasionally a few rounded, morphological subunits could be detected on some uranyl oxalate contrasted TNV satellite particles (Pl. 2, fig. 4). The average diameter of these subunits was approximately 40 to 50 Å.

![Fig. 5. Histograms showing the widths (a) and the heights (b) of shadowcast IgG from normal human serum at pH 8.0.](image)

**Production of IgG molecules**

The fractionation of antiserum against TNV satellite by zone electrophoresis on a large-scale column (Porath, 1964) and by molecular sieving on a Sephadex G-150 column resulted in a homogenous suspension of IgG. The homogeneity was well documented by immunoelectrophoresis (Pl. 1, fig. 1), analytical ultracentrifugation (Fig. 1 b) and electron microscopy.

**The virus+antibody complex**

The virus complexes which formed between TNV satellite and its IgG-antibody were obtained in the front running fractions, well separated from non-specific IgG, by particle sieving on a short column packed with 10% agarose (Fig. 2). IgG alone was retarded on this column. The very big precipitates which formed at the equivalence zone were difficult to recover and study by electron microscopy, and complexes in slight antigen excess were therefore used. In this way we were able to obtain much smaller immunological complexes, often consisting of a few virus particles joined by IgG molecules which were suitable for electron microscopic examination.

The large precipitates of TNV satellite joined by IgG antibodies in the equivalence zone formed dense complexes which prevented the resolution of the individual IgG molecules. In the small immunological complexes produced at slight antigen excess
we were able to detect individual antibody molecules connected to the periphery of the virus capsid (Pl. 3). IgG antibody molecules then formed irregular bridges between several virus particles (Pl. 3, fig. 1, 2). The length of these IgG molecules varied between 100 Å and 150 Å, and the estimated breadth was approximately 30 to 40 Å on the UAQC-contrasted specimens. Plate 3, fig. 3, 4, shows two adjacent virus particles, bridged by one or more antibody molecules at their ends. IgG antibody molecules, combined by the end to single virus particles, were also observed (Pl. 3, fig. 5).

Negatively contrasted IgG antibody molecules appeared to contain a few rounded subunits, with a diameter between 30 and 40 Å (Pl. 3, fig. 1, 2). These rabbit IgG molecules were compared with purified and shadow cast IgG from normal human serum, which also appeared to contain a few subunits (Pl. 3, fig. 6). The latter molecules have about the same differences in configuration: some of them are elongated, while others are kinked or curved. The mean value of the width of IgG was 113 Å as calculated from prints of 683 shadow-cast IgG from normal human serum, and the mean value of the height of 699 IgG molecules was 41 Å (Fig. 5, a, b). There could be a few, possibly three, subunits arranged in different manners on these IgG molecules (Pl. 3, fig. 6). The width of the shadow-cast subunits varied between 50 Å and 60 Å (uncorrected for the metal cap).

**DISCUSSION**

Protein molecules have a great tendency to aggregate when preparing specimens for electron microscopy. Therefore the high-pressure spray gun was constructed to distribute the molecules on the specimen grid and so minimize aggregation.

Purified TNV satellite particles often crystallized on the mica surface when concentrated virus suspensions (> 0.1 %) were applied. This crystallization probably occurred when the microdroplets containing the virus and the volatile buffer were dried down on the mica, and the surface tension of the droplets forced the virus together. TNV satellite then crystallized in a monolayer of a rather high degree of order (Fridborg et al. 1965). When the high-pressure spray gun was used to apply TNV satellite on the specimens at a concentration between 0.1 and 0.01 %, the virus particles were well separated from each other. (Pl. 2, fig. 3).

Negative contrast suggested that the virus has icosahedral symmetry. Especially the fivefold symmetry detected on Pl. 2, fig. 2 clearly demonstrates this arrangement of the virus capsid according to Caspar & Klug (1962). The findings were substantiated by shadow-casting technique. The similarity in shape of the shadows which formed by shadow-cast TNV satellite particles supported the idea that the arrangement of the virus capsid is icosahedral. While negative contrast studies seemed to reveal capsomeres it is difficult to evaluate their number and arrangement; there were some indications of 5-coordinated capsomeres on a few virus particles (S. Höglund, unpublished).

This extremely small virus with its rigid and symmetrical shape was employed as an antigen, thus minimizing the interference from the structural features of the virus capsid in evaluating the conformation of the antibody molecules combined to the virus. The studies of IgG in virus antibody complexes are often obscured by the contrast agent adhering to the virus capsid, or by the varying relation in height of the layer of the contrast agent and the height of the observed molecule, when the negative-contrast technique is employed. When calculating the length of the attached antibody molecules it is difficult to establish whether the antibody is combined to the discernible periphery of the virus capsid or above or below this. The maximal length of the IgG
molecules was therefore used for those estimations from the electron micrographs giving two-dimensional images.

The appearance of the IgG antibody molecules in the virus antibody complexes seems to be variable. Some antibody bridges probably consisted of two or more parallel IgG molecules, thus giving the appearance of a broad junction (about 60 Å) between the virus particles. The curved structure of the IgG molecules within the virus antibody complexes may or may not be real, depending on the nature of the physical forces to which the complexes are subjected during drying of the electron microscopy specimens. Lafferty & Oertelis (1963) suggested that the antibody combining sites are situated at or very close to each end of the extended molecule, which is in close agreement with our findings. The same appearance was also observed when the T2 phage was used as an antigen (Höglund, 1966; 1967a). Valentine & Green (1967) have previously detected V-shaped antibody molecules, using an immunological system with a synthetic divalent DNP hapten. Therefore it seems that IgG can have different configurations when acting as an antibody, perhaps due to different spatial arrangements of the antigenic sites. Grossberg, Markus & Pressman (1965) also suggested from chemical studies that the conformation of the antibody molecule might be changed at the combination to a hapten group.

Previous studies on negatively contrasted IgG antibody molecules suggested that IgG could consist of a few subunits of more or less regular shape. Those gave the appearance of rounded projections on phosphotungstate-contrasted IgG (Höglund 1966; 1967a, b) or on UAc-contrasted IgG as shown in this study. These findings are also consistent with our observations of shadow cast IgG. However, we still do not know whether this is also the real shape of IgG in vivo.

We could assume that IgG contains a triad of hypothetical subunits, two of which possess antibody activity, combined to each other by disulphide bonds as Noelken et al. (1965) suggested. Since the metal cap of the shadow-cast IgG molecules was estimated to be 50 Å, then the mean value of the metal cap at each subunit is approximately one third of 50 Å. The calculated diameter of the shadow-cast subunits, 50 to 60 Å, must be the upper limit because of the added metal cap, and the estimated value of 30 to 40 Å of the negatively contrasted subunits must be the lower limit for the diameter of those, as previously discussed. These estimated dimensions could then give a molecular weight of IgG compatible with the reported ones, using a partial specific volume of 0.72. The subunits could perhaps be arranged in a flexible structure of IgG, which was also indicated by other physicochemical data (Noelken et al. 1965), or it might perhaps reflect different, rather rigid configurations of IgG molecules, although these molecules appeared highly purified by the physicochemical analysis employed in this study. The observed differences in configuration of IgG could perhaps also account for the great difficulty encountered in crystallizing these molecules for X-ray crystallographic studies.

I am much indebted to Professor A. Tiselius for his great interest in this work. I also wish to thank Drs E. Basset, B. Strandberg and D. H. Walker, Jun. for stimulating discussion and criticism. Mrs K. Uisk provided technical assistance.

This investigation was supported by research grants, 67:13, from the Swedish Cancer Society and AI 07382 from the National Institutes of Health, U.S. Public Health Service.
REFERENCES


(Received 8 September 1967)
EXPLANATION OF PLATES

PLATE 1

Fig. 1. Immunoelectrophoresis of rabbit anti-TNV satellite IgG, developed against sheep anti-rabbit globulin. IgG was immunologically homogeneous.

Fig. 2. Ouchterlony gel diffusion analysis of TNV-satellite virus and antibody (IgG). Trough contained 0.2% virus. Wells contained IgG serially diluted from 0.2% (from left to right). Equivalence was reached at about 0.2% virus and 0.05% IgG.

Fig. 3. Three selected prints of TNV satellite negatively contrasted with UAe. They illustrate (a) two-fold, (b) threefold and (c) fivefold symmetry. This was confirmed stroboscopically using 180°, 120° or 72° rotation respectively (above).

PLATE 2

Figs. 1, 2. TNV satellite negatively contrasted by UAe at pH 4.6.

Fig. 3. TNV satellite particles shadow-cast with Pt. The shapes of the shadows are pointed (p) or more obtuse (o). The insert clearly demonstrates this obtuse shadow shape.

Fig. 4. TNV satellite contrasted by uranyl oxalate at pH 6.3. A few rounded subunits are visible on some virus particles.

PLATE 3

Figs. 1-5. TNV satellite combined with IgG at slight antigen excess, previously freed of non-specific IgG by chromatography (Fig. 2b).

Figs. 1, 2. Some virus particles bridged by irregularly curved IgG molecules, which seem to consist of rounded subunits.

Figs. 3, 4. Adjacent TNV satellite particles, apparently bridged by a few attached antibody molecules.

Fig. 5. An IgG-antibody molecule is visible (arrowed), which is connected to a virus particle by one of its ends, thus radiating out from the virus capsid.

Fig. 6. A study of shadow-cast IgG-molecules from normal human serum at pH 8.0. These seem to consist of a few rounded subunits, arranged in elongated or irregular configurations. The arrows indicate IgG molecules which possibly contain three such subunits.