STUDIES ON THE TOBACCO MOSAIC VIRUS NUCLEIC ACID

by

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INTRODUCTION

Investigation Objectives

The purification and crystallization of tobacco mosaic virus (TMV) by Stanley (1) in 1935 provided a system in which large amounts of virus could be obtained in a highly purified form. Since that time TMV has been intensively investigated by many workers. Interest in the virus was greatly enhanced in 1956 when Fraenkel-Conrat (2) and Gierer and Schramm (3, 4) reported strong evidence that the ribonucleic acid (RNA) portion of the virus was infective by itself. This implied that the RNA was functionally involved in the hereditary system of the virus. The structural codes for both the RNA and the specific viral protein must then reside in the RNA.

Thorough physical and chemical characterization of the RNA is a prerequisite to an understanding of the mechanisms involved in its biological functions. Conflicting reports have been given concerning some of the basic properties of the infective unit. The objectives of the investigations to be described were to determine directly the size of the infective element by partition cell ultracentrifugal analysis and to provide additional information concerning the physical and chemical structure of the RNA.
Brief Review of Literature on Tobacco Mosaic Virus

X-ray diffraction and electron microscopic studies reveal TMV to be a helical grooved rod with a mean diameter of 150 Å and a length of 3000 Å (5, 6, 7, 8, 9, 10). The molecular weight of TMV is between $30-50 \times 10^6$ (11, 12, 13, 14). From light scattering data, Boedtker and Simmons (15) have recently calculated the molecular weight of a highly purified TMV to be $40 \times 10^6$. This is in agreement with the molecular weight calculated on the basis of the protein subunits to be discussed below.

The virus is composed of protein and ribonucleic acid (16, 17). It has been demonstrated that under alkaline conditions the protein is degraded to subunits with approximate molecular weights of 90,000 (18, 19). These subunits can be made to polymerize again into rods resembling the virus. The carboxypeptidase experiments of Harris and Knight (20, 21) suggest on the basis of released threonine that the chemical subunit has a weight of 17,000. X-ray diffraction studies indicate an axial repeat period of 69 Å (9, 10) and diffraction patterns of mercury substituted TMV show 49 subunits for each 69 Å of length (22). These findings are consistent with a chemical subunit of 17,000 m.w. in size and with TMV of particle weight $40 \times 10^6$.

The amino acid composition of TMV has been investigated quite thoroughly (23, 24, 25, 26). Sequential analysis of
the amino acids of the subunits is being pursued at the University of California (26).

Five to six per cent by weight of the virus is nucleic acid. Knight and Woody (27) in their recent investigations of many different preparations quote a value of 5.1%.

The location of this RNA in the intact virus is of considerable importance and has been examined by electron microscopy of partially degraded virus and by X-ray diffractions. Hart (28) has removed protein from the ends of TMV in a controlled fashion. Electron micrographs of such material show fibers 30-40 Å in diameter extending from the ends of the undegraded portion. These fibers are sensitive to ribonuclease but not to trypsin. As a corollary, central holes may be seen in native protein without nucleic acid. Electron micrographs (29) of alkali degraded TMV reveal a beaded structure in which a filament 30-40 Å in diameter joins the undegraded units of protein. Intact TMV, however, has a central hole of 20 Å radius according to X-ray data (30, 31). At 40 Å radius the virus shows a high electron density in contrast to re-aggregated protein which shows a minimum (31, 32). The conclusion is that the sugar phosphate backbone of RNA lies at this radius.
Review of Literature on TMV-RNA

The base ratios of RNA have been obtained by several investigators (24, 33, 34). Various strains of TMV which differ in their amino acid composition have very similar base ratios. Regularities, such as exist for deoxyribonucleic acid (DNA), have not been found. The study of pancreatic ribonuclease digests of RNA show the existence of a large number of different nucleotide sequences (35, 36, 37).

Cohen and Stanley (38), using a heat-salt method for the separation of RNA from protein, obtained a molecular weight of 300,000 for the RNA which could be degraded to units of 60,000 m.w. Using a modification of Cohen and Stanley's isolation procedure, Northrup and Sinsheimer (39) and Hopkins and Sinsheimer (40) were first to provide evidence that all of the RNA in one virus particle could be obtained as a single molecule with a weight between 1.7 and 2.0 x 10^6. The above value was determined from light scattering data. Combining data from sedimentation rates with those from specific viscosity, Gierer (41) calculated a molecular weight of 2 x 10^6 for RNA dissociated from protein with water-saturated phenol. The size of the radiosensitive volume for RNA agrees with these results (42). Hart (43) has reported electron microscopic evidence for the existence of RNA as a single molecule in the virus particle.
The configuration of the RNA depends upon the solvent. It appears to contract with increasing salt concentration (40). Gierer in a seminar on the characteristics of TMV-RNA at the California Institute of Technology in September, 1958, claimed that reversible changes occur upon mild heating, application of urea, and change of solvents. The kinetics of enzyme degradation of RNA (41) suggest a single stranded molecule in contrast to a double stranded molecule such as described by Thomas for DNA (44).

Review of Literature on Infective RNA

Infective RNA has been isolated by the use of sodium dodecyl sulfate (SDS) plus mild heat (2, 45) and by the use of water-saturated phenol (3, 4).

The claims for the infectivity are subject to certain limitations. That the biological activity of the virus is not a function of the protein by itself has been clearly demonstrated (2, 18). Various types of chemical changes in the protein of intact TMV do not destroy its infectivity. Approximately 70% of the amino groups may be acetylated without loss of infectivity (46, 47). Similarly, the oxidation of sulfhydryl groups with iodine does not decrease the specific infectivity (48). Portions of the protein can be removed by carboxypeptidase (20), incubation at pH 9 (29), and by SDS (49) without destruction of infectivity.
Fraenkel-Conrat et al. (45) cite six lines of evidence for associating the infectivity with RNA. The infective principle could not be centrifuged under conditions which effectively sedimented dilute solutions of TMV. Electron microscopic investigation of centrifuged RNA for full length TMV particles did not reveal a sufficient number of them to account for the infectivity. In very low concentrations, crystalline ribonuclease completely destroyed the biological activity. A thousand fold increase in enzyme concentration had no effect on TMV activity. RNA solutions lost their infectivity rapidly at 36°C in contrast to the stability of intact TMV. Treatment with anti-TMV-γ-globulin inactivated TMV suspensions but had appreciably no effect on RNA. Finally, a microbiuret test and an analysis by two dimensional chromatography of a hydrolyzed nucleic acid sample gave limits of 0.5 and 0.1% protein contamination.

No protein has been detected either by chemical or serological methods in phenol prepared RNA (3, 4). The reliability of the tests indicated that less than 0.1% by weight of RNA could be protein and less than 0.02% could be native protein. Since the infectivity is only 4% of an equal weight of TMV, it is not possible to exclude the presence of some amino acids in the infective unit.

Takahashi et al. (50) have subjected RNA to chromatography and shown that the fractions that tend to have the highest specific activity also have a positive protein reaction.
with brom phenol blue. The results of their experiments, however, are not conclusive.

Before the infectivity of RNA was established, it had been shown that SDS-prepared RNA mixed with protein subunits at pH 6 caused a repolymerization of the proteins into nucleic acid containing rods which were biologically active (51). Reconstituted viruses have been reported with 30% of the activity of intact viruses (52). These results have been used to support the contention that the low efficiency of infection for RNA does not reflect a minor component of the RNA but rather reflects the low plating efficiency for the bulk of the material.

Conflicting values have been given for the minimal size of the infective unit. It has been claimed that the sedimentation characteristics of infective RNA are not compatible with a molecular weight of $2 \times 10^6$ (45, 52). Gierer (53), on the other hand, claims on the basis of data obtained from differential centrifugation and from the kinetics of the loss of activity of RNA upon digestion by ribonuclease that the molecular weight does correspond to $2 \times 10^6$. Recently, Schuster and Schramm (54) have treated RNA with nitrous acid which converts cytosine, guanine, and adenine into uracil, xanthine, and hypoxanthine respectively. They found that the alteration of any base out of 3300 nucleotides was lethal. This would argue against small units carrying infectivity. Fraenkel-Conrat (52) has suggested that phenol-
prepared RNA has the larger size but that SDS prepared RNA does not. The work to be reported here should resolve some of these differences.
MATERIALS AND METHODS

Preparation of Virus Stocks

Dr. Wildman of the University of California at Los Angeles kindly supplied enough tobacco plants infected with the common variety of TMV (55) to permit the isolation of a stock solution. All experiments were performed with this strain of virus. Turkish tobacco plants (Nicotiana tabacum var. Turkish) were inoculated by lightly rubbing the leaves with pads of cheesecloth dipped into a 1.0 μg./ml. suspension of TMV containing 20 mg./ml. carborundum. Approximately one month later the leaves were harvested and stored in a deep-freeze (-14°C) until needed.

The purification of the TMV originally was accomplished by differential centrifugation of the expressed tobacco juice (15). Because TMV, so prepared, tended to dimerize, a purification method, demonstrated by Dr. Simmons of the University of California at Los Angeles in October, 1957, was adopted. The procedure described below routinely yielded monodisperse TMV preparations.

The frozen infected tobacco leaves, stored in polyethylene bags, were pounded with a wooden mallet until well pulverized. One hundred grams of the pulverized leaves were placed in a pint Mason jar and 100 ml. of cold 0.04M NH₄OH plus a few drops of capryl alcohol to prevent foaming were
added to the jar. The above operations were carried out in a 4°C cold room. The remainder of the procedure was performed at room temperature except for centrifugations which were carried out at 1-2°C. The Mason jar was fastened to an Omnimix blender furnished with sharpened blades. The Mason jar was usually lowered into a lukewarm water bath to facilitate the initial movement of the frozen leaves in the blender. The blender blades were allowed to spin relatively slowly until the whole mass of leaves was moving. After this was accomplished, the blender was brought up to speed and the contents were blended two to three minutes.

The blended leaves were quickly transferred to a 400 ml. beaker, in which they were stirred with a mechanical stirrer. Capryl alcohol was again added. In later preparations the blended material was first strained through cheesecloth and squeezed dry. The pulp was discarded. After pH electrodes were placed in the contents, 25 gms. of Amberlite IRC 50 (16-50 mesh) in hydrogen form was added. The pH of the solution always dropped rapidly from the initial value to 5 or 6. The pH was quickly brought back to 7.3 by the addition of Dowex 1-X8, 50-100 mesh resin in the amine (OH-) form. The Dowex was added in approximately 0.5 gm. batches

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1 The resin used in these preparations was the analytical grade Dowex 1-X8 Anion Exchanger, 50-100 mesh Chloride form from the Dow company. The resin was converted to the OH form as follows:

1) 400 gms. Dowex 1-X8 was stirred (Continued next page)
at 10-20 second intervals during which the pH increase was observed. Addition of large quantities caused the pH to overshoot 7.3. This process was carried out as rapidly as possible to avoid aggregation. The pH was kept at 7.3 ± 0.2 by periodic addition of Dowex until the pH remained constant for a period of 5-10 minutes. The time required for this was approximately 30 minutes.

The material was then centrifuged in a No. 845 rotor for thirty minutes at 5000 r.p.m. in an International Refrigerated Centrifuge. A maximum relative centrifugal force of 4000 developed at the tip. This served to sediment the resins and most of the remaining pulp. The supernatant was filtered through fluted No. 410 S and S filter paper into a graduated cylinder. One-tenth volume of 0.1M Versene pH 7.0 was added and the pH of the solution was immediately brought back to pH 7.5 by addition of 1.0M NaOH dropwise. The virus was spun down in the refrigerated Spinco Model L with the 30-rotor at 28,000 r.p.m. for 45 minutes. One-half of the supernatant was removed, a drop of capryl alcohol added, and

(Continued from previous page) in one liter of 10% NaOH for one-half hour.

2) The suspension was filtered on a Buchner funnel under aspiration.
3) (1) and (2) were repeated two more times.
4) The Dowex was placed on a column and washed with boiled distilled water until the effluent was pH 9.
5) The capacity of the resin was approximately 0.8 meq./gm. as checked by titrations with 1N HCl using Neutral Red as an indicator.
the tube stoppered. After shaking the contents vigorously to remove green debris from the pellet, the remainder of the supernatant was poured off. A virus pellet remained which was usually clear but occasionally had a greenish central spot. The pellet was rinsed with 0.001M Versene pH 7.5 and the walls were wiped with tissue paper. After the pellets were dissolved in 30 ml. of 0.001M Versene pH 7.5, the suspensions were combined in one test tube and stirred for half an hour at room temperature or left to redissolve in the cold room overnight. Following a centrifugation for 30 minutes at 12,000 r.p.m. in the Model L Spinco with the 30-rotor to remove debris, the virus was again spun down at 28,000 r.p.m. for 45 to 60 minutes. The virus was resuspended in 0.001M Versene pH 7.5 and subjected to another low speed (12,000 r.p.m.) spin. A very clear virus solution was usually obtained by this time which appeared as a single hypersharp peak in the Schlieren photograph of sedimentation. The yield varied between 2-4 mg./gm. leaves.

The virus at approximate concentrations of 10 mg./ml. was stored in 0.001M Versene pH 7.5 at 4°C until used. Usually the virus was used within a week or two after preparation.

Preparation of RNA

RNA isolated from the protein either by Fraenkel-Conrat's detergent method (45) or by Gierer and Schramm's phenol
method (3, 4) was used in all experiments.

In sodium dodecyl sulfate (SDS) preparations of RNA, 20-40 ml. of a 1.0% solution of TMV in 0.01M phosphate pH 7.5 were used routinely. A typical preparation involving a 30 ml. TMV solution will be described. If the stock solution of TMV was in 0.001M Versene, it was spun down and redissolved in 0.01M phosphate pH 7.5. It was found that RNA yields were very low when the phosphate buffer was not used. The TMV was adjusted to a 1.0% concentration with 0.01M phosphate. Thirty μl. of 0.1M Versene pH 7.0 was added to 30 ml. of the solution to adjust it to 10^-4M in Versene. After the solution was warmed to 50°C in a water bath, its pH was adjusted to 8.5 with 0.1M NaOH. Following the addition of 7.5 ml. of a 5% SDS solution, the TMV solution was held at 50 ± 0.1°C for 5 minutes. The opalescent solution cleared during the five minutes of mild heating. The solution was quickly cooled to 26°C in an ice bath and 18.75 ml. of saturated (NH₄)₂SO₄ was added (0.33 saturation). The (NH₄)₂SO₄ was saturated at room temperature. After a few minutes, the protein precipitate was spun down in a small angle head Servall at 4°C. The supernatant was left in a 4°C cold room overnight to permit the RNA to precipitate. All subsequent operations were performed at 4°C. The precipitate was sedimented in the small angle head Servall (15 minutes, Variac at 80) and redissolved in 7 ml. cold deionized water. After the addition of 14 ml. cold ethanol
plus 2 drops of 3M pH 5 acetate, the precipitate was again spun down. The sediment was resuspended in 4 ml. of cold deionized water and was again precipitated with 8 ml. of cold ethanol plus a drop of the acetate. After centrifugation, the pellet was dissolved in 4 ml. cold deionized water. Following a low speed spin to eliminate all water-insoluble material, the RNA solution was subjected to centrifugation at 40,000 r.p.m. in a Spinco Model L with a 40-rotor for one to two hours in order to get rid of contaminating proteins. A yield of 10.5 mg. of RNA at a concentration of 2.63 mg./ml. was obtained from the 30 ml. TMV solution. The RNA was usually diluted to 1.2 mg./ml. with cold deionized water, frozen in an acetone-dry ice bath, and stored in a -60°C deepfreeze.

The procedure for preparing RNA with phenol is given below. All operations were carried out in the cold room at 4°C. To 10 ml. of a 6% TMV solution in 0.02M phosphate pH 7.3 in a 50 ml. separatory funnel, 10 ml. of water-saturated phenol was added. The mixture was shaken vigorously for 10 minutes after which it was centrifuged for 5 minutes in a small angle head Servall to separate the water phase from the phenol phase. The water phase was pipetted off and placed back in the separatory funnel. The phenol extraction was repeated two more times with only 5-8 minute shaking periods. The phenol phases were pooled in the separatory funnel, and 1-2 ml. of buffer were added. After
extraction, the buffer was added to the RNA solution. An equal volume of ether was then added to the RNA solution and shaken for 5 minutes. The heavier, RNA-rich water phase was drained into a beaker. The ether extraction to remove traces of phenol was repeated two more times. The ether was removed by bubbling dry nitrogen through the solution for 20-30 minutes. Traces of protein were removed by centrifugation at 40,000 r.p.m. in the Spinco Model L for one hour. In the early experiments, the RNA was precipitated with two volumes of ethanol, spun down in the small angle head Servall, and resuspended in 0.02M phosphate pH 7.3. For later experiments the alcohol precipitation was repeated an additional three times. The resulting preparations were considerably more stable than the once precipitated preparations.

The RNA concentration was usually adjusted to 1.2 mg./ml. with 0.02M phosphate pH 7.3 and stored in the same manner as the SDS preparations.

The stability of frozen samples as judged by ultracentrifugal analysis and residual infectivity indicated that preparations did not change appreciably over a period of several weeks.

The Biological Assay System

Biological assays were performed on the leaves of *Phaseolus vulgaris* L. var. Pinto (pinto beans), *Nicotiana
tabacum L. var. Xanthi-nc (Xanthi), and the Holmes hybrid L. L., each of which produces local lesions upon infection with TMV or TMV-RNA.

Pinto bean seeds were originally germinated and grown in three inch cups containing fumigated vermiculite as soil in an air-filtered greenhouse under uncontrolled temperatures. Later it was discovered that seeds, germinated and grown under climate controlled conditions, produced plants which were twenty times as sensitive and much more uniform in their response to infection than those grown in the air-filtered greenhouse. The Earhart Laboratories at the California Institute of Technology provided facilities which were kept at 26°C for 9 hours followed by 15 hours at 20°C. Auxiliary lights were used in the morning and evening to provide a 16 hour day and an 8 hour night. Air pollutants were removed by an elaborate filtering system.

The pinto beans were grown in this laboratory and routinely removed to the air-filtered greenhouse two weeks later.

Twenty-four hours before use, the plants were placed in the dark. The leaves were then dusted evenly with carborundum from a salt shaker. One drop of the test solution, kept in test tubes immersed in an ice bath until inoculation time, was placed on the center of the leaf by means of a glass capillary with a 5 mm. inside diameter. The drop was spread evenly over the surface of the leaf with a glass spatula applied with uniform pressure. The opposite leaf
received the same treatment with a control solution. Ten to twenty replicates were made of each test. Five to seven days later, the lesions were counted under a low power microscope.

Xanthi and Holmes L. L. seeds, generously supplied by Dr. Takahashi from the University of California and Dr. Newmark from the University of Kansas respectively, were germinated in flats containing vermiculite for soil. After the seedlings reached a height of one or two inches, they were transplanted to individual four inch cups. Approximately four weeks later they were ready for use. The assay procedure was the same as that described above except that adjacent half-leaves were used for the control and test solutions. Four to six leaves per plant were utilized.

In all cases, the concentration range yielding lesions proportional to the concentration was established. Figures 1, 2, and 3 show the lesion response as a function of RNA concentrations for pinto beans, Xanthi, and Holmes respectively. Control and test solutions were diluted with 0.02M phosphate, pH 7.3 to fall within appropriate concentration ranges.

Analytical Ultracentrifugation Techniques

All analytical centrifugation was performed with a Model E Spinco Ultracentrifuge and its standard equipment.
Figure 1. Lesion response of pinto beans to ribonucleic acid. Linear range: 5-20 µg. RNA/ml.
The graph shows a relationship between the concentration of ribonucleic acid (µg/ml) and the number of lesions per leaf. The concentration of ribonucleic acid increases along the x-axis, while the number of lesions per leaf increases along the y-axis. The data points indicate a positive correlation, with higher concentrations of ribonucleic acid leading to a greater number of lesions.
Figure 2. Lesion response of Xanthi to ribonucleic acid. Linear range: 0.1-0.5 μg. RNA/ml.
Figure 3. Lesion response of Holmes to ribonucleic acid.

Linear range: 0.05-1.0 μg. RNA/ml.
RNA was ordinarily centrifuged at 5°C either in 0.02M phosphate pH 7.3 or in 0.2M salt plus 0.001M phosphate pH 7.5.

Uncorrected sedimentation constants were determined from velocity sedimentation experiments by the well known relationship, \( s = \frac{1}{\omega^2} \frac{d\ln x}{dt} \). It was found experimentally, in agreement with the calculated value, that the \( s_5 \) had to be multiplied by a factor of 1.5 to obtain \( s_{20}' \). Velocity sedimentation rates were determined by means of either Schlieren optics or ultraviolet absorption optics. For the former, 1-1.1 mg./ml. of RNA were used, and for the latter, 20-50 \( \mu \)g./ml. These will be called respectively Schlieren and UV concentrations.

It was assumed that sedimentation rates obtained at 20 \( \mu \)g./ml. were not appreciably different from those obtained by extrapolation to infinite dilution, where no molecular interactions occur.

Experiments involving the Waugh partition cell were carried out as described by Yphantis and Waugh (56). With this cell, the sedimentation rate for a biologically active substance can be determined in the presence of biologically inactive materials. The cell is a conventional 12 mm. 4° analytical cell modified to contain a moveable partition. The partition is held against its stops in the middle of the cell by two rubber springs. As increasing centrifugal force is applied, the partition moves toward the bottom of the cell against the force exerted by the rubber springs. Free
sedimentation occurs while the partition is at the bottom of the cell. After the centrifugal force has decreased sufficiently at the end of a run, the rubber springs slowly move the partition back to its rest position without disturbing the distribution of molecules attained during centrifugation. The material remaining above the partition (supernatant) and the material below the partition (subnatant) can each be withdrawn quantitatively and subjected to appropriate analysis.

The equation by which the sedimentation constant is determined from biological activity data is given below. It is an approximation of the exact solution given by Yphantis and Waugh (57).

\[
\frac{Q-1}{\lambda} = \tau - \frac{r^2}{2}
\]

where

\[
\tau = 2s \int \omega^2 dt
\]

\[
\lambda = \frac{r^2}{r^2 - a^2}
\]

Q is the ratio of activity remaining in the supernatant after centrifugation to that of the original solution placed in the cell; \(s\) is the sedimentation constant; \(\omega\) is the centrifugal speed in radians per second; \(t\) is the time in seconds; and \(r_p\) and \(a\) are respectively the radial distances
from the center of the rotor to the rest position of the partition and to the meniscus of the solution in the cell.

Examination of the equation shows that $Q$, $a$, $r_p$, and $\int \omega^2 dt$ need to be determined in order to calculate the sedimentation constant. The values for $a$ and $r_p$ are determined from Schlieren photographs. $\int \omega^2 dt$ may be obtained graphically from a plot of $\omega^2$ versus $t$. $Q$ values are determined by biological assays and spectrophotometry.

Experiments were performed both at Schlieren and UV concentrations. The procedures will be described individually.

Initially it was discovered that the centerpiece of the Waugh cell inactivated the RNA upon contact. The solution to this problem was obtained by immersing the centerpiece for one minute in Desicote (manufactured by Beckman Instrument Co.), rinsing with carbon tetrachloride, and allowing it to air dry. The biological activity of RNA placed in the coated cell at 1.2 mg./ml. for two hours at 5°C was statistically identical to that of the control solution. The cell was periodically checked in this manner.

A typical partition cell experiment at Schlieren concentrations began with the removal of a RNA sample from the deepfreeze and allowing it to thaw in an ice bath. The rotor, cell, and centrifuge chamber were cooled to 5°C prior to their use. After the weight of the empty Waugh cell was determined, it was filled by means of a 1.0 ml. syringe and
a No. 22 needle. The partition was pushed to the bottom of the cell with the needle and held there while the solution was expelled into the cell. The filled cell was weighed and prepared for centrifugation. As the rotor was accelerated, the speed was recorded at one minute intervals. The cell was observed for a check on proper operation through the Schlieren optical system. The final speed of 56,100 r.p.m. was maintained for 10-20 minutes. Just before deceleration, a Schlieren photograph was taken. The speed was again recorded in one minute intervals during deceleration. No brake was used from 56,100 to 39,500 r.p.m.; the medium brake was used from 39,500 to 28,000 r.p.m.; the fast brake was used from 28,000 to 6000 r.p.m.; and no brake was used thereafter. Pictures were obtained at 13-18,000 r.p.m. for the determination of a and after the plate had returned to its rest position (2000 r.p.m.) for the determination of r_\text{p}. The diffusion pump was turned off at 26,000 r.p.m., which was approximately 10 minutes before the rotor came to rest. After the rotor had been gently removed and tilted with the cell in its upright position, the cell was carefully removed and placed on a stand for emptying. The supernatant, drawn into a cold syringe without disturbance of the partition, was placed in a test tube immersed in an ice bath. The cell was weighed again before the sediment was resuspended and removed with the subnatant.
Aliquots of the supernatant and subnatant were used to determine the optical densities. The optical density of the original solution was also obtained if this had not been done previously. Appropriate dilutions for the assay of the supernatant and the original RNA solution were then made. The original RNA solution, as a control, was assayed against two dilutions of the supernatant. Usually the supernatant was diluted Q and 2 Q times that of the control solution.

Theoretically, the subnatant could also be used to determine the sedimentation constant, but there was strong evidence that RNA centrifuged against the bottom of the cell lost its infectivity. As a consequence, the subnatant was used only to calculate the recovery of ultraviolet light absorbing materials.

Shortly after experiments were initiated at UV concentrations, it became apparent that at these low concentrations erratic inactivation was a severe problem. The rubber springs, cell centerpiece, micro-absorption cells, and occasional test tubes were found to inactivate RNA severely. On the assumption that trace metals were responsible, various attempts were made to circumvent the inactivation. Techniques were finally found which greatly reduced the inactivation and nearly eliminated its erratic nature. Since inactivation could not be eliminated entirely, rigid control experiments were performed so that appropriate corrections could be made.
The procedure finally adopted may now be described. The centerpiece of the Waugh cell was given two more coats of Desicote. The cell was always assembled and soaked with 0.1M Versene pH 7 for 24 hours before use. It was then disassembled, washed with distilled water, rinsed thoroughly with deionized distilled water, air dried, and reassembled for immediate use. At no time were the operator’s hands allowed to come in contact with areas available to the RNA solutions. Tweezers, covered with surgical tubing, were used to place the partition and rubber springs in the centerpiece. All glassware, including pipettes and absorption cells, were soaked in nitric acid for 24 hours, rinsed with deionized distilled water, and air dried before use. Micro-pipettes and micro-absorption cells were rinsed with deionized distilled water followed by distilled alcohol and air dried under aspiration immediately before and after use.

The actual centrifugation procedure was identical to that described for the Schlieren experiments except for the modifications to be discussed. The final centrifuging speed was 42,040 r.p.m. The braking speeds were changed at 29,500, 21,000, and 6000 r.p.m. respectively in the order previously given. The optical density had to be determined directly on the samples to be used for assay. Finally, control experiments were performed immediately before and after each analytical centrifugation by subjecting aliquots of the control solution to every procedure used in the analytical
run itself. The only difference was that the control solutions were spun at only 4000 r.p.m., a speed at which the partition moved to the bottom of the cell but no appreciable sedimentation occurred. As nearly as possible the time span was made identical to that of the actual experiment. The original solution was then assayed against the supernatants of the two control experiments in addition to those mentioned previously.

Initially ultraviolet absorption pictures were obtained during the partition cell experiment. During the search for sources of inactivation of the RNA, a 4000 r.p.m. centrifugation was carried out with and without the ultraviolet exposures normally used, and the exposed RNA demonstrated less than one-half the biological activity of the unexposed RNA. The somewhat erratic inactivation of the RNA by the cell made it difficult to determine whether this difference was actually due to UV inactivation. Combination of the inactivation cross section for RNA as calculated from data given by McLaren and Takahashi (58) with that of the measured intensity of the ultraviolet light emitted by the hydrogen lamp of the centrifuge suggested that the exposure used should inactivate less than one per cent of the RNA. As an added precaution, however, the ultraviolet light was not used during partition cell experiments. The sedimentation constant from velocity sedimentation photography was obtained in the conventional manner on a separate aliquot of the RNA.
Equipment and Techniques for Light Scattering Experiments

The literature on the application and theory of light scattering is quite extensive. Doty and Edsall (59) gave a fairly extensive review of the subject in 1951. More recently, Stacey (60) has written a book on the theory of light scattering and its applications in physical chemistry.

The two basic equations by which the molecular weight is determined from light scattering are given by

\[
\frac{Kc(1 + \cos^2 \theta)}{R(\theta)} = \frac{1}{M} + 2Bc
\]

and

\[
\frac{Hc}{\tau} = \frac{1}{M} + 2Bc
\]

where

\[
K = \frac{2\pi^2 n_0^2 (\frac{dn}{dc})^2}{\lambda^4 N}
\]

\[
R(\theta) = \frac{I(\theta)}{I_0} \frac{r^2}{V}
\]

\[
H = \frac{16\pi}{3} K
\]

In the equations, \(c\) is the concentration in gm./ml.; \(M\) is the molecular weight; \(B\) is the second virial coefficient; \(R(\theta)\) is Rayleigh's ratio or the reduced intensity; \(\tau\) is the turbidity of the scattering solution; \(n_0\) is the refractive index of the solvent; \(n\) is the refractive index of the solution; \(\lambda\) is the vacuum wavelength of the primary light beam;
N is Avogadro's number; \( \frac{dn}{dc} \) is the refractive increment of the solute; \( I(\theta) \) is the intensity of scattered light at an angle \( \theta \) with respect to the primary beam; \( V \) is the volume of the solution viewed at a distance \( r \); and \( I_0 \) is the intensity of the incident beam.

Equation 4 has the advantage that it gives the reciprocal of the molecular weight independent of the shape of the particle if it is extrapolated both to zero angle and zero concentration (60). Zimm (61) has shown the best way to accomplish this is to plot the left side of equation 4 against \( \sin^2 \frac{\theta}{2} + kc \). The constant \( k \) is chosen so that the data for different concentrations are separated sufficiently.

The extrapolation of \( Hc/r \) to zero concentration yields the correct reciprocal molecular weight only if the particle is smaller than \( \frac{\lambda}{2\theta} \). For larger particles correction factors which depend on the shape of the particle must be used.

The light scattering experiments to be described were performed with a modified Brice-Phoenix Light Scattering Photometer Model 1000-D (Photometer No. 1750-D). This photometer was designed and calibrated by the manufacturer to be used for the determination of turbidity and, consequently, the molecular weight by use of equation 5. The equation from which the turbidity is determined from data obtained with the Brice-Phoenix Photometer No. 1750-D under standard conditions is given by
\[ \tau = c a \frac{G(90)}{G(0)} \]

where \( \frac{G(90)}{G(0)} \) is the scattering ratio and is equal to the ratio of the galvanometer deflections obtained for the light scattered from the solution at 90° to that for the transmitted light at the 0° position, appropriately including the transmittances of the neutral filters used in the determinations; \( a \) is the constant relating the working standard to the furnished opal glass reference standard; and \( C \) is the factory determined calibration relating the instrument to the opal glass reference standard. The constant \( C \) involves the product of the diffuse transmittance of the opal glass reference standard and a diffusor correction factor, the depth of the scattering solution viewed as determined by the width of the beam, a correction for reflection of the primary beam at the emergent face of the cell, and a correction for incomplete compensation of refraction effects. The latter is a function of the refractive index of the solvent and is given in the form of a table by the manufacturer.

The standard instrument was modified in two ways. A magnetic stirrer was installed so the scattering solution could be stirred while observations of the scattered light were being made. The magnetic stirrer was mounted in the center of a recess on the cell support table. A hole was drilled through the center of the table to accommodate the
shaft by which the stirrer was attached to the motor. The stirrer for the scattering cell consisted of a glass enclosed wire. The diameter of the stirrer was 2 mm.

The second modification consisted of converting the equipment for use with a small cylindrical scattering cell (C-105) containing a minimal volume of 10 ml. The Phoenix Precision Inst. Co. provides a kit of three narrow diaphragms to convert the instrument for use with the small cylindrical cell. The height of the beam with these was such that the minimal volume that could be used was 20 ml. To reduce this essential volume to 10 ml., the length of the second diaphragm was reduced centrally from 1.5 cm. to 0.5 cm. The scattering cell was raised an appropriate amount by a plastic spacer placed on the cell table. The cell was raised 0.3 cm. less than the height at which the scattering ratios were altered.

An additional calibration factor is needed when the cylindrical cell is used which relates the narrow beam geometry to the standard beam geometry. This factor was obtained by determining the scattering ratio, \( r = \frac{g(0)}{g'(0)} \), for a Ludox solution in the usual manner with the standard 1.2 cm. diaphragms and a Brice-Phoenix scattering cell No. D-101. The standard diaphragms were then replaced by the narrow beam diaphragms. A new scattering ratio \( r' \) was determined with cell No. C-105 using the same scattering medium employed above. The calibration factor for the new cell is given
by $p_1$. The factor $p_1$ converts the scattering ratio obtained with the cylindrical cell to that which would have been obtained with the standard cell under standard conditions for which the photometer was calibrated by the manufacturer.

In addition to $p_1$, the working standard constant, $a$, was redetermined frequently since any realignment, such as the replacement of a lamp, may cause a slight change in its value. This constant was determined by observing the ratio of galvanometer deflections when the working standard was in its usual position to that when the reference standard opal glass was in the center of the table. The photometer was set to view the primary beam (0°).

The Brice-Phoenix instrument is so designed that the working standard is in the beam in addition to the scattering cell whenever 0° readings are made. Therefore the scattering ratio $\frac{G(90)}{G(0)}$ is not the ratio of absolute intensities in these two positions. It is a ratio already related to the calibration constants of the equipment and must be used with equation 9. In order to determine molecular weights from Zimm plots, Rayleigh's ratio of equation 4 had to be determined as a function of angle. The intensity of scattered light as a function of angle was determined by observing galvanometer deflection at intervals between 25 and 135°. These intensities were converted to $R(\theta)$ by its relationship to $R(90)$ as given by
Prom equations 1, 5, and 8, it is seen that \( R(90) \) could then be obtained from the turbidity by the equation

\[
R(\theta) = \frac{I(90)R^2}{I_0 V} \frac{I(\theta)}{I(90)V(\theta)} = R(90) \frac{G(\theta)}{G(90)} \frac{V(90)}{V(\theta)}
\]

From equations 4, 5, and 8, it is seen that \( R(90) \) could then be obtained from the turbidity by the equation

\[
R(90) = \frac{37}{16\pi}
\]

The ratio of the volumes viewed at 90° to that viewed at an angle \( \theta \) should vary as \( \sin \theta \) under ideal conditions. In practice there may be small deviations from this relationship because of optical imperfections in the cell. To determine corrections for these deviations, a dilute solution of Fluorescein (99% transmittance at 525 \( \text{m} \mu \)) was placed in the scattering cell. A cellophane filter, which absorbed most of the light of wavelength 436 \( \text{m} \mu \) but passed the fluorescent light produced by the Fluorescein, was placed in front of the photometer to filter out the scattered light. The galvanometer deflections caused by the fluorescent light were observed as a function of the angle. Any deviation of \( \frac{G(90)}{G(\theta)}^F \) from \( \sin\theta \) reflected deviations of the optical conditions from the ideal. The values of \( \frac{G(90)}{G(\theta)}^F \) obtained as indicated were used in the place of \( \frac{V(90)}{V(\theta)} \) in equation 10.
Since the light scattered by the solvent \((x)\) itself must be subtracted from that scattered by the solution \((s)\), the final equation for \(R(\theta)\) may be written from equations 9, 10 and 11 to be

\[
R(\theta) = \frac{3}{16\pi} C a \frac{R}{r_f} \frac{G(90)_F}{G(0)_F} \frac{r_f}{I(90)} I(\theta)
\]

where

\[
r_f = \frac{G(90)_s}{G(0)_s} - \frac{G(90)_x}{G(0)_x}
\]

\[
\frac{I(\theta)}{I(90)} = \frac{G(\theta)_s - G(\theta)_x}{G(90)_s - G(90)_x}
\]

Equation 4 may now be rewritten in the form it was used:

\[
\frac{T \gamma(\theta)c}{r_f} \frac{I(90)}{I(\theta)} = \frac{1}{M} + 2Bc
\]

where

\[
T = \frac{16\pi K}{3 C a \frac{R}{r_f}}
\]

\[
\gamma(\theta) = (1 + \cos^2 \theta) \frac{G(\theta)_F}{G(90)_F}
\]

The constants in equation 16 had the following values:

\(C = 2.175; \frac{R}{r_f} = 1.44; a = 0.0494;\) and with a value of 0.194 m.//gm. for \(\frac{dn}{dc} (40)\), \(K = 6.16 \times 10^{-7}\).
To free the cell and solvent of dirt is probably the most important technique in light scattering because of the heavy contributions extraneous large particles make to the scattered light. A snugly fitting nylon cover with a small hole in the center was constructed. A nylon plug was made to fit the V-shaped hole. Doubly distilled water was filtered through a "HA" millipore filter into the cell. The cell was rotated so that the water came in contact with all parts of the cell several times except for the nylon cover. The cover was removed briefly and the water poured out. The process was repeated until the dissymmetry \( \frac{G(15)}{G(135)} \) indicated a minimum of dirt. The cell was rinsed with the appropriate filtered buffer and a measured volume of buffer was added to the cell. The light scattering envelope of the solvent was obtained by determining the galvanometer deflections at 0°, every 5 degrees from 25 to 90, and every 10° thereafter to 135°. The 0° deflection was periodically checked for drift.

If the experiment was to be performed at 5°C, all solutions were filtered into the cell in a cold room (2-4°C). The cell was carried to the photometer in a dry beaker surrounded by ice. In the photometer housing, the cell was kept cold by the circulation of refrigerated ethylene glycol through the cooling jacket. Nitrogen and dry air jets were used to prevent condensation of moisture on the outside of the cell. Two inlets were made for this purpose. Black rubber tubing was used inside the photometer housing.
After the solvent scattering envelope was obtained, the solvent in the cell was poured back into the filtering funnel and the RNA at a high concentration was added to the solvent. These were filtered into the cell under a few pounds pressure of nitrogen. The scattering envelope of the solution was then obtained. One-third to one-half of the solution was poured into a test tube and an equal amount of buffer was added to the cell. The scattering envelope of the diluted solution was obtained. This procedure was repeated one or two more times. The concentrations of the various samples were then determined from the optical density.

For degradation experiments at 25°C, the cell was cleaned with the small glass enclosed stirrer in it. The cleaned solvent was allowed to equilibrate at the jacket temperature before its envelope was taken. Concentrated RNA was added to the cell through a syringe adapted with a millipore filter. The solution was immediately stirred with the magnetic stirrer and the zero time envelope was recorded. The scattering envelope was obtained periodically for several days. The temperature of the cell was maintained by the circulation of water through the jacket from a constant temperature water bath. Bacterial growth was prevented by the addition of a drop of chloroform to the solvent. The chloroform was found to have no appreciable effect on the solvent scattering envelope.
EXPERIMENTS AND RESULTS

Studies of Undegraded RNA

The physical-chemical characterization of RNA in its undegraded or intact state is essential to an understanding of its structure and function. However, the term undegraded is a relative term with respect to an isolated biological compound. For the investigations to be described, it will be used for RNA which had the properties it initially possessed upon isolation.

The phosphorus content of SDS RNA was determined by a modification of Allen's (62) procedure. The average

The phosphorus analysis was carried out as follows:

1) The 100 μl. of RNA at approximately 1000 μg./ml. was added to a 10 ml. Kjeldahl flask. The pipette was rinsed with 200 μl. of distilled deionized H₂O which was added to the Kjeldahl. An additional 200 μl. of distilled deionized H₂O was added.

2) 100 μl. of a standard phosphorus solution at 99.9 μg./ml. was added to another Kjeldahl. 400 μl. of distilled deionized H₂O was added as in (1).

3) 500 μl. of distilled deionized H₂O was added to a third Kjeldahl.

4) The above procedures were carried out in duplicate.

5) After 2 ml. of 60% perchloric was added to each of the six Kjeldahl flasks, they were refluxed 12 minutes under a hood.

6) The contents were quantitatively transferred to six 10 ml. volumetric flasks by carefully rinsing 4 times with distilled deionized H₂O and adding these to the volumetrics.

7) 2 ml. 5% ascorbic acid was added to each volumetric and mixed with contents.

8) 1 ml. 2.5% ammonium molybdate (Continued next page)
readings at 825 mµ for the sample, standard, and blank were respectively 0.7141, 0.785, and 0.012. The UV absorption spectra of 75 µl. of the sample in 2.6 ml. of distilled deionized water, 0.02M phosphate pH 7.3, and 0.2M salt plus 0.001M phosphate pH 7.5 respectively were obtained at 37°C. These spectra are shown in Figure 4. The absorption of 0.865 at 257 mµ in distilled deionized water is equated to unity in the graph.

From the phosphorus analysis it was calculated that the RNA sample contained 9.43 x 10^{-2} gm. P/l. Absorptions of 27.32, 24.64, and 30.96 were obtained in 0.02M phosphate pH 7.3, 0.2M salt plus 0.001M phosphate pH 7.5, and deionized distilled water respectively for the sample analyzed. The extinction per mole phosphorus, $\xi(P)$, was then calculated from the equation,

$$\xi(P) = \frac{30.96}{c1}$$

where $E$ is the absorbance of the sample; $c$ is the phosphorus content in gm./liter; and $l$, the width of the absorption cell, is equal to 1.0 cm. Assuming 8.97% by weight of RNA is phosphorus, the sample contained 1.05 mg. RNA per ml. From this value the absorbances ($E^{1%}$) may be calculated. (Continued from previous page) in 0.05N H2SO4 was added to each volumetric and mixed.
9) The volumetrics were brought to volume with distilled deionized H2O and placed in a 60°C H2O bath for 20 minutes.
10) The absorption of the solutions were determined at 825 mµ.
Figure 4. Ultraviolet light absorption spectra of SDS RNA
1) RNA in 0.2M NaCl + 0.001M phosphate pH 7.5
2) RNA in 0.02M phosphate pH 7.3
3) RNA in deionized distilled H₂O.
75 μl. of RNA in 2.6 ml. of the solvent at 37°C.
The absorption in H₂O at 257 μm. equated to unity.
The spectra of phenol RNA which had been reprecipitated with two volumes of alcohol and redissolved in deionized distilled water were obtained at 37° in the three solvents mentioned above. These spectra are given in Figure 5.

The extinction of phenol RNA was not determined by phosphorus analysis because the RNA was routinely prepared in phosphate buffer. Reprecipitation from a phosphate buffer would not guarantee the absence of inorganic phosphorus. In its place, the relative sugar contents of samples of SDS RNA and phenol RNA whose absorptions were the same in 0.2M salt plus 0.001M phosphate pH 7.5 at 10°C were determined by the phloroglucinol method (63). ¹ (The phenol RNA had been

---

¹The sugar analysis was carried out as follows:
1) 0.5 ml. of SDS RNA at 250 µg./ml. was placed in each of two test tubes.
2) 0.5 ml. of phenol RNA (adjusted to the same absorption in 0.2M salt as that of the SDS RNA) was added to each of two test tubes.
3) 0.5 ml. of distilled deionized H₂O was added to each of two test tubes.
4) 4 ml. of a 0.1% solution of FeCl₃ in a mixture of 1 part concentrated HCl and 6 parts of glacial acetic acid was added to each test tube. The contents were mixed and the tubes, covered with marbles, were placed in a boiling water bath for 50 minutes.
5) After the tubes were cooled to room temperature, 0.5 ml. of 0.25% phloroglucinol solution in a mixture of 1 part concentrated HCl, 1 part H₂O and 2 parts glacial acetic acid was added to each tube. After the contents were mixed, the tubes were allowed to stand at room temperature for 20 minutes.
6) The tubes were immersed in a boiling water bath for exactly 4 minutes.
7) After 10 hours at room temperature, the absorption at 630 mλ was determined.
Figure 5. Ultraviolet light absorption spectra of phenol RNA

1) RNA in 0.2M NaCl + 0.001M phosphate pH 7.5
2) RNA in 0.02M phosphate pH 7.3
3) RNA in deionized distilled H₂O

75 µl. of RNA in 2.6 ml. of the solvent at 37°C.

The absorption in H₂O at 257 µm equated to unity.
precipitated with alcohol and redissolved in distilled deionized water.) Average spectrophotometer readings at 630 μm were 0.020, 0.173, and 0.177 respectively for the blank, phenol RNA, and SDS RNA. Hence the SDS RNA had 1.026 times as much sugar as the phenol RNA. On the assumption that the sugar and phosphorus content of RNA are independent of the mode of preparation, the extinction of phenol RNA in 0.2M salt plus 0.001M phosphate at 10°C is 1.026 times that of the SDS RNA at this same temperature.

The temperature dependence of the absorptions of SDS RNA and phenol RNA in the three solvents mentioned above were determined with a Beckman DK-2 equipped with a temperature regulating device. Closed adsorption cells were used in these experiments. The solutions were allowed to equilibrate with the jacket temperature for 10 minutes before a spectrum was obtained for all temperatures greater than room temperature. The temperatures below that of the room were attained by pre-cooling the holder and cells in the cold room. Readings were taken as the cells warmed up. After the highest temperature had been attained, the spectra were again determined as the cells cooled down. Within experimental error all absorption changes were reversible.

From the determined extinction of SDS RNA at 37°C, the extinction as a function of temperature could be calculated and is given in Figure 6. In the same manner, from the extinction of phenol RNA in 0.2M salt plus 0.001M phosphate
Figure 6. The extinction of SDS RNA as a function of temperature.

▼ RNA in deionized distilled H₂O
○ RNA in 0.02M phosphate pH 7.3
□ RNA in 0.2M NaCl + 0.001M phosphate pH 7.5.
at 10°C and the relationship of absorptions in the three solvents at 37°C as given in Figure 5, the extinction as a function of temperature was obtained and is presented in Figure 7.

Because the phosphorus, relative sugar content, and absorption determinations are each subject to 1 or 2% error, the extinctions as given in the figures at best do not have greater accuracy.

RNA, isolated either by SDS or phenol, was subjected to ultracentrifugal analysis at Schlieren concentrations. Figure 8 shows Schlieren photographs of representative phenol and SDS preparations of RNA centrifuged at 56,100 r.p.m. at 1.2 mg./ml. These samples were analyzed as soon as possible after isolation and were kept at 5°C or less throughout experimentation. Ultraviolet absorption pictures of both types of RNA preparations at 20 μg./ml. are also shown in Figure 8. Densitometer tracings of these photographs are given in Figures 9 and 10.

The sedimentation constant of RNA at 20 to 50 μg./ml. in 0.02M phosphate pH 7.3 at 5°C is 18.9 (Table 1). In 0.2M salt plus 0.001M phosphate pH 7.5, phenol RNA has an S₂₀ of 22.4. At 1.2 mg./ml. an average value of 12.6 is obtained for S₂₀ as shown in Table 2. For comparative purposes the sedimentation rate for RNA at 0.5 mg./ml. prepared by heating as described by Hopkins and Sinsheimer (40) was determined to be 20.8 at 5° in 0.3M acetate pH 5.7. On the
Figure 7. The extinction of phenol RNA as a function of temperature

▽ RNA in deionized distilled H₂O
⊙ RNA in 0.02M phosphate pH 7.3
□ RNA in 0.2M NaCl + 0.001M phosphate pH 7.5.
Figure 8. Schlieren and ultraviolet light absorption photographs of undegraded samples of SDS and phenol RNA

1) Phenol RNA at 1.2 mg./ml. at 56,100 r.p.m.
   Pictures at 8 minute intervals

2) SDS RNA at 1.2 mg./ml. at 56,100 r.p.m.
   Pictures at 8 minute intervals

3) Phenol RNA at 20 μg./ml. at 56,100 r.p.m.
   Pictures at 4 minute intervals

4) SDS RNA at 20 μg./ml. at 56,100 r.p.m.
   Pictures at 8 minute intervals
Figure 9. Densitometer tracing of ultraviolet light absorption photographs of SDS RNA.
Figure 10. Densitometer tracing of ultraviolet light absorption photograph of phenol RNA.
Table 1. Sedimentation constants for RNA at UV concentrations

<table>
<thead>
<tr>
<th>Mode of preparation</th>
<th>Concentration (µg./ml.)</th>
<th>Solvent</th>
<th>S (5°C)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenol</td>
<td>50</td>
<td>0.02M (\text{PO}_4)(^-) pH 7.3</td>
<td>18.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>19.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>18.7</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>18.8</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>19.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>18.5</td>
</tr>
<tr>
<td>&quot;</td>
<td>20</td>
<td>&quot;</td>
<td>18.7</td>
</tr>
<tr>
<td>&quot;</td>
<td>16</td>
<td>&quot;</td>
<td>19.3</td>
</tr>
<tr>
<td>SDS</td>
<td>25</td>
<td>&quot;</td>
<td>18.3</td>
</tr>
<tr>
<td>phenol</td>
<td>20</td>
<td>0.2M NaCl + 0.001M</td>
<td>22.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>(\text{PO}_4)(^-) pH 7.5</td>
<td>22.1</td>
</tr>
</tbody>
</table>

\(^a\)The average value in the 0.02M phosphate pH 7.3 is 18.9. This value corrected to 20° is approximately 29.
Table 2. Sedimentation constants for RNA at Schlieren concentrations

<table>
<thead>
<tr>
<th>Mode of preparation</th>
<th>Concentration (mg./ml.)</th>
<th>S (5°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenol</td>
<td>1.2</td>
<td>12.1</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>11.3</td>
</tr>
<tr>
<td>&quot;</td>
<td>0.96</td>
<td>12.0</td>
</tr>
<tr>
<td>SDS</td>
<td>1.2</td>
<td>13.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>13.9</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.3</td>
<td>12.8</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>12.6</td>
</tr>
</tbody>
</table>

assumption that 20-50 μg./ml. is equivalent to infinite dilution, a $S_{20}$ of 34 is obtained in 0.2M salt upon application of the viscosity correction factor of 1.5.

Analysis of the densitometer tracings indicates that approximately 70% of the material has the sedimentation constant quoted above. The slower components are represented by the trailing edges in Figures 9 and 10. There is the suggestion of a faster component with a $S_{5}$ of 34 in the phenol preparations of RNA. A boundary corresponding to this $S$ can be observed in both Schlieren and UV absorption photographs.
The weight average molecular weights of the RNA in 0.02M phosphate pH 7.3 were determined by means of light scattering. Aliquots of the samples studied were checked in the ultracentrifuge to insure their similarity to those analyzed above. Figures 11, 12, and 13 are Zimm plots of the scattering envelopes for phenol and SDS RNA.

Values of $2.15 \times 10^6$, $1.9 \times 10^6$, and $2.0 \times 10^6$ are obtained for the molecular weights from the extrapolated value of $R_c (1 + \cos^2 \theta)/R(\theta)$. The extrapolations of the scattering envelopes for SDS RNA are not very accurate because of the concavity of the curves.

From the initial slope of the zero concentration line and the intercept, the radius of gyration, $\rho_g$, can be calculated from the expression,

$$\frac{\text{slope}}{\text{intercept}} = \frac{16\pi^2}{3} \rho_g^2 \left( \frac{n}{\lambda_0} \right)^2$$

The values for $n$ and $\lambda_0$ are the same used in equation 6.

From Figure 11, $\rho_g$ is calculated to be 600 Å in 0.02M phosphate pH 7.3.

Studies of the Degradation of RNA

The lability of RNA, both with respect to its infectivity and its structure, at room temperature is well known (3, 4, 45). Whether this instability is caused by extrinsic
Figure 11. Zimm plot for phenol RNA

1) $1.17 \times 10^{-4}$ gm./ml.
2) $0.73 \times 10^{-4}$ gm./ml.
3) $0.482 \times 10^{-4}$ gm./ml.
4) $0.294 \times 10^{-4}$ gm./ml.
\[
\frac{K_c(1+\cos^2 \theta)}{R(\theta)} \times 10^7
\]

\[
\sin^2 \frac{\theta}{2} + 1000 C \text{ (gm/ml)}
\]
Figure 12. Light scattering envelopes for phenol RNA
1) $1.08 \times 10^{-4}$ gm./ml.
2) $0.653 \times 10^{-4}$ gm./ml.
3) $0.425 \times 10^{-4}$ gm./ml.
Figure 13. Light scattering envelopes for SDS RNA

1) $1.18 \times 10^{-4}$ gm./ml.
2) $0.75 \times 10^{-4}$ gm./ml.
3) $0.285 \times 10^{-4}$ gm./ml.
$K_e (I + \cos^2 \theta)^7$

$\sin \theta / 2 + 500C$

Graph showing the relationship between $\sin \theta / 2 + 500C$ and $K_e (I + \cos^2 \theta)^7$. The graph includes data points and labeled axes.
or intrinsic factors is not known. Since the investigation of the degradation process should yield information concerning both the structure of the intact molecule and the nature of the degradation, a series of experiments involving the degradation of RNA at 25°C were undertaken.

The kinetics of the degradation of DNA upon enzymatic digestion was determined by means of light scattering by Thomas (44). He was able to demonstrate that the kinetics agreed with a double stranded DNA. The kinetics of the degradation of RNA at 25°C should give similar information for the structure of RNA. The degradation of RNA was followed as described in the section on materials and methods. In Figure 14 the reciprocal of the molecular weight is given as a function of time the RNA was kept at 25°C, and it is clear that the reciprocal of the molecular weight is proportional to the time for the duration of the experiment.

To obtain information concerning the relationship of high molecular weight RNA to that of the degradation products, ultracentrifugal analyses of RNA at various stages of degradation were made. RNA at 1.2 mg./ml. was incubated at 25°C in the presence of a drop or two of chloroform to prevent bacterial growth. Initially and at periodic intervals thereafter, aliquots of RNA were removed, ultracentrifugally analyzed, and checked for residual biological activity.

Figures 15 and 16 show the centrifuge patterns of SDS and phenol RNA respectively as obtained at various stages of
Figure 14. Reciprocal molecular weight of phenol RNA as a function of time at 25°C.
Figure 15. Ultracentrifuge patterns of SDS RNA kept at 25°C for various periods of time.
Figure 16. Ultracentrifuge patterns of phenol RNA kept at 25°C for various periods of time.
Figure 16. (Continued)
degradation. Analysis of these patterns indicates that material initially sedimenting with the leading component appears as slower sedimenting material. The proportion of material present in these slower components increases as a function of time the RNA was held at 25°C. Careful observation of the patterns shown in Figure 16 suggest that the slower moving components tend to occur as several discretely sedimenting boundaries. The $S_5$ values for the slow components range from 2 to 10.

The parallel loss of infectivity of the RNA was determined by biological assay. Dr. P. Cheo performed the assays during the time that aliquots of RNA were being centrifugally analyzed. The RNA at 20 µg./ml. was assayed on pinto beans against a standard 0.8 µg./ml. TMV solution. The loss of infectivity of the phenol RNA is given as a function of time at 25°C in Figure 17 together with the decrease in the area remaining under the leading component in the Schlieren photographs. There appears to be a complete correlation between the two. A similar study of SDS RNA was inconclusive because of the difficulty with which the proportion of area remaining under the leading peak could be determined.

Waugh Partition Cell Experiments

In order to determine the sedimentation rate of the infective unit, Waugh partition cell experiments were
Figure 17. Decrease in residual infectivity and proportion of areas remaining under leading peak in Schlieren photographs as a function of time RNA was kept at 25°C.

▼ Area remaining under leading peak
○ Infectivity retained by sample.
performed on undegraded and degraded samples of RNA. The validity of using the cell with RNA at 1.2 mg./ml. was checked by sedimenting all the material below the rest position of the partition, allowing the partition to return, and checking the supernatant for residual ultraviolet absorbing material and for infectivity. Only a very small amount of absorbing material with a spectrum very different from RNA was recovered. Even if all the absorption was due to RNA, less than 0.4% of the material was above the partition. In addition, the sedimenting peak appeared undisturbed even after the plate had returned past it. Finally, no infectivity could be demonstrated in the supernatant.

The sedimentation rate for TMV as determined by biological activity was 208 S as opposed to 189 S determined photographically on the same sample of TMV. These may be considered to be the same within the statistical error involved in the biological assay.

The experiments may be divided into two groups. In the first group, all centrifugation was carried out at Schlieren concentrations, and in the second group centrifugation was performed at UV concentrations.

For a given sample, three sedimentation rates could be calculated. These were obtained from the Schlieren photograph, from the proportion of ultraviolet light absorbing material remaining above the partition (Q_UV), and from the residual biological activity (Q_infectivity). For undegraded
RNA, samples which had been freshly prepared or stored at -60°C were used. Degraded RNA was obtained as described previously. The residual infectivity was determined by assaying the degraded RNA against a portion of the same stock which had been kept below 5°C after removal from the deep-freeze. Samples with residual activities ranging from 4 to 61% of the initial value were analyzed. All experimental data and derived quantities for these experiments are recorded in Table 3.

The value for λ (equation 3) was determined from a and rp, which was in all cases 6.500 cm. That the partition had returned to this position at the end of the run was checked photographically. The accuracy with which the recovery of UV absorbing material could be determined was not very high because it was dependent on cell weights determined at three different times. It was difficult to avoid rapid condensation of moisture on the cold cell during the weighing process. Furthermore, quantitative recovery of material from the subnatant depended upon complete resuspension of sedimented materials. This in itself was not too readily accomplished in the presence of the partition. For these reasons, any value for the recovery between 90 and 110% was considered satisfactory.

In general, if a given solution was assayed on two sets of 10 plants or leaves, the lesion counts could be expected to agree within 10 to 20%. This rather unsatisfactory
Table 3. Results of partition cell experiments on infective RNA at 1 mg./ml.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Per cent survival</th>
<th>UV recovery (cm.)</th>
<th>$\int \omega^2 dt \times 10^{10}$ for Q</th>
<th>Dilution $\frac{Q_{inf}}{Q_{def}}$</th>
<th>$S_{(5^\circ C)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Phenol</td>
<td>100</td>
<td>91</td>
<td>5.963</td>
<td>4.52</td>
<td>6982/8518</td>
</tr>
<tr>
<td>2. Phenol</td>
<td>100</td>
<td>101</td>
<td>5.884</td>
<td>6.38</td>
<td>2473/3143</td>
</tr>
<tr>
<td>3. Phenol</td>
<td>100</td>
<td>100</td>
<td>5.865</td>
<td>3.80</td>
<td>169/227</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Phenol</td>
<td>100</td>
<td>99.7</td>
<td>5.874</td>
<td>5.65</td>
<td>227/185</td>
</tr>
<tr>
<td>5. SDS</td>
<td>100</td>
<td>92.4</td>
<td>5.832</td>
<td>6.70</td>
<td>678/1340</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Phenol</td>
<td>10.</td>
<td>5.902</td>
<td>6.29</td>
<td>394/1949</td>
<td>3.65</td>
</tr>
<tr>
<td>7. Phenol</td>
<td>54</td>
<td>5.846</td>
<td>6.70</td>
<td>1784/1924</td>
<td>3.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The ratio is given as the total number of lesions obtained from the supernatant over the total number of lesions produced by the initial sample.

\(^b\) The dilution is given as the factor by which the dilution of the control exceeds that of the test solution.
Table 3. (Continued)

| Preparation | Per cent | UV recovery (cm.) x 10^10 | Ratio of \( \int \omega^2 dt \) for Q lesion Dilution | \( Q_{inf} \) | \( Q_{UV} \) Boundary UV Inf S(5°C) |
|--------------|---------|--------------------------|---------------------------------|---------|--------|-----|-----|-----|-----|
| 8. Phenol    | 61      | 110                      | 5.888 5.71                      | 40124/326 | 2.0   0.194 | 12.2 | 8.1 | 16.7 |
| 9. SDS       | 59      | 100                      | 5.884 5.91                      | 300122/1682 | 3.0  0.262 | 6.52 | 0.546 | 7.2  | 12.1 |
| 10. SDS      | 43      | 97                       | 5.888 6.52                      | 300122/1682 | 1.0  0.170 | 12.0 | 8.4 | 12.4 |
| 11. SDS      | 4       | 113                      | 5.879 6.19                      | 12/71      | 1.5   0.046 | 11.5 | 7.5 | 15.5 |
| 12. SDS      | 40      | 94                       | 5.864 6.20                      | 12/71      | 1.5   0.047 | 11.5 | 7.5 | 15.5 |

\( a = \int \omega^2 dt \) means the integral of the square of the angular velocity with respect to time.
property of the assay may largely be attributed to the variations in the pressure with which the solution was applied and in the amounts of carborundum applied to the leaf. No means of precisely controlling these variables was found. For this reason it was necessary to plan the experiments so that the Q values were below 0.4, preferably between 0.1 and 0.25. In these regions a relatively large error in Q produces a relatively small error in the sedimentation constant. For example, if $Q = 0.25 \pm 20\%$ and $\lambda = 5.5$, $S = 13 \pm 7\%$. At $Q = 0.5$, the two errors are approximately the same. Theoretically, the accuracy of the sedimentation rate should be even better at $Q = 0.1$. In practice, the accuracy of the assay decreases because less material is available for assay, and hence the determination of $S$ is not improved. It was desirable to determine the sedimentation rate for as great a range in $Q$ as possible in order to establish whether infective units of various sizes existed. As a compromise, Qs between 0.05 and 0.5 were used. It is clear from the above discussion that not all experiments listed in Table 3 are equally reliable. For example, in Experiment 11 a low Q value was offset by an assay which was very poor because only a small amount of highly degraded RNA was available. In Experiment 3 the combination of a relatively poor assay and a high Q value lead to a less reliable sedimentation constant.
Table 4. Results of partition cell experiments on infective phenol RNA at 50 μg/ml.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>UV recovery (cm.) x 10^10</th>
<th>Ratio of lesions for controls</th>
<th>Ratio of lesions for Q Dilution</th>
<th>Q&lt;sub&gt;inf&lt;/sub&gt;</th>
<th>Q&lt;sub&gt;UV&lt;/sub&gt;</th>
<th>Boundary UV Inf</th>
<th>S (5°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>5.867</td>
<td>2.22</td>
<td>6750/8700</td>
<td>4821/3134</td>
<td>0.492</td>
<td>0.592</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1573/3166</td>
<td>853/1211</td>
<td>0.636</td>
<td>0.550</td>
</tr>
<tr>
<td>2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100</td>
<td>5.869</td>
<td>3.36</td>
<td>2210/3145</td>
<td>481/1009</td>
<td>0.251</td>
<td>0.340</td>
</tr>
<tr>
<td>3</td>
<td>90</td>
<td>5.979</td>
<td>3.49</td>
<td>4162/6941</td>
<td>712/3817</td>
<td>0.080</td>
<td>0.395</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>614/4552</td>
<td>1.35</td>
<td>0.188</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Ratio is given as total number of lesions obtained from both control supernatants divided by the total number of lesions produced by the initial sample.

<sup>b</sup>Ratio is given as total number of lesions obtained from test supernatant divided by total numbers produced by the initial sample.

<sup>c</sup>The control or initial sample was diluted the factor given times as much as the supernatant.

<sup>d</sup>This sample was badly degraded for unknown reasons.
Table 4. (Continued)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>UV a $\int w^2 dt$</th>
<th>Recovery (cm.)</th>
<th>x $10^{10}$ for controls</th>
<th>lesions</th>
<th>lesions' $S^{(5^\circ C)}$</th>
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<tr>
<td>4</td>
<td>5.833</td>
<td>3.93</td>
<td>3222/4367</td>
<td>2166/2115</td>
<td>5</td>
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<td>0.284</td>
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<td>0.388</td>
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<td>15</td>
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<td>5</td>
<td>5.928</td>
<td>4.19</td>
<td>1412/3697</td>
<td>580/1871</td>
<td>6</td>
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<td>0.323</td>
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<td>23</td>
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</table>

Average 19.0 15 19.6
The "boundary" sedimentation constants were determined from the final position of the leading peak, $\int \omega^2 dt$, and $a$.

The unweighted averages of the sedimentation rates at $5^\circ C$ are respectively 13.7 and 12.4 for the biological activity and the leading boundary. For the ultraviolet absorbing materials of undegraded RNA, a value of 11.6 is obtained. For degraded samples, the sedimentation rate for the UV absorbing material is a function of the degree of degradation.

Because molecular interaction is not negligible at Schlieren concentrations, experiments at UV concentrations were initiated after an assay sensitive enough for this purpose was obtained. Assays were made either on Xanthi, Holmes L. L., or pinto beans which were grown in Barrhert Laboratories as described earlier. The $Q$ values for these experiments were corrected for inactivation of the RNA during the experimental procedure. The amount of inactivation was determined by dividing the infectivities of the supernatants of the two control runs (made at 4000 r.p.m.) by that of the initial uncentrifuged sample. The ratio ($R$) so obtained gave the fraction of the initial activity remaining in the controls. On the assumption that the RNA in the supernatant of the 42,040 r.p.m. run was inactivated by the same amount, the $Q$ value actually obtained from the test solution was $R$ times smaller than the value that would have been obtained in the absence of inactivation. The experimental $Q$ value multiplied by $1/R$ was, therefore, used for $Q_{\text{infectivity}}$. 
The data and derived quantities for these experiments are listed in Table 4. The average value for the sedimentation constant of the infective unit was 19.6 as compared to 19.0 for the boundary.
DISCUSSION OF RESULTS

Physical-Chemical Properties of TMV-RNA

The molecular weight of RNA prepared either by means of SDS or phenol is $2 \times 10^6$ as determined by light scattering. This is the same value obtained by Northrup and Sinsheimer (39) and Hopkins and Sinsheimer (40) for RNA prepared by heating the virus to 100°C for a short period of time. Gierer (41) has determined the molecular weight of phenol RNA by combining sedimentation data with that of viscosity. The value he reported is in agreement with the light scattering data.

An extrapolated value of 30 S was given by Gierer for the sedimentation constant of phenol RNA in 0.02M phosphate. The results reported here substantiate this value. In buffers of higher ionic strength, such as 0.2M salt plus 0.001M phosphate pH 7.5, the extrapolated value of the sedimentation constant is approximately 34. The same values are obtained for SDS RNA and heat-prepared RNA.

Both the light scattering data and the sedimentation patterns of SDS RNA suggest that more degradation occurs in making an SDS preparation than in making a phenol preparation.

It is significant that RNA prepared by any of three methods has the same sedimentation constant and the same molecular weight. This in itself is good evidence that this
high molecular weight RNA is not an experimental artifact but represents the intact molecule as obtained from the virus. This corresponds to all of the RNA present in one virus particle on the basis of a 5.1% RNA content of a virus particle with a weight of $4.0 \times 10^6$ (27).

If the hyperchromic effects described for the RNA are attributed to the rupture of hydrogen bonds and the expansion of the molecule as is commonly done, the results suggest that the hydrogen bonds reform and rupture reversibly. This is true for both SDS and phenol RNA. Within experimental error, the absorption properties of the RNA are independent of the two modes of preparation. It is not known whether hydrogen bonds reform in a specific or in a random manner. The hyperchromic effects are in agreement with the reported changes in the radius of gyration upon changes in the solvent ($4.0$). That is, the radius of gyration is largest in solvents which cause RNA to exhibit its highest extinction. This correlation circumstantially is in agreement with the theory that absorption increases may be associated with the expansion of the molecule.

It is important to observe that SDS RNA and phenol RNA are very similar in their basic properties. No discrepancies or differences, such as reported by Fraenkel-Conrat (52), were found in RNA prepared by these methods. There may be slight differences in the manner in which RNA prepared by the two methods breaks down at room temperature. These
differences may reflect an extrinsic factor which is not present to the same extent in both preparations.

Analysis of the degradation of RNA at 25°C provides additional information concerning the structure of intact RNA. Charlesby (64) has derived an expression for the weight average molecular weight ($M_w$) of a single stranded molecule as a function of the probability, $p$, that a given bond will be broken. The expression is valid if the initial weight average molecular weight is equal to the number average molecular weight, $M_n$, and is given by

$$
\frac{M_w(p)}{M_{no}} = \frac{2}{(pu)^2} (e^{-pu} - 1 + pu)
$$

where $pu$ is the average number of scissions per initial molecule. If it is assumed the probability that a given bond will be broken is proportional to the time, $pu$ will be equal to $kt$. As long as $kt$ is small, equation 19 may be approximated by

$$
\frac{M_{no}}{M_w(t)} = 1 + Kt
$$

On the assumption that the phenol RNA used in the degradation experiment was initially nearly monodisperse and that the number of bonds broken is proportional to the time at 25°C, the kinetics of the degradation of the RNA is in agreement with that of a single stranded polymer. It does not agree
with a double stranded molecule for which the reciprocal molecular weight would be proportional to $t^2$ (44).

However, this result does not necessarily imply that the RNA breaks down to the nucleotide level in this manner. The basic degradation product might consist of a fairly large number of nucleotides without changing the form of the kinetics as obtained. $M_w$ was followed down to a value of 400,000. At this point background scattering became equivalent to that of the RNA and the process could not be followed any longer. This is still an order of magnitude larger than a value of 50,000, for example.

In fact, sedimentation patterns of degraded RNA suggest that it may break up in a non-random fashion into units of specific size. The slowest components observed in these preparations would still represent molecular weights of the order of $2.5-5.0 \times 10^6$ if Gierer's (44) expression, $m = 1100 s^{2.2}$, relating the sedimentation constant to molecular weight is used for the calculation. It would appear that degradation at 25°C does not continue to the nucleotide level. If so, not all bonds in the RNA molecule are equivalent. Whether this non-equivalence reflects physical or chemical differences at specific sites is not known.
Properties of Infective RNA

The properties of the infective unit are identified with those discussed above to the extent that the biologically active material has the same sedimentation rate as that of the bulk of RNA and loses its infectivity at 25° at the same rate that the intact RNA breaks down into smaller units. Neither observation permits the unqualified identification of the infective unit with that of the bulk of the RNA. However, they do suggest that the size of the infective unit is similar to that of the high molecular weight RNA and that whatever causes the degradation of high molecular weight RNA also causes the destruction of infectivity. It is also clear from both types of experiments that smaller or slower components at no time are associated with biological activity that can be detected by biological assay. If slower components were infective, the sedimentation rate for the biological activity should be much slower in degraded samples because a much larger proportion of the RNA exists in the slow components of such samples. For the same reason, the infectivity should disappear at a slower rate than that at which high molecular weight RNA decays if the above were true. Gierer (53) has arrived at the same conclusions by other means.

No attempt was made to determine whether amino acids or other unusual compounds are associated with the infective
unit. With respect to this problem, it can only be said that all preparations used were completely inactivated when incubated with small amounts of ribonuclease at 5°C.
SUMMARY OF RESULTS

Ribonucleic acid, isolated from the common strain of tobacco mosaic virus by either sodium dodecyl sulfate or water-saturated phenol, has a molecular weight of \(2 \times 10^6\) and a sedimentation rate of \(34 S\) in 0.2M salt. The ultraviolet light absorption properties of this RNA are a marked function of temperature and solvent, increasing with temperature and decreasing with ionic strength. The extinctions in terms of moles phosphorus per liter are 8130 and 8250 for SDS RNA and phenol RNA at 37°C in 0.2M salt plus 0.001M phosphate pH 7.5.

Analysis of the degradation of RNA suggests that at least portions of the molecule are single stranded and that the RNA may decay in a non-random fashion into discretely sedimenting units.

The sedimentation rate of the infective unit is statistically identical to that of intact RNA. The biological infectivity is lost at the same rate that intact or high molecular weight RNA decays. These results demonstrate that smaller components are not infective and suggest that the high molecular weight RNA is probably the infective unit.
REFERENCES


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