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ISOLATION AND CHARACTERIZATION OF A VIRUS-SPECIFIC RIBONUCLEIC ACID FROM NICOTIANA TABACUM PLANTS INFECTED WITH TOBACCO MOSAIC VIRUS

by

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GRADUATE COLLEGE

I hereby recommend that this dissertation prepared under my direction by Dawn Masunaga Mitchell

entitled Isolation and Characterization of a Virus-Specific Ribonucleic Acid from Nicotiana tabacum Plants Infected with Tobacco Mosaic Virus.

be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy

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After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

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ABSTRACT

The nucleic acids from TMV-infected plants have been studied for the presence of unique forms of RNA associated with virus replication. The RNA was isolated by phenol extraction of leaves infected six to seven days and was fractionated by chromatography twice on cellulose powder, being eluted in plain buffer. The RNA was characterized by the techniques of analytical isopycnic centrifugation, thermal denaturation, annealing with radioactively labeled TMV-RNA, and polyacrylamide gel electrophoresis. Furthermore, to localize the RNA in the cell, the nucleic acids were obtained from several cell fractions, were fractionated by cellulose chromatography, and were analyzed by isopycnic centrifugation.

The RNA in the buffer fraction appeared as a single band with a large shoulder in Cs_2SO_4 equilibrium density gradients. The RNA in the shoulder, having a buoyant density intermediate between single- and double-stranded RNA, was eliminated by exposure to low concentrations of RNase prior to isopycnic centrifugation. The concomitant increase in double-stranded RNA indicated that the single-stranded portion of a partially single-stranded, partially double-stranded molecule was removed, producing RNase-resistant double-stranded RNA. In contrast, no

RNase-resistant RNA was found in extracts from uninfected leaves.

A sharp increase in the optical density over a very narrow temperature range with a midpoint at 68° C, and a slight increase over a broad low temperature range were seen in the thermal denaturation profiles. The sharp transition established that the RNA had a hydrogen-bonded double-stranded molecular structure and the melting at low temperatures suggested a single-stranded component.

A significant increase in RNase-resistance over the control was conferred to H^3-labeled viral RNA by heat denaturation and annealing in the presence of RNA from the buffer fraction. Thus, the double-stranded forms were virus-specific and contained strands complementary to TMV-RNA.

RNA, which behaved like partially single-stranded, partially double-stranded RNA when treated with RNase, barely penetrated into 2.0% polyacrylamide gels, indicating that it had a high molecular weight. The relative electrophoretic mobility was approximately half that of RNase-resistant RNA with twice the molecular weight of TMV-RNA (2×10^6 daltons). Heat denatured RNA from the buffer fraction migrated in polyacrylamide gels with the same mobility as TMV-RNA indicating that one or both strands of the double-stranded molecules were as large as TMV-RNA.

Double-stranded RNA was not seen in the SDS-solubilized nuclear fraction or the Triton-X-100 solubilized chloroplast fraction of TMV-infected leaf cells, but was present in the mitochondrial, ribosomal, and cytoplasmic fractions. The greatest amount and largest proportion of partially single-stranded partially double-stranded RNA with high molecular weight appeared in the mitochondrial fraction (14,000 x g) providing evidence that TMV-RNA replication might be associated with mitochondria or cosediments with them.

Thus, a ribonucleic acid with physicochemical properties consistent with those expected of a "replicative intermediate," consisting of a double-stranded core and associated single-strands, in addition to double-stranded RNA with twice the molecular weight of TMV-RNA was isolated from extracts of TMV-infected tobacco plants and was localized in the mitochondrial fraction.

INTRODUCTION

Tobacco mosaic virus (TMV) is a rod-shaped macromolecule consisting of ribonucleic acid (RNA) and protein. Upon infection of a host plant, the viral components are reproduced yielding more virus. The mechanisms of this reproductive process are incompletely understood. It is the aim of this study to gain insight into this process by isolating unique forms of RNA which might be intermediates in the replication of the viral RNA.

The RNA is the hereditary component of TMV. During reproduction it is necessary that more viral RNA is made which is identical to the infecting RNA strand. It is postulated that the infecting strand is used as template for the production of the progeny viral RNA. In the replication model upon which this investigation is based, the appearance of two types of intermediates in TMV-RNA replication is predicted. The infecting or "plus" strand is thought to be used as a template for the synthesis of a complementary "minus" strand. This intermediate, called replicative form (RF) is a double-stranded RNA molecule with complementary plus and minus strands. The second intermediate results when the "minus" strand of the replicative form serves as template for the synthesis of new "plus" strands. Asymmetrical synthesis of "plus"

strands, rather than symmetrical synthesis of both strands of the replicative form, is postulated as an economical means of producing "plus" strands without a surplus of "minus" strands. As a result of this asymmetric synthesis, a branched molecule, termed replicative intermediate (RI), consisting of a double-stranded core and one or more associated nascent single-strands, is proposed.

A fractionation procedure, chromatography on cellulose powder with elution by buffer containing various amounts of ethanol, was developed for the isolation of the RF and RI of the RNA bacteriophage R17 (Franklin 1966). The RNA is separated according to its size and relative content of hydrogen-bonded secondary structure. RNA molecules with a highly ordered hydrogen-bonded secondary structure, such as RF and RI, elute in plain buffer, whereas smaller single-stranded molecules elute in buffer and ethanol.

In the present study, RNA was isolated by phenol extraction of large amounts of plant tissue which had been infected with TMV for six or seven days. It was expected that the virus was in a rapid phase of multiplication and that intermediates in RNA synthesis might be present at a detectable concentration. The RNA was fractionated by two passages through the cellulose column. The RNA component eluting in the plain buffer fraction was examined by analytical isopycnic centrifugation in Cs_2SO_4,

thermal denaturation, annealing with viral RNA, and by electrophoresis on polyacrylamide gels. It was found that this fraction from TMV-infected leaves contains double-stranded RNA with twice the molecular weight of TMV-RNA and another species of RNA with the following properties: a buoyant density intermediate between that of double- and single-stranded RNA, partial susceptibility to low concentrations of RNase yielding double-stranded RNA, and a thermal denaturation profile resembling that of double-stranded RNA, showing melting at low temperatures which is indicative of partial single-strandedness. Furthermore, this RNA confers RNase-resistance specifically to H^3-labeled TMV-RNA demonstrating the presence of a base sequence complementary to viral RNA, and migrates on polyacrylamide gels with very low mobility indicating that it has a high molecular weight, and after thermal denaturation migrates with the mobility of TMV-RNA. The results of these physicochemical analyses are consistent with those characteristics expected of a replicative intermediate consisting of a double-stranded core and associated single strands. In addition, it was found that this RNA species and double-stranded RNA were present in the greatest amount in the mitochondrial fraction of infected plants, indicating that TMV-RNA replication was associated with mitochondria or perhaps a replication complex which cosedimented with them.

LITERATURE REVIEW

This dissertation is concerned with the replication of the ribonucleic acid (RNA) of tobacco mosaic virus (TMV). TMV is a slender rod-like macromolecule consisting of protein and RNA. The virus is 3000 A long and has an outer radius of 90 A and an inner radius of 20 A (Caspar 1964). The protein, in the form of 2130 identical subunits, is arranged in a helix having a pitch of 23 A (Franklin, Caspar, and Klug 1959). Each protein subunit is composed of one chain of 158 amino acids (Tsugita et al. 1960). The RNA is a single strand of 6400 nucleotides; its molecular weight is 2×10^6 daltons, which is five per cent of the molecular weight of the virus (Knight and Woody 1958). The RNA alone was infective which demonstrated that the hereditary material of the virus is its RNA (Fraenkel-Conrat 1956, Gierer and Schramm 1956). As the genetic material, the RNA directs the reproduction of the specific nucleic acid and protein components of TMV.

General Scheme of RNA Virus Replication

There are two kinds of viruses, distinguished by the type of nucleic acid which serves as their genetic material, DNA (deoxyribonucleic acid) or RNA. Since cells have DNA as their genetic material, the reproduction of the

genetic material of DNA viruses can occur by mechanisms present in the host cell. However, uninfected cells have no known mechanisms for using RNA as template and reproducing the genetic material of RNA viruses. The way in which some of the RNA viruses circumvent this obstacle and mediate the replication of their RNA has been partially elucidated. Mechanisms by which viruses containing single-stranded RNA reproduce appear to have a basic similarity, whether the viruses infect bacterial, plant, or animal cells. Evidence has accumulated which serves as the basis for the following review of RNA virus replication.

The events of viral replication presented in Fig. 1 represent a generalized scheme of the life cycle of an RNA virus (from Martin 1967). Infection is initiated when the virus is introduced into a susceptible cell. During the penetration of virus into the interior of the cell, the protein coat is removed, releasing viral RNA into the cell's cytoplasm. The released viral RNA acting as the messenger then directs the synthesis of proteins, such as RNA dependent RNA polymerase which effects the replication of viral RNA, and/or the production of coat protein. The virus-induced RNA dependent RNA polymerase uses the infecting or parental viral RNA as a template for production of more viral RNA. The newly synthesized viral RNA molecules may then act as messengers for the synthesis of more protein, as template for the synthesis of further

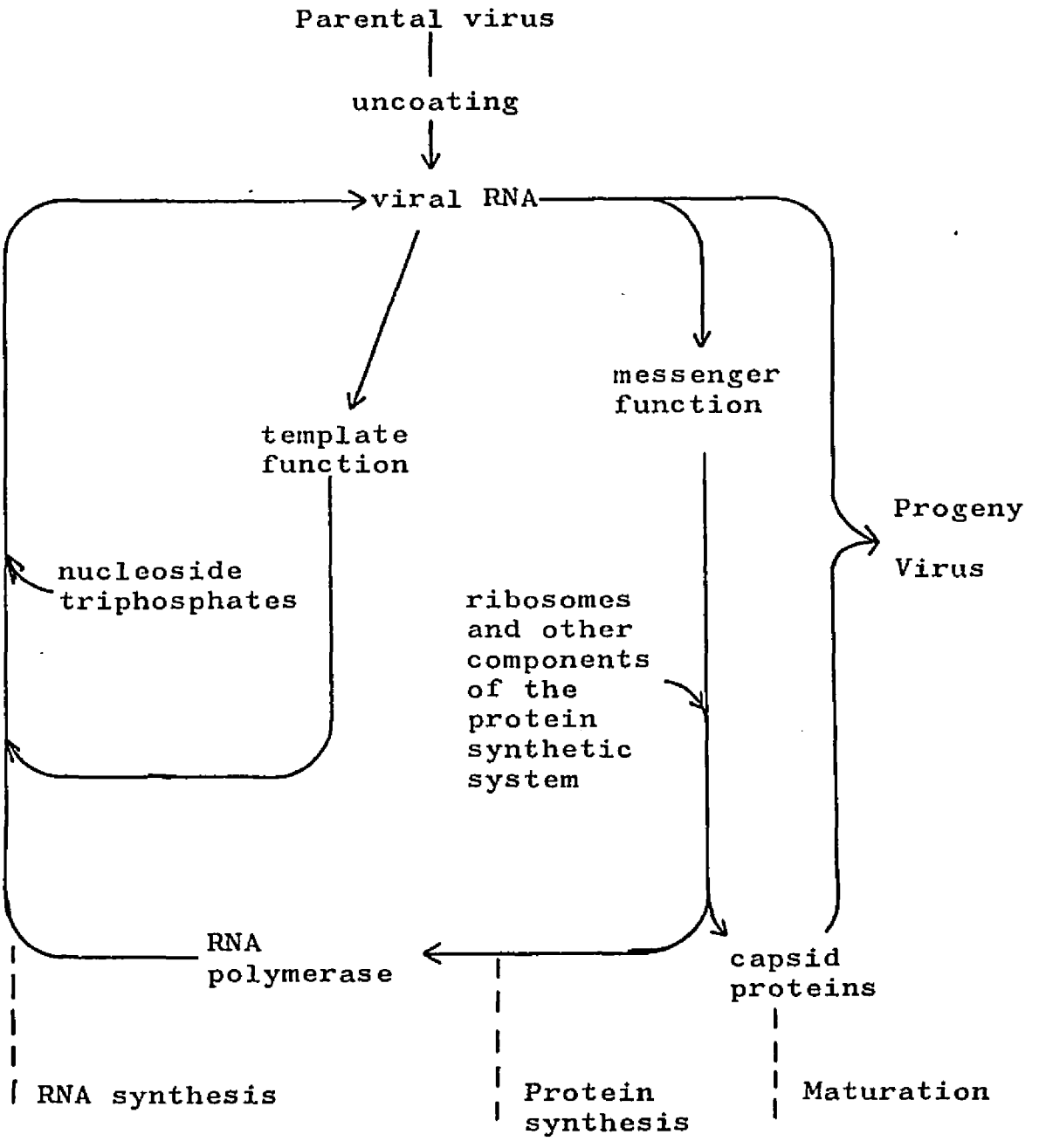


Fig. 1. Events during the replication of small RNA-containing viruses (Martin 1967).

generations of viral RNA, or be enclosed by coat protein to form new virus particles.

General Scheme of Viral RNA Replication

Several proposals have been made concerning the mechanism by which parental or infecting viral RNA effects the synthesis of progeny RNA. Some of the hypotheses implicated DNA in RNA virus synthesis. One suggestion was that viral RNA induced a DNA intermediate which would serve as template for viral RNA. Another hypothesis was that DNA of the host cell or host cell DNA which had been modified in some way was the template for the synthesis of viral RNA (R. Dulbecco as quoted in Erikson and Franklin 1966). Other proposals were based on the alternative concept that viral RNA served as template for its own synthesis. One scheme was the "direct copying" of the parental RNA by an enzyme capable of recognizing its base sequence directly, producing progeny RNA like the parent (Spiegelman and Haruna 1966). A highly probable mechanism was suggested for the replication of viral RNA when it was found that the parental single-strand of the DNA virus, θ-X-174, entered a double-stranded replicative form (RF) after infection (Sinsheimer et al. 1962). The two strands of the RF have a complementary base sequence as dictated by the principle of base pairing proposed by Watson and Crick (1953). According to their model, adenine pairs with thymine in DNA or

with uracil in RNA, and guanine pairs with cytosine because of highly specific hydrogen bonds between the respective base pairs. Due to the restriction in pairing imposed by the hydrogen bonding, the sequence of bases along one chain of polynucleotides can serve as template for the sequence of bases on the other; the sequences are complementary. The complementary base pair model predicts two steps in viral RNA replication. The infecting or "plus" strand is thought to be used as template for the synthesis of "minus" strand. The resulting molecule is a double-stranded RNA with complementary "plus" and "minus" strands and is called "replicative form" (RF). In the second step, the "minus" strand of the "replicative form" serves as template for the synthesis of new plus strands. Preferential synthesis of the plus strand of the double-stranded molecule was postulated as an economical mechanism of producing viral RNA without making a surplus of minus strands (Weissmann et al. 1964). As a consequence, a branched molecule, termed "replicative intermediate" (RI), consisting of a double-stranded core and one or more nascent single-strands, was proposed (Fenwick, Erikson, and Franklin 1964). The selective synthesis of plus strands in which a nascent, growing plus strand displaces its preexisting counterpart from the duplex is known as semiconservative, asymmetric replication (Fig. 2a). In the alternative mechanism of conservative, asymmetric replication (Fig. 2b), the

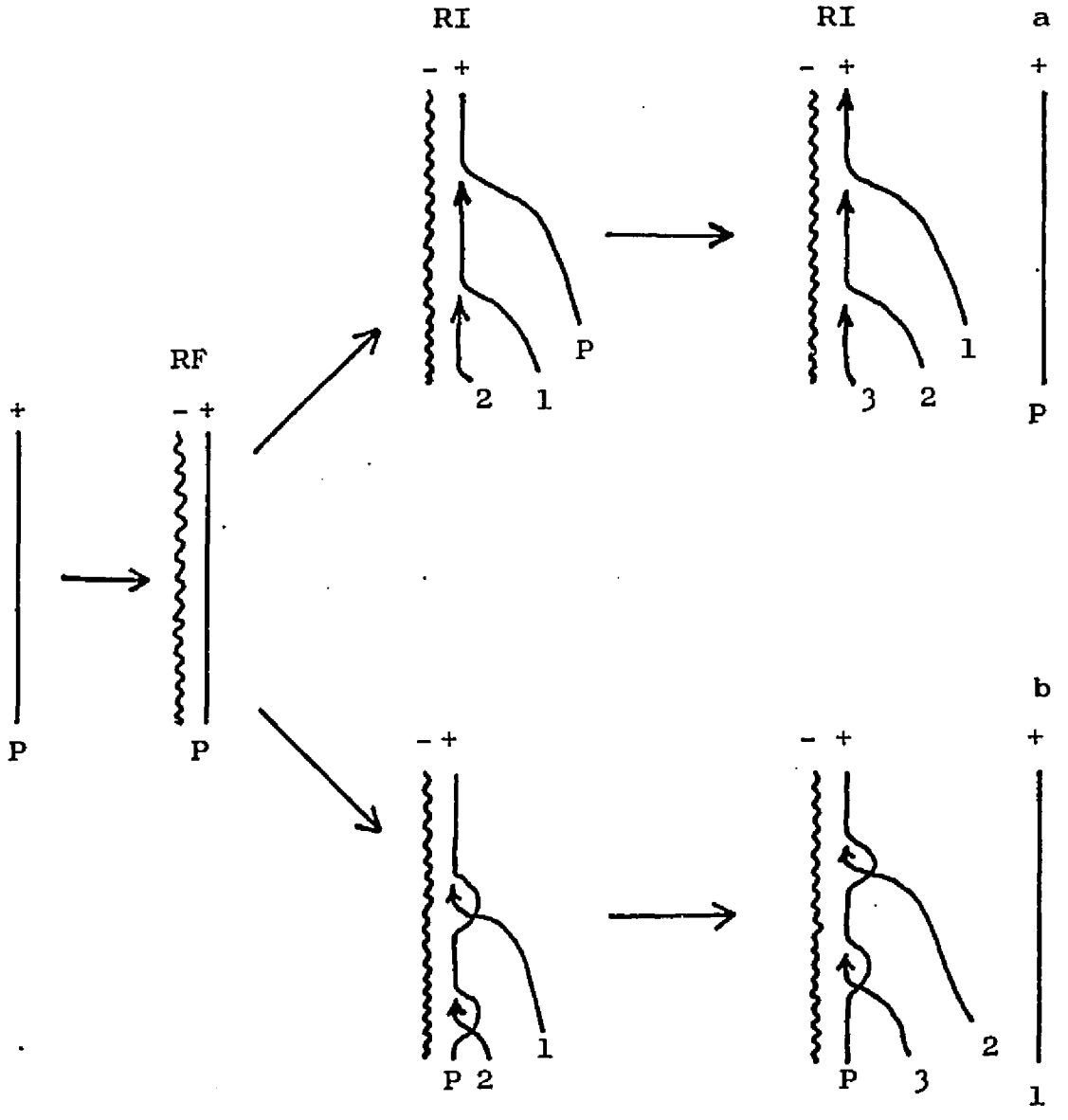


Fig. 2. Diagrammatic representation of the base pair model of viral RNA replication: semiconservative asymmetric replication (a) and conservative asymmetric replication (b).

P = parental, "plus" strand.
RF = replicative form, consisting of complementary "plus" and "minus" strands.
RI = replicative intermediate. The original "plus" strand (P) is displaced by a new plus strand in semiconservative replication (a) and is not displaced by the new plus strand in conservarive replication (b).

original plus strand is displaced only locally by the growing point of the nascent plus strand.

In support of the complementary base pair model, RNA replicative forms have been described in plant, animal, and bacterial cells infected with RNA viruses (Mandel et al. 1964, Baltimore, Becker, and Darnell 1964, Kelly and Sinsheimer 1964, Weissmann et al. 1964). The first replicative form was isolated by Montagnier and Sanders (1963) from Krebs II ascites cells infected with encephalomyocarditis virus. The RF was relatively resistant to digestion by ribonuclease (RNase), a property expected of double-stranded RNA and had a sedimentation coefficient of 20s, which is the value anticipated for a double-stranded molecule of 4×10^6 molecular weight (the viral RNA being 2×10^6 daltons). It had a buoyant density in Cs_2SO_4 lower than that of single-stranded RNA which was expected, and displayed a large increase in absorbance at 260 mμ upon heating which is characteristic of double-stranded nucleic acid. The RF of encephalomyocarditis virus was infectious, even after RNase treatment and without prior denaturation.

Support for the scheme of viral RNA replication involving a replicative intermediate was provided by the characterization of a replicative intermediate of R17 bacteriophage, which infects E. coli (Fenwick et al. 1964). R17 viral RNA has a molecular weight of 1.1×10^6 and a sedimentation coefficient of 27s, whereas a complete duplex

would have a molecular weight of 2.2×10^6 and would sediment at 14s. Replicative intermediate sedimented as a broad band in a sucrose density gradient. The sedimentation coefficient ranged from 14s to 30s. The RNA in the broad band, RI, was only partially digested with RNase; the RNase-resistant RNA sedimented in a sucrose density as a homogeneous peak as 14s. It was proposed that these molecules consisted of RNase-resistant double-stranded cores (14s) to which were attached RNase-sensitive, nascent, single-stranded viral RNA. The longer the nascent single-stranded portion, the faster the molecule sedimented (Erikson, Erikson, and Gordon 1966). In order to determine whether RI was involved in phage RNA replication, R17 infected E. coli cells were given a very short exposure of radioactively labeled RNA precursors such as H^3-uridine. Upon sucrose density gradient analysis of the RNA, radioactivity appeared first in the RI region, and could be chased, by giving excess unlabeled uridine, into the 27s region of the gradient where phage RNA sediments. This result suggested that RI was a precursor of phage RNA. In addition, when parental labeled R17 viral RNA was used to infect cells, it was incorporated intact into RI (Erikson and Franklin 1966). Equivalent RNA forms have been observed in poliovirus-infected HeLa cells (Baltimore 1968, Girard 1969), MS2 infected E. coli (Kelly and

Sinsheimer 1967), and alfalfa mosaic virus infected tobacco (Pinck, Hirth, and Bernardi 1968).

TMV: Release of Viral RNA

It would be reasonable to assume that the replication of the ribonucleic acid of tobacco mosaic virus would follow the general scheme of RNA virus replication presented in Fig. 1 above. TMV infection is initiated when the virus is introduced into a cell. The contact and union of virus and leaf cell is instantaneous and irreversible; when inoculated tissue was washed immediately with water, infection was not reduced when compared with unwashed tissue (Siegel unpublished, quoted by Wildman 1959). After virus is introduced into a cell, there is evidence that the virus is uncoated and viral RNA is released (Hamers-Casterman and Jeener 1957). RNase infiltration of tobacco leaves *in vacuo* either before infection with TMV or within two hours afterward, completely inhibited virus multiplication. By two hours, the infection had reached an RNase resistant condition. Since RNA enclosed in coat protein is not susceptible to RNase whereas free RNA is, separation of the RNA from protein in the initial stage of infection was inferred.

A similar conclusion was reached by Siegel, Ginoza, and Wildman (1957) based on measurements of the sensitivity to ultraviolet light of tobacco mosaic virus and virus

nucleic acid after inoculation into plants. Infective centers initiated by two strains of intact TMV showed lags of 2-1/2 and 5 hours during which no change in sensitivity to UV light occurred. When infective centers were initiated by the respective RNAs of the strains, they became resistant to UV inactivation with little or no lag. The lag in resistance to UV exhibited by the TMV-induced infective centers indicated that 2-1/2 and 5 hours respectively were required to free the RNA from the coat. Other workers have shown that virus is detected sooner and local lesions appear earlier when plants are inoculated with viral RNA rather than with virus (Fraenkel-Conrat, Singer, and Veldee 1958, Schramm and Engler 1958).

TMV: Evidence for Non-Involvement of a DNA Intermediate

It had been speculated that a DNA template was involved in viral RNA synthesis. Sänger and Knight (1963) showed that actinomycin D did not inhibit uracil-2-C^{14} incorporation into TMV-RNA at concentrations which inhibited the incorporation of uracil into normal tobacco leaf RNA. Actinomycin D is known to inhibit specifically DNA-dependent RNA synthesis by combining with the DNA template. When RNA fractions from a sucrose density gradient were tested for infective TMV-RNA, infectivity was found under the 28s peak. Thus, the authors concluded

that the synthesis of TMV-RNA occurs without the benefit of a DNA template.

A similar conclusion was reached on the basis of experiments with mitomycin C which induces the breakdown and cross linkage of DNA. Plants infected with TMV and controls were treated with mitomycin C and given P^{32} labeled orthophosphate. Upon analysis of their RNAs on a methylated albumin kieselguhr (MAK) column, it was found that P^{32} incorporation into RNAs of uninfected leaves was considerably reduced. However, incorporation of P^{32} by infected leaves into the 28s RNA fraction which contains TMV-RNA was not inhibited by mitomycin C (Itoh and Hirai 1966). It was concluded that the integrity of the host DNA was not required for TMV-RNA replication.

Reddi and Anjaneyalu (1963) demonstrated that the DNA of the host tissue was not likely to be involved directly with the production of new viral RNA. DNA isolated from healthy and mosaic-diseased tobacco leaves had similar purine and pyrimidine compositions. The rate of incorporation of P^{32} into DNA was not stimulated as a result of TMV infection. If DNA served as template in TMV-RNA synthesis, a postulated intermediate would be a DNA-RNA hybrid; however, Reddi and Anjaneyalu (1963) were unable to find evidence for such a hybrid in infected plants.

Template Function: Double-Stranded Virus-Specific RNA

It was established that DNA was not an intermediate in TMV-RNA replication; thus it becomes probable that viral RNA serves directly as template for further viral RNA synthesis. Three important studies demonstrated the existence of a unique species of RNA in tobacco cells infected with TMV (Burdon et al. 1964, Shipp and Haselkorn 1964, and Ralph et al. 1965). This molecular species had properties of a double-stranded RNA and was designated as the replicative form. Characteristic properties were: (1) resistance to high concentrations of RNase; (2) a sharp thermal transition to an RNase-sensitive state, the temperature at the midpoint of the transition (T_m) depending on the salt concentration; and (3) the capacity, following denaturation, to reanneal specifically with P^{32}-TMV-RNA. The buoyant density in Cs_2SO_4 was 1.601 g/cm^3 which was less than that of corresponding single-stranded TMV-RNA (1.640 g/cm^3) (Burdon et al. 1964). The RNase-resistant material proved to be very heterogeneous upon sucrose density gradient analysis. Most of the material appeared in the 4s-10s region with a small peak in the 15s-20s region of the gradient indicating that most of the RNase-resistant material was of low molecular weight. The material was resistant to DNase and was sensitive to alkaline hydrolysis under conditions that did not degrade

DNA (Shipp and Haselkorn 1964). Double-stranded RNA with characteristics similar to those described above was obtained by Ralph et al. (1965), except that the buoyant density in Cs_2SO_4 was reported as 1.620 g/cm^3 compared to the value of 1.675 g/cm^3 for TMV-RNA. Weissmann et al. (1965) determined the nucleotide composition of the replicative form of TMV-RNA and its two component strands and found the values to be in agreement with those expected for a double helix consisting of a parental type viral RNA strand and its complement.

RNase-treated double-stranded RNA (RF), purified by chromatography on Sephadex G-75 and hydroxyapatite columns was viewed by electron microscopy. The modal length was found to be 1.8 μ. The expected length, on the basis of 6400 nucleotides and 3.04 A per base, was 1.95 μ. Since the number of branches per unit length of double-stranded RNA decreased with decrease in field density of RNA, it was concluded that few, if any, truly branched molecules were present (Wolstenholme and Bockstahler 1967).

Woolum, Shearer, and Commoner (1967) studied the biosynthesis of virus-specific ribonuclease-resistant RNA. The amount of RNase-resistant RNA "complex," minus strand, and plus strand present in the leaf at different times after inoculation was determined. Assuming that the amounts of plus and minus strand in the complex were equal, they concluded from the observed kinetics of incorporation

of uracil C^{14} into the plus strand of the RNA complex and the kinetics predicted for a precursor of TMV-RNA that at least some of the plus strand found in the "complex" later became incorporated into virus.

The intracellular location of double-stranded TMV-RNA was investigated by Ralph and Clark (1966). RNase-resistant P^{32}-labeled TMV-duplex RNA appeared to be associated with mitochondrial components and could not be detected in chloroplasts or nuclei from tobacco leaves inoculated with TMV. In a later study, synthesis of double-stranded viral RNA by fractionated cell-free extracts from infected leaves was reported (Ralph and Wojcik 1969). Formation of double-stranded RNA, indicating some *in vitro* TMV-RNA replication, was found to occur in a cytoplasmic fraction containing mitochondria or light chloroplasts lacking starch grains. *In vitro* RNA synthesis by the nuclear fraction was totally inhibited by actinomycin D and no double-stranded RNA was detected in the extracts, suggesting that the nucleus was not the site of *in vivo* TMV-RNA synthesis.

A recent paper presents results which seem to contradict the evidence for both the existence of double-stranded RNA and the cytoplasmic site of synthesis (Reddi 1969). After infecting tobacco with P^{32}-labeled TMV-RNA, thirty-four per cent of the parental viral RNA was associated with the "nuclear pellet" of infected tobacco

leaf homogenates. In this study, the leaf tissue was frozen prior to cell fractionation, so the existence of intact nuclei in the preparation is questioned. Parental labeled TMV-RNA, isolated at all periods after inoculation, was found to be RNase sensitive and presumably single-stranded. It was inferred that parental labeled viral RNA formed no RNase-resistant duplex with a complementary strand or did so only transiently.

In Vitro Synthesis of TMV-RNA

The ability of TMV-RNA to act as template for its own synthesis *in vitro* has been demonstrated by several laboratories (Cochran et al. 1962, Cornuet and Astier-Manifacier 1962, Kim and Wildman 1962, Karasek and Schramm 1962). They utilized as criteria of synthesis the incorporation of radioactively labeled RNA precursors into acid precipitable polyribonucleotides and the increase in infectivity of their preparations. Extracts of tissue infected with TMV were used as the source of polymerases, primer TMV-RNA and in some cases the substrates. A TMV-RNA synthesizing preparation isolated from nuclei or ruptured nuclei was interpreted as effecting *de novo* synthesis of infectious RNA (Cochran et al. 1962). At the same time, Cornuet and Astier-Manifacier (1962) isolated a partially purified enzymatic system from the nuclear fraction of TMV-infected leaves and reported its ability to synthesize

viral RNA. A third group extracted the DNA fraction from the nuclei of TMV-infected mesophyll cells (Kim and Wildman 1962). Their incubation mixture, consisting of the DNA fraction and all four nucleotide triphosphates without primer, produced only a slight increase in infectious material. Infectivity was sensitive to both RNase and DNase. Karasek and Schramm (1962) obtained *in vitro* TMV-RNA synthesis by a ribonucleotide polymerase from tobacco leaves. They suggested that unfinished strands of viral RNA which were finished *in vitro* rather than newly formed infectious RNA contributed to the increase in infectivity of the extracts. Ralph and Matthews (1963) were unable to repeat the results of the *in vitro* syntheses of infectious TMV-RNA by using preparations made by the methods described in the previous studies. Their critical interpretations of the previous experiments, particularly because the increases in infectivity were at best only one or two fold in magnitude, suggest that if TMV-RNA synthesis did occur *in vitro* it was extremely inefficient.

TMV: Existence of Free Viral RNA

Several kinds of experiments have provided evidence for the notion that free infective viral RNA is present in TMV-infected cells. Cochran and Chidester (1957) reported that free infective RNA was present along with virus particles in mosaic-diseased plants. Extracts of

TMV-infected Turkish tobacco plants, ground in liquid nitrogen, were passed through a carboxymethylcellulose column. The infectious material which emerged from the column first was susceptible to RNase and did not consist of intact TMV rods. The infectivity detected was therefore attributed to the presence of free RNA in the extracts. The results could not be confirmed by Whitfeld et al. (1960), who demonstrated that the infectivity of the RNA fraction emerging from the column first was sedimentable at 100,000 x g and hence was ascribed to intact virus, which had come through an overloaded column. The "susceptibility to RNase" observed by Cochran and Chidester (1957) was attributed to the high ratio of enzyme to virus which results in a marked inhibition of lesion production on N. glutinosa.

Other experiments, however, favor the possibility that free RNA may persist during some stages of infection by TMV. The infectivity of RNA extracted from leaf homogenates which were pretreated at 37° for one hour to destroy postulated free RNA was less than that of RNA obtained by direct phenol extraction of infected leaves. The difference was attributed to the free RNA, since the infectivity of directly extracted RNA was attributed to both free RNA and RNA extracted from virus. In later stages of infection most of the RNA was contained in TMV (Engler and Schramm 1959).

Other evidence for free viral RNA in TMV-diseased leaves was provided by Diener (1962). Infected leaves were extracted with sodium chloride buffered at pH 9-10. Differential centrifugation and density gradient centrifugation enabled him to obtain an infectious fraction from the nucleic acid zones separate from the TMV zone. The infectious nucleic acid was abundant in plants two to three days after infection and little was obtained from plants three weeks post infection. Several types of controls were done to show that the infectivity of the nucleic acid fraction was not due to degradation of TMV or contamination with TMV.

Infectious ribonucleic acid was present in a ribosomal fraction of tobacco leaves infected with tobacco mosaic virus. Ribosomes were almost completely freed of intact virus particles by treatment of the preparations with virus specific antiserum which was free of measurable RNase. After extraction of these purified ribosomes with phenol, 70-160 times more infectivity was obtained than had been predicted on the basis of the level of intact virus contamination known to be still present in the preparation. The extra infectivity was ascribed to infectious TMV-RNA associated with ribosomes (van Kammen 1961).

Basler and Commoner (1956) gave evidence that excess nucleic acid which accumulated before TMV appeared represented precursor to TMV nucleic acid. Prior to the

appearance of free TMV, the nucleic acid of the buffer insoluble fraction of infected leaf homogenates exceeded that of a comparable fraction from uninfected tissue by an amount which was slightly in excess of the nucleic acid later found in the maximum amount of TMV formed by the tissue. As free TMV appeared, this excess nucleic acid disappeared, indicating a possible precursor-product relationship.

Additional evidence that TMV-RNA can exist free and in a functional state *in vivo* was provided by Siegel, Zaitlin, and Sehgal (1962). They isolated defective strains of TMV which induced the synthesis of nonfunctional virus proteins, which failed to aggregate and form nucleoprotein rods. The infective principle consisted of free nucleic acid and behaved in a manner similar to that of infectious nucleic acid isolated from ordinary TMV in its instability and manner of fractionation from leaf homogenates.

The general scheme of the replication of a ribonucleic acid virus and of viral RNA has been outlined. Following the outline, a review of previous studies relative to the replication of TMV has been presented. It began with the adsorption of virus and uncoating to release viral RNA. Investigations of the role of TMV-RNA in template function have demonstrated the existence in infected tobacco plants of a double-stranded

RNase-resistant virus-specific molecule called the replicative form. Evidence that viral RNA is released and can exist free in the cell has been reviewed. In the replication of viral RNA, a RNA molecule characterized as having a double-stranded core with nascent single-stranded "tails" has been isolated from bacterial and animal cells infected with virus; such an intermediate was postulated for TMV-RNA replication. The present work was undertaken to determine whether evidence for the existence of a replicative intermediate in TMV-infected cells could be obtained.

MATERIALS AND METHODS

Preparation of Infected Plants

Greenhouse grown, potted Nicotiana tabacum L. var. Turkish Samsun plants, approximately eighteen inches tall were used for all phases of this study. All the leaves were inoculated with a solution of 0.2 mg/ml tobacco mosaic virus (TMV) strain U_1 and 50 mg/ml Celite in M/15 Sorenson's phosphate buffer, pH 7.0. After inoculation, the leaves were rinsed with tap water. The plants were maintained under artificial lights which consisted of fluorescent tubes and 200 watt incandescent bulbs, giving an intensity of 400 foot candles 20 inches above the bench. The photoperiod was sixteen hours and the average temperature was 27° C.

Extraction and Fractionation of Ribonucleic Acid

Usually one hundred grams of leaves infected for six or seven days with TMV were ground to a fine powder in liquid N_2 in a large chilled mortar and pestle. Two volumes (200 ml) of one per cent sodium pyrophosphate pH 8.0 and two volumes (200 ml) of freshly distilled phenol (Schlegel 1960) were then poured into the mortar and the resulting slurry was mixed and allowed to melt. Just prior to use, the phenol was washed with STE buffer (0.1 M NaCl,

0.05 M Tris pH 8.0 [reagent grade, Sigma Chemical Co., St. Louis, Mo.] and 0.001 M Na_2EDTA [ethylenediaminetetraacetic acid]) (Franklin 1966). The slurry was transferred to large plastic centrifuge tubes and was centrifuged at 6000 rpm for 5 min. in a Sorval (RC-2) Refrigerated Centrifuge, SS-34 rotor. The aqueous phase, containing the nucleic acids, was removed and shaken with one-half volume washed phenol. Centrifugation was repeated to separate the buffer and phenol layers. The upper aqueous layer was carefully removed into large plastic centrifuge tubes and the nucleic acids were precipitated by adding two volumes of 95% ethanol and about 0.3 ml 3 M acetate buffer pH 4.0. After remaining overnight at 0° C, the precipitated nucleic acids were centrifuged at 10,000 rpm for 10 min. and suspended in a small volume of boiled STE buffer, pH 6.85. After addition of 95% ethanol making the final solution 35% with respect to ethanol, the RNA was chromatographed at room temperature (25°) on a cellulose column (Franklin 1966).

The cellulose column was prepared by suspending Whatman cellulose CF-11 in STE buffer, pH 6.85. After removing fine particles by repeated decantation, the cellulose was poured into a column (1" x 16") and washed with STE containing 1% 2-mercaptoethanol and 0.01 M Na_2EDTA. The column was then washed with STE (pH 6.85):ethanol, 65:35 (v/v) until the effluent OD_{260} was the same as that

of the buffer. The RNA sample was applied to the column and stepwise elution proceeded with decreasing concentrations of ethanol in STE. Successive elution of RNA in 65%, 85%, and 100% STE was monitored with a recording ultraviolet meter (Gilson Medical Electronics Model UV-265 IF with Texas Instruments, Inc. Rectilinear Recorder Model RR). The fraction eluting in 100% STE was collected by a fraction collector using a volumetric fractionating device (GME V-10). In some cases the material eluting at 100% STE was chromatographed a second time; this fraction was adjusted to 35% with ethanol and chromatographed on a smaller column (5/8" x 10") of cellulose which had been pretreated as above. Following elution with 65% and 85% STE, the fraction eluting from the second column in 100% STE was collected and concentrated by precipitation with two volumes of ethanol and a few drops 3 M acetate buffer pH 4.0. After remaining overnight at 0° C, the RNA was sedimented at 10,000 rpm for 10 min. and suspended in STMg buffer (0.2 M NaCl, 0.01 M Tris pH 7.6 and 0.01 M $MgCl_2$) (Bellamy et al. 1967). Alcohol was removed by bubbling N_2 through the solution. The concentration of the RNA solution was determined from the optical density at 260 mμ obtained using a Gilford Analytical Spectrophotometer, on the basis that in a 10 mm light path a 1 mg RNA/ml solution had an optical density of 25 at 260 mμ.

Franklin (1966), working with R17 bacteriophage, demonstrated that double-stranded RNA present in extracts of infected cells eluted from the cellulose column in 100% STE. The replicative intermediate, a partially double-stranded, partially single-stranded RNA molecule, also eluted with the 100% STE fraction presumably due to its double-stranded portion. In order to show that double-stranded RNAs from TMV-infected tissue also eluted in 100% STE, RNA isolated from infected plant tissue and collected as the 100% STE fraction was characterized by analytical isopycnic centrifugation in Cs_2SO_4, before and after RNase treatment. Double-stranded RNA is much more resistant to degradation by RNase and has a lower buoyant density in Cs_2SO_4 than single-stranded RNA. RNase treatment prior to isopycnic centrifugation was performed as follows: approximately 2 µg of RNA suspended in STMg was digested with 0.2 µg/ml pancreatic RNase A (Worthington Biochemical Corp., Freehold, N. J.) at room temperature for 20 min. Sometimes, RNase was removed by pronase treatment (0.5 mg/ml, pre-incubated at 50° C for 30 min., Calbiochem, Los Angeles, Calif.) at room temperature for 10 min. This RNase-treated RNA preparation and a sample of RNA not treated with RNase were routinely subjected to analytical isopycnic centrifugation analysis.

Analytical Isopycnic Centrifugation in Cs_2SO_4

Analytical isopycnic centrifugation was performed according to procedures described by Szybalski (1968), using a Spinco Model E analytical Ultracentrifuge. Cs_2SO_4 (Pierce Biochemical Co., Rockford, Ill.) was purified by heating to 700° C overnight, dissolving in deionized water, boiling with activated charcoal and filtering through acid-washed filter paper. After precipitating with AR grade absolute ethanol (4° C), filtering, and drying at 50° C, the salt in a 65% aqueous solution had an OD_{260} of 0.020. Prior to use, a saturated, aqueous solution of Cs_2SO_4 was filtered through a 1.0 μ filter (Millipore, Bedford, Mass.). A homogeneous solution consisting of saturated Cs_2SO_4 solution, STMg buffer, and approximately 2 μg RNA was adjusted to a mean density of 1.633 ± 0.008 g/cm^3. The density of the solution was determined with the aid of a Zeiss Abbe Refractometer at 25° C. The relationship between density (ρ) and refractive index (n_D^{25}) for Cs_2SO_4 solutions in the range of density 1.6-1.8 g/cm^3 at 25° C is:

$$\rho = 13.6986\ n_D^{25} - 17.3233$$ (Vinograd and Hearst 1962).

Standard 4°, single sector, 12 mm cells with Kel-F centerpieces and a 1° negative wedge window were used. The RNA-Cs_2SO_4 solution was introduced into the cell and centrifuged for 18-20 hours at 44,000 rpm in an AN-D rotor.

UV absorption photographs were taken on Kodak commercial film to show the band position. The centrifuge was then stopped and 2.5 µl of Cs_2SO_4 (ρ = 1.633 g/cm^3) containing 1 µg TMV-RNA was added to each cell to serve as a density marker. The solutions were again centrifuged to equilibrium (44,000 rpm, 18-20 hours). The rationale for the procedure was that if TMV-RNA were put in the cell with the 100% STE fraction, the presence of contaminating single-stranded TMV-RNA in the preparation would have been obscured (Bockstahler 1967). Densitometer tracings of the photographs of the equilibrium distribution of RNA were made using a Beckman model RB Analytrol densitometer equipped with a film densitometer accessory.

The buoyant density of the RNA was calculated according to the methods of (1) Erikson and Szybalski (1964) and (2) Vinograd and Hearst (1962).

1. $$\rho = \rho_o + \frac{\omega^2}{2\beta}\{r^2 - 1/2\ (r_M^2 - r_B^2)\}$$

ρ = density of the band at r

ρ_o = original density of the solution

$\frac{\omega^2}{2\beta}$ = 0.01489, using the value of 14.03×10^{-10} for $1/\beta$, the buoyancy coefficient and 21.23×10^6 sec^{-2} for ω^2, the angular velocity at 44,000 rpm.

r = radial distance of band

r_M = radial distance of meniscus

r_B = radial distance of the cell bottom

By this equation, the buoyant density of TMV-RNA was found to be 1.640 g/cm^3 in agreement with that reported by Burdon et al. (1964).

2. $\rho = \rho_o + \frac{\omega^2}{\beta} (\bar{r}_o) (\Delta r)$

ρ = density of unknown RNA

ρ_o = density of marker RNA (1.640 g/cm^3 for TMV-RNA)

$\frac{\omega^2}{\beta}$ = 0.02978 using the value of 14.03×10^{-10} cgs for $1/\beta$, the buoyancy coefficient and 21.23×10^6 sec^{-2} for ω^2, the angular velocity at 44,000 rpm

$\bar{r}_o$ = mean of the radial distances of the marker and unknown bands

Δr = difference between the radial distances of the marker and unknown bands.

When the buoyant density of double-stranded RNA was calculated by both methods, one utilizing the value for marker RNA and the other without using marker, identical values were obtained.

Thermal Denaturation

For thermal denaturation, approximately 7.5 µg of RNA per ml were dialyzed overnight against two changes of 500 ml PE buffer (0.001 M $K\text{-}PO_4$ pH 6.6 and 0.001 M Na_2EDTA, neutralized) (Franklin 1967a). Two ml of the dialyzed RNA solutions were placed in 10 mm stoppered cuvettes.

Thermal transition profiles were obtained by simultaneously recording the temperature and absorbance at 260 mµ of the RNA solutions with the Gilford Model 2000 Automatic Recording Photometer fitted with Dual Thermospacer Model 2180, Linear Thermosensor Model 207, and Auxiliary Recording Channel Model 212. A temperature gradient with a rate of increase of 0.25° per min. was generated with the aid of a Haake Temperature Circulator. The data were corrected for the expansion of water with uracil in PE buffer as a blank and plotted as optical density at 260 mµ as a function of temperature (Mandel and Marmur 1968).

Annealing with Radioactively Labeled TMV-RNA

Radioactively labeled TMV-RNA was donated by Alan Jaworski. It was obtained from plants infected with TMV for two days, which were given 100 µc uridine-5-H^3 (International Chemical and Nuclear Co.) via a doubled thread inserted through the main stem (Hirai and Wildman 1967). The radioactive uridine in one ml 0.01 M KH_2PO_4 was chased into the plant by successive additions of two

one ml portions of 0.01 M KH_2PO_4, over a period of two days. TMV was purified by grinding the leaves with an equal volume of M/15 phosphate buffer, clarification at 10,000 rpm for 30 min. and sedimentation at 30,000 rpm for one hour (Spinco Model L-2 Ultracentrifuge). The pellet was suspended in versene phosphate buffer (0.001 M Na_2EDTA in M/15 phosphate). The RNA was prepared by phenol extraction of the virus, a procedure similar to phenol extraction of leaf tissue, except for washing with three volumes of ether to remove the phenol prior to precipitation with two volumes of 95% ethanol at 0° C (Schuster 1964). The RNA was sedimented at 7000 rpm for 5 min. and resuspended in STMg buffer.

Double-stranded RNA from the 100% STE fraction was purified by three passages through a cellulose column. The absence of single-stranded RNA contamination was confirmed by Cs_2SO_4 isopycnic centrifugation analysis.

Radioactively labeled ribosomal (rRNA) was obtained from tobacco leaves which were floated in petri dishes for four days on 10 ml 0.01 M KH_2PO_4 containing 100 μc uridine-5-H^3/ml. Phenol extraction and ethanol precipitation were performed essentially as described above, followed by precipitation of the rRNA with 2 M LiCl (Baltimore 1966).

Mixtures of H^3-labeled TMV-RNA and double-stranded RNA, or H^3-labeled leaf rRNA and double-stranded RNA were made up to a final volume of 0.30 ml in STMg. Labeled RNA

alone served as controls. These solutions were tightly stoppered, heated for ten min. at 100° C to denature the RNA, immediately placed in a 85° C constant temperature water bath, and slowly cooled to 35° C over a period of 14 hours, to allow complementary RNA strands to anneal. The annealing mixtures were cooled to 0° C, adjusted to 1.0 ml with STMg containing 10 μg pancreatic RNase A, and 1 μg ribonuclease T_1 (Sankyo Co., Ltd. Tokyo). The mixtures were incubated for one hour at 37° C to degrade non-annealed RNA, then iced. RNA was precipitated by the addition of carrier leaf RNA (200 μg in 0.1 ml STMg buffer), followed by 0.11 ml cold 50% trichloroacetic acid (TCA) (Robinson et al. 1964). After three min. at 0° C, the acid-insoluble material was collected by filtration through 0.5 μ cellulose acetate filters (Celotate EH, Millipore) and washed five times with five ml portions of cold 5% TCA to remove RNase digestion products. Filters were placed in glass scintillation vials and dried at 70° C for 30 min.; 0.5 ml Nuclear Chicago Solubilizer (NCS) was added to each vial. The vials were capped, heated at 50° C for 20 min., then cooled. After addition of 10 ml 0.5% PPO (2,5, diphenyloxazole) + 0.05% POPOP (1.4-bis-2-[5-phenyloxazolyl] benzene) in toluene, the samples were counted in a Packard Model 3320 Tri-Carb Scintillation Spectrometer (Larsen 1969). The efficiency, as determined

with the Automatic External Standard, was 36% when the gain was 53% and the window was 50-1000.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out following the procedures described by Bishop, Claybrook, and Spiegelman (1967). Acrylamide and N,N'-methylene bisacrylamide (Eastman-Kodak Organic Chemicals, Rochester, N. Y.) were recrystallized from chloroform and acetone, respectively (Loening 1967). Sodium dodecyl sulfate (SDS, Sigma Chemical Co.) was recrystallized twice from 95% ethanol. Stock solutions were prepared as follows:

a. Acrylamide solution:

   acrylamide monomer--15g
   N,N'-methylene bisacrylamide--0.75 g
   H_2O to 100 ml

b. 3E buffer

   0.12 M Tris--14.53 g
   0.06 M Na acetate--8.16 g
   0.003 M Na_2EDTA--1.116 g
   H_2O to 994 ml, pH was adjusted to 7.2 with 6 ml glacial acetic acid

c. Ammonium persulfate solution

   ammonium persulfate, reagent grade--10 g
   H_2O to 100 ml

d. Electrophoresis buffer

   333 ml 3E buffer
   2.0 g SDS
   H_2O to 1000 ml

All solutions were stored in a refrigerator (4° C), except solution c which was prepared just before using.

Two per cent acrylamide gels were prepared by mixing 3.34 ml solution a, 8.34 ml solution b, 13.13 ml H_2O and polymerizing with 0.166 ml solution c and 0.017 ml N,N,N'N'-tetramethylethylenediamine(TMED). The gel solution was mixed, degassed by suction, transferred into stoppered Plexiglas tubes (0.7 cm I.D.) and allowed to polymerize at room temperature. After polymerization, the gels were transferred to 500 ml of solution d and allowed to swell for 72 hours. Swollen gels, sucked into 0.7 cm I.D. Plexiglas tubes and supported by a dialysis membrane stretched across the lower end, were inserted into the Canalco Model 6 Electrophoresis Apparatus. Electrophoresis buffer (solution d) was put into both the upper and lower electrode vessels. The gel columns, suspended between the two vessels, completed the circuit. Gels were prerun at room temperature for one hour at 5 ma per gel using a Beckman Duostat Power Supply set for current regulation. Samples of RNA from the 100% STE fraction in STMg buffer containing 10% sucrose were layered on top of the gel columns. RNase resistance of the RNA was determined by treating with 0.5 µg pancreatic RNase/ml for 20 min. at room temperature before layering the RNA onto the gel columns. Electrophoresis was carried out at room temperature using 5 ma per column for 18 hours.

After electrophoresis, the gels were transferred to a rectangular quartz cell (5 mm x 8.5 mm x 100 mm) and

scanned by transmitted ultraviolet light (260 mμ) in a Gilford Model 240 Spectrophotometer with Gel Scanner Model 2410 and Heathkit Servo Recorder Model EUW 20A.

Preparation of Cell Fractions

Fifty grams of leaves, infected with TMV for one week, were ground at 2° with 100 ml extraction medium in a meat grinder, then with a mortar and pestle. The extraction medium contained 0.4 M sucrose, 0.05 M Tris pH 7.8, 0.01 M KCl, 0.01 M $MgCl_2$ and 4 mM mercaptoethanol (Zaitlin, Spencer, and Whitfeld 1968). The brei was filtered through two layers of wet Miracloth (Chicopee Mills, Inc.) to remove cell debris and the filtrate was centrifuged at 3000 rpm (1000 x g) for 5 min. to sediment chloroplasts and nuclei. The supernatant was centrifuged at 13,000 rpm (14,000 x g) for 30 min; the resulting pellet, resuspended in suspending medium (extraction medium without sucrose), was considered to be the "mitochondrial" fraction. Ribosomes were sedimented from the 14,000 x g supernatant by centrifugation at 45,000 rpm (160,000 x g) for one hour in a Spinco Model L-2 Ultracentrifuge using a #50 Ti rotor and were resuspended in suspending medium yielding the ribosomal fraction. The 160,000 x g supernatant will be referred to as the cytoplasmic fraction.

The 1000 x g pellet, containing chloroplasts and nuclei, was resuspended in suspending medium containing

5% Triton-X-100 (alkylphenoxy-polyethoxyethanol, Rohm and Haas), which solubilized the chloroplasts, and centrifuged at 1000 x g for 5 min. The green supernatant was the chloroplast fraction. Nuclei in the Triton insoluble residue in the 1000 x g pellet were disrupted by the addition of 0.5% SDS to the suspending medium, yielding the nuclear fraction.

Each fraction was extracted with two volumes of phenol, chromatographed on the cellulose column, and centrifuged to equilibrium in Cs_2SO_4 for detection of double-stranded RNA as described above.

RESULTS

Chromatography on Cellulose

Nucleic acids obtained by phenol extraction of healthy and virus-infected tobacco leaves were fractionated by chromatography on cellulose powder by stepwise elution with 65%, 85%, and 100% STE buffer in ethanol. Fractionation of RNA by cellulose chromatography is based on changes in the chemical activity of nucleic acids caused by the presence of varying amounts of ethanol in buffer (Barber 1966). High molecular weight RNAs appear to be separated according to their size and relative content of hydrogen bonded secondary structure (Franklin 1966). If nucleic acids are loaded onto a cellulose column in 65% buffer:35% ethanol solution, soluble RNA, other low molecular weight RNAs, and DNA fail to absorb. High molecular weight single-stranded RNA (ribosomal RNA and single-stranded viral RNA) can be eluted with 85% buffer. RNA molecules with a higher order of secondary structure such as replicative form (RF) and replicative intermediate (RI) elute only when the column is washed with 100% buffer.

Figure 3 illustrates the chromatography of RNA from TMV-infected tissue. A large amount of material is eluted with the 65% STE front and in the 85% STE fraction. The material eluting in 100% STE buffer is a small portion of

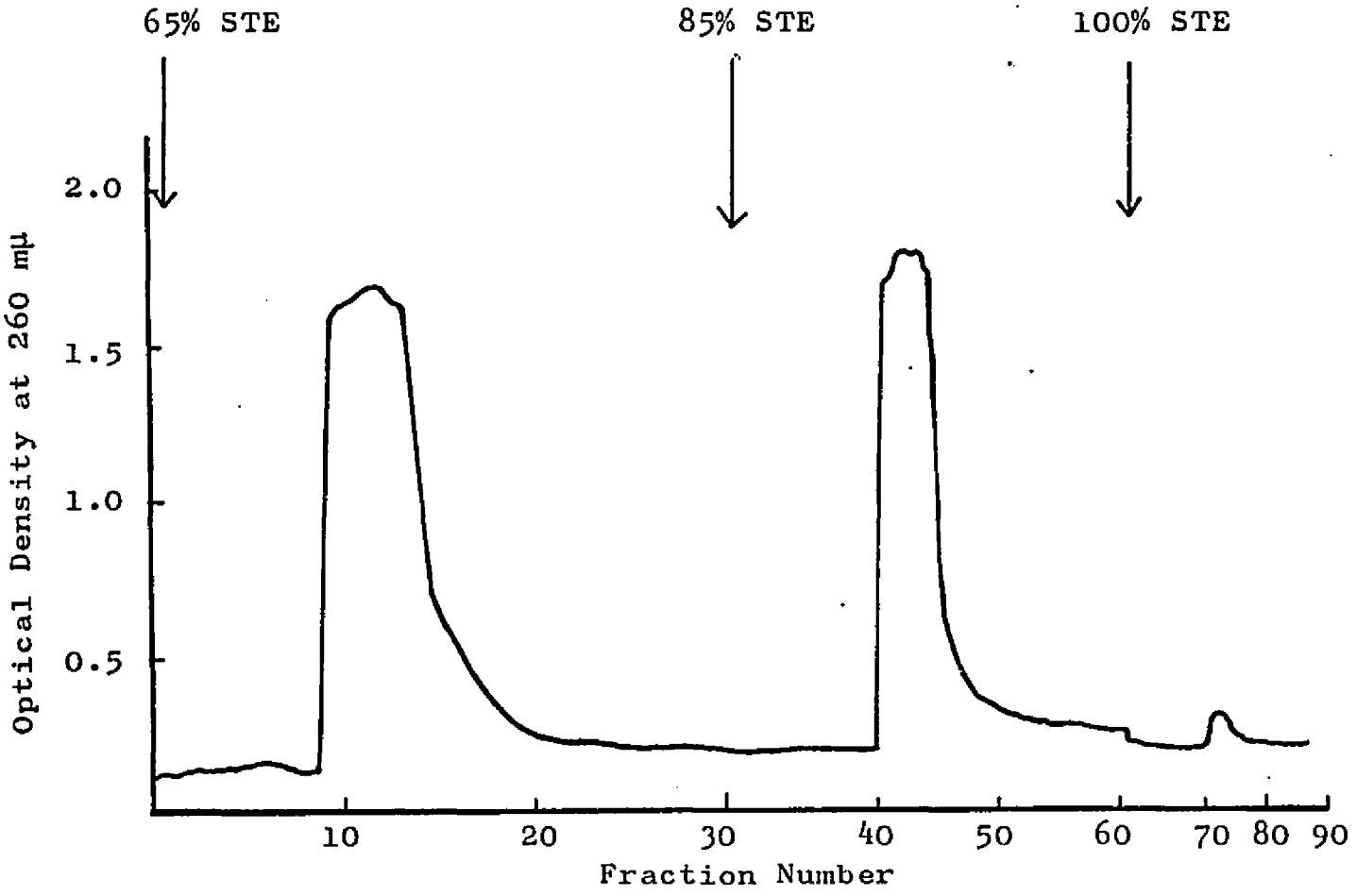


Fig. 3. Chromatography on cellulose powder of nucleic acids extracted from TMV-infected N. tabacum -- Nucleic acids were extracted with phenol and 1% sodium pyrophosphate pH 8.0 from TMV-infected (six to seven days post-infection) leaves pulverized in liquid nitrogen and were precipitated from the aqueous phase with two volumes of ethanol (95%). The precipitated nucleic acids were loaded onto a column (1" x 16") in 65% STE:35% ethanol and were eluted stepwise with decreasing concentrations of ethanol in STE buffer.

the total RNA from TMV-infected plants. It was collected in approximately sixteen six ml fractions. Note the decreased interval between fractions after a buffer change. The rate of flow of the effluent increased with decreasing concentrations of ethanol in buffer. The RNA in the 100% STE fraction was found to be a mixture of double-stranded RNAs and contaminating single-stranded RNA. In order to remove the contaminating single-stranded RNA, the 100% STE fraction was adjusted to 65% STE with ethanol and re-chromatographed on cellulose. The single-stranded RNA subsequently eluted in the 85% STE fraction; the RNA in the 100% STE fraction was further characterized by equilibrium density gradient analysis, thermal denaturation, annealing studies, and polyacrylamide gel electrophoresis.

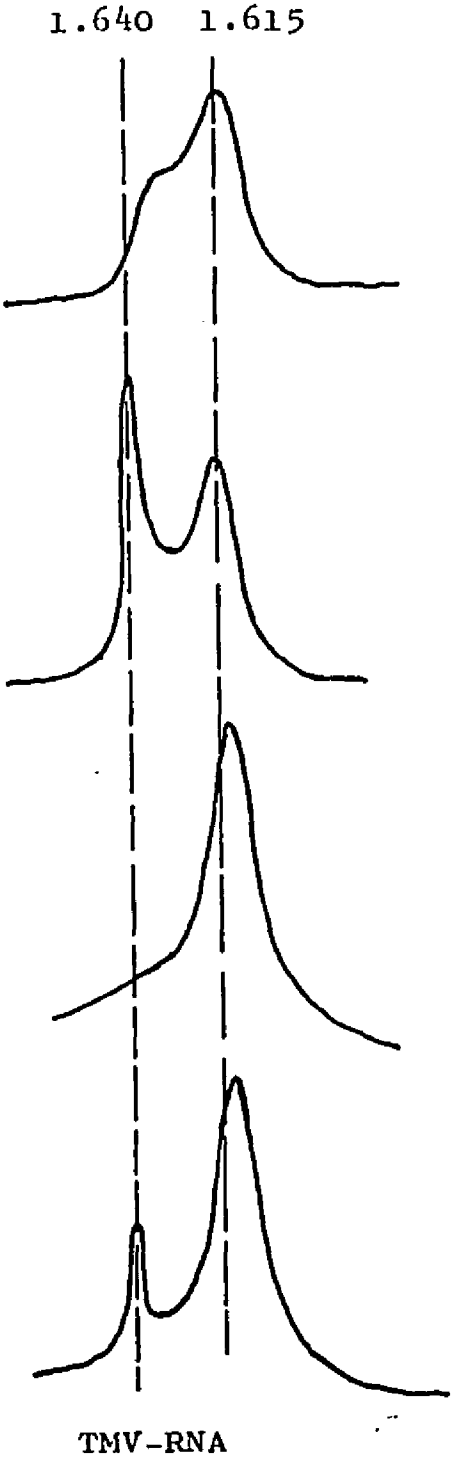
RNA obtained from uninfected tobacco plants eluted from the cellulose column in a similar manner to the RNA from infected plants, except that the amount of RNA eluting in 100% STE was much smaller. When chromatographed a second time, most of the RNA from the 100% STE fraction appeared in the 85% STE eluate. The small amount of optically dense material which eluted in 100% STE the second time was examined by isopycnic centrifugation analysis.

Analytical Isopycnic Centrifugation

RNA from TMV-infected leaves fractionated by two successive passages through a cellulose column and collected as the 100% STE fraction was examined by analytical isopycnic centrifugation in a Cs_2SO_4 density gradient. Analytical isopycnic centrifugation is used to determine the buoyant density of a macromolecular species. The density of the solution is selected to correspond roughly to the buoyant density of the macromolecule. Upon application of a centrifugal field, a density gradient is generated and the macromolecule forms a narrow band in a unique position in the liquid column. The buoyant density of the macromolecule is the same as the density of the solution at this position. Characteristically, single-stranded ribonucleic acid has a greater buoyant density in Cs_2SO_4 solutions than double-stranded RNA.

Microdensitometric traces of UV absorption photographs of RNA banded by analytical isopycnic centrifugation are presented in Fig. 4. The density profile of the banded RNA (Fig. 4a) shows a single peak with a large shoulder representing a fraction of RNA with a distinctly higher buoyant density than the RNA of the main peak. The RNA in the main peak has an estimated mean buoyant density of 1.615 gm/cm^3. This value was calculated using the value of 1.640 gm/cm^3 as the density of the TMV-RNA marker, the sharp peak which is adjacent to the shoulder and partially

Fig. 4. Analytical isopycnic centrifugation in Cs_2SO_4 of RNA from the 100% STE fraction -- RNA (2 µg) from the 100% STE fraction in 0.7 ml STMg buffer and Cs_2SO_4 (mean density 1.633 ± 0.008) was centrifuged (25° C) for 18-20 hours at 44,000 rpm. The band position was determined (a), the ultracentrifuge was stopped and TMV-RNA (1 µg) in 2.5 µl Cs_2SO_4 solution (mean density 1.633) was added to the cell. Equilibrium was reached again in 18-20 hours at 44,000 rpm (b). RNA from the 100% STE fraction was treated with RNase (0.2 µg/ml, 20 min., room temp.) and pronase (0.5 mg/ml, 10 min., room temp.) prior to addition of Cs_2SO_4 and centrifugation to equilibrium (c) and the subsequent addition of TMV-RNA (d).



a. 100% STE fraction

b. 100% STE fraction + TMV-RNA

c. 100% STE fraction treated with RNase prior to centrifugation

d. RNase-treated 100% STE fraction + TMV-RNA

Fig. 4. Analytical isopycnic centrifugation in Cs_2SO_4 of RNA from the 100% STE fraction.

obscures its presence in Fig. 4b. TMV-RNA forms a precipitate which appears as a hypersharp band in the Cs_2SO_4 density gradient (Lozeron and Szybalski 1966). The TMV-RNA marker was introduced into the sample after the gradient had attained equilibrium so that the position of the RNA band could be determined (Fig. 4a); centrifugation was performed a second time with marker (Fig. 4b). The buoyant density of the fraction of RNA which appears as a shoulder of the main peak in Fig. 4a is approximately 1.630 gm/cm^3. Between 15 and 36 per cent of the RNA in the 100% STE fraction was present in the shoulder, varying for different preparations of RNA from TMV-infected leaves. In samples which contained a large shoulder of higher buoyant density, two bands could be distinguished by visual examination of the photograph.

When the RNA from the 100% STE fraction was treated with a low concentration of RNase A (0.2 μg/ml) and analyzed by isopycnic centrifugation, the density profile of the banded RNA appeared as in Fig. 4c. The RNA bands as a single symmetrical peak; the fraction of RNA in the shoulder with higher buoyant density has been eliminated. The buoyant density of the RNA could be obtained after marker TMV-RNA was added (Fig. 4d); the RNA has a buoyant density of 1.613 gm/cm^3. The buoyant density of the double-stranded replicative form of TMV was reported by Burdon et al. (1964) to be 1.601 gm/cm^3 which is less than

that of single-stranded TMV-RNA (1.640). Ralph et al. (1965) reported values of 1.628 and 1.620 as the buoyant density of RNase-resistant material, compared with 1.675 for TMV-RNA. Although the buoyant density obtained for RNase resistant RNA in these experiments is 1.613 and this value differs from that found in the previous papers, it is nevertheless in accord with the observation that double-stranded RNA has a lower buoyant density than single-stranded RNA.

The RNA fraction in the shoulder having a buoyant density of 1.630 gm/cm^3, intermediate between that of single- and double-stranded RNA was eliminated by RNase. Since single-stranded RNA is susceptible to low concentrations of RNase whereas double-stranded RNA is resistant, one can infer that the single-stranded portion of a partially single-stranded partially double-stranded molecular species was degraded. The double-stranded component of such a molecular species should remain after RNase treatment and contribute to the peak representing double-stranded RNA. One can see upon closer examination of Fig. 4 that the height of the peak of double-stranded RNA (1.613) in Fig. 4c is increased compared to that in Fig. 4a. The increase in the amount of double-stranded RNA in the RNase treated sample (Fig. 4c) is proportional to the amount of RNA in the shoulder prior to RNase (Fig. 4a), suggesting that the latter contributes to the former.

One can conclude that the RNA in the shoulder is partially single-stranded and partially double-stranded and removal of its single-stranded portion by RNase treatment yields double-stranded RNA.

A molecule characterized as being the replicative intermediate of R17 bacteriophage (Erikson, Fenwick, and Franklin 1964) was described as being a partially single-stranded partially double-stranded RNA. The replicative intermediate (RI) was a population of molecules composed of double-stranded cores and associated single-stranded "tails." The RNase resistant double-stranded cores remaining after removal of the single-stranded branches can be represented by two different structures depending upon whether replication is by a semiconservative or a conservative mechanism (Fig. 2). If replication is semiconservative, a double-stranded structure consisting of a complete minus strand and pieces of plus strand which remain hydrogen bonded to the complementary strand might result. On the other hand, double-stranded RNA consisting of complete minus and plus strands would be expected if replication were by a conservative mechanism. Molecules of the latter type would be indistinguishable from replicative form (RF) which precedes RI in either replication scheme. Replicative form is a double-stranded RNA which is composed of one strand of viral RNA (plus strand)

and one strand of RNA complementary to the viral RNA (minus strand).

The buoyant density of the double-stranded RNA in RNase-treated samples was generally less by about 0.002 gm/cm^3 (1.615 vs. 1.613) than prior to RNase treatment. Note the position of the peak in Fig. 4c and d relative to the 1.615 band in Fig. 4a and b. Among the reasons for this shift in density might be that RNase eliminated the more dense single-stranded components of RI which contribute to the peak of double-stranded RNA.

Profiles similar to those in Fig. 4 were obtained for each preparation of RNA from infected leaves. For convenience and in keeping with the established nomenclature for double-stranded RNAs associated with RNA virus infection, the RNase-resistant double-stranded RNA will be referred to as replicative form (RF), and the partially single-stranded partially double-stranded RNA with buoyant density intermediate between single and double stranded RNA will be referred to as replicative intermediate (RI). The evidence so far presented indicates that the RNA from the 100% STE fraction is a mixture of RF and RI.

The RNA which was extracted from uninfected leaves with phenol, fractionated by cellulose chromatography and eluted in 100% STE was also examined by analytical isopycnic centrifugation. In most cases, only a very small amount of material was found which appeared as a sharp band

of UV absorbing material having a buoyant density greater than that of TMV RNA. In this respect the material behaved like ribosomal RNA (Szybalski 1968). When the RNA was passed through the cellulose column two times, only a minute amount was recovered; it banded in a series of diffuse lines. Both the ribosomal RNA and the minute bands were eliminated by treatment with a low concentration of RNase prior to equilibrium density gradient centrifugation.

Thermal Denaturation

Thermal denaturation profiles were determined for TMV-RNA, RNase-resistant double-stranded RNA (RF), and the RNA from the 100% STE fraction (RF and RI). Nucleic acids with hydrogen-bonded double-stranded secondary structure undergo a sharp transition from an ordered helical form to a random coil structure when heated above a critical temperature. This transition, called denaturation is accompanied by an increase in the optical density of the nucleic acid solution (hyperchromicity) and is characterized by the temperature at its midpoint, T_m (denaturation temperature).

Figure 5 presents a comparison of the thermal denaturation profiles of TMV-RNA, RNase-resistant double-stranded RNA, and RNA from the 100% STE fraction. As seen in Fig. 5, the single-stranded viral RNA shows hyperchromicity over a very broad temperature range. This

Fig. 5. Thermal denaturation of TMV-RNA, RNA from the 100% STE fraction, and RNase-resistant RNA -- RNase-resistant RNA was prepared by treating the 100% STE fraction with RNase (0.2 μg/ml, 20 min., room temp.) and pronase (0.05 mg/ml, 10 min., room temp.) and re-extracting with phenol. Approximately 7.5 μg of each sample of RNA were dialyzed against a 1000-fold excess of PE buffer (0.001 M $K\text{-}PO_4$ pH 6.6, 0.001 M Na_2EDTA, neutralized) prior to denaturation. Correction was made for the expansion of water.

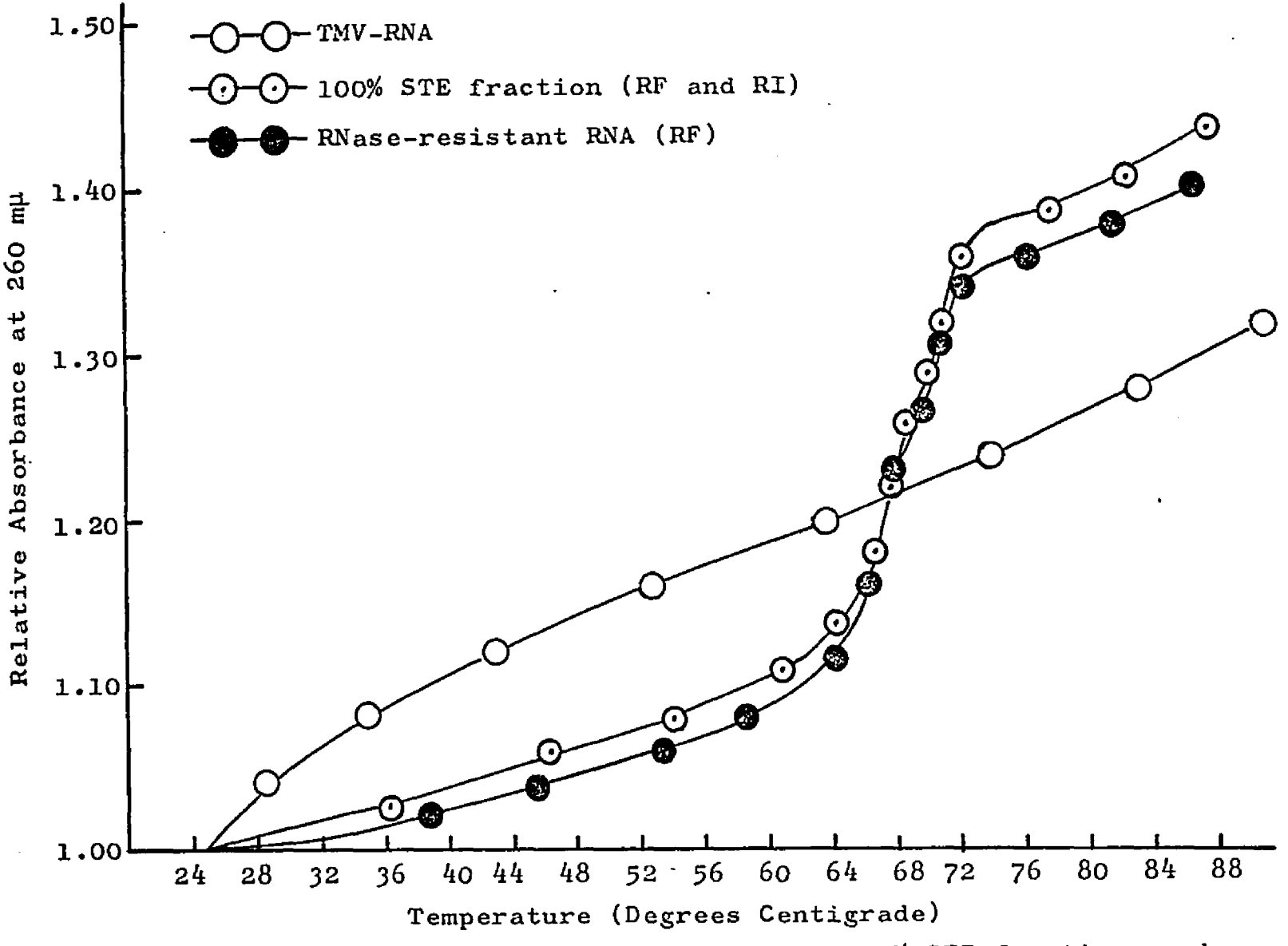


Fig. 5. Thermal denaturation of TMV-RNA, RNA from the 100% STE fraction, and RNase-resistant RNA.

increase is attributed to melting which is the removal of hydrogen-bonded regions from single-stranded nucleic acid (Boedtker 1960). The RNase-resistant RNA (RF) denatures over a very narrow temperature range, which is characteristic of double-stranded RNA (Franklin 1967b). The thermal denaturation profile of the RNA from the 100% STE fraction, consisting of RF and RI, resembles that of RF showing a very sharp increase in optical density which verifies its double-stranded nature. Note, however, the slight increase in optical density over a broad range of low temperatures; this increase is indicative of the single-stranded component of the replicative intermediate. Denaturation of the double-stranded RNase-resistant RF and the RNA of the 100% STE fraction occurs over a range of 10-13 degrees. The denaturation temperature for both samples in PE buffer (0.001 M K-PO_4 pH 6.6 and 0.001 M Na_2EDTA) is approximately the same, 68° C. The increase in hyperchromicity is 35% for RF and 37% for RNA from the 100% STE fraction (RF and RI).

Hyperchromicity curves for both RNase-treated and untreated samples, instead of displaying a smooth sigmoid curve, show a slight, but reproducible discontinuity in the curve. As discussed above, the RNase-resistant double-stranded RNA might consist of complete duplex molecules and molecules composed of an intact strand hydrogen-bonded to pieces of complementary strand. These two types of

molecules might denature at slightly different temperatures and cause the irregularity in the curve. Similar discontinuities occur in the thermal denaturation profiles of DNAs exhibiting polydispersity in molecular size (Mandel and Marmur 1968).

Equilibrium density gradient centrifugation had shown that between 15 and 36 per cent of the RNA from the 100% STE fraction had properties of a partially single-stranded partially double-stranded RNA. Since the majority of RNA in the "RI" sample was of the RF type, the thermal denaturation profile reflects the large proportion of double-stranded RNA; only the small initial rise at low temperatures is evidence of the single-stranded component.

Annealing Experiments

RNA from the 100% STE fraction was annealed with radioactively labeled TMV-RNA in order to determine whether it was virus-specific. The capacity of double-stranded RNA to reanneal specifically with the homologous viral RNA is based on the concept that only complementary base sequences can be annealed to form a stable RNase-resistant double helix. Double-stranded RNA (RF and RI), purified by three passages through the cellulose column, was denatured by heat and annealed in the presence of H^3-TMV-RNA or with H^3-ribosomal RNA from tobacco. The samples were treated with high concentrations of RNase A and T_1 to remove

single-stranded RNA which had not annealed. The RNase-resistant RNA was collected on filters and the radioactivity was determined. The results of such an experiment are presented in Table 1. One can see that the presence of RI with ribosomal RNA, under conditions of maximum re-annealing, did not confer upon the rRNA an increase in RNase-resistant radioactivity. However, a significant increase in RNase-resistant radioactivity over that displayed by TMV-RNA alone was obtained when RI was annealed with radioactively labeled TMV-RNA.

Since each mixture contained approximately 2.5 μg double-stranded RNA, theoretically one-half of the sample or 1.25 μg would be minus strands which could anneal with the large excess of plus strands in the mixture. On that basis, one might expect 1250 cpm above the residual radioactivity to become RNase-resistant if annealing were 100% efficient. The data show 810 cpm in one case and 396 cpm in the other above the radioactivity of the control H^3-TMV-RNA indicating an efficiency of 65% and 35% respectively. Thus one can conclude that the RNA from the 100% STE fraction is virus-specific and contains minus strands which anneal with radioactively labeled TMV-RNA and form double-stranded RNA.

Table 1. Annealing of RNA from the 100% STE fraction with H^3-labeled RNA

H^3-labeled RNA	Unlabeled RNA	RNase-resistant radioactivity after annealing			
		Expt. 1		Expt. 2	
		cpm[a]	cpm over control	cpm[a]	cpm over control
TMV-RNA (25 µg, 25,000 cts)	--	1887		304	
TMV-RNA (25 µg, 25,000 cts)	RI (2.5 µg)	2697	810	700	396
rRNA (25 µg, 39,000 cts)	--	1569			
r RNA (25 µg, 39,000 cts)	RI (2.5 µg)	1559			

[a]Background counts per minute were subtracted.

Annealing mixtures were made up to a final volume of 0.30 ml in STMg buffer (0.2 M NaCl, 0.01 M Tris, pH 7.6, 0.01 M $MgCl_2$), heated for 10 min. at 100° C, placed in an 85° C water bath and cooled overnight. RNase resistance (10 µg RNase A/ml, 1 µg RNase T_1/ml, 37° C, 1 hour) was assayed by addition of carrier leaf RNA (200 µg) and TCA (5%) precipitation of counts onto cellulose acetate filters.

Polyacrylamide Gel Electrophoresis

RNA, which was obtained from TMV-infected leaves and eluted as the 100% STE fraction from a cellulose column, was subjected to electrophoresis on 2.0% acrylamide gels which were swollen in buffer for three days. Fractionation of RNA of high molecular weight on polyacrylamide gels is based on differences in molecular weight. The pore size of the gel can be controlled over a wide range for high resolution of RNA molecules.

A densitometric trace (260 mμ) of a composite electropherogram shows the migration of TMV-RNA and RNA from the 100% STE fraction after electrophoresis for 18 hours at 5 ma/gel (Fig. 6a). Note the RNA which migrates 3.2 cm, the same distance as RNase-resistant double-stranded RNA (RF) presented in Fig. 6b, and the suggestion of a larger, more heterogeneous RNA (RI) which migrates approximately 1.6 cm. Under the conditions of electrophoresis for 18 hours at 5 ma/gel TMV-RNA would migrate approximately 13 cm. Since the gel was only 10 cm long, a composite electropherogram was presented. TMV-RNA and RF appear on the same gel after about nine hours of electrophoresis at 5 ma. However, longer times of electrophoresis were required to obtain even a suggestion of penetration of RI into the gel or separation of RF and RI components. The relative electrophoretic mobilities (REM) depicted in

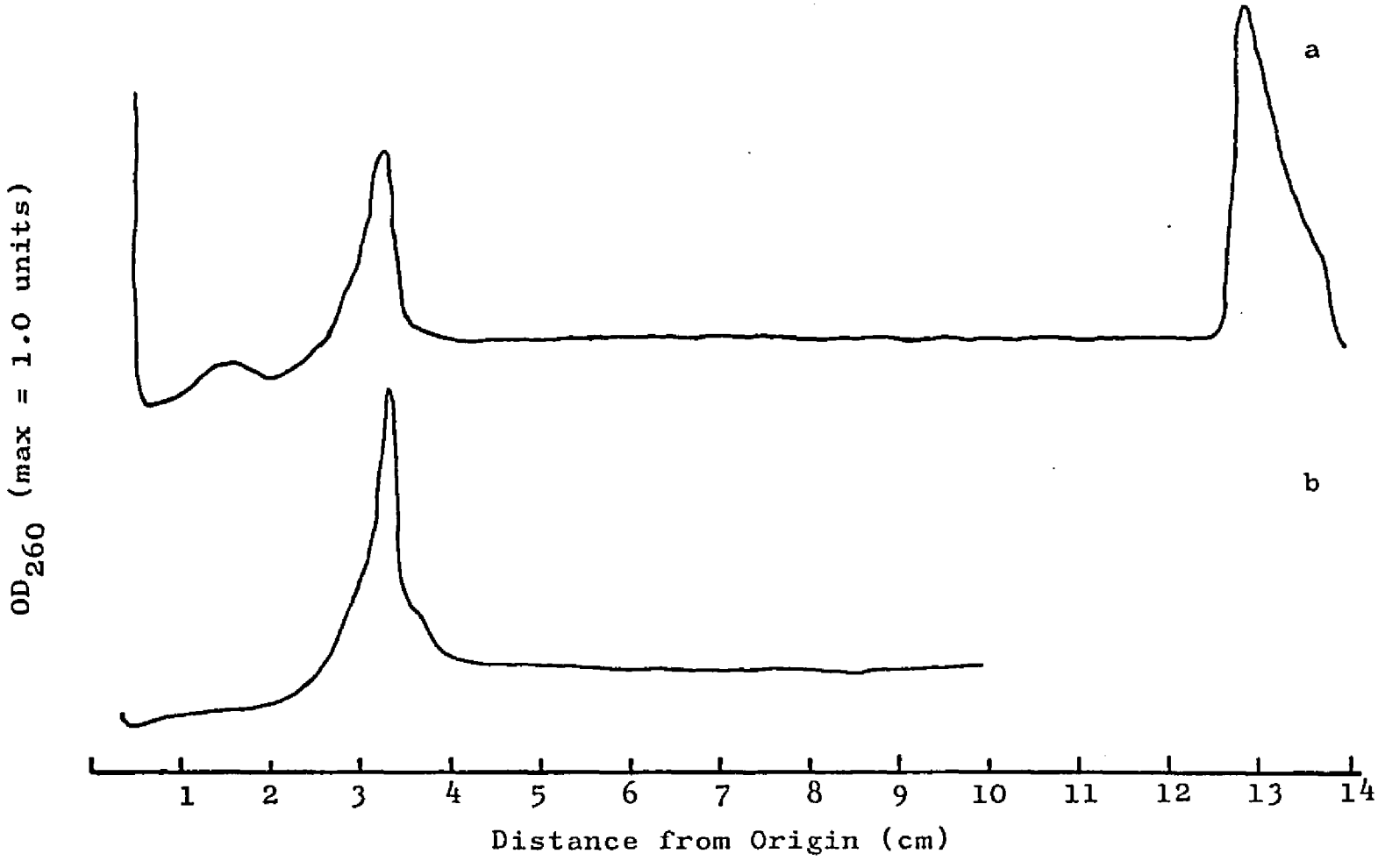


Fig. 6. Composite diagram of the migration of RNA from the 100% STE fraction and marker TMV-RNA (a) and RNase-resistant RNA (b) under standard conditions of electrophoresis (gels containing 2% acrylamide and swollen for 72 hours, electrophoresis at room temp., 5 ma per gel, 18 hours) -- RNA from the 100% STE fraction was treated with RNase (0.5 µg/ml, 20 min., room temp.) prior to electrophoresis (b). After electrophoresis, gels were scanned by transmitted ultraviolet light (260 mµ).

Fig. 6a represent the average REM from at least ten different gels on different preparations of RNA.

A microdensitometric trace of an electropherogram of RNase-resistant RNA is shown in Fig. 6b. The peak was quite narrow and was generally taller than the corresponding peak in a sample of RNA not treated with RNase indicating that the RNase-resistant RNA was more homogeneous than the double-stranded RNA prior to RNase treatment. The increase in height of the peak could be attributed to the double-stranded RNA remaining after removal by RNase of nascent single strands from an RI-type of component.

The relative electrophoretic mobility (REM) of TMV-RNA and RF vs. the logarithm of their molecular weights is presented in Fig. 7. The molecular weight of TMV-RNA is 2×10^6 daltons (Knight and Woody, 1958); a complete duplex (RF) would be expected to have a molecular weight of 4×10^6. For RNAs between 3×10^5 and 2.3×10^6 daltons, the REM was linearly related to the logarithm of the MW (Bishop, Claybrook, and Spiegelman 1967). Assuming a linear relationship for REM at molecular weights greater than 2.3×10^6 and for RNAs with double-stranded and/or branched molecular conformations, a straight line was drawn between the REM of TMV-RNA and RF. By extrapolation, the mean molecular weight of the RI component was estimated to be about 4.5×10^6 daltons. The variation in the amount and mobility of the RI component for different preparations

Fig. 7. Estimation of the molecular weight of the "replicative intermediate" component from the relative electrophoretic mobility (REM) on polyacrylamide gels -- A linear relationship between the REM and the logarithm of the MW of TMV-RNA and RNase-resistant RNA (RF) is assumed. The range in REM obtained for all components appears near the bottom of the figure.

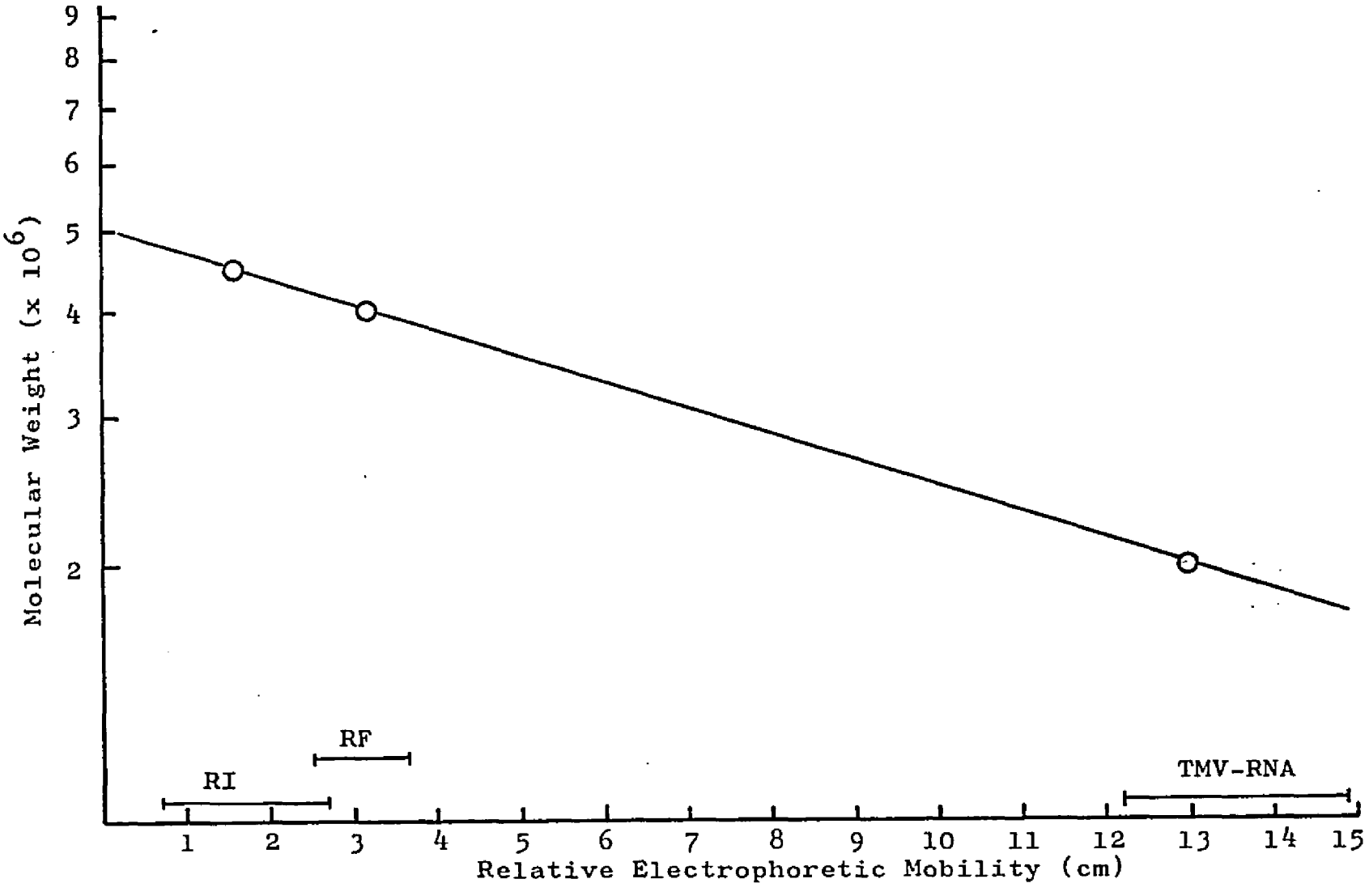


Fig. 7. Estimation of the molecular weight of the "replicative intermediate" component from the relative electrophoretic mobility (REM) on polyacrylamide gels.

is reflected in the range of MW which is estimated to be between $4.2\text{-}4.8 \times 10^6$. Since a RI molecule with one completed nascent single strand would have a MW of 6×10^6, the estimate of MW from REM is low. Several reasons for the discrepancy between estimated and theoretical MW can be presented: (1) the relationship between REM and log MW may not apply for double-stranded and/or branched molecules; (2) the molecules in the RI region of the gel might represent RI as it is in the infected cell, a double-stranded molecule with one or several short tail(s); (3) larger RI molecules could not enter the pores of the acrylamide gels, either because of their size or their branched conformation. Support for the last notion is the observation that occasionally the height of the RF peak in an RNase treated sample was higher than could be accounted for the sum of the RF and RI peaks of the comparable sample which was not treated with RNase. One might infer that the excessive height of the peak was due to RI molecules which were too large or too branched to enter the gel before RNase treatment, but afterwards contributed to the peak. Variation in electrophoretic mobility, presented as range of REM in Fig. 7, can be ascribed to several factors: actual heterogeneity among the molecules themselves, e.g., RI; differences in time for electrophoresis (TMV-RNA); slight variations in gel concentration; and distortions in length such as differential stretching or shrinking.

In order to determine the size of the single-stranded components of the double-stranded RNA forms, heat denatured samples were subjected to electrophoresis on 2.0% polyacrylamide gels. RNA from the 100% STE fraction (RI and RF) and RNase-resistant RNA (RF), prepared by treating the 100% STE fraction with 0.2 μg pancreatic RNase/ml, extracting with phenol, and dialyzing against PE buffer overnight, were used for thermal denaturation studies, then were subjected to electrophoresis after addition of 10% sucrose and 0.2% SDS. After electrophoresis for 1-1/2 hours, both samples of RNA showed a large peak of optically dense material with the same REM as TMV-RNA, providing evidence that one or both strands of the double-stranded molecules were as large as TMV-RNA itself.

One can conclude from the electrophoresis of the 100% STE fraction that polyacrylamide gels do not provide a convenient means of separating or characterizing RNA with molecular weights above 2×10^6. Although some separation between RNA which migrated like RNase-resistant RNA and a RNA component having a lower mobility, which behaved like RI was observed, it was not quantitative. Polyacrylamide gels might prove to be useful for characterizing the single-stranded components of which RI and RF are comprised.

Cell Fractions

Since double-stranded RNAs, namely RI and RF, are thought to be intermediates in the synthesis of TMV-RNA, the localization of double-stranded RNA in subcellular fractions was investigated in an effort to identify the intracellular site of TMV-RNA synthesis. Leaf homogenates of TMV-infected leaves were fractionated by differential centrifugation and differential solubility in detergents into five fractions. The 1000 x g pellet was separated into a SDS-solubilized nuclear fraction and the Triton-X-100 solubilized chloroplast fraction. The 1000 x g supernatant was centrifuged at 14,000 x g to pellet mitochondria, starch grains and small chloroplasts, and subsequently at 160,000 x g to obtain the ribosomal pellet and cytoplasmic fraction. RNA, which was extracted by phenol from each cell fraction, was fractionated by cellulose chromatography and its buoyant density in Cs_2SO_4 determined. Microdensitometric traces of UV absorption photographs of RNA, including marker TMV-RNA, banded by analytical isopycnic centrifugation are presented in Fig. 8. Evidence for double-stranded RNA, which has a characteristically lower buoyant density than the marker TMV-RNA (ρ = 1.640 gm/cm^3) cannot be seen in the nuclear or chloroplast fractions (Fig. 8a and b). The optically dense material in the nuclear fraction was of higher buoyant density than the TMV-RNA and its nature is unknown; the material in the

Fig. 8. Analytical isopycnic centrifugation in Cs_2SO_4 of RNA isolated from leaf-cell fractions -- TMV-infected (seven days post-infection) leaves were ground with a mortar and pestle and the cell-free extract was fractionated into nuclear, chloroplast, mitochondrial, ribosomal, and cytoplasmic fractions. Each fraction was extracted with phenol. The nucleic acids obtained were fractionated by chromatography on cellulose and examined by analytical isopycnic centrifugation after adding TMV-RNA (2.5 μg) as marker. RNA was treated with RNase (0.2 μg/ml, 20 min., room temp.) and pronase (0.5 mg/ml, 10 min., room temp.) prior to addition of Cs_2SO_4 and TMV-RNA (d, f, and h).

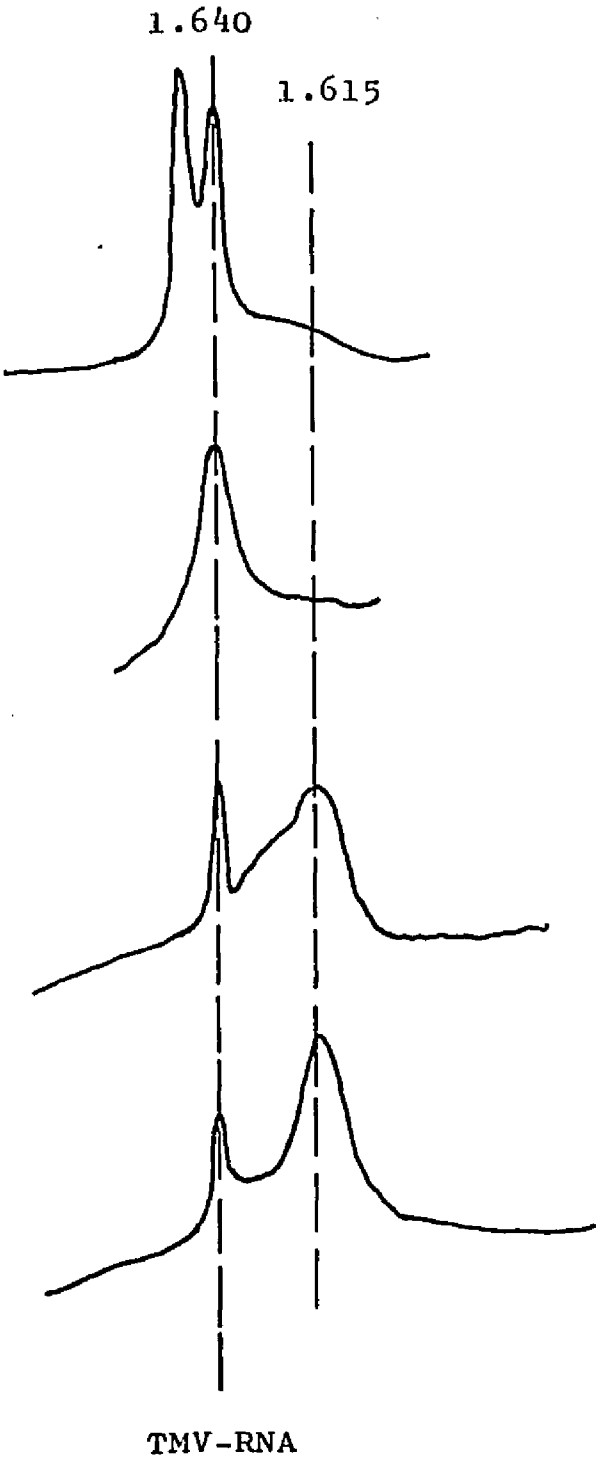
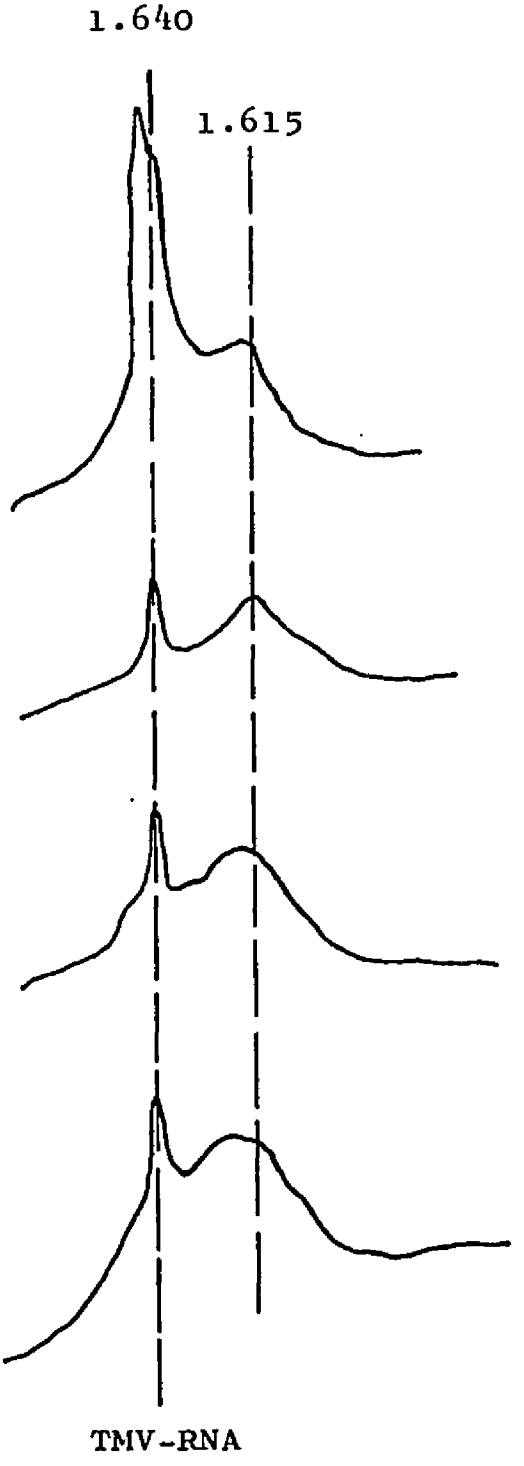


Fig. 8. Analytical isopycnic centrifugation in Cs_2SO_4 of RNA isolated from leaf-cell fractions.



e. ribosomal fraction + TMV-RNA

f. RNase-treated ribosomal fraction + TMV-RNA

g. cytoplasmic fraction + TMV-RNA

h. RNase-treated cytoplasmic fraction + TMV-RNA

Fig. 8.--Continued

chloroplast fraction appeared as a diffuse broad area of the gradient, only the marker could be seen as a peak. Neither of the fractions obtained at 1000 x g showed any material with the buoyant density of double-stranded RNA.

RNA with the buoyant density of double-stranded RNA from the mitochondrial, ribosomal, and cytoplasmic fractions plus marker can be seen in Fig. 8c, e, and g; the RNase-resistant RNA from each fraction plus marker is shown in Fig. 8d, f, and h, respectively. Note the shoulder of RNA with buoyant density of 1.630 gm/cm^3 in the mitochondrial fraction prior to RNase treatment (Fig. 8c); the shoulder is eliminated by RNase (Fig. 8d). The ribosomal fraction (Fig. 8e) is heavily contaminated with ribosomal RNA (more dense than TMV-RNA), since the RNA from the cell fractions was passed through the cellulose column only once. Ribosomal RNA is eliminated by RNase-treatment (Fig. 8f) and a broad band of RNase-resistant RNA remains (ρ = 1.615). Double-stranded RNA in the cytoplasmic fraction appears as a broad band both before (Fig. 8g) and after (Fig. 8h) RNase-treatment.

Closer examination of the densitometric profiles of the RNA can give an indication of the size and heterogeneity of the double-stranded RNA in each fraction. Density heterogeneity may be structural or compositional in origin and band width is inversely related to molecular weight (Meselson, Stahl, and Vinograd, 1957). Note the

mitochondrial fraction and the shoulder of 1.630 gm/cm^3 buoyant density on the 1.615 band (Fig. 8c) which is removed by RNase treatment (Fig. 8d). Low concentrations of RNase degrade the single-stranded component of the partially single-stranded partially double-stranded molecules in the shoulder and essentially eliminate the structural heterogeneity due to "strandedness," yielding molecules of the double-stranded replicative form type. These molecules band upon equilibrium density gradient centrifugation in a narrow peak, homogeneous in strandedness and composition (Fig. 8d). Since the band width is fairly narrow, one can say that the molecular weight of the macromolecular species is fairly high. On the other hand, examination of the RNA from the ribosomal and cytoplasmic fractions, both before and after treatment with RNase (Fig. 8e and f, g and h) can lead to a different conclusion. Although the 1.615 peak of double-stranded RNA is skewed to the side of higher buoyant density, the shoulder appears to be less prominent than that of the RNA of the mitochondrial fraction. RNase treatment does not affect the shape of the peak significantly. The RNA in both samples bands in a rather broad flat peak, before and after RNase treatment. The width of the peak cannot be attributed to structural heterogeneity because after RNase treatment, structural heterogeneity as the presence of "branches" on some molecules has been eliminated.

Therefore, the broad band width probably can be ascribed to the low molecular weight of the RNA. One could conclude that the RNA was of low molecular weight if one assumed that broad band width was due to compositional heterogeneity inasmuch as small pieces of RF might exhibit such heterogeneity. On the basis of either reason, the conclusion is reached that the double-stranded RNA in the ribosomal and cytoplasmic fractions is probably of low molecular weight.

Besides the evidence that the double-stranded RNA in the mitochondrial fraction is of high molecular weight, the proportion of RNA having a buoyant density of 1.630 gm/cm^3 is greater in the mitochondrial than either the ribosomal or cytoplasmic fraction. In addition, a greater quantity of RNA elutes in 100% STE from the mitochondrial than the other cell fractions. Thus, the appearance of the highest proportion and largest total amount of RNA with buoyant density intermediate between single- and double-stranded RNA in the mitochondrial fraction suggest that the TMV-RNA replication system might be associated with the mitochondria or cosediments with them.

DISCUSSION

The purpose of this study was to elucidate some aspects of the replication of the ribonucleic acid of tobacco mosaic virus. To this end, an attempt was made to confirm the presence in infected tissue of unique forms of RNA which might be involved in the RNA replicative process, to more fully characterize these forms, and to localize them within the cell.

Previous reports had indicated the presence in infected plants of an RNase-resistant form of RNA, presumably double-stranded. This RNA was obtained only after treatment of the plant extracts with high concentrations of RNase. One of the objectives of the present work was to isolate unique forms of RNA from infected tissue before RNase treatment so that these presumed intermediates of the RNA replicative process might be examined in a more nearly native state. Precautions were taken to prevent RNase degradation during isolation procedures and a technique that permits preferential purification of double-stranded forms of RNA was employed. It was found that extracts of TMV-infected cells contain double-stranded RNA with twice the molecular weight of TMV-RNA and, in addition, a species of RNA with the following properties: (1) it is found only in infected plants; (2) it elutes from a cellulose column

in a manner expected of double-stranded RNA; (3) it has a buoyant density in Cs_2SO_4 intermediate between that of double- and single-stranded RNA; (4) it is partially susceptible to low concentrations of RNase and appears to yield double-stranded RNA after RNase treatment; (5) its thermal denaturation profile resembles that of double-stranded nucleic acid and shows melting at low temperatures which is indicative of single-strandedness; (6) it confers RNase-resistance specifically to H^3-TMV-RNA demonstrating the presence of a base sequence complementary to viral RNA; (7) it migrates on polyacrylamide gels with very low mobility indicating that it has a high molecular weight and after thermal denaturation migrates with the mobility of TMV-RNA; (8) it has a heterogeneous sedimentation behavior; in a preliminary experiment RNA from the 100% STE fraction was subjected to sucrose density gradient analysis and showed a distribution between approximately 12s and 22s. The properties of this RNA from TMV-infected tissue are analogous to those described for the replicative intermediate of R17 bacteriophage and poliovirus; the replicative intermediate was a population of RNA molecules composed of double-stranded cores and associated single-stranded "tails" (Erikson and Franklin 1966, Bishop and Koch 1969). On the basis of this physicochemical evidence, a partially double-stranded, partially single-stranded replicative

intermediate (RI) is believed to be involved in the replication of the RNA of TMV.

A technique used to characterize the components of RNA isolated from TMV-infected leaves and fractionated by cellulose chromatography was Cs_2SO_4 analytical isopycnic centrifugation. One RNA component has a buoyant density of 1.630 gm/cm^3 which is between that of single- (1.640 gm/cm^3) and double-stranded RNA (1.615 gm/cm^3). This component having an intermediate buoyant density was susceptible to low concentrations of RNase, presumably being converted to a double-stranded form. The intermediate buoyant density and the susceptibility to RNase may be explained by the presence of single-strands on a partially single-stranded, partially double-stranded replicative intermediate.

The difference between the buoyant density of the RI and the single-stranded TMV-RNA (0.010 gm/cm^3) is less than that between RI and the double-stranded RNA (0.015 gm/cm^3). These results are in contrast to those obtained for the buoyant density in Cs_2SO_4 of the intermediate RNA forms of the R17 bacteriophage; the buoyant density of the RI was closer to that of the double-stranded RNA (RF) than to single-stranded RNA (Erikson 1966). The 15s MS2 phage-specific RNA having the characteristics of a replicative intermediate had a similar behavior in a CsCl density gradient to the RI of R17 bacteriophage (Kelly, Gould, and

Sinsheimer 1965). Some structural feature peculiar to the RI of TMV might account for its buoyant density being closer to that of single- rather than double-stranded RNA. Such a feature might be the point where the single strand diverges from the double strand. The structure at the branch point would be different depending upon whether replication occurred by a semiconservative or by a conservative mechanism (see Fig. 2). It is possible that the mechanism of replication of the RNA of a plant virus might differ from that of a bacterial virus and might account for the difference in the buoyant density behavior.

A more likely explanation to account for the buoyant density of the RI of TMV being closer to single-stranded RNA than to double-stranded RNA is suggested by the recent publication of Bishop and Koch (1969), who examined the poliovirus replicative intermediate. They found that the buoyant density of the replicative intermediate relative to the single-stranded or double-stranded marker varied from preparation to preparation. This variation was attributed to different degrees of degradation during isolation and purification. Thus, it is possible that a preferential selection of samples with buoyant density closer to that of single-stranded RNA was made. Another factor which might have affected the banding position of the replicative intermediate is the precipitation of TMV-RNA which occurs in Cs_2SO_4. The

precipitation of the marker might have caused partial coprecipitation of the replicative intermediate due to its single-stranded components.

A more detailed analysis of the buoyant density of the replicative intermediate of TMV might permit one to distinguish among these possibilities. Centrifugation in CsCl in the presence of the dye ethidium bromide might allow greater resolution of RI inasmuch as the single-stranded MS2 RNA is separated from the double-stranded RNA by 0.042 gm/cm^3 (Kelly and Sinsheimer 1967). Another suggestion is to use a different marker RNA in place of TMV-RNA in order to eliminate the possibility of coprecipitation of the RI (Szybalski 1968). It is possible to prevent the precipitation of TMV-RNA in Cs_2SO_4 by incorporating formaldehyde (0.5-2.0%) which does not affect the banding of double-stranded RNA (Lozeron and Szybalski 1966). However, formaldehyde was not used in these experiments because the effect of formaldehyde on the banding of double-stranded RNA with "tails" has not been elucidated.

The variation in the degradation of replicative intermediate which occurs during isolation and purification has been mentioned. A comparison of microdensitometer traces of various preparations of RNA banded by isopycnic centrifugation reveals that the amount of RNA with intermediate buoyant density varies. This variability was

attributed to different degrees of loss of the single-stranded component of the RI. It is possible that improvements in technique might reduce this loss. One suggested improvement would be the incorporation of an RNase adsorbant such as bentonite in the extraction procedure (Singer and Fraenkel-Conrat 1961). Another suggestion for enhancing the RI fraction relative to the RF is to precipitate RI with 2 M LiCl or 1 M NaCl in which RF is soluble (Bishop and Koch 1967). Such a fractionation might not be feasible because the concentration of RNA must be high to obtain quantitative precipitation of RI by salts (Erikson and Gordon 1966). It is probable that when large quantities of RI are extracted from greater amounts of infected tissue, improvements in fractionation can be achieved.

Further characterization of the fractionated RNA from TMV-infected leaves was accomplished by thermal denaturation. This technique depends on the change in the optical density of the nucleic acid solution that accompanies the change in the secondary structure upon heating of the nucleic acid. Thermal denaturation profiles of the RNA from the 100% STE fraction showed a sharp increase in optical density at 260 mμ which one would expect for double-stranded nucleic acid (Franklin 1967b). In addition to this sharp increase, there was a rise in OD over a broad range of low temperatures which was attributed

to melting of the H-bonded regions of single-stranded RNA and provided evidence for the single-stranded components of the replicative intermediate. The results obtained can be compared for their similarity with those for the replicative intermediate of R17 bacteriophage. The RI of R17 showed a much more pronounced melting at low temperatures which was evidence of the 22-28 per cent single-stranded component in the population of RI (Franklin 1966). For TMV, at most 36% of the RNA from the 100% STE fraction represented RI, of which a fraction was single-stranded, so the effect was proportionately less. It would be reasonable to assume that a RI fraction enriched by methods suggested above, would show a proportionate increase in melting at low temperatures when heated.

One of the distinguishing features of a thermal denaturation profile of nucleic acid is the denaturation temperature, T_m, which is the midpoint of the hyperchromic shift. The T_m, under standard conditions of denaturation, was shown to be linearly related to the base composition (expressed in terms of the mole percentage of guanine plus cytosine bases) of DNA (Marmur and Doty 1962). For double-stranded RNAs, a compilation of some T_m's and base compositions has been made (Billeter, Weissmann, and Warner 1966). The double-stranded RNAs with their respective mole percentage of GC and T_m in SSC (0.15 M NaCl, 0.015 M Na citrate) were as follows: wound tumor (37.6%, 90-92°);

reovirus (44%, 95°); encephalomyocarditis-RF (47%, 96°); MS2-RF (52%, 103°). A positive relationship between GC and T_m seems to exist although it is not linear. TMV-RF does not fit, as the T_m should be 94.5° according to its GC content of 43.4% (Weissmann et al. 1965); however, a T_m of 97° was reported (Burdon et al. 1964). This value was determined from the loss of resistance to RNase upon heating of double-stranded RNA. It remains to be shown whether the replicative intermediate would have a T_m of 94.5° in SSC as determined from the hyperchromic shift. Values for T_m obtained by determining the loss of RNase resistance which accompanies the disruption of ordered secondary structure cannot be compared directly with T_m calculated from the hyperchromic shift (Bishop and Koch 1967). For this reason, the T_m of 68° determined in PE buffer (ionic strength = .012 [Franklin 1967a]) could not be correlated to the previously reported values of 97° and 74° obtained in SSC and 0.01 x SSC respectively (Burdon et al. 1964) to obtain a relationship between T_m and $\log_{10} [Na^+]$. It is possible that further studies on the denaturation behavior of RNase-resistant double-stranded RNA under conditions of different ionic strengths would enable one to establish the relationship between T_m and $\log_{10} [Na^+]$ (Mandel and Marmur 1968).

In order to establish whether the RNA isolated in the 100% STE fraction was virus-specific, its ability to

anneal with radioactively labeled viral RNA and to confer RNase-resistance on the labeled material was determined. The extent to which the RNA annealed was between 35 and 65 per cent, demonstrating that the double-stranded forms actually contained strands complementary to TMV-RNA. The type of product resulting when either the RF or RI of R17 bacteriophage was annealed with radioactively labeled phage RNA was similar (Iglewski and Franklin 1967). The specificity of annealing was utilized by Weissmann et al. (1965) to determine the nucleotide composition of the minus strand of the RF of TMV. P^{32}-labeled RF was heat-denatured and reannealed in the presence of a large excess of unlabeled TMV-RNA to displace the labeled plus strand from the duplex with unlabeled plus strand. Analysis of the composition of the radioactive nucleotides remaining in the RNase-resistant duplex yielded values in agreement with those calculated for a strand complementary to TMV-RNA. It remains to be shown whether both the RI and RF of TMV anneal with TMV-RNA in the same way and yield the same type of product. It might be possible to determine the size of the annealed RNA by sedimentation through a sucrose density gradient or by electrophoresis on polyacrylamide gels.

Electrophoresis on polyacrylamide gels was utilized in an attempt to separate the double-stranded forms of RNA and to characterize their molecular size. By this technique, three components could be distinguished. TMV-RNA

could be distinctly separated from RNA which migrated with the relative electrophoretic mobility of RNase-resistant (double-stranded) RNA. Difficulty was encountered in distinguishing the third component from the double-stranded RNA. The heterogenous third component migrated with a low relative electrophoretic mobility indicating that it had a high molecular weight. The elimination of this third component by RNase and the concomitant increase observed in the peak representing double-stranded RNA provided evidence that the third component represented double-stranded RNA with "tails." Poliovirus RNA is the same size as TMV-RNA and an analysis of poliovirus replication products showed no migration of RI in 2.25% acrylamide gels under conditions of electrophoresis for 3.75 hours at 5 ma per gel (Noble, Kass, and Levintow 1969). However, RI entered 1.6% gels after one hour of electrophoresis and migrated as a diffuse band. Under the conditions of the present experiments, 2.0% gels which were swollen for 72 hours thus increasing their pore size, and electrophoresis for 18 hours at 5 ma per gel, a third heterogeneous, presumably RI type of component, migrated approximately 1.6 cm into the gel. The low mobility of the RI may be due to the unusual conformation of the molecule or its large size in relation to the pore size of the gel. Upon removal of the single-stranded "tails" and/or reduction in molecular size by RNase, the RI was converted to a RNA with higher

mobility. The relative electrophoretic mobility of the single-stranded, double-stranded (RF), and branched double-stranded RNA (RI) was in accord with the order of those reported for the products of the Qβ replicase reaction (Bishop, Claybrook, Pace, and Spiegelman 1967).

Although polyacrylamide gels have a limited resolving power for molecules of very high molecular weights, concentrated gels might be useful for resolving the component strands of RF and RI after denaturation. The possibility has been mentioned that RNase-treated RI might consist of an intact minus strand and pieces of plus strand hydrogen-bonded to it. Resolution of the size of the plus strand pieces would reveal the distance between replication points and thereby the number of replication points or sites of polymerase molecules per replicative intermediate.

In order to localize the double-stranded forms of RNA in the leaf cell, nucleic acids were isolated from the nuclear, chloroplast, mitochondrial, ribosomal, and cytoplasmic fractions and banded by isopycnic centrifugation. Double-stranded RNA was detected in the mitochondrial, ribosomal, and cytoplasmic fraction. The highest proportion and the largest total amount of RNA with buoyant density intermediate between single- and double-stranded RNA was found in the mitochondrial fraction, suggesting that TMV-RNA may be synthesized in a cytoplasmic organelle, possibly mitochondria or less dense

chloroplasts, or in association with a large "replication complex" which would sediment with the large particle fraction. A "replication complex" isolated from poliovirus infected cells was an aggregate of a replicative intermediate with, on the average, four molecules of viral RNA polymerase (Girard, Baltimore, and Darnell 1967). A larger complex envisioned as replicative intermediate and polymerase enzymes associated with ribosomes was found in R17 bacteriophage infected E. coli. This complex was isolated from the large polysome fraction (Hotham-Iglewski, Phillips, and Franklin 1968).

The conclusion that TMV-RNA synthesis occurs in a "mitochondrial" fraction is in contradiction to the cytological, microspectrophotometric and autoradiographic evidence that the RNA of the virus is synthesized in the nucleus or nucleolus or in the chloroplasts (Schlegel, Smith, and de Zoeten 1967, Reddi 1964). These experimental approaches compare infected vs. uninfected tissue and do not demonstrate that synthesis is virus-specific. The various reports of the site of TMV synthesis (reviewed by Schlegel et al. 1967) might be reconciled if in fact TMV-RNA synthesis occurs in the mitochondrial fraction, but TMV protein synthesis and virus maturation occur in the cytoplasm or nucleus.

It is possible that by establishing the cellular site of virus synthesis one could study not only the

cellular components associated with virus multiplication but also the mechanisms of viral RNA synthesis. It would be possible to isolate an *in vitro* virus synthesizing system from that cellular fraction of virus infected plant tissue. Cell free extracts capable of synthesizing actinomycin D resistant RNA which was partially resistant to RNase were reported for turnip yellow mosaic virus in chinese cabbage (Ralph and Wojcik 1966), bromegrass mosaic virus in barley (Hiruki 1969), broadbean mottle virus in broadbean (Semal 1969), barley leaves infected with bromegrass mosaic virus (Semal and Hamilton 1968), and TMV in tobacco (Ralph and Wojcik 1969). It would be interesting to test whether the double-stranded RNAs, RF and RI, could serve as template for viral RNA synthesis in such an *in vitro* system. Double-stranded RNAs, isolated by phenol or detergent treatment of the *in vitro* synthesizing system of Qβ-RNA could not serve as template for the Qβ-replicase (Weissmann, Feix, and Slor 1968). It was suggested that double-stranded RNAs as isolated did not correspond to the replicative intermediates functional *in situ* and that "manipulations required for their isolation may convert them into the more or less complete duplexes they appear to be in the purified state" (Spiegelman et al. 1968). It remains to be shown whether the double-stranded RNAs isolated from TMV-infected tissue or from an *in vitro* synthesizing system either with or without exposure to

phenol or detergent are actually playing the roles of mandatory intermediates in the replication of TMV-RNA.

One of the biological properties of viral RNA is its ability to initiate an infection. It is of interest to determine whether the double-stranded RNAs from TMV-infected tissue are capable of initiating an infection, either before or after denaturation. Both the replicative form and replicative intermediate of poliovirus were intrinsically infectious when assayed on HeLa cells (Bishop and Koch 1969). In contrast, the RF and RI of bacteriophage R17 have little or no apparent biological activity; only after denaturation are they able to initiate infection (Amman, Delius, and Hofschneider 1964). Essential to the demonstration that double-stranded RNAs are infective is the complete elimination of contamination by single-stranded viral RNA. Chromatography on cellulose has proved to be useful for separating double-stranded RNAs from TMV-RNA and permits their isolation from plants without prior treatment with RNase. Prevention of exposure to nuclease during purification was critical to the preservation of maximal infectivity of poliovirus RF (Bishop and Koch 1967). Since the distinction between animal and bacterial RNA viruses lies in the intrinsic infectivities of their RF's and RI's, determining the infectivity of the double-stranded RNAs of a plant virus might provide insight into the relationships among the RNA

viruses. In addition, infectivity studies might further clarify the relationship of double-stranded RNAs to replication and elucidate the nature of the replication complex.

SUMMARY AND CONCLUSIONS

The nucleic acids from TMV-infected plants have been studied for the presence of unique forms of RNA associated with virus replication. They were obtained by chromatography twice on a cellulose column and analyzed using the techniques of isopycnic centrifugation, thermal denaturation, annealing with viral RNA, and polyacrylamide gel electrophoresis. Furthermore, to localize the RNA in the cell, the nucleic acids were obtained from several cell fractions, were fractionated by cellulose chromatography, and were analyzed by isopycnic centrifugation in Cs_2SO_4.

It was concluded from the banding in Cs_2SO_4 equilibrium density gradients and the susceptibility to low concentrations of RNase that the RNA fraction eluting from the cellulose column in plain buffer contained partially single-stranded, partially double-stranded RNA in addition to RNase-resistant double-stranded RNA. A single peak with a shoulder was seen in UV absorbance photographs of the RNA banded in a Cs_2SO_4 equilibrium density gradient. Using the value of 1.640 g/cm^3 for the marker TMV-RNA, the buoyant density of the RNA in the main peak was 1.615 g/cm^3, and that of the RNA appearing in the shoulder was approximately 1.630 g/cm^3, a value intermediate between that of single- (1.640) and that of double-stranded (1.615) RNA. The

fraction of RNA in the shoulder was eliminated by treatment of the RNA in the buffer fraction with a low concentration of RNase prior to isopycnic centrifugation; the RNase-resistant double-stranded RNA banded in a symmetrical peak of increased height. The increase in the peak height of the double-stranded RNA was proportional to the amount of RNA in the shoulder prior to RNase treatment, indicating that the single-stranded portion was removed by RNase, producing double-stranded RNA which contributed to the increased peak height. In contrast, no RNase-resistant RNA eluted in the buffer fraction of nucleic acids from healthy plants. Only one peak of RNA, with a buoyant density greater than TMV-RNA, was seen by isopycnic centrifugation in Cs_2SO_4. This band probably represented ribosomal RNA and was eliminated by treatment with RNase prior to centrifugation or by chromatography on cellulose a second time.

It was concluded from the thermal denaturation properties that the RNA had a hydrogen-bonded double-stranded molecular structure and some single-stranded components. The hyperchromicity curve resembled that of RNase-resistant RNA; a sharp change in optical density occurred over a very narrow temperature range, the mid-point being 68° C. In addition to the sharp thermal transition, a slight increase in optical density over a broad range of low temperatures was noted in the hyperchromicity curves. This increase was attributed to

melting or removal of hydrogen bonded regions in a single-stranded component of a partially single-stranded partially double-stranded RNA. The hyperchromicity curve showed a slight discontinuity in its sigmoid shape; the cause was not investigated.

The RNA from the buffer fraction conferred RNase-resistance to H^3-labeled viral RNA to establish that the RNA from the buffer fraction was virus-specific and contained base sequences complementary to TMV-RNA. RNA from the buffer fraction was denatured by heat, then annealed in the presence of H^3-labeled viral RNA. A significant increase in RNase-resistant radioactivity over the control of labeled viral RNA alone was obtained, indicating that RNA from the buffer fraction had annealed with the viral RNA. The efficiency of annealing was between 35 and 65 per cent.

The RNA which behaved like a partially single-stranded partially double-stranded molecule displayed a low mobility in 2.0% polyacrylamide gels; the conclusion was reached that the RNA had a high molecular weight. Three RNA bands, including TMV-RNA which was used as a standard, were detected by electrophoresis of the RNAs of the buffer fraction in 2.0% polyacrylamide gel. When electrophoresis was carried out for 18 hours at 5 ma/gel, the marker TMV-RNA migrated 13 cm; a second band migrated 3.2 cm, the same relative electrophoretic mobility (REM) as RNase-resistant

RNA in a parallel electropherogram. The third component which exhibited a greater variation in amount and mobility than the other two components, penetrated into the gel about 1.6 cm. Assuming a molecular weight (MW) of 4×10^6 for RNase-resistant RNA (second component), by an extrapolation of the linear relationship of the REM and MW, the molecular weight of the third component was determined to be between 4.2 and 4.8×10^6 daltons. The third component was eliminated by exposing the RNA to RNase prior to electrophoresis. A simultaneous increase in the height of the peak of RNase-resistant RNA was usually observed; it was attributed to double-stranded RNA which remained after removal of RNase-sensitive single-strands from a partially single-stranded, partially double-stranded molecule.

It was concluded that one or both strands of the double-stranded molecules were as large as TMV-RNA from the electrophoretic behavior on polyacrylamide gels. When the RNA from the buffer fraction was heat denatured and subjected to electrophoresis on polyacrylamide gels, it had the same REM as TMV-RNA.

The appearance of the largest proportion and greatest amount of partially single-stranded partially double-stranded RNA in the mitochondrial fraction led to the conclusion that the TMV-RNA replication system is associated with mitochondria or cosediments with them. Double-stranded RNA which bands at a lower buoyant density

than single-stranded marker TMV-RNA in Cs_2SO_4 equilibrium density gradients was not found in the SDS solubilized nuclear fraction or in the Triton-X-100 solubilized chloroplast fraction of TMV-infected leaf cells. Double-stranded RNA appeared in the mitochondrial, ribosomal, and cytoplasmic fractions. The mitochondrial fraction contained the largest proportion and greatest amount of RNA which banded with a buoyant density of 1.630 g/cm^3, characteristic of the partially single-stranded, partially double-stranded RNA. Moreover, the band width of isopycnically analyzed RNase-resistant RNA from the mitochondrial fraction was narrow, indicating that the molecular weight of the RNase-resistant RNA was high. The width of the banded RNase-resistant RNA in the cytoplasmic or ribosomal fraction was broader suggesting that it was of low molecular weight.

Thus, it was concluded that in addition to double-stranded RNA with twice the molecular weight of TMV-RNA, a high molecular weight, virus-specific, partially single-stranded partially double-stranded RNA can be obtained from TMV-infected plants. The physicochemical properties are consistent with those expected of a replicative intermediate consisting of a double-stranded core and associated single-strands. On this basis it is believed that a replicative intermediate is involved in TMV-RNA replication

and that viral RNA replication is associated with mitochondria or cosediments with them.

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