5-1966

A Study of the Factors Influencing the Synthesis of Tobacco Mosaic Viral RNA in a Partially Purified Synthesizing System

Agnes P. Fok
Utah State University

A STUDY OF THE FACTORS INFLUENCING THE SYNTHESIS OF TOBACCO MOSAIC VIRAL RNA IN A PARTIALLY PURIFIED SYNTHESIZING SYSTEM

by

Agnes P. Fok

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Plant Nutrition and Biochemistry

Approved:

Major Professor

Head of Department

Dean of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

1966

ACKNOWLEDGMENTS

The author wishes to express her appreciation to the members of her graduate committee for the help and encouragement they have given her in this study.

Sincere thanks are extended to Dr. George W. Cochran for his guidances and suggestions which have made this study possible; special thanks are conveyed to Dr. David Walker for his encouragement and whole-hearted support in this research program.

Thanks and appreciation are extended to Dr. Harris Van Orden, Dr. Orson Cannon and Dr. Amrik S. Dhaliwal for serving on the guidance committee and for their help which was invaluable to the author.

The author also wishes to thank all the members of the virus laboratory, Mr. John Chidester, Mr. Calvin Lamborn, Dr. Bagher Forghani, Mrs. A. S. Dhaliwal, and Miss Lon-Lon Hsu for their kind assistance.

Agnes P. Fok

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Research on biosynthesis of tobacco mosaic virus (TMV) ribonucleic acid (RNA) in vitro has been reported by Cochran, et al. (1); Karasek and Schramm (2); Kim and Wildman (3); Cornuet and Astier (4); and Tongur and Balandin (5-7). It has been postulated that the replication of a number of viruses containing single-stranded RNA is accompanied by the formation of a virus-specific double-stranded helical RNA, the replicative form. This has been demonstrated both for animal and bacterial viruses including MS2 (8). The double helical structure of purified replicative form of MS2 was established by X-ray diffraction studies (9). One of the strands was shown to be a viral RNA strand of the parental type ("plus" strand), the other being complementary to it ("minus" strand) (10). Studies on Escherichia coli infected with RNA phages suggest that a structure containing both a "plus" and a "minus" strand is an obligatory intermediate in viral reproduction (10).

If the formation of a double-stranded replicative form is a feature common to all RNA viruses, it would be expected to occur in plant virus replication. Burdon, et al, have shown that tobacco leaves infected with TMV contain a TMV-specific double-stranded RNA with properties similar to those replicative forms of MS2 phage (11). Similar observations have been concurrently made by Shipp and Haselkorn (12). Haruna and Spiegelman (13) demonstrated that an RNA-dependent RNA polymerase purified from Escherichia coli infected with

a RNA bacteriophage (Qβ) generates in vitro a polynucleotide of the same molecular weight as viral RNA; the polymerase cannot distinguish the newly synthesized polynucleotide from the starting viral RNA. Evidence has been presented from in vitro synthesis of viral RNA in our laboratory which suggested that one or more enzymes, RNA polymerases, link the four nucleotides (ATP, UTP, CTP, GTP) to form new viral RNA units with viral RNA present as a primer and template and magnesium ions participating as a catalyst (1, 14). Lodish and Zinder (15) have suggested that two polymerase enzymes are involved in the synthesis of f2 bacteriophage. One completes the structure of double-stranded viral RNA by synthesizing the negative strand. The other one reacts with the completed double-stranded RNA to produce a new positive strand. We assume that our TMV-RNA replicative mechanism is a double-stranded structure in association with two similar specific TMV-RNA polymerase enzymes. During the isolation and purification of the TMV-RNA replicative mechanism in our laboratory there has been little opportunity to evaluate the various factors affecting the viral RNA synthesis. It is, therefore, the objective of this research to determine the conditions of pH, ionic environment, and temperature which give optimum synthesis of new viral RNA. The rate of synthesis can be followed spectrophotometrically because the linkage of the nucleotides results in a pronounced hypochromic shift at 260 mu.

MATERIALS AND METHODS

Preparation of Whole Virus and Virus RNA

Young Turkish tobacco plants were dusted with 600 mesh carborundum and then inoculated by rubbing with a small sponge carrying tobacco mosaic virus solution. Plants were usually harvested twenty to twenty-five days after the inoculation when mosaic symptoms were appearing in the new growth.

Infected leaves were ground with sand using a mortar and pestle. The virus was isolated and purified using a chomatographic method developed by Venekamp and Mosch (16). TMV-RNA was prepared using the phenol method of Gierer and Schramm (17).

Preparation of Standard Sodium Chloride Solution

The standard sodium chloride solution was prepared using 0.15 M sodium chloride, 0.015 M sodium citrate and 0.002 M $MgCl_2$ with the pH of the solution finally adjusted to pH 7.0.

Preparation of Sucrose Density Gradient

Commercial sucrose was purified by washing with 50% ethyl alcohol in a fritted glass Buchner funnel. The 50% alcohol was followed by 95% alcohol. The residual 95% alcohol was removed from the sucrose crystals by shallow pan evaporation.

Two sucrose solutions were prepared. One was 57.7% with a density of 1.273 and the other 4% with a density of 1.027. The sucrose

gradients were formed by layering progressively less dense sucrose mixtures in centrifuge tubes. This was done by placing micrometer feeds on two syringes that were positioned at angles so that the delivering hypodermic needle points could form a common mixed drop that would flow down the wall of the centrifuge tube and layer in the proper position. The syringes were driven manually by two persons according to a pre-determined schedule in a stepwise manner to give the desired linear gradient. The limits of the gradients were determined by the density values of the sucrose solutions used. The TMV-RNA replicative form isolated from infected Scotia bean leaves were placed on top of the sucrose columns and were centrifuged at 50,000 rpm for one hour.

Preparation of Phosphate Gradient

Two phosphate solutions of pH 8.5 and of 3.5 were used. These two solutions were connected to a gradient pump which automatically mixed the two phosphate solutions to give a gradient in pH. The gradient solution was carried to the top of a cellulose column on which the TMV-RNA replicative form isolated from infected Scotia bean leaves was placed.

Incubation Temperature

Before the optimum temperature was identified an incubation temperature of 37° C. was used because this is apparently the physiological temperature at which most enzymes function satisfactorily and replicative RNA appears to function best at this temperature (18-21).

Isolation and Purification of the TMV-RNA Replicative Mechanism

The polyethylene glycol (PEG) - sodium chloride chromatographic method developed by Venekamp and Mosch (16) was modified. Scotia bean tissue infected with TMV was ground with enough polyethylene glycol and sodium chloride crystals to give a final mixture containing 10 and 4 percent of these chemicals, respectively. The macerate was mixed with dry cellulose powder to make a thick, stiff mixture. This mixture was placed above a 3-inch-thick cellulose column in a chromatographic tube that had been preconditioned by the passage of a 10 percent PEG 4 percent NaCl solution. The macerate was then washed with this same solution until the washing solution came through in a clear condition. The macerate was washed further with solutions having decreasing PEG and NaCl concentrations, in a stepwise manner, until a solution containing 5 percent PEG and no NaCl was used. This solvent appears to elute the TMV-RNA replicative mechanism which can be recovered from the solvent by centrifugation at 12,000 rpm using the Sorvall centrifuge. The pellet was suspended in standard sodium chloride solution which was again centrifuged at 30,000 rpm using the ultracentrifuge. The pellet was again resuspended in standard salt solution for later use.

The most current method in use in the virus laboratory at Utah State University is described as follows: Scotia bean leaves infected with TMV were ground with enough polyethylene glycol, sodium chloride and monobasic potassium phosphate to give a final concentration of 10 percent, 4 percent and 0.1 M respectively. The pH of

the macerate was adjusted to 5.0 with concentrated hydrochloric acid. The macerate was then mixed with dry cellulose powder and was placed on a two-inch-thick cellulose column 8 inches in diameter. The macerate was then eluted with twelve liters of solution containing 10 percent polyethylene glycol, 4 percent sodium chloride and 0.1 M monobasic potassium phosphate at pH 5.0. The effluent from this elution was discarded. The macerate was again eluted with a second twelve-liter solution containing 5 percent polyethylene glycol and no sodium chloride at pH 5.0 and likewise the effluent was discarded. A third twelve-liter solution containing 5 percent polyethylene glycol, 0 percent sodium chloride and 0.1 M dibasic potassium phosphate at pH 9.1 was used and the effluent was brought to 10 percent polyethylene glycol and 4 percent sodium chloride pH 7.0 and was centrifuged at 7,000 rpm for 35 minutes in the Sorvall rotor (type SGA). The pellets were discarded. The supernatant from this centrifugation was saved and passed through a four-inch-diameter cellulose column with the TMV-RNA replicative mechanism being adsorbed. This was then eluted with 5 percent polyethylene glycol and 0 percent sodium chloride. The TMV-RNA replicative mechanism was finally concentrated by centrifuging in the Spinco #30 rotor for one hour at 30,000 rpm. The pellets from this final procedure were suspended in standard sodium chloride solution. These pellets contained the TMV-RNA replicative mechanism. Infectivity assay on Scotia bean leaves revealed the large reddish concentric zonate lesions that we have constantly associated with the replicative form of the TMV-RNA during the past

two years. Tests for synthetic activity gave an exponentially increasing synthesis rate.

Preparation of the Nucleotide Solution

The reaction in which one viral RNA strand is synthesized by the polymerase requires the following molar ratio for the triphosphate nucleotides in tobacco mosaic virus: guanosine triphosphate (GTP), 1625; adenosine triphosphate (ATP), 1950; cytidine triphosphate (CTP), 1137; uridine triphosphate (UTP), 1788 (22). Converting this molar ratio to a simpler ratio the concentration of nucleotides solution was calculated in u moles per ml was GTP, 0.32; ATP, 0.4; CTP, 0.24 and UTP, 0.36; (23) magnesium ion in form of $MgCl_2$ was added to a concentration of 0.220 ug/ml.

A quantity of 200 ml of nucleotides and magnesium chloride solution was made and was divided to aliquots of 10 ml. All aliquots were adjusted to specific pH values from 4 to 8.9. Immediately after preparation all solutions were distributed into 0.5 ml quantities and stored in a deep freeze until used. Nucleotide triphosphates were obtained as sodium salts from Nutritional Biochemical Corporation and were dissolved in water.

Spectrophotometric Analysis

Cary 14 and Gilford 2000 spectrophotometers were used in these experiments. The transfer of an undisturbed density gradient to a spectrophotometer flow cell for analysis was achieved by constructing

a plastic container to receive the centrifuge tube and to seal against its rim. The upper part of the plastic container was connected to a 5-pound air pressure source to give a positive displacement of liquid from the centrifuge tube. A hole was punctured at the bottom of the centrifuge tube where a plastic capillary tube was installed to carry the solution through a peristaltic type pump to the flow cell in the spectrophotometer and then out again for fraction collecting procedures. The gradient liquid was displaced downward by the positive action of the air pressure at a rate determined by the speed of the peristaltic pump. In a usual analysis the absorption in 1 cm-path-length flow cell at 260 mu was recorded with the spectrophotometer chart drive operating at 1/15 inch per minute. These techniques gave reproducible accuracy to the sucrose density gradient analyses.

The effluents were fractionated manually. The absorption of the gradient was plotted automatically on the recording chart of the spectrophotometer. All the fractions were assayed for synthetic activity.

The analysis of the phosphate gradient was done in a similar manner, although a Beckman gradient pump was used, and the experiment was performed on a larger scale. A phosphate pH gradient from the pump was delivered to the top of a cellulose column that already carried the TMV-RNA replicative forms. The effluent passed through the spectrophotometer and was collected by a fractionator. At the same time the spectrophotometer was set so that every fraction of the effluent was scanned automatically from 300 to 210 mu. The

plotted curves then gave the absorbance of each fraction. The fractions that had the highest absorbances were centrifuged at 30,000 rpm for an hour and the pellets were resuspended in standard sodium chloride solution. Then these selected fractions were assayed for synthetic activity.

Assay of Synthetic Activity

Four spectrophotometer cuvettes of 2-mm-path-length were sterilized and cleaned with acid potassium dichromate solution and were rinsed thoroughly to remove any trace of the acid. Then 0.5 ml of nucleotide solution having a given pH value was transferred to each of the three cuvettes. Quantities of 0.05 to 0.1 ml of the TMV-RNA replicative mechanism, depending on the concentration of the particular preparation, were added with stirring to the cuvettes containing the nucleotide incubation mixture. Then the cuvettes were sealed with two layers of "Saran" wrap and one layer of polyethylene plastic by tying carefully with rubber bands. The cuvettes were inserted in the photo compartment of the Gilford spectrophotometer which automatically plots the change of absorbance for four samples one after the other in any desired interval of time. Distilled water was added to the fourth cuvette which was also sealed and was placed in the number one position in the photo compartment. This served as a control for automatic zeroing of the spectrophotometer. The changes in absorbance with time were recorded.

The entire photo compartment was kept at a constant temperature, usually 37°. Temperature was also automatically recorded on the chart.

The vernier of the instrument was turned fully counterclockwise to give the most sensitive setting. The absorbance scale was set and calculated so that one unit chart width was equivalent to 0.88 unit of absorbance when the ratio switch was at 0.1 position.

The samples were incubated for varying periods of time which were determined by the changes in absorbance recorded for a particular sample in a given experiment.

Assay of Infectivity

The assay of infectivity was that developed in this laboratory by Lamborn (24). Primary leaves of 10-day-old growth chamber grown Scotia bean plants were treated by immersing the plants in 45° C. water for one minute. This treatment increases susceptibility and gives increased size and number of lesions. The leaf halves were removed from the midribs and placed on one percent agar in aluminum pans. Next they were dusted lightly with 600-mesh carborundum. Since the actual virus concentrations are usually unknown, a series of 10-fold dilutions are usually assayed for meaningful data. In a given assay, usually 6 half leaves are infected with the same inoculum and the average lesion count on the leaves is used as a measure of the infectivity. One drop of inoculum was delivered using a capillary glass tube to the upper surface of each slightly wilted half leaf. The leaves supported on a half-inch-thick polyurathane sponge were passed under the cylindrical surface of the oscillating metal probe of a Branson Sonifier. This induces many small wounds by agitating the carborundum particles on the leaf surface. The wounds serve as

inoculation points for virus or viral RNA entry. A post inoculation treatment consisted of holding the inoculated leaves between dry paper towels for 17 hours at 31° C. sealed between glass plates. The inoculated half leaves were placed back on the agar surface in glass-covered aluminum trays. These were held under continuous illumination at approximately 28° C. Lesions that developed in about two days were counted.

General Experimental Procedure

Determination of the necessary reacting components for synthesis of TMV-RNA

To determine the reacting components in the extracts containing the TMV-RNA replicative mechanisms, the various fractions obtained from sucrose density gradient centrifugation and from phosphate pH gradient chromatography were tested for synthetic activity in the presence of the substrate, the ribose 5' triphosphates, the method of assay for synthetic activity has already been described. The amount of each fraction or combination of fractions used was determined from the results of the infectivity assay. The fractions or combination of fractions that gave the highest hypochromic shift during the assay for synthetic activity were identified as probable necessary component or components for the synthesis of tobacco mosaic viral RNA.

Determination of best environment for the replicative mechanism isolated from infected Scotia bean leaves

To determine the best ionic conditions, part of the TMV-RNA replicative mechanism was suspended in one ml of solution containing 0.15 M sodium chloride, 0.015 M sodium citrate, and 0.002 M magnesium chloride at pH 7.0; another part in 0.15 M sodium chloride and 0.015 M sodium citrate at pH 7.0; and a third part in 0.15 M sodium chloride and 0.002 M magnesium chloride at pH 7.0. The TMV-RNA replicative mechanism (0.05 ml) was added to three cuvettes each containing 0.5 ml of nucleotide solution at pH 7.0. These mixtures were incubated at 37° C. in the photo compartment of the spectrophotometer for the same period of time to determine which solution had the highest rate of synthesis. When the most favorable ionic condition was determined, further work was done to examine the influence of the concentration of the sodium chloride. The TMV-RNA replicative mechanism isolated from infected Scotia bean leaves was suspended in solutions having the same concentrations of sodium citrate and magnesium chloride but with concentrations of 0.1 M, 0.05 M and .010 M sodium chloride. Each of these solutions was assayed for synthetic activity. In addition the TMV-RNA replicative mechanism was also suspended in a solution containing 0.16 M sodium chloride, and then tested for synthesis of TMV-RNA with a pH 7.0 nucleotide solution at 37° C.

Determination of optimum pH

The optimum pH was determined by testing 0.05 ml of the replicative mechanism suspended in solution having the best ionic conditions

with the nucleotide solutions at pH levels ranging from pH 4 to 9. The synthetic mixtures were incubated at 37° C. while the absorbance of the samples were automatically recorded. The optimum starting pH value could be determined by comparison of the degree of hypochromic shift.

Determination of ratio of substrate to replicative mechanism

The ratio of substrate to replicative mechanism yielding optimum synthesis was determined by varying the concentration of the substrate, and replicative mechanism while testing for synthetic activity. In this experiment the replicative mechanism was suspended in the best ionic environment at optimum pH level and optimum temperature.

Correlation of the magnitude of the observed hypochromic shift in synthesis experiments with quantity of viral RNA synthesized

To correlate the magnitude of the observed hypochromic shift in synthesis experiments with quantity of viral RNA actually synthesized, the synthetic mixture was transferred from the cuvette to a small cellulose nitrate centrifuge tube holding approximately 0.5 ml of solution. Distilled water was added to fill the tube to 2 mm from the top. The tube was centrifuged in a swinging bucket rotor at 20,000 rpm for an hour to remove the more massive replicative mechanism. The less massive synthesized single-stranded viral RNA remaining in the supernatant was precipitated by adding an equal volume of 100 percent alcohol to give a final concentration of 50 percent alcohol. The solution was again centrifuged in a glass tube at 12,000

rpm to sediment the precipitated RNA. The resulting pellet was suspended in solution containing salts that provided the best ionic environment. The quantity of RNA in solution was determined by measuring the absorbance of the solution at 260 mu.

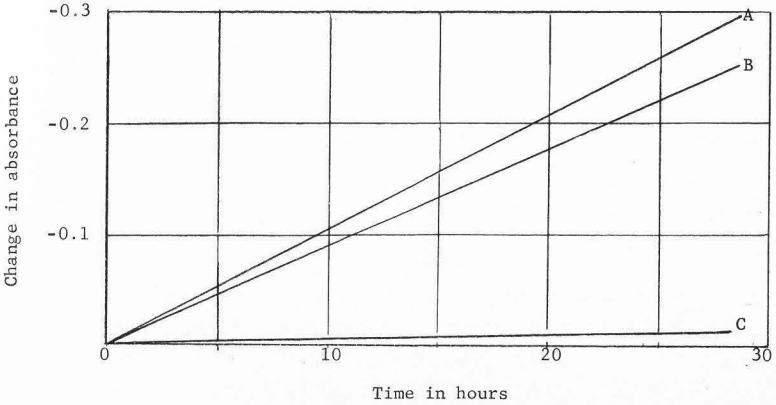
EXPERIMENTAL RESULTS AND DISCUSSION

The TMV-RNA replicative mechanism was concentrated by centrifuging at 30,000 rpm in a Spinco 30 rotor for one hour. The resulting supernatant was centrifuged at 50,000 rpm for one hour in a Spinco 50 rotor to pellet down any replicative mechanism that failed to sediment at 30,000 rpm. The 50,000 rpm pellet was suspended in distilled water and tested for synthetic activity. The activity is shown in C in Figure 1. The concentrated TMV-RNA replicative mechanism (sedimented at 30,000 rpm) was also resuspended in distilled water and tested with nucleotide solution at pH 7.0 and 7.8. The results are shown as A and B in Figure 1. The results of this experiment indicate that centrifuging at 30,000 rpm sediments all of the TMV-RNA replicative mechanism and that pH apparently had an effect on synthesis rates. This was further tested later.

In the determination of the most favorable ionic conditions for storage of the TMV-RNA replicative mechanism it was observed that 0.15 M sodium chloride, 0.015 M sodium citrate and 0.002 M magnesium at pH 7.0 gave the highest degree of hypochromic shift. It was therefore assumed that these ionic conditions had given the highest relative rate of synthesis. These results agree generally with the work of others in this field (8, 12, 25, 26). Weissmann (8) showed the resistance of the replicative RNA to ribonuclease (RNAse) increased rapidly with increasing ionic strength. He found that magnesium chloride was effective in enhancing RNAse resistance. Our experimental

data are shown in Figure 2. The solution containing 0.15 M sodium chloride, 0.015 M sodium citrate and 0.002 M magnesium chloride pH 7.0 will now be referred to as standard sodium chloride solution.

Avery, et al., demonstrated that deoxyribonucleic acid (DNA) suspended in physiological salt solution (0.5 to 1 mg/ml of sodium chloride) retained its activity during storage at 2-4° C. for three months while the activity rapidly decreased to zero after a few days of storage in distilled water (26). We also observed that the TMV-RNA replicative mechanism lost its synthetic activity after storage in distilled water at 2-4° C. for thirty days and the results are shown in Figure 3. The same TMV-RNA replicative mechanism stored in standard sodium chloride solution retained most of its activity. The concentration of sodium chloride appeared to be important. Concentrations lower or higher than 0.15 M appeared to lower the rate of synthesis as shown in Figure 4.



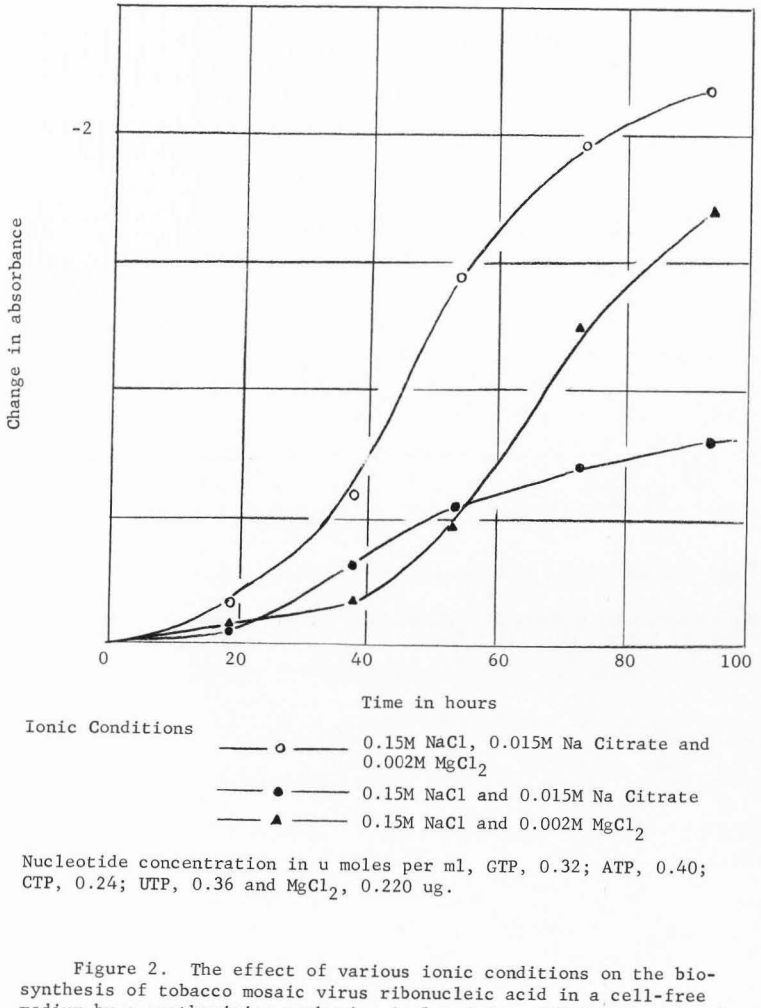
Nucleotide concentrations in u moles per ml: GTP, 0.32; ATP, 0.40; CTP, 0.24; UTP, 0.36 and $MgCl_2$, 0.220 ug.

Curve A: TMV-RNA replicative mechanism primed with nucleotide solution at pH 7.0.

Curve B: TMV-RNA replicative mechanism primed with nucleotide solution at pH 7.8.

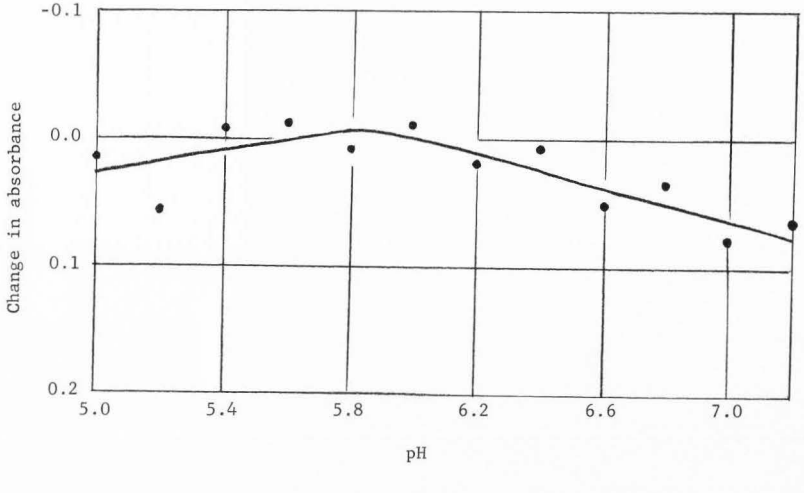
Curve C: Supernatant from the preparation of the replicative mechanism primed with nucleotide solution at pH 7.0.

Figure 1. Biosynthesis of tobacco mosaic virus ribonucleic acid in a cell-free medium by a synthesizing mechanism isolated from TMV-infected Scotia bean leaves when primed with nucleotides and magnesium ions.



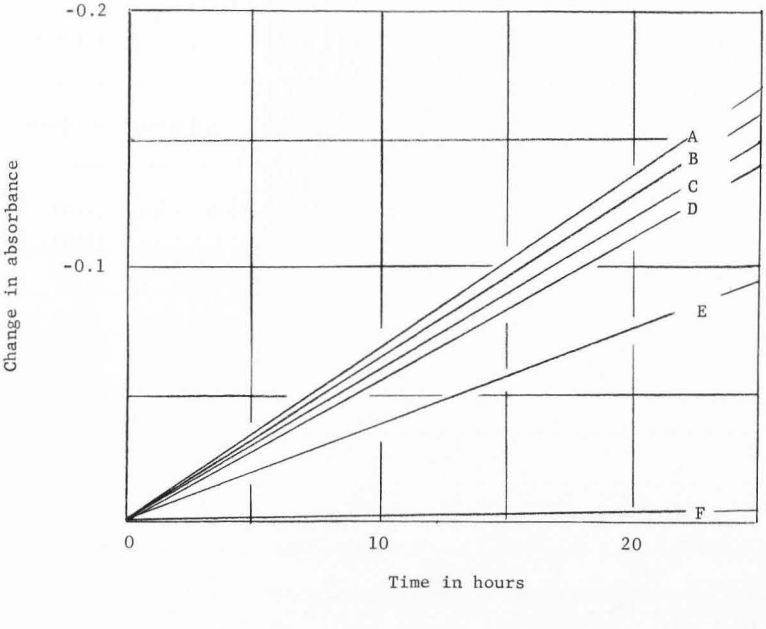
Nucleotide concentration in u moles per ml, GTP, 0.32; ATP, 0.40; CTP, 0.24; UTP, 0.36 and $MgCl_2$, 0.220 ug.

Figure 2. The effect of various ionic conditions on the biosynthesis of tobacco mosaic virus ribonucleic acid in a cell-free medium by a synthesizing mechanism isolated from TMV-infected Scotia bean leaves when primed with nucleotides and magnesium ions.



TMV-RNA replicative mechanism stored in distilled water at 2-4° C. for 30 days was incubated with nucleotide substrate ranging in pH from 5.0 to 7.4 at 37° C. for 48 hours. Nucleotides and magnesium ion levels were the same as those indicated in previous experiments.

Figure 3. The inability of 30-day-old water suspended TMV-RNA replicative mechanism to utilize the ribose 5' nucleotide substrate for the synthesis of new viral RNA at varying pH levels.



The replicative RNA mechanism was suspended in solutions containing the same amount of sodium citrate (0.015M, pH 7.0) and magnesium chloride (0.002M) but different amount of sodium chloride. The concentration of NaCl of sample A, B, C, D, and E in moles are 0.15, 0.1, 0.05, 0.01 and 0.16 respectively. Sample F contained only nucleotides and magnesium ions. The nucleotide and magnesium ion concentration of all these samples were the same as those used in previous experiments. The starting pH was 7.0.

Figure 4. The effect of sodium chloride levels on the activity of the replicative mechanism of TMV-RNA in nucleotide solutions.

A preliminary experiment was undertaken to determine the limiting extremes of pH. It was found that there was very little synthesis when the nucleotide solutions were below pH 5.0 or above pH 8.1 as shown in Figure 5.

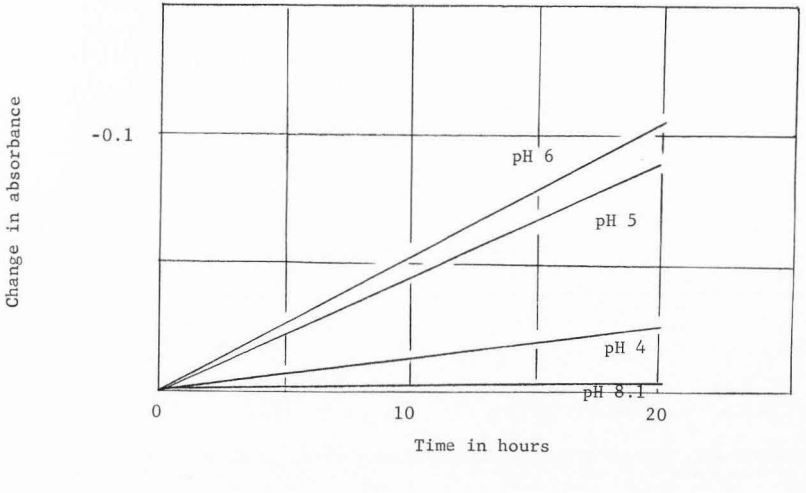
Next a series of experiments were initiated to determine the initial pH value giving optimum synthesis. The isolated replicative mechanism stored in standard sodium chloride solution was incubated for 40 hours at 37° C. after the addition of nucleotide-magnesium chloride solution mixture that had been adjusted to the desired pH levels. The results of the first experiment, shown in Figure 6a, indicate that initial pH values between 7.0 to 7.2 were apparently the most favorable. However, an initial pH level of 6.3 also resulted in considerable synthesis as judged from the degree of hypochromic shift. Similar results were found in subsequent experiments and are shown in Figure 6 b to d.

These experiments were repeated using a modified technique. Instead of examining only three samples at one time in the Gilford spectrophotometer, numerous synthesis mixtures at varying pH levels were incubated in a water bath at 37° C. in a small sealed glass test tubes for 48 hours. The tubes were also wrapped in aluminum foil to insure temperature uniformity and to prevent condensation of water on the upper seal. The absorbance of the mixtures were measured before and after incubation and the differences were recorded as the degree of hypochromic shift. The optimum range of pH was again observed between 7.0 to 7.2 as shown in Figure 6b.

A third experiment was conducted with the replicative mechanism coming from storage in a more dilute ionic environment (0.05 M sodium chloride .015 M sodium citrate and 0.002 M $MgCl_2$). The rather low amount of hypochromic shift in this experiment suggests that the ionic environment for the storage of the replicative mechanism may have been too dilute. Starting pH values of 7.0 and 6.15 seemed to give the best synthesis in this one experiment. The results are shown in Figure 6c.

The results of a fourth experiment that gave somewhat similar results are shown in Figure 6d. In this experiment the replicative mechanism was a fraction from a sucrose density gradient separation of infected Scotia bean extracts.

In these determinations the initial pH's were known but the final pH values were higher because one of the synthesis products is alkaline pyrophosphate. Because synthesis shifts the pH toward alkalinity, the increasing pH values may tend to slow down synthesis rates. One obvious answer to this problem is to use a suitable buffer. Our initial tests were conducted without such a buffer because we did not want to introduce another variable that had to be evaluated. One experiment utilizing a phosphate buffer system gave no synthesis. Perhaps the large excess of phosphate ions inhibited synthesis by shifting the reaction equilibrium in the direction opposite of synthesis. Before a buffer system can be chosen for this synthesis reaction, the effect of the buffer on the synthesizing system must be studied.



Nucleotides and magnesium ion levels were the same as those indicated in the previous experiments.

Figure 5. Effects of starting pH levels on subsequent synthesis of TMV-RNA from ribose 5' triphosphate nucleotides by a partially purified replicative system isolated from TMV infected Scotia bean leaves.

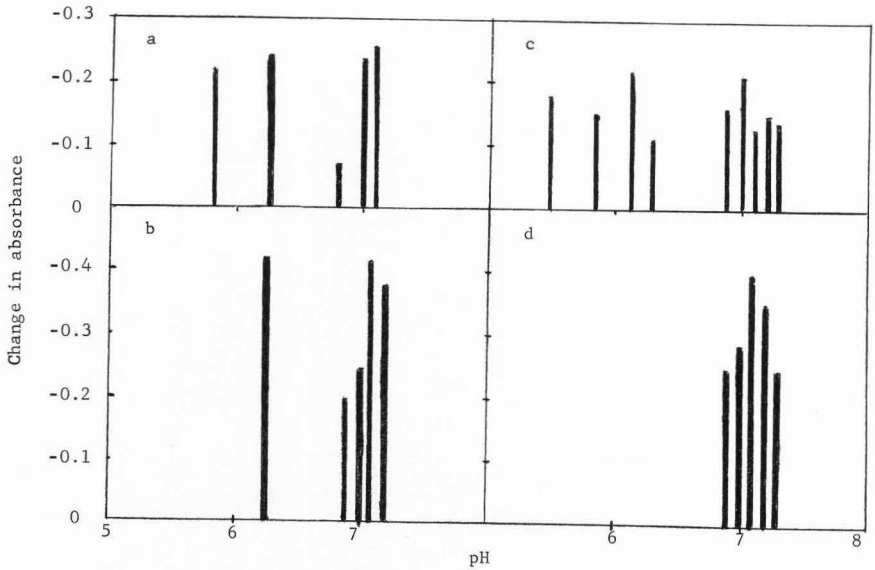
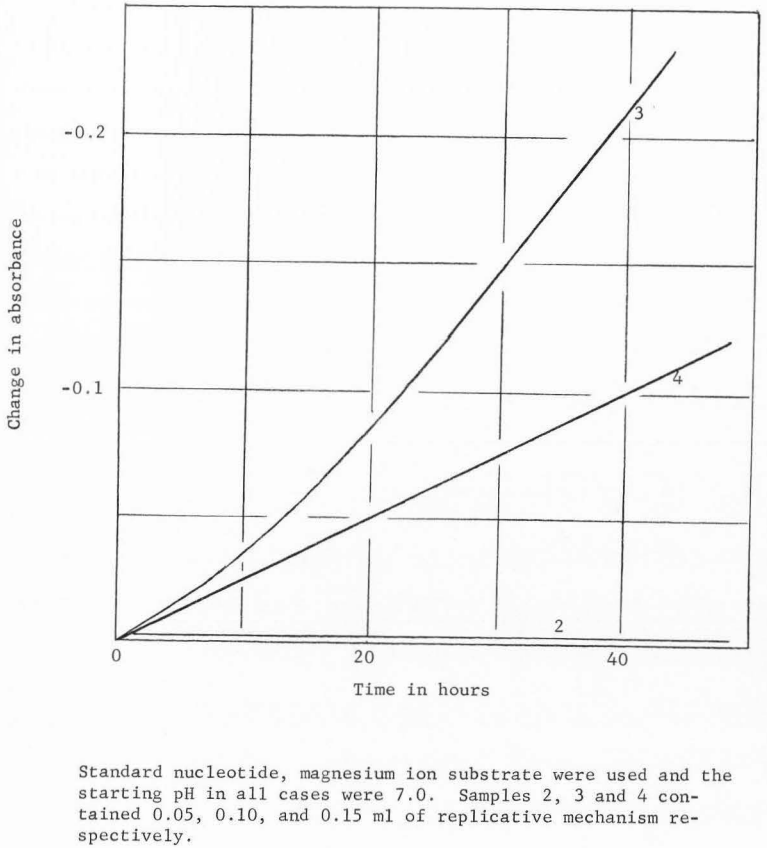


Figure 6. Determination of optimum pH for the biosynthesis of TMV-RNA from ribose 5' nucleotides by a partially purified synthesizing mechanism isolated from TMV infected Scotia bean leaves. Nucleotides and Mg^{++} concentrations were the same as in previous experiments.

A preliminary experiment was performed to study the ratio of substrate to TMV-RNA replicative mechanism giving optimum synthesis. The results shown in Figure 7 suggested that there is a certain ratio of replicative mechanism to substrate that gives most rapid synthesis. This was a preliminary experiment with a particular replicative mechanism preparation. These results should not be generalized for every preparation because the concentration of the isolated replicative mechanism depends on the amount of infected leaves, on the duration of the infection and on the amount of standard sodium chloride solution that the preparation was suspended in.



Standard nucleotide, magnesium ion substrate were used and the starting pH in all cases were 7.0. Samples 2, 3 and 4 contained 0.05, 0.10, and 0.15 ml of replicative mechanism respectively.

Figure 7. A study of the ratio of replicative mechanism to substrate in one experiment.

Attempts were made to correlate the amount of single-stranded viral RNA synthesized with the magnitude of the hypochromic shift observed in several experiments. In our experiments we postulate that the starting conditions are replicative mechanism and nucleotide-magnesium chloride solution mixture and the ending conditions are replicative mechanism and nucleotide-magnesium chloride solution mixture plus newly synthesized viral RNA.

A procedure was followed in which we hoped to differentially centrifuge the replicative mechanism out of the solution without sedimenting the newly synthesized RNA. In a second step the newly synthesized RNA was to be precipitated with 50 percent ethyl alcohol and thereby separated from the unused nucleotides. It was hoped that the isolated, newly synthesized RNA could be measured spectrophotometrically and that these measurements could be correlated with the degree of hypochromic shift observed in these experiments. As it often happens, the practice does not confirm the theory. The chief problems were the lack of a clear cut separation in the differential centrifugation of the RNA types and our inability to transfer such small amounts of reacting mixtures without excessive losses. Also a solubilization of cellulose nitrate from centrifuge tubes by ethyl alcohol contributed to the difficulties of the technique. It did not appear that these problems could be overcome so that the proposed objective could be attained.

Since the degree of hypochromic shift was the criteria for determining the most favorable ionic environment, optimum pH and temperature, one must be sure that the hypochromicity was not due to some cause other than synthesis of viral RNA. In a 2 mm-path-length cuvette filled with a highly absorbing liquid such as our nucleotide mixture, a lowering of the liquid level by evaporation can induce an effect that may be confused with hypochromicity due to viral RNA synthesis. An experiment was devised to study the relation of evaporation to the change of absorbance of a solution. A typical curve of the evaporation effect is shown in Figure 8. From point A to point B on the curve, absorbance increased. This is a concentration effect caused by loss of solvent which confines the solutes into smaller and smaller volumes. This effect continues as long as the solvent level remains above the top of the spectrophotometer light beam. However, when the meniscus reaches the light beam at point B a drastic change occurs because the empty cuvette space now occupying part of the beam area transmits increasing amounts of radiation with increasing evaporation. This more than cancels the concentration effect. This effect is peculiar to 1 and 2 mm-path-length cells. In longer path-length cells such as 10 mm the transmission of light becomes much less because of higher refraction losses at the quartz-air interfaces.

We are certain that the hypochromic shifts observed in our experiments are not caused by this evaporation phenomenon. Firstly, we can check the cuvettes visually to note if evaporation had occurred. Secondly, if evaporation had occurred the recorded curve would have

resembled that shown in Figure 8. If it did not, then it represents a recording of true synthesis. Thirdly, if the evaporated water was replaced to bring the cuvette liquid volume back to the original level, the spectrophotometer recorder pen should return to the original levels. We have found in our experiments where both synthesis and evaporation occurred that the pen could never be brought back to the original levels with the replacement of the lost water. This, therefore, was accepted as evidence of a hypochromic shift resulting from true synthesis.

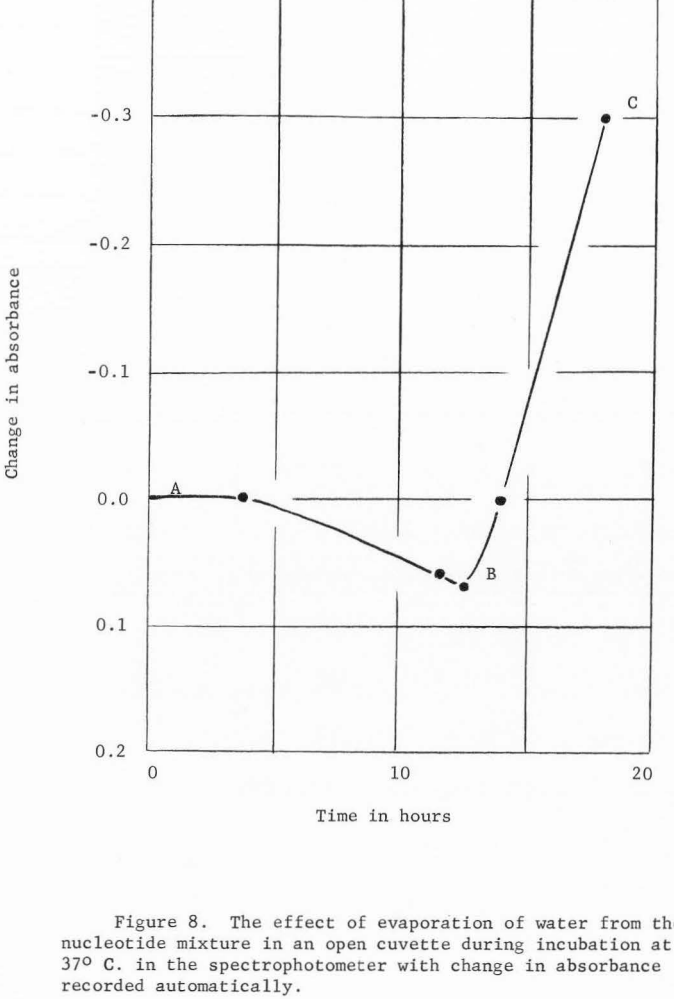
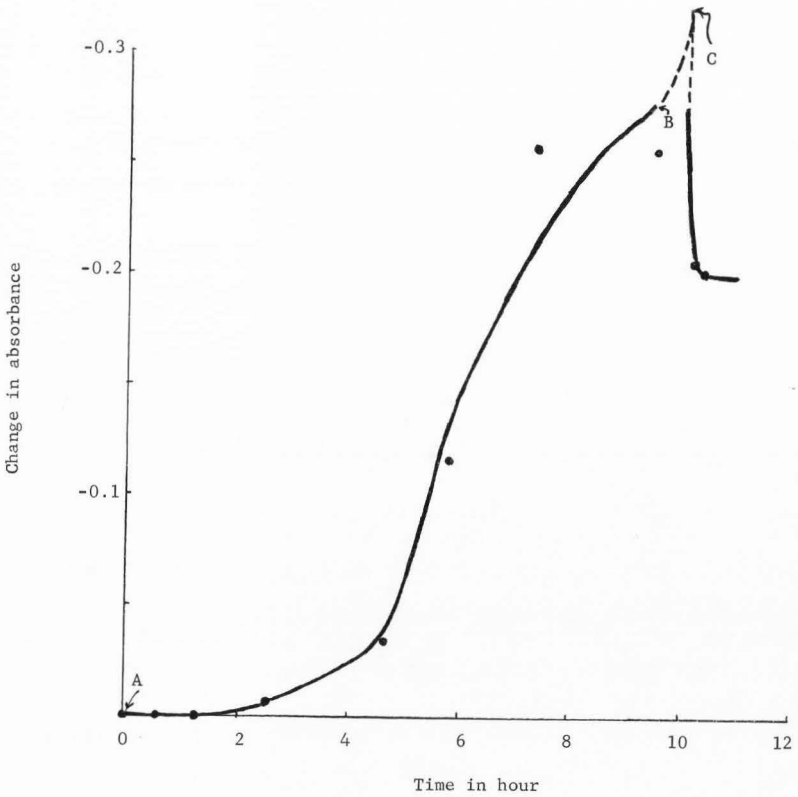


Figure 8. The effect of evaporation of water from the nucleotide mixture in an open cuvette during incubation at 37° C. in the spectrophotometer with change in absorbance recorded automatically.

In order to show that single-stranded RNA is the product of the synthesis of tobacco mosaic virus RNA, a ribonuclease (RNAse) test was performed. The results are shown in Figure 9. The synthesis mixture was allowed to continue to completion when incubated at 37° C. after which 0.1 ug of RNAse was added. The immediate decrease in absorbance was the result of dilution. This effect was reversed very quickly by the action of the enzyme in digesting the single-stranded viral RNA. The increased absorbance suggests that the RNA was broken into fragments that absorbed more light.



The Mg^{++} and nucleotide levels were the same as in previous experiments. Curve from point A to B is a typical TMV-RNA synthesis curve. At point B 0.1 ug of RNAse was added and there was an immediate absorbance decrease due to dilution. However, this was reversed immediately by the attack of RNAse in reducing the single-stranded viral RNA to oligonucleotides. This attack is reflected by the increase in absorbance beginning at point C on the curve.

Figure 9. Action of pancreatic ribonuclease on the product of the TMV-RNA synthesis reaction.

SUMMARY AND CONCLUSION

The factors influencing the synthesis of tobacco mosaic viral RNA in a partially purified system were studied.

Isolation of the Necessary Reacting Components from Extracts Containing the Replicative Mechanism

In isolating the components for the synthesis of tobacco mosaic virus from the extracts by sucrose density gradient techniques, it was observed that much synthetic activity was lost. The use of phosphate pH gradient chromatography separation techniques appeared to give somewhat better results. However, other techniques will be tried to achieve this purpose in continuing the research.

Determination of the Most Favorable Ionic Environment

The ionic concentrations of storage that appeared most favorable for highest activity of the replicative form of tobacco mosaic virus were found to be 0.15 M sodium chloride, 0.015 M sodium citrate and 0.002 M magnesium chloride.

The Determination of Optimum pH Level

The most favorable pH for synthesis appeared to be near 6.3 and in the 7.0 to 7.2 range. Various workers in this field have also used neutral or slightly alkaline pH levels (3, 12-13, 18, 20). More work is needed to complete this study when a preparation of the

replicative mechanism has been more highly purified. In addition the use of a suitable buffer system will probably give a more constant pH value throughout any given synthesis.

The Determination of the Ratio of Substrate to Replicative Mechanism

The ratio of substrate to replicative mechanism could not be determined precisely until our replicative mechanism has been highly purified and standardized. Each preparation of replicative mechanism varies in the amount of enzyme activity. This object will be achieved when a standard purified preparation is prepared.

The Relation of the Amount of RNA Formed to the Magnitude of Hypochromic Shift

Our proposed methods did not permit us to measure the amount of RNA formed with sufficient accuracy for the small samples involved. Perhaps the incorporation of radioactive nucleotides will give us the precision needed for these determinations.

LITERATURE CITED

(1) Cochran, G. W., A. S. Dhaliwal, G. W. Welkie, J. L. Chidester, M. H. Lee and B. K. Chandrasekhar. Biosynthesis of infectious tobacco mosaic ribonucleic acid in a cell-free medium. Science. 138:46, 1962.

(2) Karasek, M., and G. Schramm. A ribonucleotide polymerase from tobacco leaves and the formation of viral ribonucleic acid in vitro. Biochem. Biophys. Res. Comm. 9:63, 1962.

(3) Kim, Y. T., and S. G. Wildman. Synthesis of infectious tobacco mosaic virus RNA by cell free extracts from TMV-infected tobacco leaves. Biochem. Biophys. Res. Comm. 8:394-401, 1962.

(4) Cornuet, P. and S. Astier. Essais de biosynthèse enzymatique in vitro du pouvoir infectieux du virus de la mosaique du tabac. Acad des. Sci. Compt. Rend. 255:3076-3078. 1962a.

(5) Balandin, I.G., E. B. Khoroshutina, and V. S. Tongur. Study of the mechanism of the synthesis of RNA in extracts from leaves of Nicotiana glutinosa infected with tobacco mosaic virus. Doklady Akad. Nauk., USSR, 155, 201-203.

(6) Balandin, I.G., E. B. Khoroshutina, and V. S. Tongur. Synthesis of tobacco mosaic RNA in vitro. Abstracts of papers of the Ivanovsky Centennial Conferences on Viruses, p. 33-34, 1964.

(7) Tongur, V. S., I. G. Balandin, E. D. Vyshepan, and E. B. Khoroshutina. Synthesis of RNA in cell free homogenates of leaves infected with tobacco mosaic virus. Vopr. Virusology 2, 142-143, 1963.

(8) Weissmann, C. and P. Borst. Double-stranded RNA formation in vitro by MS2 phaged-induced RNA synthetase. Science 142:1188, 1963.

(9) Langridge, R., M. A. Billeter, P. Borst, R. H. Burdon and C. Weissmann. The replicative form of MS2 RNA: An X-ray diffraction study. Nat. Acad. Scie. Proc. 52:114-119, 1964.

(10) Weissmann, C., P. Borst, R. H. Burdon, M. A. Billeter and S. Ochoa. Replication of viral RNA, III. Double-stranded replicative form of MS2 phage RNA. Nat. Acad. Scie. Proc. 51:682, 1964.

(11) Burdon, R. H., M. A. Billeter, C. Weissmann, R. C. Warner, S. Ochoa and C. A. Knight. Replication of viral RNA, V. Presence of a virus specific double-stranded RNA in leaves infected with tobacco mosaic virus. Nat. Acad. Scie. Proc. 52:768, 1964.

(12) Shipp, W., and R. Haselkorn. Double-stranded RNA from tobacco leaves infected with tobacco mosaic virus. Nat. Acad. Scie. Proc. 52:401, 1964.

(13) Haruna I., and S. Spiegelman. Autocatalytic synthesis of a viral RNA in vitro. Science 150:884, 1965.

(14) Cochran, G. W., A. S. Dhaliwal, G. W. Welkie, J. L. Chidester, M. H. Lee and B. K. Chandrasekhar. Biosynthesis of infectious tobacco mosaic virus ribonucleic acid in a cell-free medium. Phytopathology 52:728, 1962.

(15) Lodish, H. F., and N. D. Zinder. Replication of the RNA bacteriophage f2. Science 152:372, 1966.

(16) Venekamp, J. H., and W. H. M. Mosch. The use of polyethylene glycol in clarification and purification of tobacco mosaic virus. Virology 22:503, 1964.

(17) Gierer A., and G. Schramm. Infectivity of ribonucleic acid from tobacco mosaic virus. Nature 177:702, 1956.

(18) Weissmann, C., M. A. Billeter, M. C. Schneider, C. A. Knight and S. Ochoa. Replication of viral RNA, VI. Nucleotide composition of the replicative form of TMV-RNA and its component strands. Nat. Acad., Scie. Proc. 53:653, 1965.

(19) Wool, Ira G., and Alan J. Munro. An influence of insulin on the synthesis of a rapidly labelled RNA by isolated rat diaphragm. Nat. Acad. Scie. Proc. 50:919, 1963.

(20) August, J. T. and Lucille Shapiro. Replication of RNA viruses I, characterization of a viral RNA - dependent RNA polymerase. J. Mol. Biology 11:257, 1965.

(21) Bonner, J., R. C. Huang and N. Maheshwari. The physical state of newly synthesized RNA. Nat. Acad. Scie. Proc. 47:1548-1554, 1961.

(22) Cochran, G. W. Secret of viral reproduction. Twenty-eighth Faculty Honor Lecture, Nov. 26, 1963. Utah State University, Logan, Utah.

(23) Holoubek V. The composition of tobacco mosaic protein after incorporation of 5-fluorouracil into the virus. Molecular Biology 6:164-166, 1963.

(24) Lamborn, C. R. An improved method for assaying infectivity of tobacco mosaic virus. Phytopathology 54:898, 1964.

(25) Ralph, R. K., R. E. F. Matthews, A. I. Matus, and H. G. Mandel. Isolation and properties of double-stranded viral RNA from viruses-infected plants. J. Mol. Biol. 11:202-212, 1965.

(26) Avery, O. T., C. M. Macleod, and M. McCarty. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. J. Expl. Med. 79:143, 1944.