tion of RNA which is almost entirely progeny RNA, and whether the initial infecting RNA strand follows the same patterns of translation remains unknown.

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† NIH special postdoctoral fellow.
‡ USPHS Career Development Awardee.
1 Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell, these PROCEEDINGS, 49, 654 (1963).
8 Penman, S., and D. F. Summers, in press.
16 Scharff, M., A. Shatkin, and L. Levintow, these PROCEEDINGS, 50, 686 (1963).
18 Rueckert, R., personal communication.

PURIFICATION, ELECTRON MICROSCOPY, AND X-RAY DIFFRACTION STUDIES OF THE SATELLITE TOBACCO NECROSIS VIRUS*

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The Rothamsted culture of tobacco necrosis virus was long known to contain spherical particles of two sizes,1 which were recently established as two serologically unrelated viruses, one called the "satellite virus" (SV) which causes no lesions and multiplies detectably only in the presence of the other virus (TNV).2 The SV is an unusually small nucleoprotein with a calculated molecular weight of 1.97·106, containing 20 per cent of RNA.3 The genetic information carried by this RNA with a molecular weight of the order of 4·106 is very limited and it was recently proposed that SV RNA could code for a maximum of 400 amino acid residues, which is consistent with the number of amino acid residues in the coat protein.
Very large single crystals of the SV were obtained as early as 1944 and examined by crystallographic methods.4,5 The present study reports extensive purification, crystallization from aqueous solution, electron microscopy, and preliminary X-ray diffraction analysis of SV.

Materials and Methods.—Virus production: SV and TNV strain b were obtained from Dr. B. Kassanis of Rothamsted. French beans, *Phaseolus vulgaris* L. var. Cutina, and tobacco, *Nicotiana tabacum* L. var. Samsun × White Burley, were used as host plants. The plants were grown in disposable pots (Jiffy pots) filled with a granular artificial growing medium (Perlite) in a greenhouse at a temperature of 17–19°C in the case of beans and 20–23°C in the case of tobacco. They received 18 hr of light per day and were fertilized with a commercial nutrient solution every second day. During the last 24 hr before inoculation the plants were kept in total darkness at 25–30°C. Bean plants were inoculated at an age of 14–16 days. Tobacco plants were most susceptible at an age of 6 weeks during the summer and 7 weeks during the winter. The time of inoculation was found to be critical; a delay of only 3–4 days reduced the number of lesions considerably. Plants to be inoculated were dusted with 600-mesh carborundum, and the inoculum was rubbed onto the leaves with the finger. The inoculum consisted of homogenized infected leaves diluted threefold in water. The plants were watered with tap water 2 min after inoculation and transferred to a room kept at a constant temperature of 17°C, where they received 18 hr of light per day from fluorescent lamps. During the first 15 hr in the constant temperature the plants were shaded by covering them with paper. Bean plants were harvested after 5 days, tobacco plants after 6–7 days. The leaves showing the most typical symptoms from a crop of plants were selected, homogenized as described below, and stored in aliquots at −25°C for future use as inoculum. The yield of SV was 1.5–2.0 mg and 5–6 mg per 100 gm fresh weight of bean and tobacco leaves, respectively.

Homogenization: The infected leaves (100 gm) were disrupted in a homogenizer together with 20 gm sand, 20 gm ice, and 250 ml of a solution containing 1·10⁻³ M magnesium sulfate and 3·10⁻³ M sodium diethyldithiocarbamate. The sap was squeezed through cloth and the residue homogenized in 125 ml of the same solution and filtered. The two extracts were combined and stored at −25°C.

Ammonium sulfate precipitation: Cell sap was thawed and stored at +20°C for 24 hr to remove the "virus-inactivating system" and centrifuged. To the supernatant solid ammonium sulfate was added to a final concentration of 1.75 M. After storage at +4°C for 3–4 hr the suspension was centrifuged and the precipitate was dissolved in 50 ml cold 1·10⁻³ M magnesium sulfate and dialyzed overnight against this solution. Insoluble material was removed by centrifugation. This procedure was repeated once or twice. The final volume of the material did not exceed 6 ml. All centrifugations were performed at 15,000 × g for 20 min.

"Particle-sieve" chromatography was performed according to Hjertén,7 on a column of agarose spheres. The virus preparation from the ammonium sulfate step was layered under buffer on top of the agarose bed. Chromatography was performed at +4°C (Fig. 1). The absorbancy of the elute fractions was measured at 260 and 280 μ at a 1-cm cuvette.

Analytical electrophoresis: An apparatus with a horizontal electrophoresis tube rotating during the run and without a supporting medium was used.8,9 With this technique it is possible to analyze very small amounts of material.

Preparative electrophoresis was performed with agarose suspension as supporting medium in columns with an inner diameter of only 6 mm. The type of column has been described by Hjertén.9,10 The sample was applied by layering and the anode was placed at the bottom of the column. After completed electrophoresis the agarose suspension was sucked out in 1-cm fractions from the top of the column. Aliquots from each fraction were diluted 100-fold in 1·10⁻³ M magnesium sulfate, and the absorbancy was determined at 260 μ. The fractions from the peak containing SV were pooled and centrifuged at 20,000 × g for 20 min to remove the agarose.

Ultracentrifugation: Sedimentation velocity analyses were made in 0.1 M Tris-acetate buffer, pH 7.9, with 1·10⁻³ M MgSO₄ in a Spinco model E analytical ultracentrifuge at 35,000 rpm. The schlieren optical system was used.

Virus concentration was determined according to Kassanis8 who reported an extinction coefficient of 6.5 (1-cm cuvette; 1 mg/ml; 260 μ). Electron microscopy: SV preparations were suspended in 0.1 M ammonium bicarbonate (pH
7.8) or 0.1 M ammonium carbonate (pH 8.8), and applied to freshly cleaved mica by the use of a modified high-pressure spray gun according to Höglund.11 The shadow casting was performed in the Siemens evaporating unit, essentially as described by Hall,13 using platinum or a platinum alloy (85% Pt + 15% Ir). The specimens were subsequently backed with carbon, floated on double-distilled water, and picked up on platinum disks with 70-μ holes. The heights of the virus particles were inferred by examining shadowed specimens containing a mixture of the viruses and 264-mu polystyrene spheres (Dow Chemical Co.).13

The negative contrast was performed with pho-photungstic acid (PTA) adjusted with ammonia14 to a slightly alkaline pH-value (7.6); the final PTA-concentration was about 1%/C. Carbon-coated platinum disks were used. The specimens were prepared at pH values above the isoelectric point which is close to pH 7.

The specimens were examined in the Siemens-Elmiskop I. The double condenser lens was used at a magnification of 40,000 or 80,000. A 400-μ condenser aperture and a 50-μ objective aperture were utilized at an operating voltage of 60 kV.

**Crystallization of SV:** The crystallization was done in capillaries of the type generally used when mounting wet protein crystals for X-ray investigations. After the virus solution was sucked into a capillary, the ends were covered with plasteline which allows for a slow evaporation. This crystallization procedure has several advantages. (1) The crystal growth and the evaporation rate may easily be controlled, and the crystals can be observed microscopically during growth. (2) Very small amounts (0.1-0.2 mg) of virus material may be used for a crystallization experiment. The method is likely to be of importance for the crystallization of other compounds, of which the amounts are very limited. (3) The crystals can be examined by X rays in the same capillary as they are grown.

**Density of SV crystals:** The density of SV crystals was determined using the flotation method on crystals in capillaries 0.5 mm in diameter. The mother liquor was first removed from the crystals by means of narrow capillaries. Then a mixture of bromoform and acetone of known density was sucked into the 0.5-mm capillary, and one end was sealed off with black wax. A glass fiber was used to detach the crystals from the walls of the capillaries, and then the crystal motion was observed with the capillaries vertical.

**X-ray diffraction:** A Philips fine-focus X-ray tube and collimators with diameters 0.20-0.60 mm were used. The photographs were taken with a Buerger precession camera, using unfiltered CuKα-radiation. “Still” photographs were taken at spindle intervals of about 20°, two different reciprocal crystallographic axes along the spindle, crystal-to-film distance of 4 cm, and exposure times of about one hour. The exposure times used when obtaining 5° precession photographs were about 20 hr with a crystal-to-film distance of 7.5 cm.

**Results.—Virus purification:** Chromatography on columns of agarose spheres of the virus preparation purified by ammonium sulfate precipitation revealed several components as shown in Figure 1. The ratio in absorbancy at 260 and 280 μ is largest in the material in the peak centered around fraction 72. It was therefore assumed that this peak contained the SV, which was confirmed by electron microscopy. The associated virus, TNV-b, was removed as an insoluble deposit in the ammonium sulfate precipitation.

![Graph](image-url)
Fig. 2.—Analysis by free zone electrophoresis of the SV-containing peak obtained from a chromatographic experiment similar to that shown in Fig. 1. The inner diameter of the electrophoresis tube: 3 mm. Buffer: 0.07 M sodium cacodylate, pH 6.33. Current: 8 mA. Voltage: about 1400 v. Temperature of the cooling water: 1.5°C. Sample vol: 15 μl. Sample concentration: 0.02% . The electrophoresis tube was scanned by TV light (280 mm) at the times indicated.

The homogeneity of the material from the agarose chromatography was tested by free zone electrophoresis. Figure 2 shows that already after 4 min of electrophoresis two peaks were observed and on prolonged electrophoresis a minor zone migrated out of the stationary main zone. The material in the stationary zone was sucked out of the electrophoresis tube and examined in the electron microscope. This highly purified SV had a great tendency to crystallize on the grids in monolayers; the angle between the rows of virus particles was about 64° (see Fig. 3).

The analytical electrophoresis revealed that the main impurities have a considerably higher mobility than SV. Consequently, the preparative electrophoresis could be performed in narrow columns loaded with large sample volumes and yet yield an efficient separation. When further increased capacity was required, two starting zones were applied in the same column. Figure 4 shows a typical diagram after preparative electrophoresis in agarose columns. The SV material was identified by electron microscopy (Fig. 6). The material contained 20 per cent RNA as tested by the orcinol method for RNA15 and a modified Folin method for protein.16

Analyses in the ultracentrifuge of an 0.88 per cent virus suspension gave an uncorrected value for the sedimentation constant of 45.7S (Fig. 5). For a small peak, not exceeding 5 per cent, the uncorrected value was 202S. The values given by Kassanis and Nixon17 for SV and an observed aggregate of 12 SV particles are 49S and 222S, respectively. The ratio between these values is 4.53 and the ratio between our S-values is 4.42.

Electron microscopy: Negatively stained SV had a diameter of about 170 Å, as seen in Figure 6, and the height deduced from the shadow lengths in shadowed preparations was...
about the same. A few empty capsids were sometimes observed on PTA contrasted specimens.

**Crystallization of SV**: SV in 1.0-1.5 M MgSO₄ at a concentration of 1.65 per cent was sucked into capillaries 1.0–1.5 mm in diameter and was crystallized at room temperature as described in Methods. It has been possible to get large SV crystals (up to 0.5 mm × 0.5 mm × 0.2 mm) in 1–2 weeks. Most crystals are rhombic plates, as shown in Figure 7, but a few somewhat smaller triclinic prisms are also formed. The crystals produced were of the same size and quality whether the material was purified only by chromatography or by chromatography and electrophoresis.

**X-ray investigation of single crystals of SV**: Selected crystals in the capillaries were oriented for X-ray investigation, and all mother liquor except a small drop around the crystal was removed. Because of the good morphology of SV crystals a unique crystallographic axis could be chosen optically, and therefore the exposure time needed for the setting of a crystal was short. Crystals grown directly after the agarose chromatography gave the same X-ray data as those grown from material which had been further purified by electrophoresis. Even with a 1-hr exposure time, reflections out to a resolution of 2.3 Å were obtained in the “still” photographs, as seen in Figure 8A, and from these photographs the directions and (for a chosen crystal class) the magnitudes of cell axes perpendicular to any particular reciprocal lattice plane were measured. The 5° precession photographs with the X-ray beam parallel to the two shortest axes chosen from a great number of still photographs led unambiguously to a strict monoclinic cell. Systematic extinctions were found for reflections where \( h + k = 2n + 1 \). The absences were found in hk0 and 0kl.
A structure containing L-amino acids cannot have symmetry elements such as glide-planes, mirror planes, or inversions. Therefore, we conclude that at least to 5 Å resolution (9° picture; see Fig. 8B) the space group is C2.

The cell dimensions measured from the precession photographs are: \( a = 319 \pm 2 \) Å; \( b = 304 \pm 2 \) Å; \( c = 185.5 \pm 1 \) Å; \( \beta = 94.022' \pm 5' \); \( V = 17.9 \cdot 10^6 \) Å³. The b-axis lies along the acute bisectrix, and the c-axis along the obtuse bisectrix of the rhombic face of a crystal.

The density of the crystals is \( 1.34 \pm 0.01 \) gm/cm³ determined as described in Methods. From the above figures and a molecular weight\(^3\) of \( 1.97 \cdot 10^6 \) the water content of the unit cell has been calculated. If we assume that there are two virus particles per unit cell, there is 72.8 per cent water by weight, and with four particles the water content is 45.5 per cent. A water content of 55 per cent has been reported for crystalline bushy stunt virus\(^8\) \( (M = 8.9 \cdot 10^6) \). The value of 72.8 per cent water for SV crystals is therefore very unlikely. Hence we assume that there are four virus particles in the unit cell.

It has been pointed out\(^19\) that small virus particles, as far as is known, exhibit icosahedral symmetry. The fivefold axes of point group 532 should reveal themselves in the X-ray photographs as "spikes," that is, the intensities tend to be stronger in certain directions. The same holds for noncrystallographic threefold and twofold axes. This feature was first shown in X-ray photographs from bushy stunt virus.\(^20\) SV which crystallizes in a monoclinic system can obviously have only one twofold axis of the crystal system coinciding with one of the 15 twofold axes of a particle with the possible 532 symmetry. No thorough investigation of the intensity distribution in X-ray photographs of SV has so far been made. However, in the precession

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**Fig. 6.**—Electron micrographs of SV negatively contrasted with 1% neutralized phosphotungstic acid. Insert gives shadowed preparations of SV virus. The prints are reversed.

**Fig. 7.**—Rhombic crystals of SV. Note the sharp edges. (Magnification × 50.)
photosgraphs streaks of high intensity can be seen in certain directions (Fig. 8C). In precession photographs the reflections in the inner region of the pattern have comparably high intensities because at low angles the whole virus particle or each of its subunits is scattering as a body. Further out, the reflections are weaker, but strong enough to be clearly visible on 12° precession photographs, that is, 3.7 Å resolution (see Fig. 8D).

Discussion.—The methods selected for purification of SV have all been described in detail in earlier publications.7-10 These methods fractionate particles according to size, shape, and surface charge, but do not differentiate empty capsids from intact particles.21, 22 The final product was therefore subjected to analytical ultracentrifugation and caesium chloride gradient centrifugation, but no protein shells were observed with these methods. In spite of this, empty capsids were sometimes observed in the electron micrographs (Fig. 6). These damaged particles most certainly arise during preparation for electron microscopy, since it was verified by equilibrium gradient centrifugation that neutralized phosphotungstic acid produced protein shells from SV virus. The over-all purification factor from bean leaves was of the order of 2,000-5,000.

Wet SV crystals were found to have a strict monoclinic unit cell, probably containing four SV particles, when crystals were grown in aqueous solution with 1·10⁻³ M MgSO₄.

Labaw23 reports that a monoclinic tetramolecular unit cell can be deduced from
electron micrographs of crystals of SV slowly grown in concentrated aqueous solutions. During the preparation for an electron microscopy investigation, the crystals dry and the cell dimensions are affected. Therefore, Labaw's values for the unit cell dimensions cannot directly be compared with our cell dimensions from X-ray work on wet crystals.

It is important to note that Labaw's and our results indicate a strict monoclinic unit cell in both dry and wet crystals, respectively. These findings differ from the X-ray diffraction results which are reported by Cowan and Crowfoot-Hodgkin on dry crystals (triclinic cell) and by Crowfoot and Schmidt on wet crystals (triclinic or pseudomonoclinic cell). There is some resemblance between our cell dimensions and those of Crowfoot and Schmidt but the discrepancies are also very clear:

<table>
<thead>
<tr>
<th>Crowfoot and Schmidt 4</th>
<th>The present investigation</th>
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<tr>
<td>( a ) 346 Å</td>
<td>319 Å</td>
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<tr>
<td>( b ) 303 Å</td>
<td>304 Å</td>
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<td>( c ) 179 Å</td>
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<td>( \beta ) 101°</td>
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<td>( \gamma ) 96°</td>
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The techniques used by Crowfoot and Schmidt differ from ours in the following respects: (1) Crowfoot and Schmidt's crystals had not been grown from pure virus material. (2) They examined a crystal, which was lying in a drop of 0.4 \( M \) ammonium sulfate. (3) In 1945 precession cameras were not available. Crowfoot and Schmidt therefore had to characterize the unit cell only from "still" photographs and \( 21/2 \)° oscillation photographs.

The present report is the first in a program aimed at resolving the structure of a small spherical virus. The complete structure of SV would ultimately inform about the genetic code. Primarily, work is in progress to determine whether SV RNA codes for its own structural protein and additional proteins and whether SV RNA is oriented in the same way in all the particles in a crystal. Some experiments aimed at preparing isomorphous heavy atom derivatives of SV have been started.

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3 Reichmann, M. E., these PROCEEDINGS, 52, 1009 (1964).
INHIBITION OF DNA AND RNA POLYMERASE REACTIONS
BY CHLOROQUINE

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Chloroquine is one of a series of 4-aminoquinoline antimalarial compounds whose biology and chemistry were studied extensively during and immediately following World War II.1-3 Early investigations by Parker and Irvin4 demonstrated the ability of chloroquine to bind to both DNA and RNA in vitro, suggesting a possible mechanism by which this drug might interfere with cellular processes in malarial parasites. Subsequently, chloroquine has been found to be effective in controlling infestations by other species of parasites,5 and recently effects of this compound on the metamorphosis of tadpoles,6 and on cell division in tissue culture7 have been reported. The mechanism of action of chloroquine is not known, although effects on various enzyme systems have been described.8,9

Several years ago, it was demonstrated that chloroquine can inhibit incorporation of radioactively labeled nucleic acid precursors in DNA and RNA of intact Plasmodium gallinaceum and P. berghei,10 and more recently it has been shown that 4-hydroxychloroquine, a closely related analogue, has a similar effect on L-fibroblasts growing in tissue culture.11 The observations that the binding of chloroquine to DNA can alter the biological12 and physical13 properties of the DNA helix suggests that this inhibition of cellular processes might indeed result from complex forma-