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# A COMPARISON OF SOME BIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF SEVERAL TOBACCO RINGSPOT VIRUS ISOLATES

By

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ABSTRACT

A COMPARISON OF SOME BIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF SEVERAL TOBACCO RINGSPOT VIRUS ISOLATES

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Tobacco ringspot virus (TRSV) is an RNA-containing plant virus which has a wide host range, including both herbaceous and woody plants. This dissertation reports a study of the biological diversity among seven isolates of TRSV which were obtained from different herbaceous and woody plants. The purpose of this research was to identify TRSV isolates which were unique in the symptoms they induced in selected herbaceous hosts, and subsequently to compare some of the biochemical properties of these selected isolates.

Seven different isolates of TRSV were collected from grape, cherry, two cultivars of blueberry, tobacco, soybean, and watermelon. These isolates were characterized on the basis of: (1) the symptoms they induced in herbaceous indicator plants, (2) sensitivity to thermal inactivation, and (3) serological properties. Three TRSV isolates which showed distinct antigenic differences and distinct differences in the symptoms they induced in the herbaceous indicator plants were selected for further comparisons. The three isolates selected for further study were isolates from blueberry, tobacco,

and soybean. One of these three isolates was shown to be different in the overall charge on its virus particles as indicated by differences in mobility in an electric field.

In vitro synthesis of the protein products coded by the RNA of the three isolates of TRSV made it possible to look for isolate-dependent differences in the molecular weight of these protein products. The RNA from the three isolates of TRSV was extracted and used to direct protein synthesis in a wheat embryo cell-free protein synthetic system. Polyacrylamide gel electrophoresis was used to separate and characterize the radioactively labeled protein products of in vitro synthesis. The number of in vitro protein products was found to be the same, regardless of the TRSV isolate used as the source of RNA in the cell-free protein synthetic system. However, an isolate-dependent difference in the molecular weight of one of the in vitro protein products was demonstrated. Evidence obtained by in vitro translation of the small RNA of TRSV indicated that the protein product which showed the isolate-dependent molecular weight difference was coded by the large RNA piece of the TRSV genome.

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# INTRODUCTION

An important characteristic of any natural population is the diversity that exists within that population. It is the differential selection of variants within a natural population that leads to evolution.

The occurrence of new variants in a group of plant viruses is of both practical and theoretical importance to the plant virologist. Newly arisen variants which induce different symptoms in the host may damage crops to a greater extent, may circumvent established control measures, and may make breeding for host resistance to infection more complicated.

Variants which differ greatly in virulence, host range, or vector specificity must have some changes in their biochemical structure to account for these changes in their biological properties. By defining the biochemical changes leading to variants which accompany differences in biological properties, we will begin to understand these viral properties which are important in disease propagation and regulation.

The related strains of a virus that infect different hosts have probably arisen, through mutation, from some common ancestral type. The strains which successfully infect new host plants are those mutants which are better adapted to these new hosts. Various

examples are known where particular host plants allow the selective multiplication of certain strains of a virus when inoculated with a mixture of strains (26,59). In nature the selective multiplication of newly arisen variants in a host plant may account for the survival and eventual predominance of these new variants.

Tobacco ringspot virus (TRSV), a member of the nepovirus group (19), has a broad natural host range, and exists as a number of serologically distinct strains (16,27). The natural host range of tobacco ringspot virus includes many economically important crops such as tobacco, blueberry, soybean, grape, cherry, and other annual and perennial crops. It also occurs in weeds associated with many of these crops (19). Although the existence of several serological strains of TRSV has been reported in the literature (16,27,45,50, 51,60), there has been no attempt to do an in-depth comparison of these strains.

Biochemical comparisons of plant virus strains in the past have dealt with the biochemistry of the molecules which make up the virus particle, namely the coat protein and the nucleic acid. An examination of the literature shows that many properties of plant viruses are associated with the composition of the coat protein of the virus. However, some examples in the literature indicate that there are other important properties of plant viruses which are not associated with the viral coat protein. This is not surprising when one considers that plant virus genomes code for the synthesis of several other virus-specific proteins which appear during the course of infection and multiplication in the plant. The following

literature review will consider: (a) the evidence which indicates that viral coat protein determines a set of important plant viral properties, (b) the evidence that other viral functions are determined by proteins other than the viral coat protein, and (c) the types of viral-specific proteins which have been identified in plant virus infection. This review of the literature will be limited to plant viruses which have RNA genomes, and which have a simple protein shell consisting of one or two polypeptides.

The terminology used throughout this dissertation will be as defined below. Isolate: a variant of a virus which was collected from a particular host, i.e., the 'Jersey' blueberry isolate of TRSV was collected from a blueberry of the 'Jersey' variety. Multipartite genome: a genome which consists of more than one piece of genetic material, i.e., TRSV has a genome which consists of two pieces of RNA, hence TRSV has a bipartite genome. The RNAs of TRSV will be referred to as RNA-1 ($2.2 \times 10^6$ daltons) and RNA-2 ($1.4 \times 10^6$ daltons).

# LITERATURE REVIEW

Like many other plant viruses, TRSV has a simple coat protein consisting of one type of polypeptide. The molecular weight of this polypeptide has been variously reported to be 57,000 daltons (36), or a tetramer of a monomer of 13,000 daltons (5). Following is a review of the biochemical and biological characteristics of plant viruses which have been shown to be a function of virus coat protein.

## Serology

The antigenicity of a plant virus particle depends on the composition of the coat protein of the virus. Von Sengbusch (56) and van Regenmortel (68) compared the serological behavior of tobacco mosaic virus (TMV) strains which differed in one or more amino acids in the protein coat polypeptide. They found that only eight of the twenty amino-acid replacements resulted in a change in the antigenic properties of the virus. The antigenic properties of a virus are a function of the coat protein, but not all coat protein changes result in a change in serological identity.

Serologically indistinguishable viruses do not necessarily produce the same symptoms on a selected herbaceous host. Gooding (16) classified one hundred naturally-occurring isolates of TRSV from tobacco as belonging to one of four serological groups. He

found no correlation between isolates within a strain and the symptoms they caused in tobacco. Some isolates of a serological strain caused mild symptoms; other serologically indistinguishable isolates caused severe symptoms. Even though it was possible to determine the serological relationships of these isolates it was not possible to use serological grouping to predict the effect of the isolate on selected host plants. Either serology was not capable of detecting biologically significant differences in the coat protein, or the differences in the symptoms induced in different host plants were due to some other change in the virus.

## Electrophoretic Mobility

The electrophoretic mobility of a virus particle is the rate of migration of that virus particle in an electric field at a particular pH. This property of a virus depends on the amino acid composition of the coat protein, and also on the three-dimensional structure of the virus particle as it affects the availability of ionizing groups of the amino acids. The virus particles of different serological strains of a virus sometimes differ in their electrophoretic mobilities, a property which reflects the surface charge density of the virus particle (13). Extensive studies done with chemically-induced mutants of TMV in which the amino acid composition of the coat protein of each mutant was known, have shown that changes in electrophoretic mobility are always a function of changes in the amino acid composition of the coat protein (56). However, the converse was not true. Many mutants which differed in their amino

acid composition, did not vary in their electrophoretic mobilities. In order to produce a change in electrophoretic mobility, an amino acid substitution must result in a net difference in charge of the virus particle surface. This may occur either by changing the conformation of the protein coat, and thereby exposing or masking charged groups, or by directly substituting amino acids with a different charge.

Electrophoretically variant strains of brome mosaic virus (BMV) have been isolated and used in combination with wild-type BMV for pseudorecombinant genetic analysis (28). Pseudorecombination is an experimental technique in which the separated segments of the multipartite virus genome, by themselves uninfectious, are brought together with complementary genome segments (from a different strain) to yield a progeny virus with a hybrid genome. BMV has a multipartite genome consisting of four RNA pieces. Using separated RNA components of wild-type and variant strains of BMV in various combinations, Lane and Kaesberg (28) were able to demonstrate that the electrophoretic mobility of these strains was inherited through RNA-3 of the virus. RNA-3 of BMV has since been shown to contain the gene for coat protein (57). Therefore, in this case, as with the chemically induced, well characterized mutants of TMV, electrophoretic mobility is thought to be solely a function of the coat protein of the virus, and not dependent on the nucleic acid which is found inside of the coat protein shell of the virus particle.

## Nematode Transmissibility

Studies of serological strains of raspberry ringspot virus, tobacco rattle virus, and tomato black ring virus, have shown that nematode transmissibility is determined by the serological make-up of these viruses (22,23,21). This is not surprising considering that transmission by the nematode vector seems to involve the reversible association of virus particles with specific surfaces in the food canal of the nematode (64). Since plant viruses do not multiply in their nematode vectors (13), the interaction between TRSV and its nematode vector (Xiphinema americanum Cobb) is probably limited to the physical contact between the virions and portions of the vector, and is therefore presumed to be a function of the protein coat of the virus. Pseudorecombinant work with raspberry ringspot virus (a nepovirus like TRSV with a genome of two separate RNA molecules) has shown that nematode vector specificity is determined by the piece of the RNA genome that carries the gene for the virus coat protein (22). Similar pseudorecombinant work done with tomato black ring virus, another nepovirus, showed that transmissibility by the nematode vector was correlated with the antigenic specificity of the virus, and was determined by the RNA segment which contains the coat protein cistron (21). Thus, nematode transmissibility seems to be a characteristic of a virus which is determined by the properties of the protein coat of the virus particle.

For viruses such as TRSV the coat protein probably reflects only about 3 to 14% of the viral genome coding capacity. Thus, current ideas on relationships among strains of TRSV, based

on properties dependent on the coat protein (serological relationships, nematode transmissibility, electrophoretic mobility, amino acid composition of the coat protein) are based rather heavily on a relatively small segment of the viral genome. Biological properties such as symptom expression or host specificity, which involve an interaction between several of the virus-coded proteins and the host plant, can be expected to reflect differences between strains that no physical or chemical methods applied to the virus coat protein could detect.

Following are several examples where host specificity and symptom expression have been shown to be independent of coat protein function.

## Host Range

The host range of different strains of the same virus are usually quite similar. A strain of a virus which has acquired the ability to infect a new host usually retains the original host range as well.

The host range of a virus strain has been reported in some cases to be independent of the coat protein properties of the virus strain (6,20,37). The host range of four strains of TMV was shown to be independent of the coat protein of these strains by the use of a technique called genome masking. Genome masking is an experimental technique in which the RNA and coat protein components of a virus are separated and then brought together with complementary components (from a different strain) to yield a reconstituted

virus. When the RNA from one of four naturally-occurring host specific strains of TMV was reconstituted with the coat protein from any of the other three strains, the host specificity of the reconstituted virus particle was the same as the host specificity of the strain from which the RNA had been isolated (37). The result of this experiment suggested that it is not the coat protein of the infecting virus particle, but the genetic information contained in the RNA which determines host specificity.

The determination of host range by a factor other than coat protein was also demonstrated by Dahl and Knight (6) who studied twelve nitrous acid-induced mutants of tomato atypical mosaic virus (a tomato mosaic strain of TMV). A host range mutant was found which could no longer infect tomato, even though the parent virus readily infected tomato. Amino acid analysis of the coat protein of this mutant demonstrated no change in the amino acid content of its coat protein as compared to the coat protein of the parent virus. Genome masking experiments using two strains of tomato atypical mosaic virus, one of which was not capable of infecting tomato, showed that the coat protein of the reconstituted virus did not affect the ability of the virus to infect tomato. The determination of host range in this case was not a function of the coat protein of the infecting virus particle, but rather was determined by the nucleic acid of the infecting particle.

Analysis of pseudorecombinants made with four naturally-occurring strains of raspberry ringspot virus strongly suggests that the virus coat protein, which is coded by the smaller of the

two viral segments, does not determine either the ability to invade Phaseolus vulgaris systemically or the ability to infect 'Lloyd George' raspberry (20). In addition, the virulence of the pseudorecombinants seemed to be determined mostly by the larger RNA segment which does not code for coat protein.

The above examples show that host range differences among strains of a virus are, in some cases, not due to properties of the coat protein, but rather are dependent on some other function of the RNA content of the virion. Whether the RNA determines host range through effects on the replication and translation of the RNA, or through the proteins (other than coat protein) which it codes, remains to be determined.

## Symptom Expression

The symptoms expressed by a plant infected with a particular virus strain are a readily detectable characteristic of that virus strain. Unique symptoms produced by a particular variant of a virus may be due to changes in the coat protein of the virus, but there are several examples in the literature in which coat protein is not the only determinant for differences in symptom expression among virus isolates.

Tobacco rattle virus (TRV) has an RNA genome consisting of two RNA pieces distributed between short and long virus particles. The coat protein is specified by the short particle RNA (53). Ghabrial and Lister (12) separated the long and short virus particles from two strains of TRV which had distinctive coat protein

composition and characteristic symptom expression in several herbaceous host plants. They mixed the long and short particles from the two strains of TRV to produce new reconstituted viruses. The symptoms produced by the reconstituted viruses clearly demonstrated that both the long and the short particles of TRV play a role in symptom expression. The coat protein gene of the short-particle RNA does not completely specify symptom expression in TRV. Some property of the long-particle RNA of TRV influences symptom expression in the host plant.

Cowpea mosaic virus (CPMV) has an RNA genome consisting of two RNA species of molecular weight $1.5 \times 10^6$ daltons and $2.3 \times 10^6$ daltons, and has a protein coat composed of two different-sized polypeptides. The available genetic evidence suggests that the coat proteins are coded by the small RNA of CPMV (38,43,72). Thongmeearkom and Goodman (66) characterized two naturally-occurring variants of CPMV as having different antigenic properties, and as inducing different symptoms in cowpea plants. Analysis of pseudo-recombinants derived from heterologous mixtures of the RNAs from these two variants agrees with other evidence that the antigenic specificity (determined by the coat protein) is a function of the small RNA. However in these same pseudorecombinant studies the authors determined that the symptoms expressed by cowpea plants were a function of the large RNA of the reconstituted virus. Similar results were seen in pseudorecombinant studies with four naturally-occurring variants of raspberry ringspot virus (RRSV) (20). The severity of symptoms induced on selected herbaceous

hosts by reconstituted viruses made from heterologous mixtures of the large and small RNAs from these four strains was shown to be a function of the large RNA. The large RNA of RRSV does not code for the coat protein of RRSV.

Some coat protein polypeptides have functions in addition to their role of coat protein. The coat protein of alfalfa mosaic virus (AMV) is thought to be necessary for translation of RNA-4 from AMV (69). A function of the coat protein in post-translational cleavage of large primary translation products is also possible. Such a function has been demonstrated for the coat protein of RNA phages (69).

Even though the coat protein of a virus may have multiple functions, the symptoms that a virus induces in a host are not always a function of coat protein. Other genetic determinants of the virus are responsible for the symptoms induced in the host plant. What types of viral-specific proteins (other than coat protein) might be synthesized which could account for these effects on the host?

## Additional Proteins Found in the Virus Particle

It has been shown that a polypeptide of about 4,000 daltons molecular weight is attached to the 5' end of the two RNA pieces of the TRSV genome (35). Similar genome proteins have been found on the RNA pieces of the cowpea mosaic (CPMV) genome (62), and the RNAs of several animal viruses (24,30). All of the genome proteins

reported thus far in the literature have a molecular weight of 4,000 to 10,000 daltons (14).

The precise function of these genome proteins is not known, but infectivity of TRSV-RNA is abolished when the genome proteins are removed by proteases (35). In contrast, the genome protein of CPMV-RNA is not necessary for the infectivity of the RNA (62). Furthermore, this genome protein has no effect on the in vitro translational efficiency of the RNA in the wheat embryo system. The molecular weight of the protein products of in vitro translation of CPMV-RNA were not affected by the removal of the genome protein (62). Because the genome protein of TRSV is necessary for infectivity of the RNA, it would be of interest to know the effects of the TRSV genome proteins on translation. This has not been reported. The lack of a function of genome proteins in the translation of CPMV suggests a replication or virus assembly function, but no evidence to support these possibilities is available.

The genome proteins of CPMV and poliovirus have been shown to be coded by the viral genome (7,14). Differences in the molecular weights of the genome proteins of two strains of CPMV have been demonstrated (7), but the contribution of the genome proteins to differences in the CPMV strains has not been established.

## Viral-Specific Proteins Found in Infected Tissue

In contrast to the coat protein and genome proteins which are structurally part of the viral particle, there are viral-specific proteins which are produced during viral reproduction in the host,

but which are not incorporated into the virus particle. These proteins are made, in vivo, in much smaller amounts than the coat proteins, and are difficult to detect in the presence of host proteins. The existence of several proteins which are coded by the viral genome, but which are not present in the virus particle, has been demonstrated in infected host tissue, but the function of these proteins has not been firmly established.

Synchronously infected protoplasts have been used in the study of the appearance of virus-specific proteins during BMV infection. Sakai et al. (52) demonstrated the synthesis of four virus-specific proteins, one of which was the coat protein, in BMV-infected protoplasts. BMV has a multipartite genome consisting of four RNA molecules separately encapsidated by a simple coat protein consisting of one type of polypeptide. The size of the four proteins synthesized in infected protoplasts accounted for virtually all of the RNA coding capacity of BMV. One of the proteins was identified as the coat protein of BMV, but the function of the other three proteins is not known. When the four RNAs of BMV were translated in the wheat embryo cell-free translating system, four proteins were produced of approximately the same size as those produced in vivo in infected protoplasts (57). It has been proposed that one of these four proteins of molecular weight $3.5 \times 10^4$ daltons functions as a subunit of the viral replicase (18), but there is little evidence to substantiate this.

Pseudorecombinant studies with the genomes of nitrous acid-induced mutants of BMV have been useful in associating specific

virus properties with each of the four RNA species of BMV (2). Since the gene product of each of these RNAs has been identified by *in vitro* translation studies it is possible to assign specific virus properties to these gene products. Many of the nitrous acid-induced mutant properties were associated with changes in the coat protein gene, but changes in local and primary lesion appearances were due to mutations in two RNA species which did not code for coat protein. Unfortunately, the function of the gene products of these two RNAs is not yet known.

The evidence from *in vivo* and *in vitro* studies with BMV indicates that three proteins, in addition to coat protein, are necessary for successful infection with BMV. Variation in any one of these four viral-specific proteins might account for strain variation in BMV. Structural variation in the RNA that resulted in changes in the efficiency of translation, replication, or coating of the virus particle could also account for the variation in these nitrous acid-induced mutants of BMV. The following discussion considers evidence for the production and function of several kinds of virus-coded proteins which are necessary in plant virus infection.

## Viral-Specific Replicases

Plant viruses whose single-stranded RNAs can serve directly as messenger RNA are thought to initiate viral replication by translation of viral RNA to produce proteins which are required for replication of the viral RNA. Ribophages, bacteriophages in which single-stranded RNA serves as a messenger RNA, code for a polypeptide

which replaces one of the subunits of the bacterial host replicase, thus making the replicase specific for the ribophage RNA (63). There is not strong evidence for the synthesis of a plant virus-coded replicase, or of a replicase subunit. Varying degrees of viral-specific replicase activity have been demonstrated in infected plant tissue. It has been possible to extract, partially purify, and demonstrate in vitro activity of an RNA replicase in plants infected with TMV (73), BMV (17), turnip yellow mosaic virus (39), cucumber mosaic virus (34), TRSV (41), and other plant viruses. Estimates of the molecular weights of these viral replicases range from 130,000 to 150,000 daltons (41). Most of these polymerases from infected plants exhibited little or no template specificity for the plant viral RNA. However, Sela and Haushner (55) purified TMV replicase from TMV-infected plants and showed that TMV-RNA was the only RNA template that was capable of stimulating incorporation into acid-insoluble product. Contrary to the evidence of Sela and Hauschner, there is recent evidence that indicates that RNA replicase activity is already present in healthy plant tissue, and that virus infection of a plant merely increases the activity of normal plant cell RNA-dependent RNA replicase (4). Even if RNA replicase activity is present in healthy plant tissue, some protein coded by the viral genome might function to increase the activity of the plant RNA replicase. Although it has been shown that replicases can selectively transcribe viral RNA, the determinants for this specificity are unknown at this time.

## Viral-Specific Proteases

A virus-specific protein of cowpea mosaic virus (CPMV) has been shown to function as a proteolytic enzyme in post-translational cleavage of other proteins coded by CPMV-RNA (43). The CPMV protease is coded by the large RNA of CPMV, and cleaves a precursor protein product of the small RNA of CPMV. Pelham (43) was not able to determine which of the two protein products (32,000 or 170,000 dalton) of the large RNA was responsible for the post-translational protease activity. This is the first report to date of protein-precursor processing in a plant virus system, but there are many examples of post-translational cleavage in entero- and picorna-viruses in animals (42,29). Although post-translational cleavage has not been demonstrated in TRSV, it is interesting to note the similarities between TRSV, CPMV, and the animal viruses that have been shown to have post-translational cleavage. Not only do these viruses all possess genome-linked proteins, but they all have been shown to have a 3'-terminal polyadenylate segment on their RNA genomes (35). These parallels suggest an underlying similarity in the way the genomes of these viruses function, and also suggest that TRSV may depend on viral-specific proteases for its replication.

## Viral-Specific Proteins in Plant Virus Inclusion Bodies

Tubular inclusion bodies containing single rows of virus particles have been observed by electron microscopy in the cytoplasm of undifferentiated cells infected with TRSV (70). Although similar in form to the inclusion bodies induced by turnip yellow mosaic

virus, optical diffraction experiments showed that the surface structure of the inclusions induced by these two viruses are not the same. This suggests that the tubular inclusion bodies induced by a virus have a specific structure and are characteristic of the virus or virus type which induces it. It is not known whether the proteins of the tubular inclusions of TRSV are virus-specific proteins. The function of the tubular inclusion bodies in TRSV infection is not known, but various researchers have speculated that they might be involved in cell-to-cell movement of the virus, or in virus replication (70,49).

Because so little is known about the nature of the tubular inclusion bodies induced by TRSV infection, it is of interest to examine the well-characterized proteins of the pinwheel inclusions present in plant cells infected with viruses belonging to the potato virus Y grouping. Dougherty and Hiebert (9) have identified several translation products of tobacco etch virus (TEV) RNA as being serologically identical to the structural proteins of the pinwheel inclusions of TEV. Purcifull et al. (44) partially purified the inclusion bodies from plants infected with five potyviruses, and made antisera to these partially purified inclusion bodies. These authors demonstrated that the inclusion bodies from different potyviruses were antigenically distinct, and suggested that the antigenic variation of the pinwheel virus proteins might be a good characteristic to use in the classification of potyviruses.

Variation among many isolates of a plant virus is a function of differences in viral-coded proteins of these isolates. In the

past, comparisons and characterizations of viral isolates have been made on the basis of one viral-coded product, namely the coat protein. This is explained by the relative ease of purifying coat protein compared to other viral-coded proteins. Though often easily made, these comparisons have been shown to be of limited use in predicting the biological behavior of virus isolates (16), probably because the comparisons are based on only one of the proteins coded by the viral genome.

This dissertation reports a method for comparing virus isolates which involves a comparison of several of the viral-coded proteins. The wheat embryo cell-free protein synthetic system was used to synthesize the proteins coded by TRSV-RNA. Using RNA from different isolates of TRSV as messenger RNA in the wheat embryo cell-free protein synthetic system, viral-specific protein products were synthesized and compared. This method allows the investigator the ability to compare directly all of the viral-specific proteins produced by a given viral isolate in the wheat embryo cell-free protein synthetic system.

Seven isolates of TRSV were chosen for study. They were characterized by the symptoms they induced in selected herbaceous plants, and by their coat protein antigenic properties. Of the seven isolates, three were markedly different in the reactions they induced in the herbaceous host plants and were selected for further comparisons. The RNA from these three isolates was extracted and used to direct protein synthesis in a wheat embryo cell-free protein synthetic system. The <u>in vitro</u> protein products coded by the RNA

of the three isolates of TRSV were analyzed by SDS-polyacrylamide gel electrophoresis in order to detect differences in the molecular weights of the *in vitro* products.

# MATERIALS AND METHODS

## Virus Isolates

The following isolates of tobacco ringspot virus (TRSV) were used in this study: Cherry isolate from Dr. Richard Stace-Smith (Agriculture Canada Research Station, Vancouver, BC V6T1X2), originally isolated from sweet cherry (Prunus avium L.) (60); Tobacco isolate, Serogroup 38, from Dr. Guy Gooding, Jr. (North Carolina State University, Raleigh, NC 27607), originally isolated from flue-cured tobacco (Nicotiana tabaccum L.) (16); Watermelon (Citrullus vulgaris Schrad.) isolate from Dr. Donald deZeeuw (Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824); Soybean (Glycine max (L.) Merr) isolate, obtained from cotyledons of infected soybean seeds received from Dr. Richard Lister (Purdue University, West Lafayette, IN 47907); 'Riesling' grape isolate from Dr. J. K. Uyemoto (Kansas State University, Manhattan, KS 66506); 'Jersey' blueberry (Vaccinium corymbosum L.) isolate from Dr. Donald Ramsdell (Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824) (45); 'Stanley' blueberry isolate obtained from 'Stanley' highbush blueberry, by triturating frozen infected blossoms in 2.0% nicotine in water, and inoculating it to Cucumis sativus 'National Pickling' seedlings.

All isolates were increased in Chenopodium quinoa Willd. Single lesions were excised and inoculated back to C. quinoa. Two such successive single lesion transfers insured that a virus isolate was not contaminated by other viruses, or by strains of the same virus. Since the soybean isolate did not form local lesions on C. quinoa, Cucumis sativus 'National Pickling' was used for making single lesion transfers of this isolate. The infected plants obtained from this first inoculation to a large number of plants were frozen at -20°C, and aliquots of this frozen tissue were used as inoculum for production of virus for further experimentation. Using this approach, the virus preparations used in the different experiments resembled the original isolate as much as possible.

## Symptom Expression on Herbaceous Indicator Plants

The following herbaceous plant species were inoculated separately with the seven isolates of TRSV: Chenopodium quinoa Willd., Chenopodium amaranticolor Coste & Reyn., Cucumis sativus L. 'National Pickling,' Nicotiana tabaccum L. 'Havana 423,' Vigna unguiculata (Cowpea SR) (8), Phaseolus vulgaris 'Prince,' Nicotiana clevelandii x Nicotiana glutinosa (Christie's hybrid), and Glycine max (L.) Merr. 'Hark.' Young, vigorously growing plants were dusted with 320-mesh carborundum and rub-inoculated with infectious sap obtained by triturating infected cucumber cotyledons in 0.01 M sodium phosphate buffer, pH 7.0. Test plants were grown in the greenhouse under fluorescent lights for a 14-hour day length at a temperature between 18°C and 30°C depending upon the season.

Sap from plants which did not show symptoms was inoculated to C. quinoa or tested serologically to detect symptomless infection.

## Thermal Inactivation Tests

Crude extracts of virus were made by triturating infected cucumber cotyledons in glass-distilled water, and then straining the sap through cheesecloth. These crude extracts were placed in thin-walled glass tubes and heated for 10 minutes in a waterbath of the desired temperature and then cooled on ice. After heat treatment the crude extracts were tested for infectivity by rub-inoculation to C. quinoa which had been dusted with 320-mesh carborundum. Plants which did not develop symptoms were tested serologically for asymptomatic infection.

## Virus Purification

Two different methods were used to obtain the purified virus used in various experiments. The first method was adapted from Stace-Smith et al. (61). Cucumber seedlings which were infected with TRSV were homogenized in a buffer of 0.05 M boric acid (containing 0.1% thioglycollate and 0.1% diethyldithiocarbamate, adjusted to pH 6.8 with NaOH) in a proportion of 1 kilogram of tissue to 1 liter of buffer. After the sap was squeezed through a double layer of cheesecloth, it was frozen at -20°C and thawed slowly at 4°C. After centrifugation at 10,000xg for 30 minutes, the supernatant was dialyzed for 16-24 hours against 5 volumes of ammonium sulfate, 200 g/l. The dialyzate was centrifuged at 10,000xg for 30 minutes, and the virus was then pelleted out of the supernatant

by centrifugation in a Beckman No. 30 rotor at 28,000 rpm at 4°C for 2 hours. The virus-containing pellets were suspended overnight at 4°C in a buffer of 0.01 M Tris, 0.1 N NaCl, pH 7.0. The resulting virus suspension was clarified in a Beckman No. 40 rotor at 15,000 rpm for 10 minutes. The supernatant was then centrifuged in a Beckman No. 40 rotor at 38,000 rpm for 90 minutes, and pellets were resuspended as before. This virus preparation was then layered onto a 0-30% linear-log sucrose gradient (3) in 0.01 M sodium phosphate buffer at pH 7.0 and centrifuged in a Beckman SW 41 rotor at 38,000 rpm for 90 minutes at 4°C. The sucrose density gradients were fractionated from the top of the gradient tube, and scanned at 254 nm using an ISCO density-gradient fractionator (Instrument Specialties Co., Inc., Lincoln, NE 68505). Of the three peaks that resulted, designated top (T), middle (M), and bottom (B), the M and B peaks were collected, diluted 1:3 (v/v) with 0.01 M sodium phosphate buffer, pH 7.0, and pelleted by centrifugation for 5 hours in a Beckman No. 30 rotor at 28,000 rpm at 4°C. The virus-containing pellets were resuspended in 0.01 M sodium phosphate, pH 7.0 buffer and stored at 4°C until further use.

The second purification method was adapted from the method of Rezaian and Francki (48). Infected 'National Pickling' cucumber seedlings were homogenized with 1 ml of 0.1 M sodium phosphate buffer, pH 7.0, and 1 ml of chloroform per gram of tissue. The homogenate was centrifuged at 10,000xg for 30 minutes and the upper aqueous buffer phase was collected. Polyethylene glycol, molecular weight 6000 (Fischer Scientific Co., Carbowax 6000), and NaCl were

added to the buffer phase to a final concentration of 6% (w/v) and 0.3 M, respectively, and the mixture was stirred and left at 0°C for 30 minutes. The precipitate was pelleted by centrifugation at 10,000xg for 10 minutes and resuspended overnight at 4°C in 0.1 M phosphate buffer, pH 7.0 with 0.01 M EDTA. The virus preparation was purified further by alternate high- and low-speed centrifugation, and sucrose density-gradient fractionation as described above for the first method of virus purification.

## Serology

### Antisera Sources

Antisera to the TRSV isolates used in this study were either gifts from other investigators or were produced in our laboratory. Antisera to the cherry, soybean, 'Riesling' grape, and 'Jersey' blueberry isolates were kindly supplied by the researchers who supplied these virus isolates. Antisera to the tobacco, watermelon, and 'Stanley' blueberry isolates were made in the following manner. The antigen used for injection consisted of virus which had been purified through sucrose density gradients as described in virus purification methods. Female New Zealand white rabbits were injected intramuscularly at weekly intervals with one volume (1-2 mls) of purified virus (1-3 mg/injection) emulsified with an equal volume of Freund's adjuvant (Difco Products Co., Detroit, MI 48232). For the first injection Freund's complete adjuvant was used; all subsequent injections were done using Freund's incomplete adjuvant. Serum was obtained 10 days after the second injection, or if the antibody

titer was not high enough at this time, serum was taken 10 days after the final injection. All antisera were titered against their homologous virus isolates using the double diffusion test in agar plates (1). The gel consisted of 0.8% agarose (Sigma Type I Agarose, Sigma Chemical Co., St. Louis, MO 63178), 0.85% NaCl, and 0.15% sodium azide. All antisera were stored either lyophilized or as whole serum at -20°C.

Spur Formation Tests

One of the criteria used to identify serological strains was spur formation tests (40). Spur formation tests are carried out in an agarose gel. Wells are cut into the gel and filled with the reactants. The antigens and antibodies diffuse into the gel from the wells, and form a precipitin line where they meet in optimal proportions. Two antigen (virus) preparations are paired and tested against an antiserum made to one of the antigens. The three reactants are put in wells arranged at the corners of an equilateral triangle. Two reactions are possible: (a) when the antigens are identical, the precipitin lines formed between each antigen and the antiserum will join smoothly (Figure 6), and (b) when the antigens have some determinants in common the precipitin line formed between each antigen and the antiserum will join, but the antibodies specific to the homologous antigen will diffuse through the heterologous precipitin band to form a spur (Figure 7).

The agarose medium used in the spur formation tests was the same as that used to determine antisera titers (see section on

antisera production). A six-hole gel pattern was cut by using a Grafar gel cutter (Grafar Co., Detroit, MI 48238). The wells were 3.5 mm in diameter and spaced 10 mm apart center to center. In these studies antigen-containing wells surrounded a central antiserum-containing well. The virus used as the antigen in these tests was prepared from infected 'National Pickling' cucumber seedlings by homogenizing seedlings in chloroform (5 ml chloroform/g tissue) containing 0.01 g sodium ascorbate/g of tissue, and breaking the resulting emulsion by centrifugation at 10,000 x g for 30 minutes. The virus-containing supernatant was made 0.1% (w/v) with sodium azide and used directly as test antigen. This crude virus preparation contained plant host comtaminants. To insure that these contaminants were not responsible for the test results, some of the results were verified using purified virus preparations as the antigen. The various antisera were diluted with a 0.85% (w/v) NaCl solution to a concentration which produced a sharp precipitin band when reacted with the various test antigen preparations in agar double gel-diffusion tests. A dilution of between 1:8 and 1:32 was used for all spur formation tests; at these dilutions there was no reaction to healthy plant sap with any of the antisera. Each antiserum was tested for spur formation between its homologous antigen and all other heterologous virus antigens. All tests were repeated at least twice.

Cross-Absorption Tests

Reciprocal absorption tests were used in addition to spur formation tests as a criterion in establishing serological differences among the isolates of TRSV. In this test an isolate-specific antiserum was reacted with heterologous virus, and all antibodies which cross-reacted with the heterologous virus were removed. The titer of residual antibodies to homologous virus in the cross-absorbed antiserum is used to quantify the serological differences among the virus isolates (33). Most antisera were fully absorbed with the addition of 16 to 32 parts of heterologous virus preparations (the antigen was prepared as for spur formation tests) to one part of undiluted antiserum. Absorption was carried out by mixing the antiserum with the absorbing virus preparation, incubating for 3 to 4 hours at 37°C and then overnight at 4°C. The antibody-antigen complexes were removed by centrifugation in a Beckman No. 40 rotor at 15,000 rpm for 10 minutes at 4°C. To determine if the cross-absorption was complete each absorbed antiserum was tested for residual antibodies to the absorbing virus in double gel-diffusion tests. If absorption was not complete the procedure was repeated using a higher proportion of absorbing antigen to antiserum. Cross-absorbed antisera were tested for residual antibodies to homologous virus using double gel-diffusion tests. Results were reported as the reciprocal of the titer of absorbed antiserum to homologous virus over the reciprocal of the titer of unabsorbed antiserum to homologous virus.

## Electrophoretic Mobility of Whole Virus Particles

The electrophoretic mobility of whole virus particles was determined by the method of Tremaine & Wright (67). Electrophoresis was carried out in a slab gel consisting of 0.75% agarose (Sigma Type I Agarose) in 0.02 M Tris, 0.02 M sodium dibasic phosphate, adjusted to the desired pH with citric acid. Gels were cast by pouring 5 ml of molten agarose solution onto a plexiglass plate (2.5 cm x 13.5 cm x 0.5 cm) and allowing the gels to cool at room temperature before cutting 1.5 mm diameter wells for the samples. Three equidistant wells were cut in the center of the gel on a line running the width of the gel.

Virus samples consisted of approximately 15 µg of purified virus in 0.005 M sodium phosphate buffer pH 7.0. Electrophoresis was carried out in a Gelman Deluxe Electrophoretic Chamber (Gelman Instr. Co., Ann Arbor, MI 48106) using a BioRad Model 400 Power Pack (BioRad Laboratories, Richmond, CA 94804) at 150 volts, 3.5 to 7 milliamps, for 5 hours. Electrophoresis tray buffer consisted of 0.02 M Tris, 0.02 M sodium dibasic phosphate, adjusted to the desired pH with citric acid. Following electrophoresis the gels were stained for 2 hours in a solution of 5% acetic acid, 5% glycerol, and 0.0125% Coomasie blue. Gels were destained in a solution of 5% acetic acid and 10% glycerol until background was reduced to a minimum.

## RNA Extraction

Ribonuclease degradation of RNA was kept to a minimum by heating all glassware to 225°C prior to use. If "baking" was not possible, glassware was soaked in a dichromate solution or in 0.1 N NaOH before use. Phenol and ethanol were redistilled, and stored at -20°C prior to use.

RNA was extracted from TRSV using a modification of the method developed by Dr. James Asher, Jr. (Department of Zoology, Michigan State University, East Lansing, MI 48824). The virus preparations used as sources of viral RNA were purified through 2 cycles of sucrose density gradients (as described under virus purification). Only the middle and bottom components which contain RNA were used in the RNA extraction procedure. Virus suspensions (about 1 mg/ml) in 0.01 M sodium phosphate buffer, pH 7.0 were thoroughly mixed with 2 volumes of 5% SDS, and 2 volumes of 80% phenol (v/v with distilled water). This solution was heated, with stirring, at 65°C for 5 minutes, and then cooled on ice. This high-low temperature treatment breaks up any hybrids in the RNA molecules and forces any DNA into the phenol phase. Chloroform (2 volumes) was added, and the solution was stirred vigorously for 5 minutes at room temperature. The addition of chloroform stabilizes the interface between the two layers, and also helps remove the RNA from the protein precipitate. The phases were separated by centrifugation in an IEC (International Equipment Co., Needham Hts., MA 02194) No. 870 rotor at 10,000 rpm, 0°C, for 15 minutes. The phenol phase was extracted with a mixture of 1.5 volumes of EDTA-acetate buffer

and 0.5 volumes of 5% SDS. The combined aqueous phases were extracted by mixing well with 1 volume of 80% phenol, and then adding one volume of chloroform with thorough mixing. The phases were separated by centrifugation (as above). This extraction procedure of the aqueous phase was repeated three times to remove any residual protein in the aqueous phase. Nucleic acids were precipitated from the aqueous phase overnight at -20°C after the addition of 1/15 volume of 3 M sodium acetate pH 6.0 and 2.5 volumes 95% ethanol. After centrifugation at 10,000 rpm in the IEC No. 870 rotor for 15 minutes at -20°C, the pellet was washed with 10 ml of 0.1 M sodium acetate in 66% ethanol to remove any residual phenol and other insoluble material. The washed pellet was air dried to remove residual ethanol. The nucleic acid precipitate was then washed thoroughly 3 times with a solution of 10 mls of 3 M sodium acetate, pH 6.0 and 0.25 ml 0.2 M EDTA. This washing procedure removes DNA, small RNA molecules, and polysaccharides from the pellet. The remaining pellet was solubilized in 12.5 ml of 0.1 M sodium acetate, 5 mM EDTA at pH 6.0. The nucleic acids were then precipitated by the addition of 2.5 volumes of 95% ethanol at -20°C overnight. After centrifugation the pellet was air dried, solubilized in glass-distilled water, and stored at -80°C until use. RNA concentrations were determined spectrophotometrically assuming $E^{0.1\%}_{260nm} = 22$.

## Gel Electrophoresis of Purified TRSV RNA

RNA preparations were analyzed for purity and integrity on agarose-formaldehyde denaturing gels according to the method described by Lehrach et al. (31). Ribosomal RNA from chick embryo muscle (a gift from Dr. James Asher, Jr., Department of Zoology, Michigan State University, East Lansing, MI 48824) and brome mosaic virus (BMV) RNA were used as molecular weight markers for RNA molecular weight determinations. Gels were cast in quartz tubes, and pre-electrophoresced at 4°C at 2 milliamps/gel for 60 minutes. Electrophoresis was carried out at 4°C at 2 milliamps/gel for 3 to 4 hours, after which gels were immediately scanned at 254 nm using an ISCO (Instrument Specialties Co., Lincoln, NE 68505) Model 1310 gel scanner.

## Separation of TRSV-RNA on Sucrose Density Gradients

The small RNA of TRSV (RNA-2) was separated from total TRSV-RNA by sedimentation in 0 to 32.5% linear-log sucrose density gradients (3) made up in 0.15 M sodium chloride and 0.015 M sodium citrate buffer pH 7.0 with ribonuclease-free sucrose (Schwarz/Mann, Orangeburg, NY 10962). Prior to separation on sucrose gradients the RNA samples were denatured for 5 minutes at 65°C in 0.075 M sodium chloride, 0.0075 M sodium citrate, pH 7.0. Unfractionated, denatured TRSV-RNA (200 µg/Beckman SW 41 rotor tube) was separated by centrifugation for 6 hours at 38,000 rpm, 4°C, and fractionated using an ISCO density fractionator. The peak-containing fractions were pooled, alcohol precipitated, and fractionated again on

sucrose density gradients. The resulting RNA preparations were analyzed for completeness of separation using agarose-formaldehyde gel electrophoresis as described in the foregoing section on RNA extraction.

## Infectivity of Purified TRSV-RNA

The infectivity of various RNA preparations was tested by rub-inoculation onto primary leaves of Vigna unguiculata (Cowpea 'SR') which had been previously dusted with 320-mesh carborundum. RNA preparations were diluted in 0.1 M sodium phosphate buffer, pH 7.5, and inoculation was accomplished by putting drops of the RNA solution onto the leaves, and then rubbing gently with a spatula. Test plants were grown in the greenhouse under fluorescent lights for a 14-hour day length at a temperature between 18°C and 30°C depending on the season. Lesions were counted 4 to 8 days following inoculation, and results were calculated as the mean number of lesions on ten or more cowpea leaves.

## Detection of Poly (A) Tracts in TRSV RNA

The presence of poly (A) tracts on the 3' ends of eucaryotic messenger RNAs and some plant and viral RNAs has been a useful characteristic for separating these RNAs from poly (A) negative, non-messenger RNAs. When this research was initiated it was not known whether the RNA of TRSV contained poly (A) tracts. Since the presence of poly (A) tracts on TRSV-RNA had potential for use in

separating TRSV-RNA from contaminating RNAs, the poly (A) containing properties of TRSV were investigated.

Oligo(dT)-cellulose (Type 2 from Collaborative Research Inc., Waltham, MA 02154) was used to detect the presence of poly (A) tracts in the RNA of TRSV. Unfractionated TRSV-RNA was made 0.4% in SDS, heated to 65°C for 2 minutes, and then quickly chilled in an ice bath. This RNA solution was diluted with 1 volume of the following solution: 0.02 M Tris-HCl pH 7.4, 2 mM EDTA, and 0.24 M NaCl. This solution of RNA in 'high salt' was then passed over an oligo(dT)-cellulose column which had been equilibrated in 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% SDS, and 0.12 M NaCl. Poly (A) RNA binds to the oligo(dT)-cellulose under these 'high salt' conditions, but RNA which does not contain poly (A) tracts, or which contains only short tracts of poly (A), does not bind. The sample was washed onto the column and the column was eluted with additional 'high salt' buffer until the eluate fractions did not contain any RNA as determined by spectrophotometric analysis at 260 nm. The column was then eluted with 'low salt' buffer (0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% SDS), and the eluate was collected in 1 ml fractions. The fractions from the column which contained RNA, as determined by spectrophotometric analysis, were pooled and precipitated with 1/15 volume of 3 M sodium acetate, pH 6.0, and 2.5 volumes of 95% ethanol at -20°C overnight. After centrifugation the pellet was air dried, dissolved in 100 µl of distilled water, and stored at -80°C until use. All RNA-containing fractions were analyzed on agarose-formaldehyde gels as described above.

## Translation of TRSV-RNA in the Wheat Embryo Cell-Free System

Wheat embryos were extracted from wheat seeds (Triticum aestivum L. 'Ionia'), and the dialyzed wheat embryo extract was prepared according to the method of Marcus et al. (32). RNA (1-2 μg) was added to 50 μl of reaction mixture containing 20 μl of dialyzed wheat embryo extract, 45 mM potassium chloride, 2.7-3.2 mM magnesium acetate ($Mg^{++}$ concentration optimum varied slightly with RNA preparation), 0.04 mM GTP, 1.4 mM ATP, 3 mM dithiothreitol, 35 mM Tris-acetate (pH 8.0), 10 mM creatine phosphate, 2.7 μg creatine phosphate kinase, $^{14}C$- or $^{3}H$-valine ($^{3}H$-valine: specific activity 1.2 Ci/mmole, $C^{14}$-valine: specific activity 246 mCi/mmole, New England Nuclear, Boston, MA 02118), a mixture of the remaining 19 amino acids to 0.034 mM, and 0.5 mM spermine tetrahydrochloride. Spermine tetrahydrochloride was added to prevent premature termination of protein synthesis. After 45 minutes of incubation at 30°C, 1.4 μg of aurin tricarboxylic acid/50 μl of reaction mix was added, and the reaction was terminated after an additional 15 minutes of incubation. Aurin tricarboxylic acid was added to prevent any further initiation of protein synthesis.

The reaction was terminated by spotting 50 μl of the reaction mix onto Whatman #1 filter paper discs and drying under a heat lamp. The synthetic ability of different reaction mixes was evaluated by measuring the amount of radioactively labeled, hot trichloroacetic acid-precipitable protein produced. After boiling the filter paper in 5% trichloroacetic acid (TCA), 10 mM valine

for 15 minutes the filter papers were rinsed 3 times with hot 5% TCA, 10 mM valine. The filter papers were then washed 2 times with 50% ether:50% ethanol, to remove residual TCA. Two final washes with 100% ether were done to remove the ethanol. After air drying, the filters were placed in counting vials with 0.8 ml Protosol mix (10 ml toluene: 9 ml Protosol (New England Nuclear, Boston, MA 02118): 1 ml distilled water), and heated at 60°C for 1.5 hours. When cool, 5 ml of counting fluid (1 liter toluene: 6.0 gm PPO: 0.15 gm dimethyl POPOP) was added and the vials were counted for radioactivity in a Searle Isocap/300 Model 6868 liquid scintillation counter.

Experiments were carried out to determine the optimum concentrations of $K^+$, $Mg^{++}$, and TRSV-RNA necessary for maximum incorporation of radioactivity into TCA-precipitable counts by the wheat embryo system.

## Analysis of Translation Products by SDS-Polyacrylamide Gel Electrophoresis

Translation products were analyzed by SDS-polyacrylamide gel electrophoresis by the method of Fairbanks et al. (10). Molecular weights of the labeled *in vitro* products were calculated by assuming a linear relationship between log molecular weight and mobility using the following unlabeled protein markers: cytochrome c, myoglobin, pepsin, ovalbumin, TRSV coat protein, bovine serum albumin, and phosphorylase a (molecular weights of 12,400, 17,200, 34,600, 43,000, 54,000, 68,000 and 94,000 daltons, respectively).

TRSV coat protein was prepared from whole virus by heating purified virus in dissociation buffer (0.05% β-mercaptoethanol, 0.05% SDS, 2 M urea in 0.05 M sodium phosphate buffer, pH 7.2) at 100°C for 90 seconds. The dissociated virus was then dialyzed extensively against the electrophoresis chamber buffer (0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA pH 7.4) to which had been added 1% β-mercaptoethanol.

The translation products were characterized by electrophoresis on 5.6% polyacrylamide- 0.21% bisacrylamide cylindrical gels (7.5 cm long) containing 1% SDS. The products (75 µl $^{3}$H-valine mix) were mixed with 9 µl of 20% SDS, 2 µl of β-mercaptoethanol, and 89 µl distilled water. Standard protein solutions, except for the TRSV coat protein, were prepared in a similar manner using 20 µl of the protein solution (1 mg/ml), 9 µl of 20% SDS, 2 µl of β-mercaptoethanol, and 135 µl distilled water. All samples for electrophoresis were denatured by heating to 100°C for 3 minutes in a boiling water bath. After denaturation, 50 µl aliquots of the samples were mixed with 2 µl of pyronin y (0.025 g/50 ml distilled water) and one drop of glycerol, and separated by electrophoresis at 8 milliamps per gel for approximately one hour. Following electrophoresis the gels were stained with Coomasie blue (1.25 g Coomasie blue in 454 ml of 50% methanol and 46 ml glacial acetic acid) for 12-16 hours, and then electrophoretically destained at 8 milliamps per gel until background was reduced to a minimum.

The gels containing the radioactively labeled protein products were sliced into 1-mm segments, mixed with 0.8 ml

Protosol mix, and heated at 60°C for 1.5 hours. After the addition of 5 ml of counting fluid, the gel segments were counted for radioactivity in a Searle Isocap/300 Model 6868 liquid scintillation counter. Stained gels containing the molecular weight standards were scanned at 590 nm using a Gilford Model 2410-S Linear Gel Transport System (Gilford Medical Instruments Co., Oberlin, OH 44074). The logarithm of the molecular weights of the protein standards were plotted against the relative mobility of these standards, and the resulting graph was used to estimate the molecular weights of the in vitro protein products.

### Dual-Label Experiments to Detect Small Differences in Molecular Weights of Protein Products Produced by Different Isolates of TRSV

Labeled protein products were made by translating the RNA from different isolates of TRSV in the wheat embryo system as described above. The protein products of some isolates were labeled with $^{14}$C-valine; protein products of other isolates were labeled with $^{3}$H-valine. Samples for SDS-polyacrylamide gel electrophoresis were prepared by mixing the $^{3}$H- and $^{14}$C- labeled protein products from different isolates of TRSV, and then separated by electrophoresis as described earlier. The gels were sliced and the radioactivity due to $^{14}$C and $^{3}$H was determined for each slice using a Searle Isocap/300 Model 6868 liquid scintillation counter.

# RESULTS

## Symptom Expression on Herbaceous Indicator Plants

Reactions of the various herbaceous indicator plants to infection by TRSV are given in Table 1. The virus isolates are listed in approximate order of virulence as judged by symptom severity on the herbaceous hosts. The symptoms expressed by a particular host, such as local lesion formation on inoculated leaves, were tabulated at the same time for all virus isolates. In some cases the difference in symptom expression between isolates was only a difference in the time of appearance or the severity of the symptoms produced. For instance, the local lesions produced by the soybean and 'Jersey' blueberry isolates on the inoculated cotyledons of Cucumis sativus L. 'National Pickling' are quite unique when the isolates are compared at 4 days post-inoculation (see Table 1 and Figure 1). However, if one compares the local lesions of the soybean isolate at 8 days post-inoculation with those produced by the 'Jersey' blueberry isolate at 4 days post-inoculation, the difference between the isolates is less pronounced. Some examples of the differences in symptom expression on the various indicator plants are given in Figures 2, 3, and 4.

The isolates of TRSV that were used in this study differed in their ability to infect various herbaceous hosts. For example, when the soybean isolate was inoculated to Nicotiana tabacum 'Burley' type, only 1/6 of the inoculated plants became infected. In contrast, the

TABLE 1.--Herbaceous host reactions[a] to seven isolates of tobacco ringspot virus (TRSV).

| Source of Virus Isolate | Host Plants | | | |
|---|---|---|---|---|
| | *Chenopodium quinoa* | *C. amaranticolor* | *Cucumis sativus* | *Glycine max* 'Hark' |
| 'Jersey' blueberry | Relatively large NL on IL, apical DB at 6 days; eventual D. | NL and CL on IL, apical leaves with LM, apical DB. | NL on IL, extreme stunting of new leaves with NL & CL. | Indistinct CL on IL with VN, DB of trifoliate leaves. |
| Watermelon | NL on IL, apical DB at 6 days; eventual D. | Same as 'Jersey' blueberry isolate, but no apical DB. | Same as 'Jersey' but no necrosis in CL on IL. | Same as 'Jersey' but new leaves not as necrotic. |
| 'Riesling' grape | Small NL on IL, apical DB at 10 days; eventual D. | Pinpoint NL on IL, apical leaves with LM. | Same as 'Jersey' blueberry isolate. | NL on IL with VN, DB of trifoliate leaves. |
| Tobacco | Pinpoint NL on IL, apical DB at 6 days, eventual D. | No lesions on IL, apical leaves with LM. | CL on IL, stunting, with systemic CL & NL on new leaves. | Indistinct CL on IL with VN, new leaves with NL. |
| 'Stanley' blueberry | NL on IL, apical DB at 6 days; eventual D. | Pinpoint CL & NL on IL, apical leaves with LM. | Same as tobacco isolate. | Same as tobacco isolate. |
| Cherry | NL on IL, apical DB at 10 days; eventual D. | Same as 'Stanley' blueberry isolate. | CL on IL, stunting & systemic mottling of new leaves. | Indistinct CL with VN on IL, CM with some NL on new leaves |
| Soybean | No visible lesions on IL, slight systemic mottle and stunting. | No symptoms on IL, slight chlorosis on terminal leaves. | Pale CL on IL, stunting and systemic mottling of new leaves. | Indistinct CL with slight VN on IL, mottle on new leaves. |

[a] IL = inoculated leaves; NL = necrotic lesions; DB = dieback; VN = veinal necrosis; CL = chlorotic lesions; NR = necrotic ringspots; LM = leaf stunting and malformation; D = death; CM = chlorotic mottle.

TABLE 1.--(cont.).

| Source of Virus Isolate | Host Plants | | | |
|---|---|---|---|---|
| | *Nicotiana tabacum* 'Havana 423' | *N. glutinosa* x *N. clevelandii* | *Vigna unguiculata* Cowpea 'SR' | *Phaseolus vulgaris* 'Prince' |
| 'Jersey' blueberry | Black NR on IL, 7-10 mm in diameter, no systemic response. | Indistinct brown NR on IL, small NL on new leaves. | Large dark red RS with green centers on IL, apical DB & LM. | CL on IL spreading along veins, apical DB. |
| Watermelon | Same as 'Jersey,' but NR less dark and smaller. | Diffuse CL on IL, no systemic response. | Dark red lesions on IL, some LM. | Distinct CL on IL, apical DB, necrosis in new leaves. |
| 'Riesling' grape | Same as 'Jersey' but with leaf distortions around NR. | Pinpoint NL on IL, pinpoint NL and NR on new leaves. | Large dark red LL with indistinct borders on IL, LM. | Light brown NL on IL, apical DB. |
| Tobacco | Tan colored, papery LL on IL, no systemic response. | No LL on IL, chlorotic ringspots on new leaves. | Same as 'Riesling' grape isolate, but LL on IL smaller. | Some brown incomplete NR on IL, no systemic response. |
| 'Stanley' blueberry | Brown NR on IL, no systemic response. | Same as 'Riesling' grape isolate. | Same as 'Jersey' blueberry isolate, smaller LL on IL. | Indefinite CL on IL, necrosis of new leaves. |
| Cherry | Same as tobacco isolate. | Same as 'Riesling' grape isolate, but new leaves malformed. | Small distinct dark red LL on IL, some LM. | Same as watermelon isolate. |
| Soybean | Symptomless infection.[b] | Symptomless infection.[b] | Same as Cherry isolate. | No visible LL on IL, some necrosis of new leaves. |

[a]IL = inoculated leaves; NL = necrotic lesions; DB = dieback; VN = veinal necrosis; CL = chlorotic lesions; NR = necrotic ringspots; LM = leaf stunting and malformation; D = death; CM = chlorotic mottle.

[b]No symptoms, but infected (determined serologically).

FIGURE 1.--'National Pickling' cucumber cotyledons four days after inoculation with three different isolates of tobacco ringspot virus (TRSV) showing differential reactions. Cotyledons were inoculated with the following isolates: (a) tobacco isolate of TRSV, (b) soybean isolate of TRSV, and (c) 'Jersey' blueberry isolate of TRSV.

FIGURE 1

FIGURE 2.--'National Pickling' cucumber seedlings four days after inoculation with three different isolates of tobacco ringspot virus (TRSV) showing differential reactions. Cotyledons were inoculated with (a) the soybean isolate of TRSV, (b) the tobacco isolate of TRSV, and (c) the 'Jersey' blueberry isolate of TRSV.

FIGURE 2a

FIGURE 2b

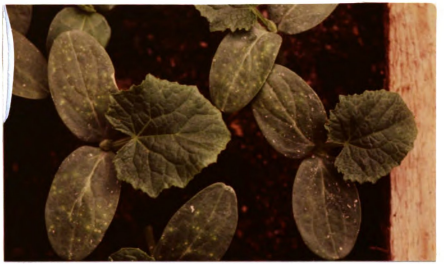


FIGURE 2c

FIGURE 3.--Nicotiana tabacum 'Havana 423' plants inoculated with three different isolates of tobacco ringspot virus (TRSV) showing differential reactions. Plants were inoculated with (a) the cherry isolate of TRSV, (b) the watermelon isolate of TRSV, and (c) the 'Jersey' blueberry isolate of TRSV.

FIGURE 3a

FIGURE 3b

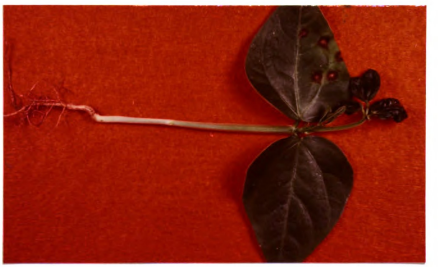
FIGURE 3c

FIGURE 4.--*Vigna unguiculata* Cowpea 'SR' plants inoculated with three different isolates of tobacco ringspot virus (TRSV) showing differential reactions. Plants were inoculated with (a) the soybean isolate of TRSV, (b) the tobacco isolate of TRSV, and (c) the 'Jersey' blueberry isolate of TRSV.

FIGURE 4a

FIGURE 4b

FIGURE 4c



tobacco and 'Jersey' blueberry isolates infected 6/6 of the N. tabacum 'Burley' plants inoculated. The TRSV isolates in this study did not, however, differ in host range. Although the efficiency of infection on some herbaceous hosts was low, especially for the soybean and cherry isolates, it was always possible to infect a fraction of the plants inoculated.

The 'Jersey' blueberry isolate induced the most severe and rapid response in most of the herbaceous hosts that were tested. In contrast, the soybean isolate generally induced relatively mild or symptomless infection in these herbaceous hosts. The symptoms induced by the remaining TRSV isolates were intermediate between the symptoms produced by the 'Jersey' blueberry isolate and the soybean isolate.

## Thermal Inactivation

The temperature of inactivation for TRSV in crude sap extracts varied for the different isolates of TRSV (Table 2), but all isolates were inactivated between 55°C and 62.5°C. All inoculated plants which showed no symptoms were serologically tested for infection using agar gel-diffusion tests (1). This was especially critical for the soybean isolate of TRSV which produces only a mild systemic mottling and stunting in Chenopodium quinoa, the test plant used in this study.

The tobacco and soybean isolates of TRSV appear to be more temperature sensitive than the other isolates used in this study. The 'Riesling' grape, watermelon, and 'Jersey' blueberry isolates, on

TABLE 2.--Thermal inactivation of tobacco ringspot virus (TRSV) isolates.[a]

| Virus Isolate | Inactivation Temperature (°C) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 45 | 47.5 | 50 | 52.5 | 55 | 57.5 | 60 | 62.5 |
| Tobacco | 2/2[b] | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 0/3 | 0/3 | --[c] |
| Soybean | 2/2 | 3/3 | 2/3 | 2/3 | 2/3 | 1/3 | 0/3 | 0/3 | -- |
| 'Jersey' blueberry | 2/2 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 0/3 |
| 'Stanley' blueberry | 2/2 | 3/3 | 3/3 | 3/3 | 3/3 | 2/3 | 2/3 | 3/3 | 0/3 |
| 'Riesling' grape | 2/2 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 0/3 |
| Cherry | 2/2 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 0/3 | 0/3 |
| Watermelon | 2/2 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 2/3 | 0/3 |

[a]Virus extracts in glass-distilled water from triturated infected cucumber cotyledons were heated for 10 minutes at the desired temperature, cooled on ice, and then assayed for infectivity by rub-inoculation to Chenopodium quinoa.

[b]Ratio of the number of host plants infected to the number of plants inoculated.

[c]Not tested.

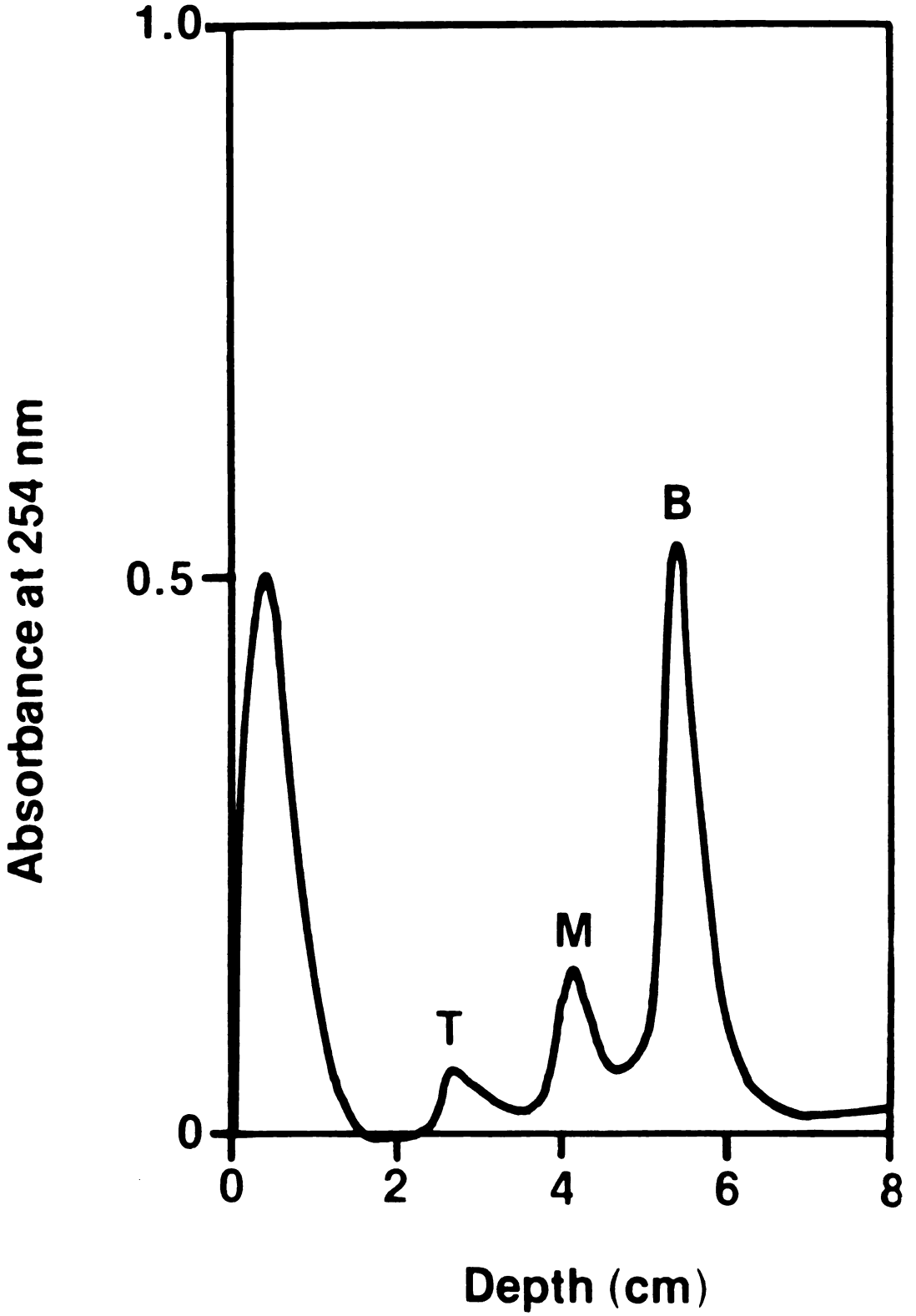
the other hand, appear to be more stable to heating than the other TRSV isolates.

## Virus Purification

The purification method of Rezaian and Francki (48) was suitable for the purification of all of the TRSV isolates except the 'Jersey' blueberry isolate. When the Rezaian and Francki (48) method was used for purification fo the 'Jersey' blueberry isolate the virus yields were almost negligible as compared to the virus yields of the tobacco isolate which had been purified in parallel with the 'Jersey' blueberry isolate. The more cumbersome Stace-Smith (61) method of purification of TRSV was found to give much better yields of the 'Jersey' blueberry isolate, and therefore this method was used to purify the 'Jersey' blueberry isolate of TRSV, unless specifically stated otherwise.

All preparations of the different isolates of TRSV contained three components (Figure 5), empty protein shells with RNA (Top, T), nucleoprotein containing RNA-2 (Middle, M), and nucleoprotein containing two pieces of RNA-2 or one piece of RNA-1 (Bottom, B). The relative proportions of these components varied between different preparations of the same virus. The yield of virus was usually 1-2 mg of purified virus per 100 g of well-infected cucumber tissue. Virus concentrations were determined spectrophotometrically, assuming $E^{0.1\%}_{1\ cm}$ at 260 nm of 7.0 for purified TRSV (48).

FIGURE 5.--Sucrose density-gradient centrifugation of 'Jersey' blueberry isolate of TRSV purified from cucumber cotyledons. Partially purified virus was layered on a 0-30% linear-log sucrose gradient, centrifuged at 38,000 rpm for 90 minutes at 4°C in a Beckman SW 41 rotor, and analyzed by an ISCO fractionator at 254 nm. The three components of the virus are (a) empty protein shells without RNA (Top, T), (b) nucleoprotein containing RNA-2 (Middle, M), and (c) nucleoprotein containing two pieces of RNA-2 or one piece of RNA-1 (Bottom, B). Plant host impurities in the preparation sedimented to give the large peak at the top of the gradient.


1.0
0.5
0
Absorbance at 254 nm
B
M
T
0
2
4
6
8
Depth (cm)

## Serology

The dilution endpoints of the isolate-specific antisera to homologous virus, as determined by precipitin line formation in double-diffusion tests in gels were as follows: cherry isolate, 1:256; tobacco isolate, 1:256; watermelon isolate, 1:256; 'Riesling' grape isolate, 1:512; 'Jersey' blueberry isolate, 1:512; 'Stanley' blueberry isolate, 1:512; soybean isolate, 1:256. When each antiserum was diluted 1:4 there was no precipitin line formed between the diluted antiserum and sap derived from healthy plant tissue. This indicated that only a small fraction of the antibodies in the antiserum were made to plant antigens.

### Spur Formation Tests

The formation of spur precipitin lines in double-diffusion tests was used as a criterion for determining serological relationships between the seven isolates of TRSV. Crude virus extracts of different virus isolates in the outer adjacent wells were reacted with antisera (in the center well) at dilutions between 1:8 and 1:32. A reaction of identity occurred in some cases (Figure 6), while typical spurs formed between some of the TRSV isolates (Figure 7). Single precipitin lines, with or without spurs, were formed in all reactions, showing that the seven isolates were serologically related, but not necessarily identical (Table 3). The formation of a spur in these tests indicated that the homologous virus possessed at least one antigenic determinant not present on the heterologous virus tested. The 'Jersey' blueberry isolate formed spurs with all of the

TABLE 3.--Spur precipitin lines formed when antisera to isolates of tobacco ringspot (TRSV) were reacted with homologous and heterologous antigens in double gel-diffusion tests.

| Heterologous TRSV Isolate Tested | Antiserum of TRSV Isolate | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cherry | 'Riesling' Grape | 'Stanley' Blueberry | 'Jersey' Blueberry | Tobacco | Watermelon | Soybean |
| Cherry | 0 | 0[a] | 0 | +[b] | 0 | + | 0 |
| 'Riesling' grape | 0 | 0 | 0 | + | 0 | + | 0 |
| 'Stanley' blueberry | 0 | 0 | 0 | + | 0 | + | 0 |
| 'Jersey' blueberry | 0 | 0 | 0 | 0 | 0 | 0 | + |
| Tobacco | 0 | 0 | 0 | + | 0 | + | 0 |
| Watermelon | 0 | 0 | 0 | + | 0 | 0 | + |
| Soybean | 0 | 0 | 0 | + | 0 | + | 0 |

[a]No spur formation evident in agar-gel double diffusion plates; confluent precipitin lines.

[b]Showed spur formation in agar-gel double-diffusion plates.

FIGURE 6.--Ouchterlony two-dimensional double-diffusion tests showing confluent precipitin lines. The center well contained antiserum to the 'Stanley' blueberry isolate of TRSV; the top, lower right, and lower left wells contained the 'Stanley' blueberry isolate of TRSV; the bottom, upper right, and upper left wells contained the watermelon isolate of TRSV.

FIGURE 7.--Ouchterlony two-dimensional double-diffusion tests showing a reaction of non-identity. The center well contained antiserum to the 'Jersey' blueberry isolate of TRSV; the top, lower right, and lower left wells contained the 'Jersey' blueberry isolate of TRSV; the bottom well contained the 'Riesling' grape isolate of TRSV; the upper right well contained the cherry isolate of TRSV, and the upper left well contained the 'Stanley' blueberry isolate of TRSV.

FIGURE 6

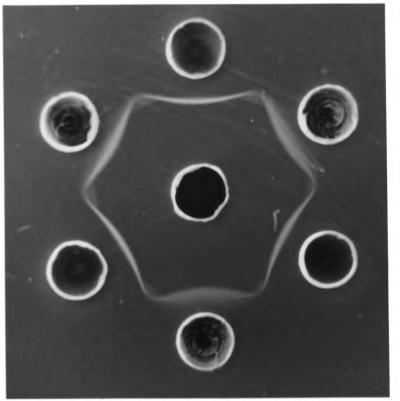
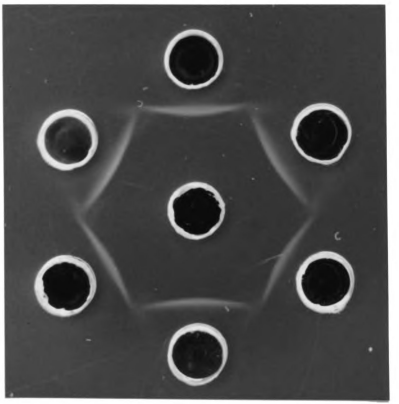


FIGURE 7



TRSV isolates when tested against the 'Jersey' blueberry isolate antiserum (Table 3). This means that the 'Jersey' blueberry isolate possesses antigenic sites not present on the other isolates. In contrast, the cherry, grape, 'Stanley' blueberry, and tobacco isolates did not form spurs when tested against any of the other isolates in adjacent wells. This is interpreted to mean that these four isolates do not possess additional antigenic sites over those present on the other TRSV isolates.

## Selection of Three TRSV Isolates for Further Study

Because of the difficulty in making detailed comparisons between all seven isolates of TRSV, three isolates which differed in symptomatology in herbaceous hosts and on their serological reactions, were chosen for further study. The 'Jersey' blueberry isolate and the soybean isolate were selected because they appeared to be the most, and least virulent, respectively, in the herbaceous host range tests. The tobacco isolate was chosen because its response on the herbaceous host range was intermediate between the 'Jersey' blueberry isolate and the soybean isolate, and because it is serologically identical to the TRSV type strain of the American Type Culture Collection (15).

## Cross-Absorption Tests

Most antisera were fully cross-absorbed with the addition of 16 to 32 parts of heterologous virus preparation. The results of the cross-absorption tests for the 'Jersey' blueberry, tobacco, and soybean isolates are shown in Table 4. Because of the dilution which

occurred during the absorption of each antiserum it was not possible to detect residual antibody to homologous virus if that residual antibody titer was lower than the dilution made during cross-absorption. For this reason the titers of the absorbed antisera which showed no reaction to homologous virus are not reported as having zero titer, but as having an undetermined titer which is less than the dilution effected by cross-absorption.

The cross-absorbed antisera contain antibodies to antigens which are found on the homologous virus, but not on the heterologous virus. The titers of the cross-absorbed antisera to their homologous viruses, represent a measure of the antigens which the homologous virus has, but which the heterologous, absorbing virus lacks. It is evident that the 'Jersey' blueberry isolate contains antigenic sites not present on the soybean and tobacco isolates (Table 4). This agrees with the results of the spur formation tests. The results of the cross-absorption tests indicate that the tobacco isolate has antigenic sites not present on the soybean isolate (Table 4), although these antigenic differences were not detected in the spur formation tests.

The antigenic differences between the isolates, as shown by cross-absorption tests, may or may not be due to the same antigens in all cases. For instance, both the soybean and the tobacco isolates are serologically different from the 'Jersey' blueberry isolates (see Table 4, 'Jersey' blueberry antiserum absorption), but this difference may be due to different antigens in each case. 'Jersey' blueberry antiserum, which had been cross-absorbed with soybean virus antigen, was found to have a titer of 1:32 against the tobacco virus antigen.

TABLE 4.--Cross-absorption tests for three isolates of tobacco ringspot virus (TRSV).

| Antiserum Absorbed | Absorbed with TRSV Isolate | Antiserum Dilution Following Absorption | Absorbed Anti-Serum Titer to Homologous Virus | Antiserum Titer to Absorbed Virus | Fraction of Titer Which Viruses Do Not Share[a] |
|---|---|---|---|---|---|
| 'Jersey' Blueberry | Tobacco | 1:32 | 1:64 | 1:512 | 64/512 |
| 'Jersey' Blueberry | Soybean | 1:32 | 1:128 | 1:512 | 128/512 |
| Tobacco | 'Jersey' Blueberry | 1:16 | <1:16 | 1:256 | <16/256 |
| Tobacco | Soybean | 1:32 | 1:64 | 1:256 | 64/256 |
| Soybean | 'Jersey' Blueberry | 1:16 | <1:16 | 1:256 | <16/256 |
| Soybean | Tobacco | 1:32 | <1:32 | 1:256 | <32/256 |

[a]Fraction = $\frac{\text{Titer of absorbed antiserum to homologous virus}}{\text{Titer of unabsorbed antiserum to homologous virus}}$

This indicates that at least some of the antigenic differences between the 'Jersey' blueberry isolate and the soybean isolate are due to antigenic sites not shared by the soybean and the tobacco isolates.

'Jersey' blueberry virus antiserum which had been cross-absorbed with the tobacco virus antigen did not react with the soybean virus antigen. Thus, the antigenic sites present on the 'Jersey' blueberry isolate, but not on the tobacco isolate, were also not present on the soybean virus isolate (at least as could be detected in the diluted cross-absorbed antiserum).

The residual antibody in the tobacco antiserum after absorption with the soybean virus antigen had a titer to the 'Jersey' blueberry virus antigen of 1:32. This indicates that at least some of the antigenic differences between the tobacco isolate and soybean isolate are due to antigenic sites not shared by the soybean and 'Jersey' blueberry isolates.

## Electrophoretic Mobility of Whole Virus Particles

Electrophoresis of whole virus particles at several different pH values showed that the migration of the virus particle depends on which isolate of TRSV was used. At pH 9.0 the virus particles of the 'Jersey' blueberry isolate migrated 6 mm toward the anode while the virus particles of the tobacco and soybean isolates migrated only 3 mm toward the anode. The difference in migration at pH 8.0 was even more dramatic. The virus particles of the tobacco and soybean isolates migrated toward the cathode 2 mm while the virus particles of the 'Jersey' blueberry isolate migrated 5 mm toward the anode.

The total charge on the virus particles of the 'Jersey' blueberry isolate was always more negative than the charge on the virus particles of the tobacco and soybean isolates.

Since it was necessary to use a different purification procedure to purify the 'Jersey' blueberry isolate, and since this difference in purification method might have had an effect on the charge of the virions, the 'Jersey' blueberry isolate was purified using the two methods of purification, and the electrophoretic mobility of the virus particles from these two preparations was compared. A measureable difference in electrophoretic mobility was evident between virus prepared by these two methods. For this reason all further electrophoretic mobility comparisons between virus isolates were made using virus which had been purified in the same manner.

The electrophoretic mobility, u, of the 'Jersey' blueberry, tobacco, and soybean isolates was calculated using the following formula:

$$u = \frac{\text{distance of migration (cm)/time of electrophoresis (sec)}}{\text{voltage applied (volt)/gel length (cm)}}$$

By convention, the sign of u is the sign of the overall charge on the virus particle at the pH being investigated. If the virus particle moves toward the anode, then the electrophoretic mobility is negative in sign. Table 5 gives the electrophoretic mobilities of the three isolates of TRSV as determined under three different conditions of pH. The total charge on the virions of the 'Jersey' blueberry isolate was always more negative than the charge on the virions of the tobacco and soybean isolates at the three pH conditions tested.

TABLE 5.--Electrophoretic mobility of whole virus for three isolates of tobacco ringspot virus (TRSV).[a]

| Source of Virus Isolate | Electrophoretic Mobility ($cm^2$ $volt^{-1}$ $sec^{-1}$) | | |
|---|---|---|---|
| | pH 7.0 | pH 8.0 | pH 9.0 |
| Tobacco | $+ 0.14 \times 10^{-5}$ | $+ 0.10 \times 10^{-5}$ | $-0.15 \times 10^{-5}$ |
| Soybean | $+ 0.14 \times 10^{-5}$ | $+ 0.10 \times 10^{-5}$ | $-0.15 \times 10^{-5}$ |
| 'Jersey' Blueberry | $+ 0.17 \times 10^{-5}$ | $- 0.29 \times 10^{-5}$ | $-0.35 \times 10^{-5}$ |

[a]Determined in 0.7% agarose in 0.02 M tris-0.02 M sodium dibasic phosphate at appropriate pH, according to method of Tremaine & Wright, Virology 31: 481-488.

## RNA Extraction and Characterization

RNA was extracted from purified preparations of the 'Jersey' blueberry, soybean, and tobacco isolates of TRSV. Spectrophotometric analysis of the purified RNA preparations showed that the ratio of the absorbance at 260 nm to the absorbance at 280 nm ranged from 1.85 to 2.08 for the various RNA preparations, indicating RNA of high purity.

Since it was considered important to have pure, covalently intact TRSV-RNA species for use as messenger RNA in the wheat embryo protein synthetic system, all of the purified TRSV-RNA preparations were analyzed by agarose-formaldehyde gel electrophoresis to assess their purity and intactness. The profiles of purified TRSV-RNA in agarose-formaldehyde gels for three different isolates of TRSV demonstrate the presence of two peaks of RNA whose molecular weight agrees closely with the published values for the molecular weight of TRSV-RNA (Figures 8, 9, 10). Molecular weight estimates of TRSV-RNA were made by plotting the logarithm of the molecular weights of the RNA standards against the relative mobility of these standards in the agarose-formaldehyde gels. The resulting graph was used to calculate the molecular weights of the two TRSV-RNAs.

The intactness of various RNA preparations was judged by the symmetry and sharpness of the RNA peaks in agarose-formaldehyde gels. Intact RNA should result in sharp, fairly narrow, symmetrical peaks. The quality of the RNA varied for different RNA preparations. Representative gel profiles of purified RNA from the tobacco, 'Jersey'

FIGURE 8.--Agarose-formaldehyde gel electrophoresis of total RNA from the tobacco isolate of TRSV. Samples of RNA were separated by electrophoresis at 4°C at 2 milliamps/gel for 3 to 4 hours, after which gels were scanned at 254 nm. The migration position of ribosomal RNAs from chick embryo muscle, which were used as molecular weight markers, is indicated by the arrows.

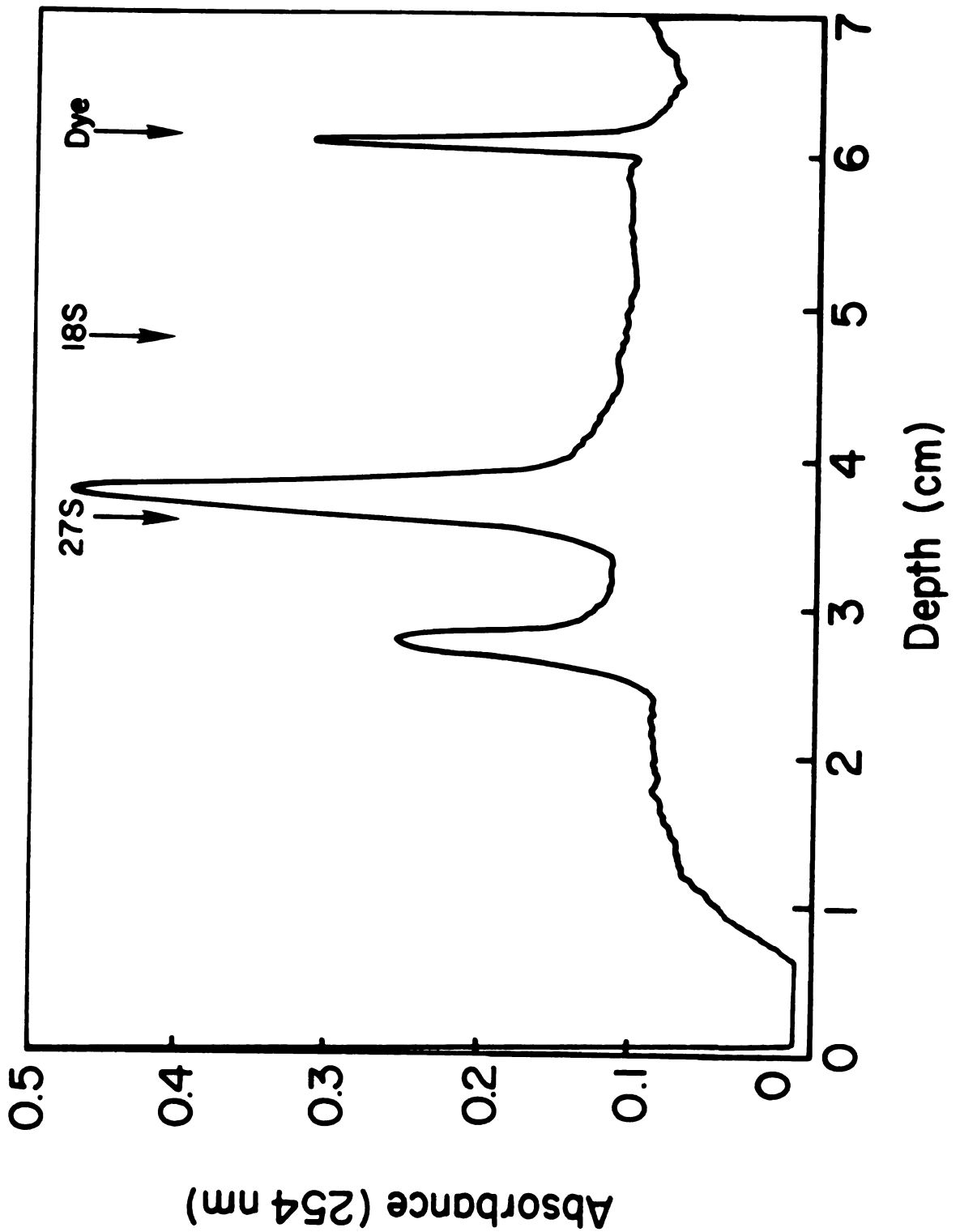

Absorbance (254 nm)
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
Depth (cm)
27S
18S
Dye

FIGURE 9.--Agarose-formaldehyde gel electrophoresis of total RNA from the 'Jersey' blueberry isolate of TRSV. Samples of RNA were separated by electrophoresis at 4°C at 2 milliamps/gel for 3 to 4 hours, after which gels were scanned at 254 nm. The migration position of ribosomal RNAs from chick embryo muscle which were used as molecular weight markers is indicated by the arrows.

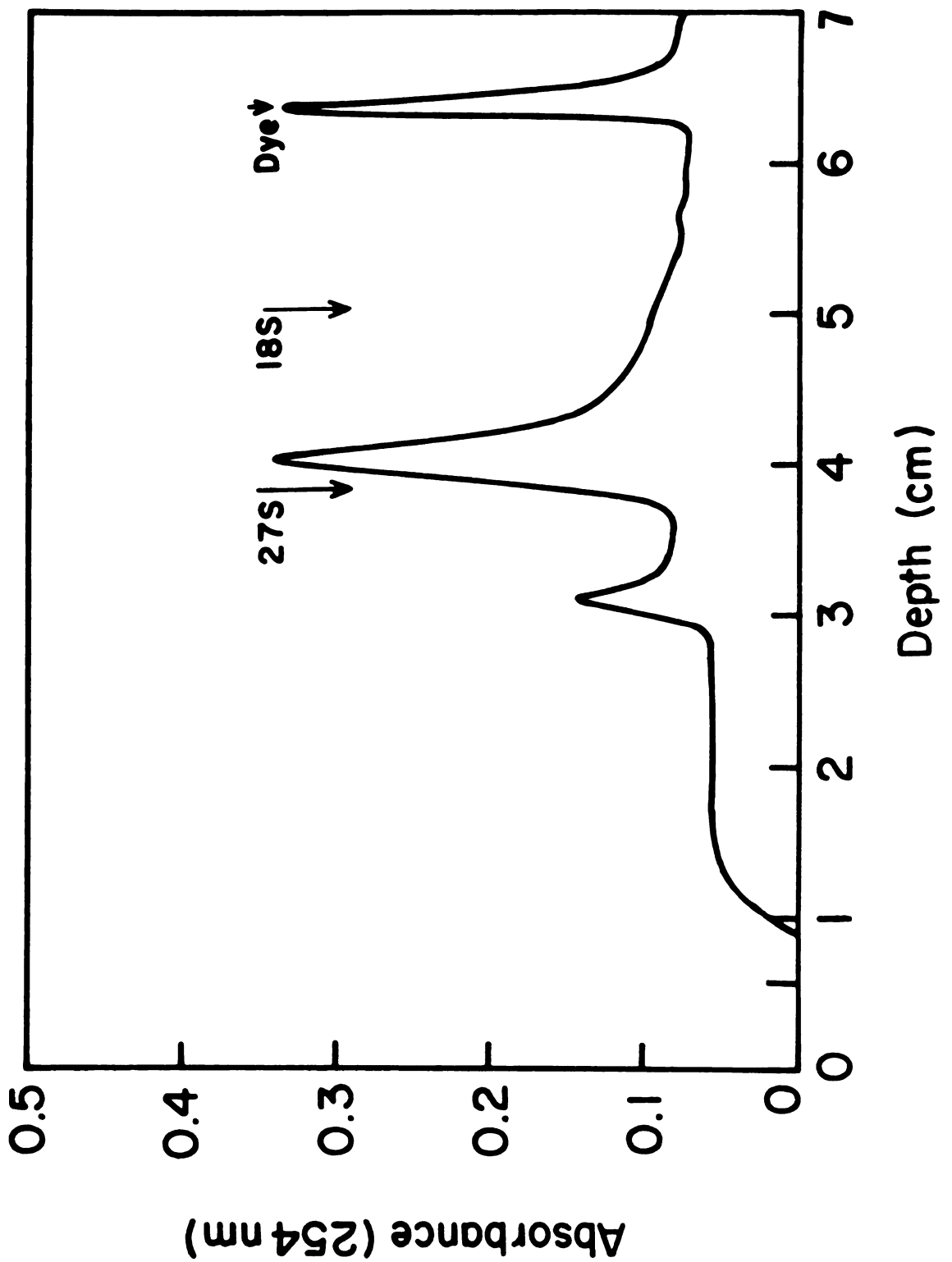
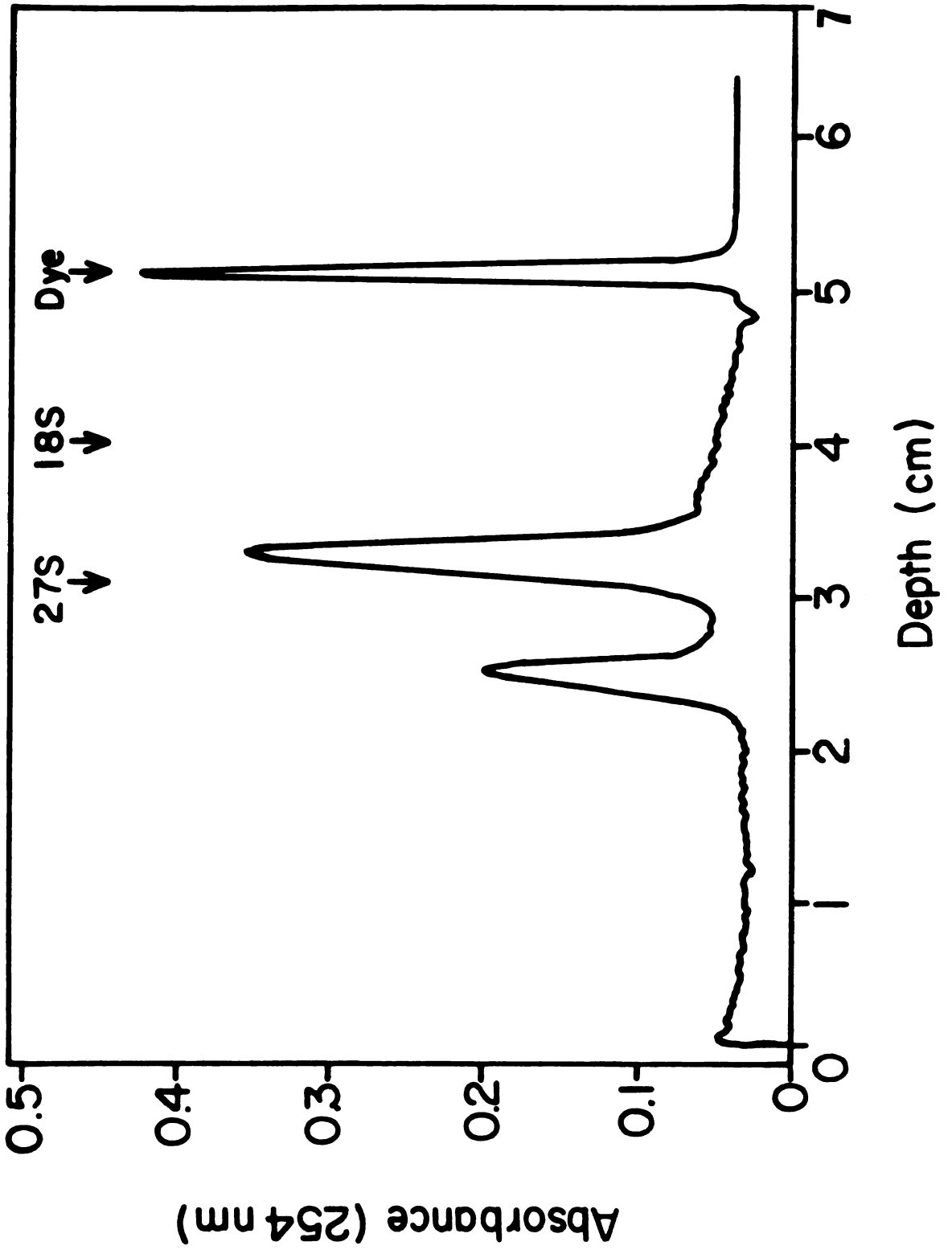

Absorbance (254 nm)
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
Depth (cm)
27S
18S
Dye

FIGURE 10.--Agarose-formaldehyde gel electrophoresis of total RNA from the soybean isolate of TRSV. Samples of RNA were separated by electrophoresis at 4°C at 2 milliamps/gel for 3 to 4 hours, after which the gels were scanned at 254 nm. The migration position of ribosomal RNAs from chick embryo muscle, which were used as molecular weight markers, is indicated by the arrows.


Absorbance (254 nm)
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
Depth (cm)
27S
18S
Dye

blueberry, and soybean isolates of TRSV are shown in Figures 8, 9, and 10.

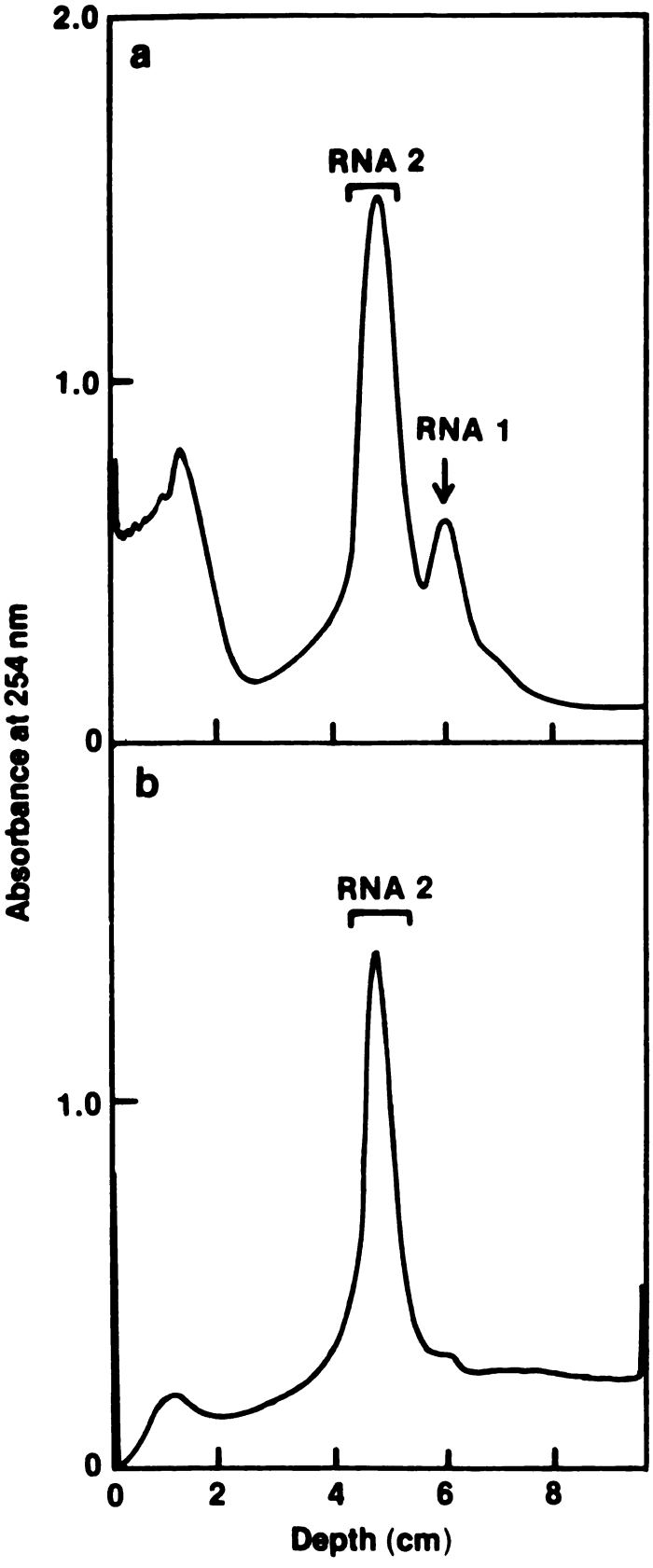
## Infectivity of Purified TRSV-RNA

The retention of biological activity in the preparations of unfractionated TRSV-RNA was considered to be an important measure of the integrity of the viral RNA. Purified preparations of TRSV-RNA which had been diluted to 1, 10, and 100 µg/ml in 0.1 M sodium phosphate buffer, pH 7.5, were tested for infectivity by inoculation to opposite, unifoliate leaves of Vigna unguiculata (Cowpea 'SR'). Since the specific activities of different RNA preparations were determined at different times, under varying greenhouse conditions, it was not possible to compare directly the biological activity of each of the RNA preparations. However, all of the preparations, at 10 µg/ml, induced the formation of an average of 49-350 local lesions/cowpea leaf (at least 10 leaves were inoculated/RNA preparation).

## Separation of TRSV-RNA on Sucrose Density Gradients

Centrifugation of 0-32.5% linear-log sucrose gradients was used for the separation of RNA-2 ($1.2 \times 10^6$ daltons) from total TRSV-RNA. Heat denaturation of the RNA preparations prior to sucrose density gradient centrifugation was necessary to eliminate the presence of a third peak in sucrose gradients which sedimented just ahead of RNA-1 ($2.2 \times 10^6$ daltons). The preparative centrifugation of heat-denatured TRSV-RNA (200 µg/Beckman SW41 rotor tube) gave a profile with two RNA peaks (Figure 11a). Fractions containing

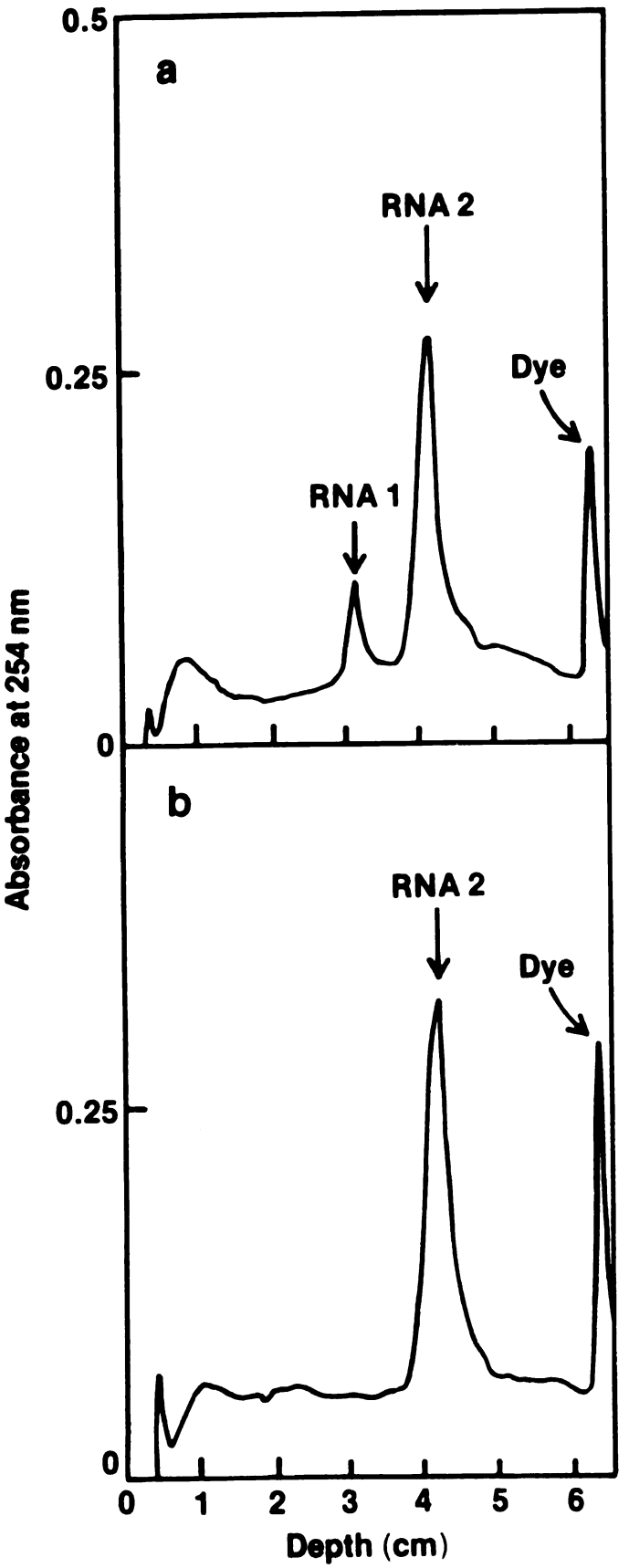
FIGURE 11.--Sucrose density-gradient fractionation of TRSV-RNA. Fractions corresponding to the RNA-2 peak in Figure 11a were pooled, as indicated by brackets, concentrated by ethanol precipitation, and refractionated from sucrose gradients to give the profile in Figure 11b. The RNA-2 peak was again collected and concentrated by ethanol precipitation.


2.0
a
RNA 2
1.0
RNA 1
0
b
RNA 2
1.0
0
0
2
4
6
8
Depth (cm)
Absorbance at 254 nm

the RNA-2 peak were collected as indicated by the brackets in Figure 11a, followed by ethanol precipitation of the RNA and recentrifugation on 0 to 32.5% linear-log sucrose density gradients. The profile of the fractionated RNA in the second sucrose density gradient is given in Figure 11b. The peak containing the small RNA was again collected (as indicated by brackets in Figure 11b), ethanol precipitated, and analyzed for purity and intactness by agarose-formaldehyde gel electrophoresis. The fact that RNA-1 was always present in relatively low concentrations compared to RNA-2, made it impossible to adequately separate RNA-1 from total TRSV-RNA.

RNA-2, fractionated by two cycles of sucrose gradients, was compared to unfractionated TRSV-RNA by agarose-formaldehyde gel electrophoresis, and by its infectivity in cowpea plants. The unfractionated TRSV-RNA showed two peaks typical of RNA 1 + 2 of TRSV in agarose-formaldehyde gels (Figure 12a). Analysis of the purified RNA-2 on agarose-formaldehyde gels (Figure 12b) showed that this RNA had been successfully separated from RNA-1 by sucrose density gradient fractionation, and the resulting RNA was covalently intact. The purified RNA-2 preparation was not infectious when inoculated onto cowpea plants at a concentration of 100 μg/ml. The preparation of unfractionated RNA from which the RNA-2 was purified produced 62 lesions/cowpea leaf when inoculated at a concentration of 10 μg/ml. This purified preparation of RNA-2 was used for analysis of *in vitro* translation in the wheat embryo cell-free system.

FIGURE 12.--Analysis by agarose-formaldehyde gel electrophoresis of unfractionated TRSV-RNA (a), and RNA-2 fractionated by two successive cycles of sucrose density gradient centrifugation (b). Samples of RNA were separated by electrophoresis at 4°C at 2 milliamps/gel for 3 to 4 hours, after which the gels were scanned at 254 nm.


0.5
a
RNA 2
0.25
Dye
RNA 1
0
b
RNA 2
Dye
0.25
0
0 1 2 3 4 5 6
Depth (cm)
Absorbance at 254 nm

## Detection of Poly(A) Tracts in TRSV-RNA

The use of oligo(dT)-cellulose column chromatography was investigated as a possible tool for further purification of TRSV-RNA. A large proportion of TRSV-RNA was bound to oligo(dT)-cellulose in buffers of high ionic strength. This suggests that TRSV-RNA molecules contain poly(A) tracts. The binding of TRSV-RNA to an oligo(dT)-cellulose column is demonstrated by the elution profile given in Figure 13. In this experiment 100 µg of denatured TRSV-RNA from the tobacco isolate, was chromatographed on an oligo(dT)-cellulose column 5 cm long and 1.5 cm in diameter as described under Materials and Methods. Upon chromatography of total TRSV-RNA about 65% of the RNA was bound and then eluted, and about 35% was not bound to the oligo(dT)-cellulose column.

The two RNA species that make up the genome of TRSV were both bound to oligo(dT)-cellulose under high ionic strength conditions. Agarose-formaldehyde gel electrophoresis of the RNA eluted under high and low ionic strength conditions showed that both RNA species of TRSV were bound equally under high ionic conditions. Figure 14 shows the agarose formaldehyde gel profiles of unfractionated TRSV-RNA (prior to chromatography on oligo(dT)-cellulose), and profiles of the RNA-containing fractions eluted under high and low ionic strength conditions. Electrophoresis of the gel whose profile is shown in Figure 14a was carried out at a different time than those in Figure 14b and 14c. Only 25% of the $OD_{260}$ absorbing material that was eluted with 'high salt' buffer precipitated with ethanol. When the material which was precipitated by ethanol was analyzed by

FIGURE 13.--Oligo(dT)-cellulose chromatography of TRSV-RNA. Poly $(A)^-$ RNA and Poly $(A)^+$ RNA, in fractions 3-4, and 12-14, respectively, were ethanol precipitated and analyzed using agarose-formaldehyde gel electrophoresis.

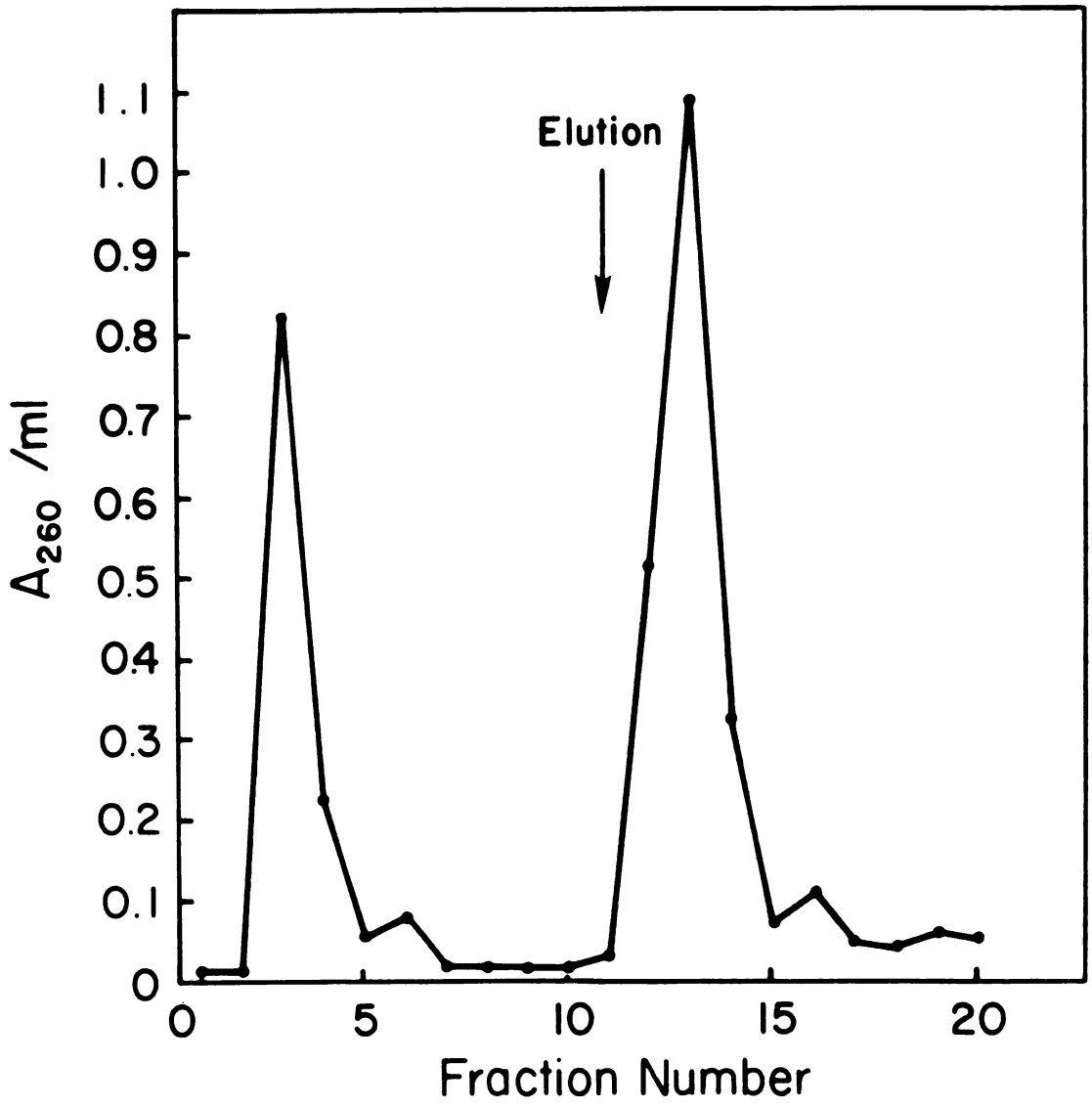
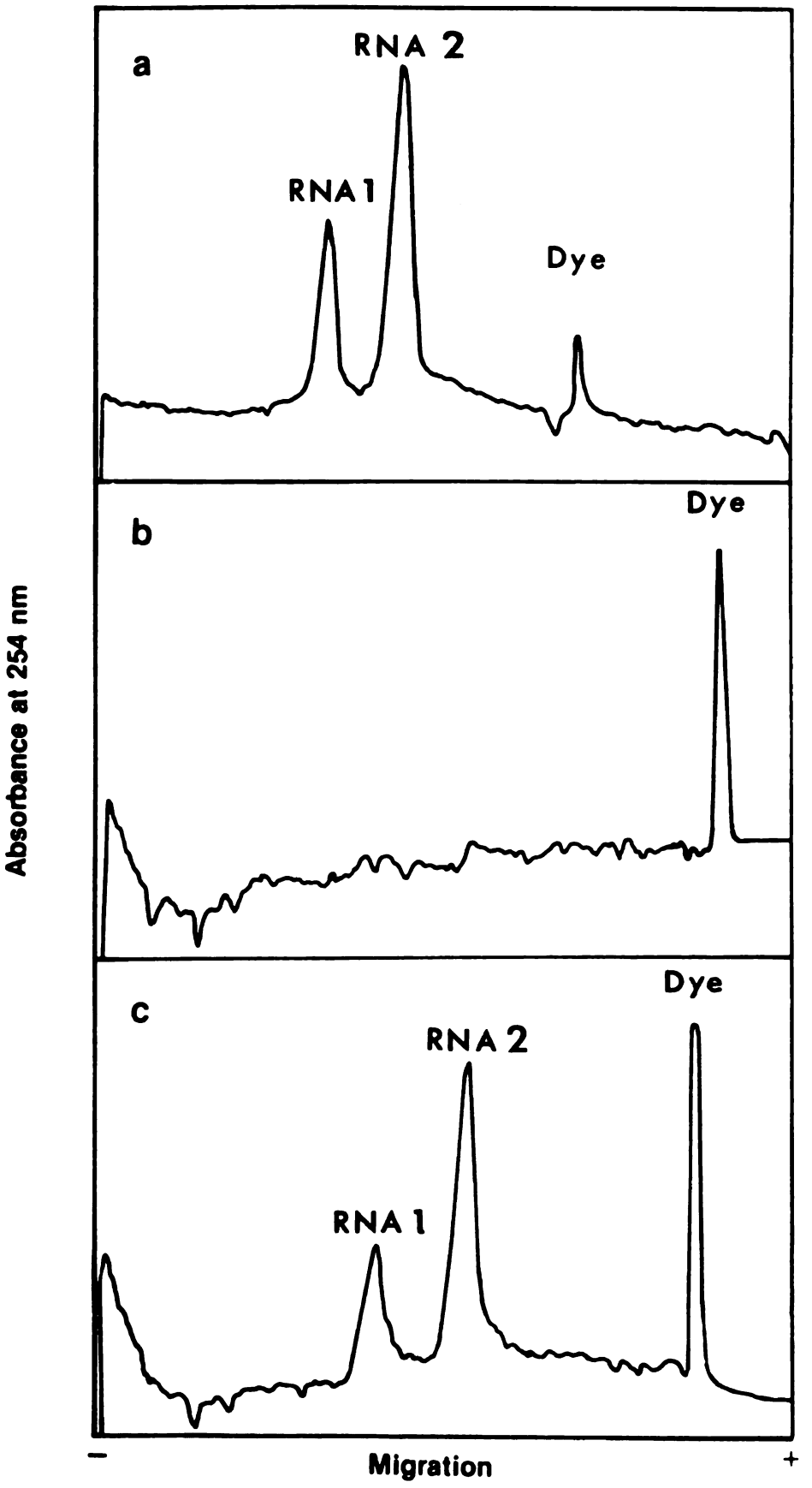

Elution
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$A_{260}$ /ml
0
5
10
15
20
Fraction Number

FIGURE 14.--Electrophoresis of RNA preparations from oligo(dT)-cellulose chromatography in agarose-formaldehyde denaturing gels. (a) Unfractionated RNA from TRSV prior to oligo(dT)-cellulose chromatography, (b) Fraction of TRSV-RNA preparation eluted with 'high salt' buffer, (c) Fraction of TRSV-RNA preparation eluted with 'low salt' buffer. Each gel contained 1-4 μg of RNA, estimated spectrophotometrically. Samples of RNA were separated by electrophoresis at 2 milliamps/gel for 3 to 4 hours, after which gels were scanned at 254 nm.

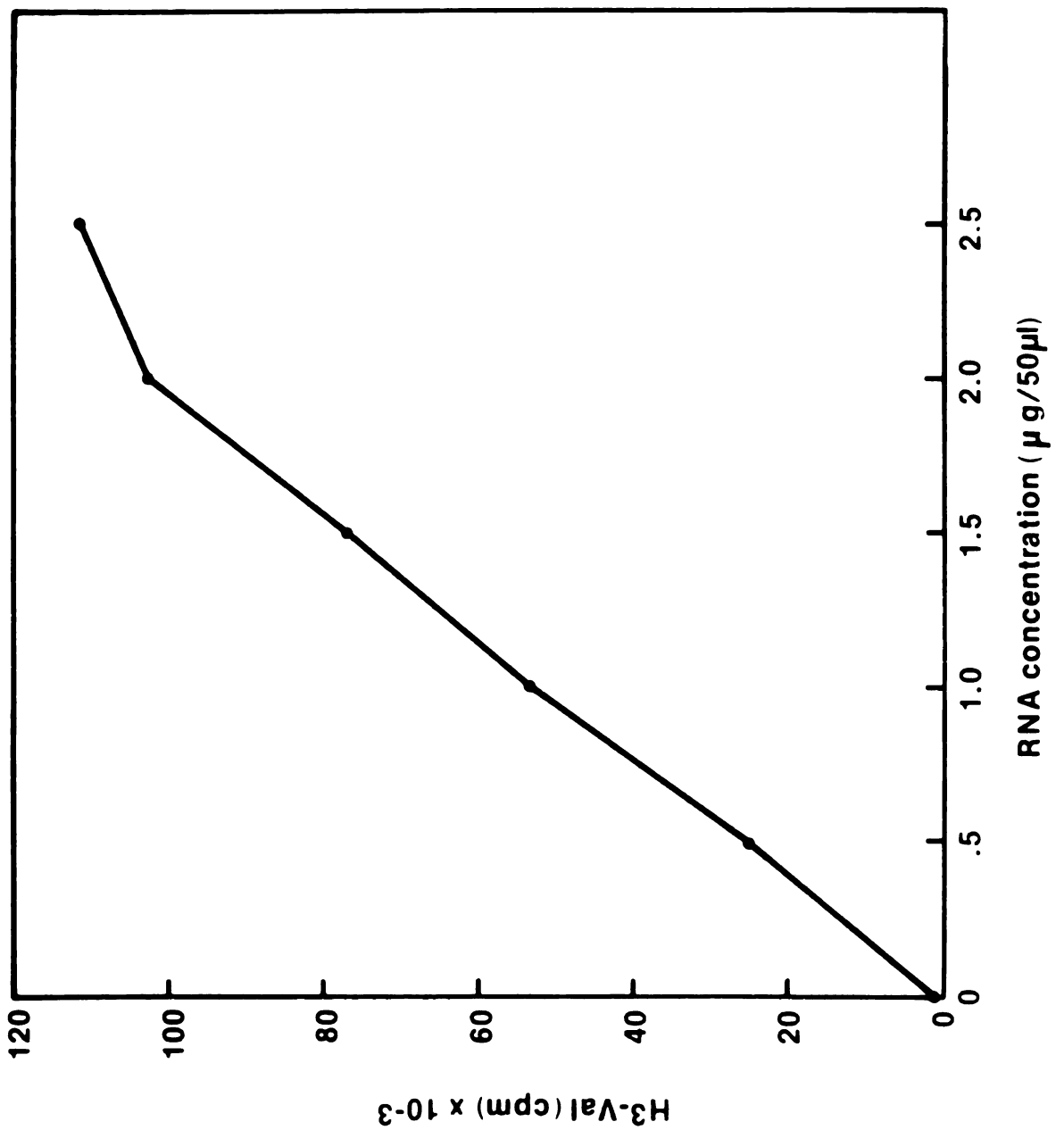

a
RNA 2
RNA 1
Dye
b
Dye
c
Dye
RNA 2
RNA 1
Absorbance at 254 nm
–
Migration
+

electrophoresis (0.25 $OD_{260}$ absorbing units/gel) there was little evidence of discrete RNA species (Figure 14b). The $OD_{260}$ absorbing material which had been eluted with 'low salt' buffer was also ethanol precipitated and subsequently analyzed by gel electrophoresis (0.8 $OD_{260}$ absorbing units/gel). Two discrete RNA species were evident in the fraction eluted with 'low salt' buffer (Figure 14c). Since the relative proportions of RNA-1 and RNA-2 remained the same after chromatography on oligo(dT)-cellulose, it was concluded that both genome RNAs of TRSV are polyadenylated in roughly equal amounts, and that oligo(dT)-cellulose chromatography could be used as a purification step in the extraction of TRSV-RNA. However, since it was possible to obtain relatively pure TRSV-RNA without oligo(dT)-cellulose chromatography this step was not included in the routine RNA purification procedure.

## Translation of TRSV-RNA in the Wheat-Embryo Cell-Free System

Addition of TRSV-RNA to the wheat embryo cell-free protein synthetic system stimulated the incorporation of $^{3}$H-valine into acid-precipitable material 50 to 100-fold, showing that TRSV-RNA is a relatively efficient messenger RNA. These results are similar to those of Fritsch et al. (11) for translation of tomato black ring virus in the wheat embryo system. When a range of concentrations of RNA were tested, incorporation was essentially linear up to a concentration of 2 μg RNA/50 μl of reaction mix (Figure 15). It has been found that low levels of plant virus messenger RNAs give improved completion of polypeptide chains in the wheat embryo system

FIGURE 15.--Incorporation of $^{3}H$-valine into protein in the wheat embryo extract in response to added TRSV-RNA. Concentrations of $Mg^{++}$ and $K^{+}$ were 3.2 mM and 45 mM, respectively.


H3-Val (cpm) x 10-3
120
100
80
60
40
20
0
0
.5
1.0
1.5
2.0
2.5
RNA concentration (μg/50μl)

(57). Therefore, in subsequent experiments TRSV-RNA concentrations of 1-2 μg/50 μl incubation mix were used, a level which was less than saturation, but which gave adequate incorporation for analysis of *in vitro* products.

The magnesium ion optimum for $^{3}$H-valine incorporation into acid-insoluble material varied for different preparations of TRSV-RNA, but was usually between 2.7 and 3.2 mM (Figure 16). The magnesium ion concentration was adjusted to the optimum concentration for each RNA preparation. The potassium ion level for maximum $^{3}$H-valine incorporation was found to be 45 mM for TRSV-RNA (Figure 17). Since there was little variation in optimum potassium requirements for different RNA preparations, this concentration of potassium was used in all synthesis reactions.

The time kinetics of $^{3}$H-valine incorporation in the wheat embryo system were determined. Translation of unfractionated TRSV-RNA directed valine incorporation for at least 70 minutes (Figure 18). The size of the *in vitro* products translated after different incubation times was not found to increase with increasing incubation time (data not shown). This experiment indicates that the routine incubation time of 45 minutes was adequate to obtain the largest possible translation products.

## Analysis of Translation Products by SDS-Polyacrylamide Gel Electrophoresis

Analysis in SDS-polyacrylamide gels of the $^{3}$H-valine labeled products of TRSV-RNA revealed 10 to 11 bands of protein with a maximum molecular weight of about 133,000 daltons (Figure 19). When TRSV

FIGURE 16.--$Mg^{++}$ requirement for amino acid incorporation directed by TRSV-RNA in the wheat embryo extract. Acid-insoluble incorporation was assayed in a 50-µl subsample as described in Materials and Methods. Concentrations of $K^{+}$ and RNA were 45 mM and 1 µg/50 µl, respectively.

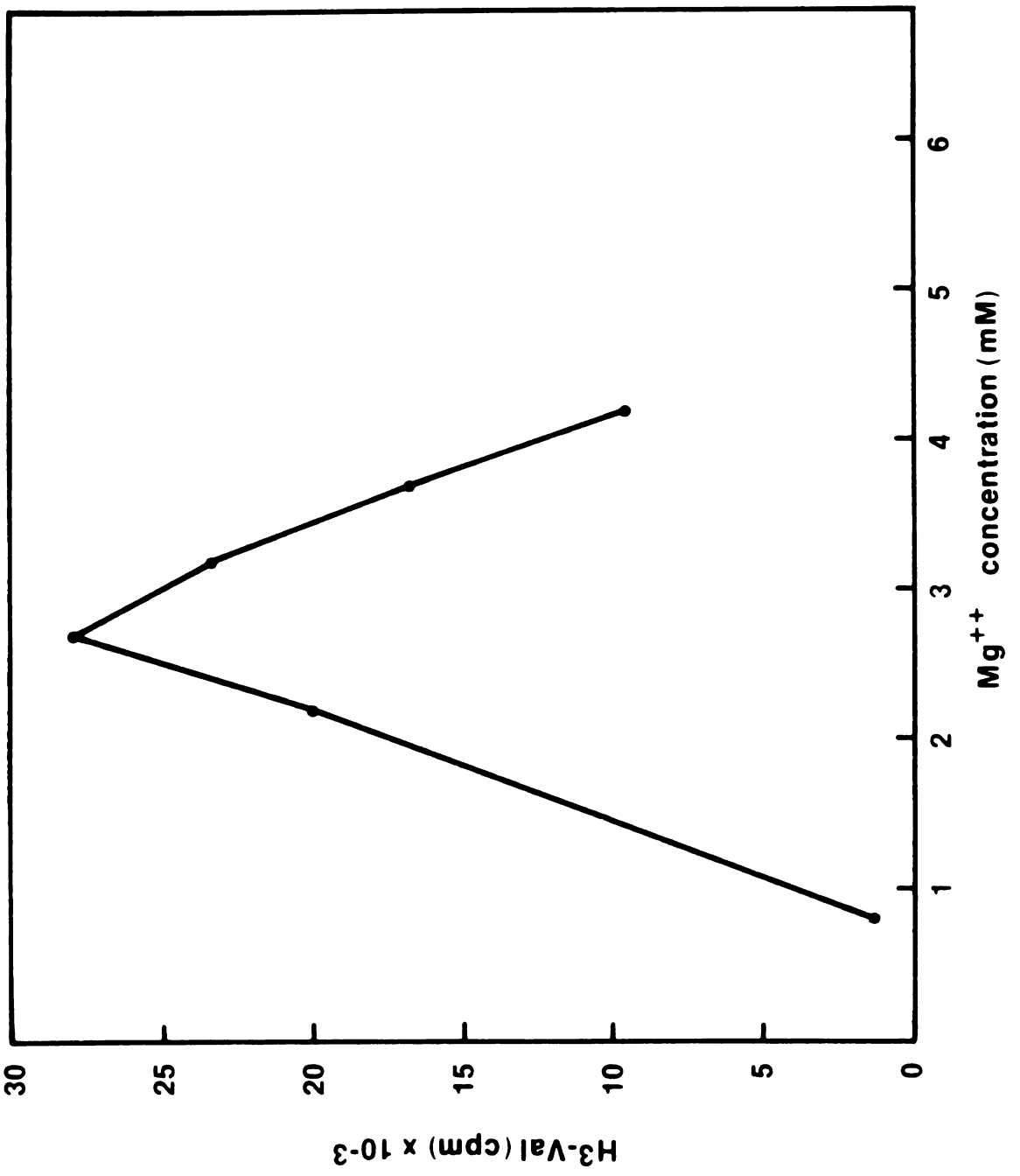

H3-Val (cpm) x 10-3
0
5
10
15
20
25
30
1
2
3
4
5
6
Mg++ concentration (mM)

FIGURE 17.--$K^+$ ion requirement for amino acid incorporation directed by TRSV-RNA in the wheat embryo system. Acid-insoluble incorporation was assayed in a 50-µl subsample as described in Materials and Methods. Concentrations of $Mg^{++}$ and RNA were 3.2 mM and 1 µg/50 µl, respectively.

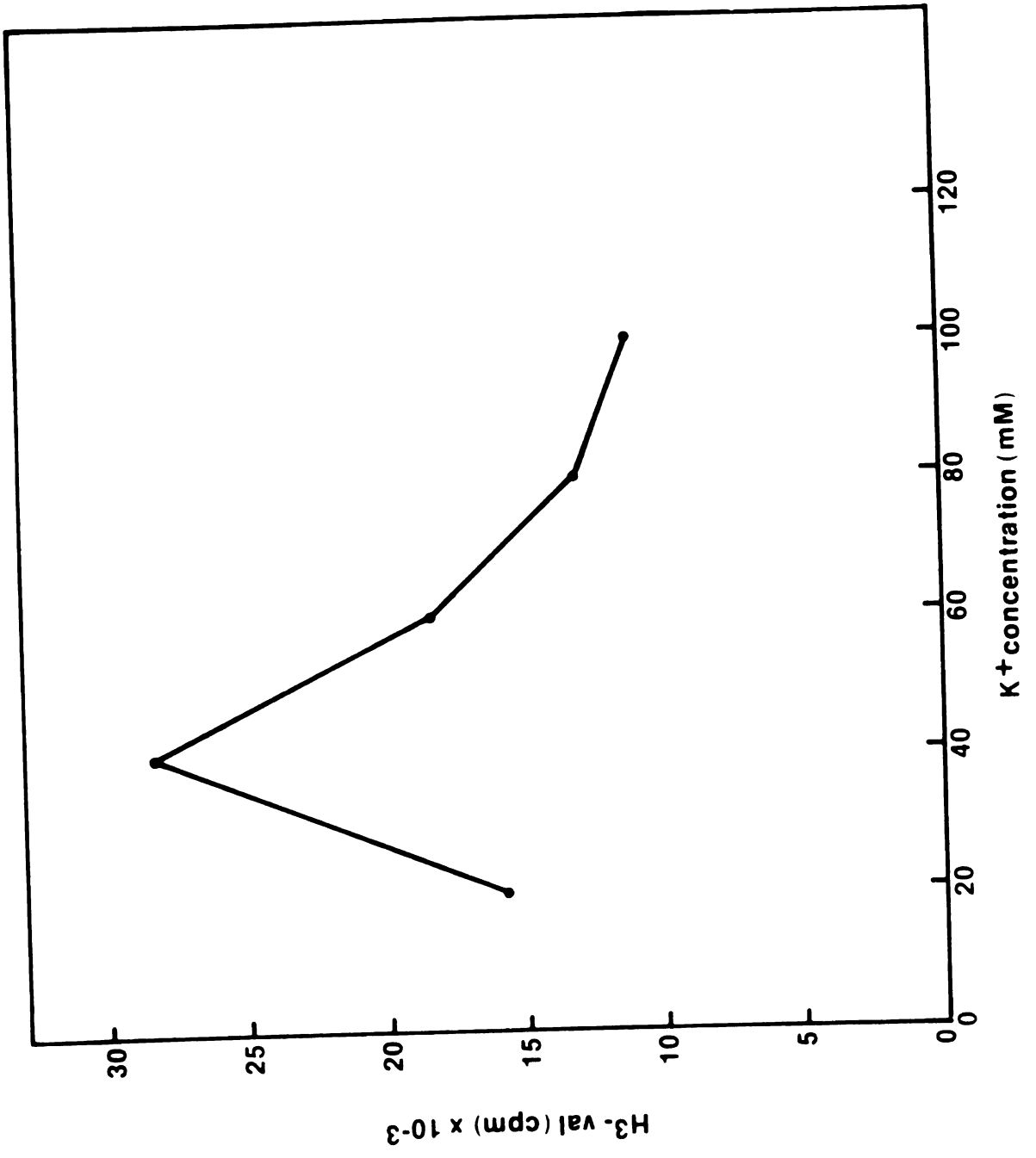
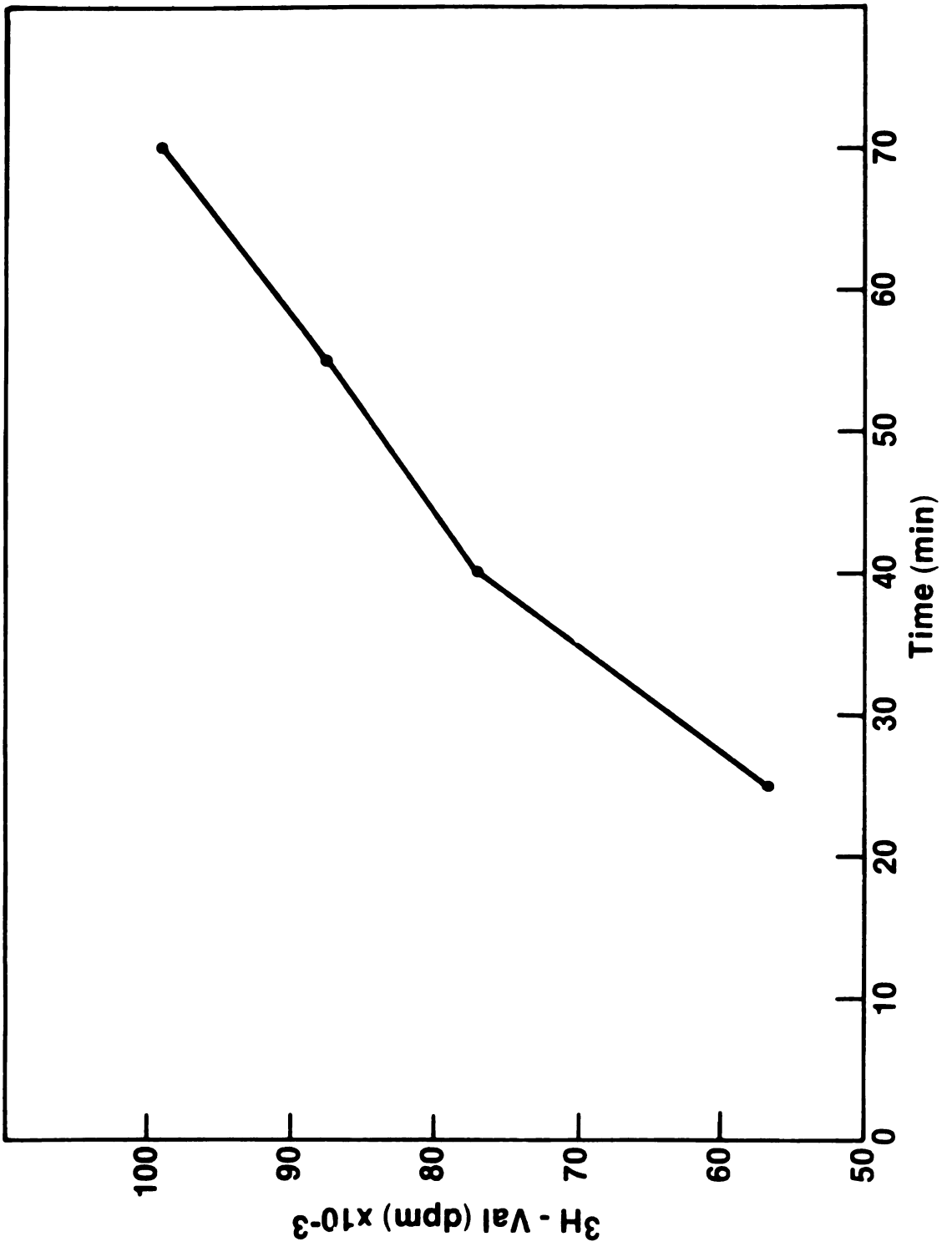

H3- val (cpm) x 10-3
0
5
10
15
20
25
30
0
20
40
60
80
100
120
K+ concentration (mM)

FIGURE 18.--Kinetics of $^{3}$H-valine incorporation directed by TRSV-RNA from the 'Jersey' blueberry isolate in wheat embryo system. Reaction mixture was made 45 mM $K^{+}$, 3.2 mM $Mg^{++}$, and RNA concentration was 2 µg/50 µl of reaction mix. Acid-insoluble incorporation was assayed in a 50 µl subsample as described in Materials and Methods.


3H - Val (dpm) x10-3
100
90
80
70
60
50
0
10
20
30
40
50
60
70
Time (min)

coat protein subunit was used as a molecular weight standard it migrated to a position similar to one of the *in vitro* protein products, but this product was not made in large amounts as might be expected of coat protein since large amounts of virus coat protein are syntheized *in vivo*. Tomato black ring virus, which is a nepovirus like TRSV, has been shown to synthesize an overwhelming amount of coat protein *in vivo* (11).

The number of protein products that were made when the RNA from three different isolates of TRSV was used as messenger RNA in the wheat embryo cell-free translation system was the same regardless of the source of the RNA (Figures 19-21). However, the relative amounts of various proteins differed from one RNA preparation to another.

## Dual-Label Experiments to Detect Small Differences in Molecular Weights of Protein Products Produced by Different Isolates of TRSV

Molecular weight estimates of proteins by SDS-polyacrylamide gel electrophoresis are only accurate, at best, to within ±5% (71). This is true even when the proteins are separated by electrophoresis at the same time, but in different gels (71). Therefore, it is very difficult to detect a difference in the molecular weight of two proteins by SDS-polyacrylamide gel electrophoresis in cylindrical gels unless the molecular weight difference is greater than 10%. For this reason a dual-label experiment was conducted in which the protein products from one isolate of TRSV, which had been labeled with $^{14}C$-valine, and protein products from another isolate labeled with

FIGURE 19.--SDS-polyacrylamide gel electrophoresis of *in vitro* translation products using RNA from the tobacco isolate of TRSV. Electrophoresis was done at 8 milliamps/gel for 60 to 90 minutes. The migration position of molecular weight markers is indicated by the arrows. Migration is from left to right.

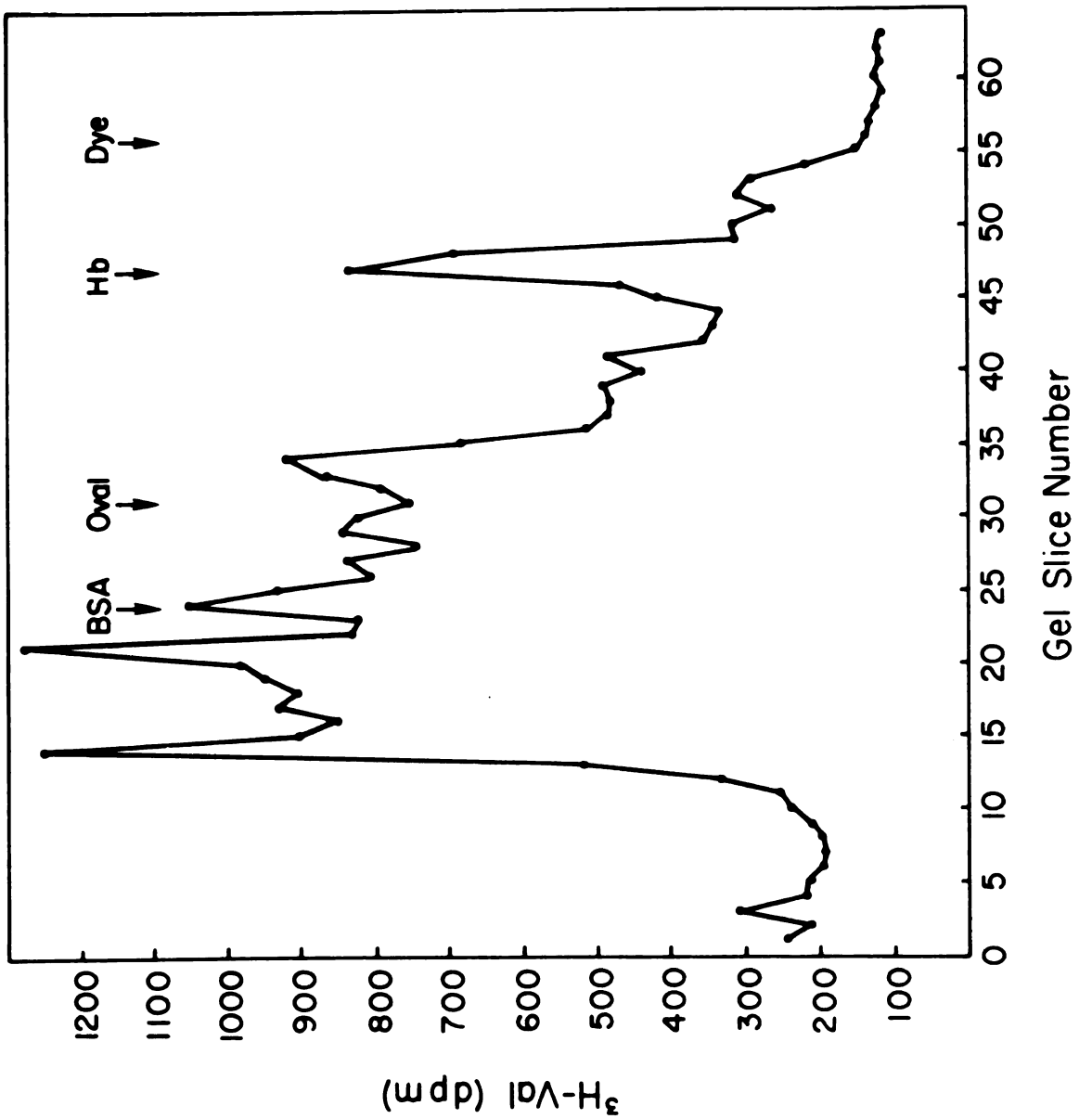

BSA
Oval
Hb
Dye
$^3$H-Val (dpm)
Gel Slice Number
100
200
300
400
500
600
700
800
900
1000
1100
1200
0
5
10
15
20
25
30
35
40
45
50
55
60

FIGURE 20.--SDS-polyacrylamide gel electrophoresis of *in vitro* translation products using RNA from the soybean isolate of TRSV. Electrophoresis was done for 60 to 90 minutes at 8 milliamps/gel. The migration position of molecular weight markers is indicated by arrows. Migration is from left to right.

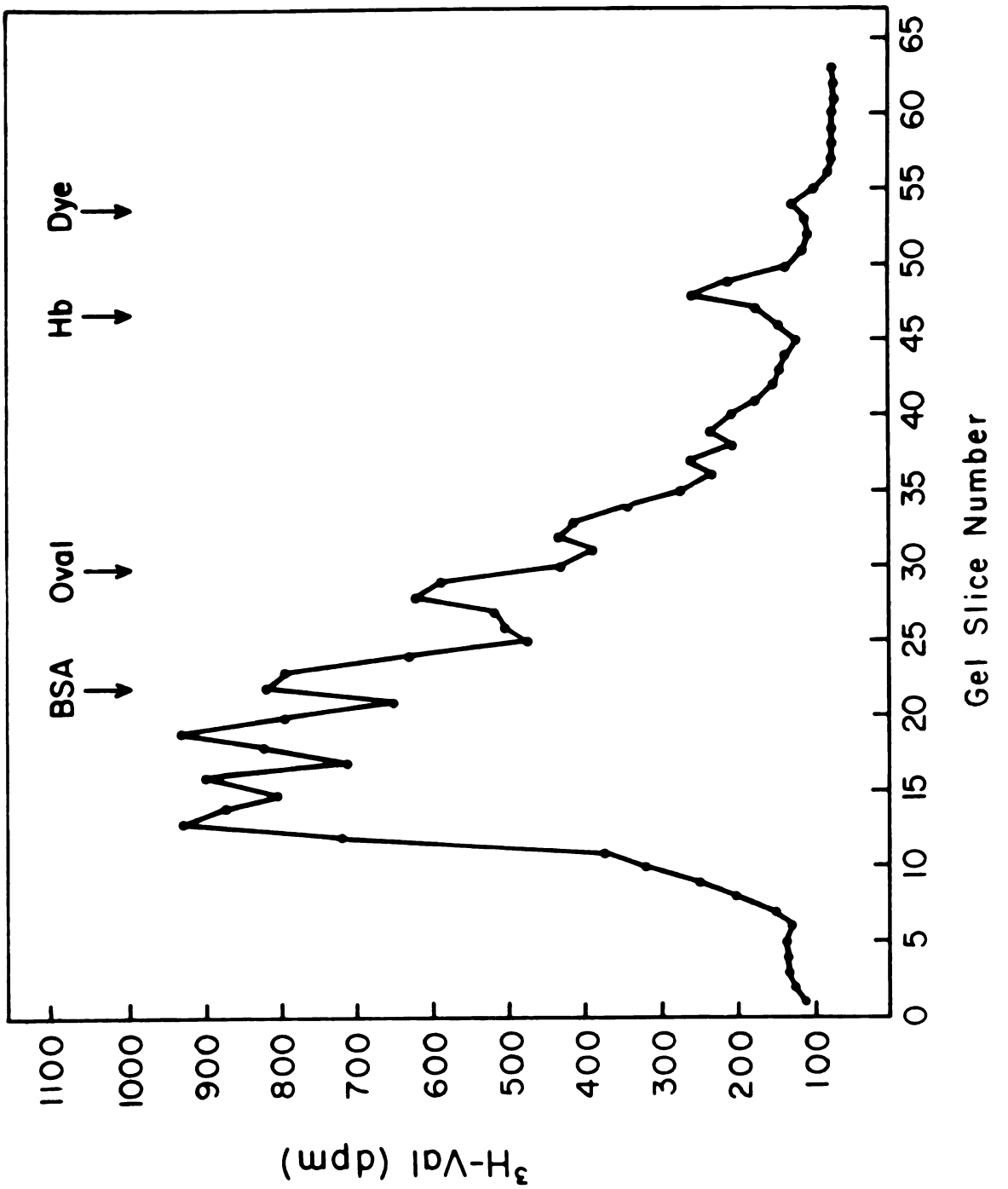
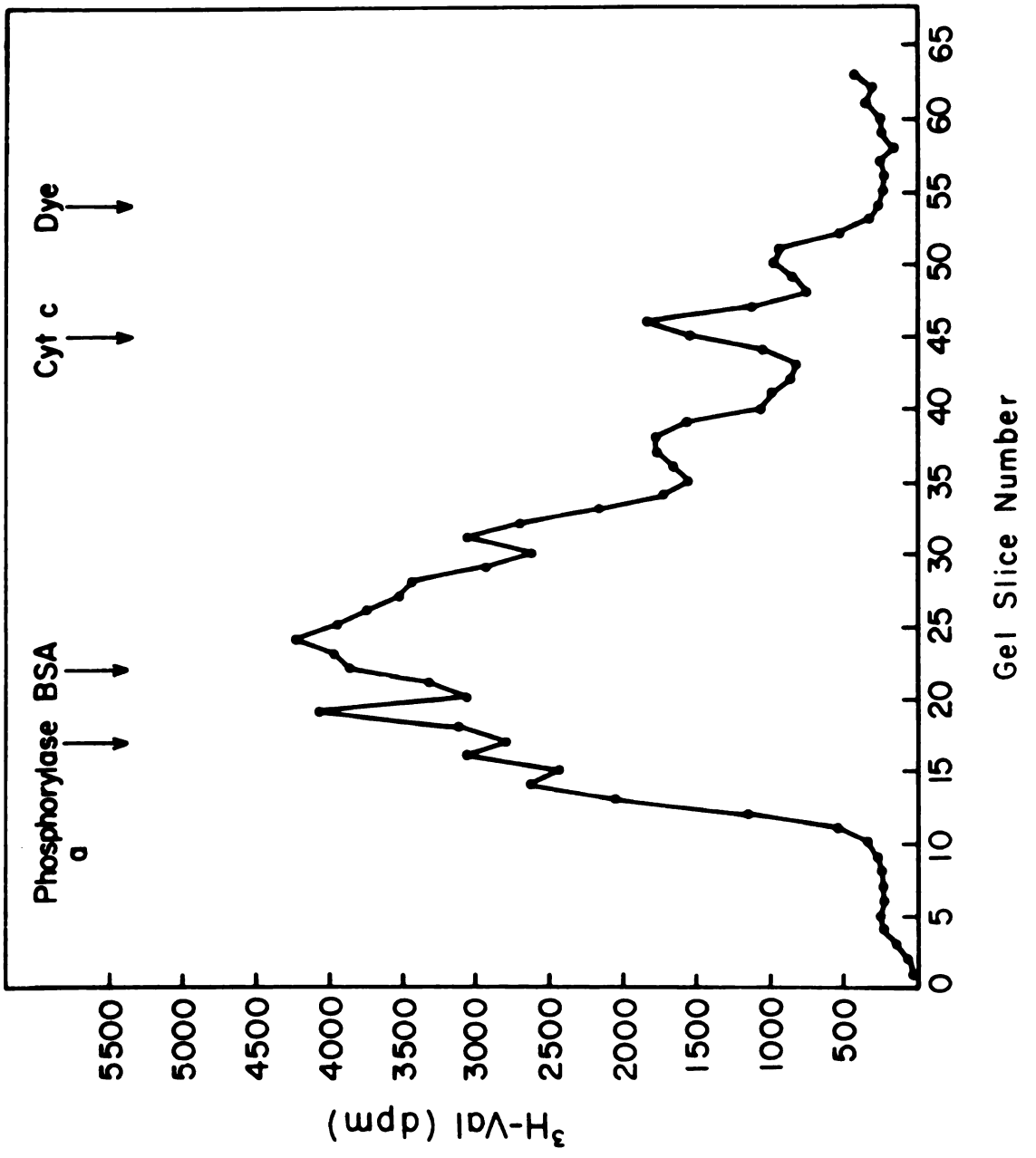

BSA
Oval
Hb
Dye
$^3$H-Val (dpm)
Gel Slice Number
0
5
10
15
20
25
30
35
40
45
50
55
60
65
100
200
300
400
500
600
700
800
900
1000
1100

FIGURE 21.--SDS-polyacrylamide gel electrophoresis of *in vitro* translation products using RNA from the 'Jersey' blueberry isolate of TRSV. Electrophoresis was done for 60 to 90 minutes at 8 milliamps/gel. The migration position of molecular weight markers is indicated by the arrows. Migration is from left to right.


Phosphorylase a
BSA
Cyt c
Dye
$^3$H-Val (dpm)
Gel Slice Number

$^{3}$H-valine, were mixed together and separated by electrophoresis on the same SDS-polyacrylamide cylindrical gel. The gels were sliced and the radioactivity from the $^{3}$H-valine products, and from the $^{14}$C-valine products was then determined for each gel slice.

The protein profiles for the set of proteins from one isolate (counted as $^{3}$H) was then plotted on the same graph as the protein profile from the second isolate (counted as $^{14}$C). This type of 'within-gel' comparison using a dual-label was done with the protein products of the 'Jersey' blueberry, soybean, and tobacco isolates of TRSV (Figures 22 to 24). Each comparison was made at least three times, but only one graph of each comparison is shown here (Figures 22-24). A close examination of Figure 22 shows that the protein profiles of the 'Jersey' blueberry and the tobacco isolate are similar except for the two protein peaks that co-migrate with the phosphorylase-a and the cytochrome-c markers. On replicate gels the migration difference between the protein which co-migrated with the phosphorylase-a marker was not evident. Also it was difficult to compare the migration of some of these higher molecular weight proteins because the protein peaks were relatively small.

When the protein products of the soybean and tobacco isolates were compared in the same gel using a dual-label there were no consistent differences in the migration of the polypeptides from the different TRSV isolates (Figure 23). However, a comparison of the products of the soybean isolate and the 'Jersey' blueberry isolate (Figure 24) showed a consistent molecular weight difference in the protein product which migrated slightly ahead of the cytochrome-c

FIGURE 22.--SDS-polyacrylamide gel electrophoresis of *in vitro* translation products of TRSV-RNA from the tobacco isolate (—) and the 'Jersey' blueberry isolate (---). *In vitro* protein products were made in the wheat embryo system using $^{3}H$-valine when translating the RNA from the 'Jersey' blueberry isolate, and $^{14}C$-valine when translating the RNA from the tobacco isolate. The protein products from these two isolates were then mixed, denatured, and separated electrophoretically in the same gel for 60 to 90 minutes at 8 milliamps/gel. The migration position of the molecular weight markers is indicated by the arrows. Gels were sliced and counted simultaneously for $^{14}C$ and $^{3}H$ radioactivity. Migration is from left to right.

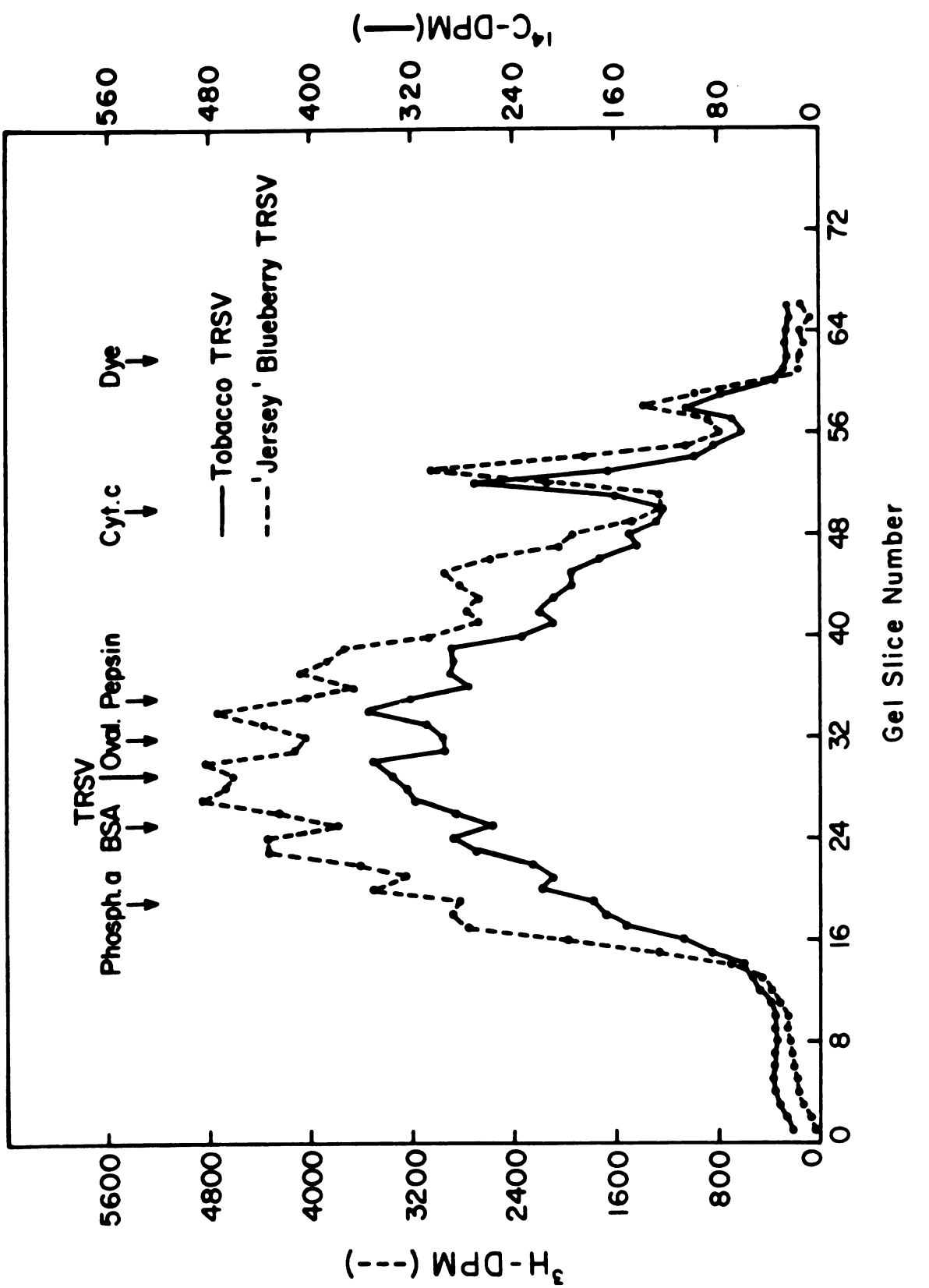

$^{14}$C-DPM(——)
560
480
400
320
240
160
80
0
Tobacco TRSV
'Jersey' Blueberry TRSV
Dye
Cyt.c
Ovd. Pepsin
TRSV
BSA
Phosph.a
5600
4800
4000
3200
2400
1600
800
0
$^{3}$H-DPM (---)
0
8
16
24
32
40
48
56
64
72
Gel Slice Number

FIGURE 23.--SDS-polyacrylamide gel electrophoresis in *in vitro* translation products of TRSV-RNA from the tobacco isolate (——) and the soybean isolate (---). *In vitro* protein products were made in the wheat embryo system using $^{3}H$-valine when translating the RNA from the soybean isolate, and $^{14}C$-valine when translating the RNA from the tobacco isolate. The protein products from these two isolates were then mixed, denatured, and separated electrophoretically in the same gel for 60 to 90 minutes at 8 milliamps per gel. The migration position of the molecular weight markers is indicated by the arrows. Gels were sliced and counted simultaneously for $^{14}C$ and $^{3}H$ radioactivity. Migration is from left to right.

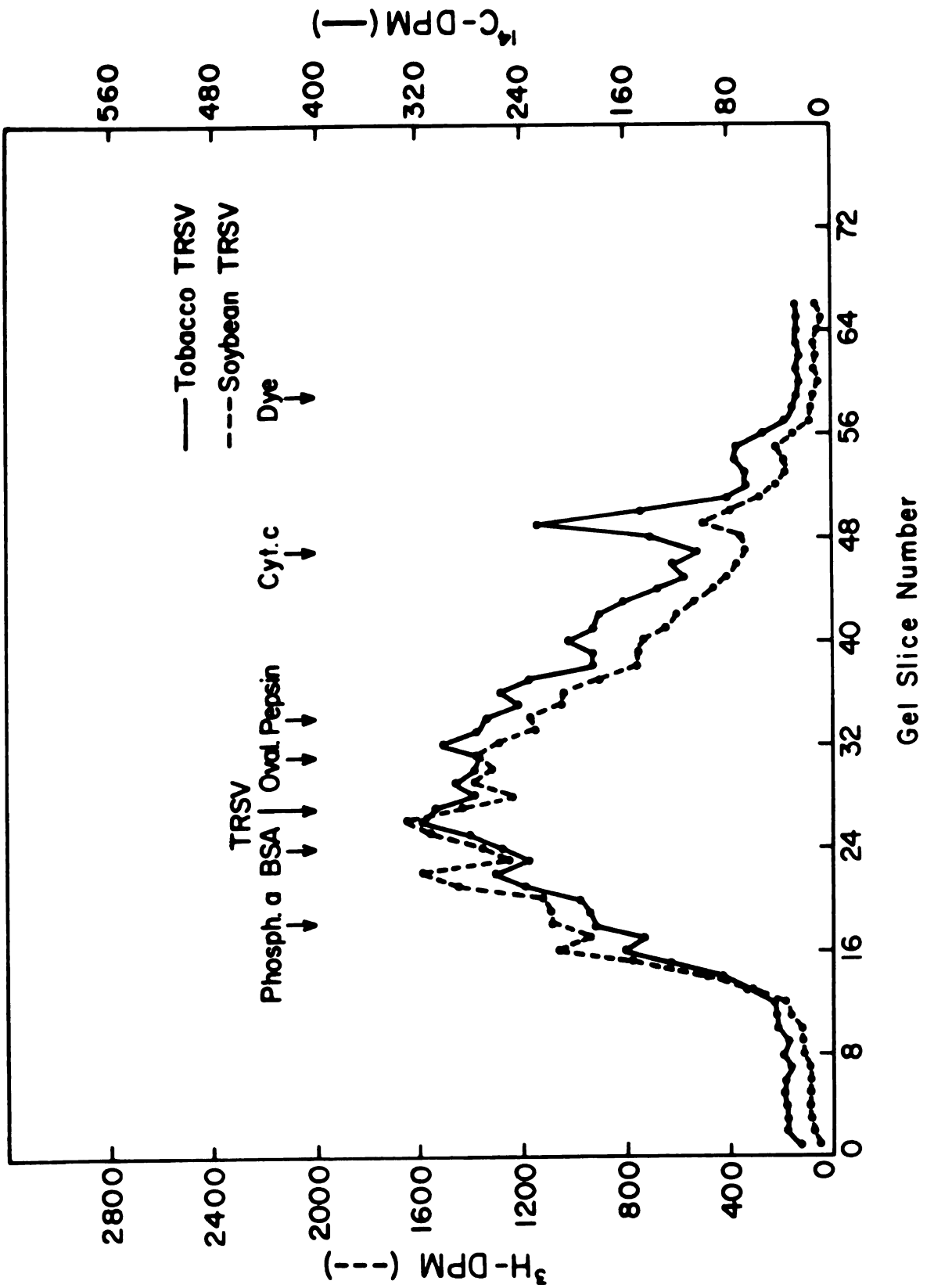
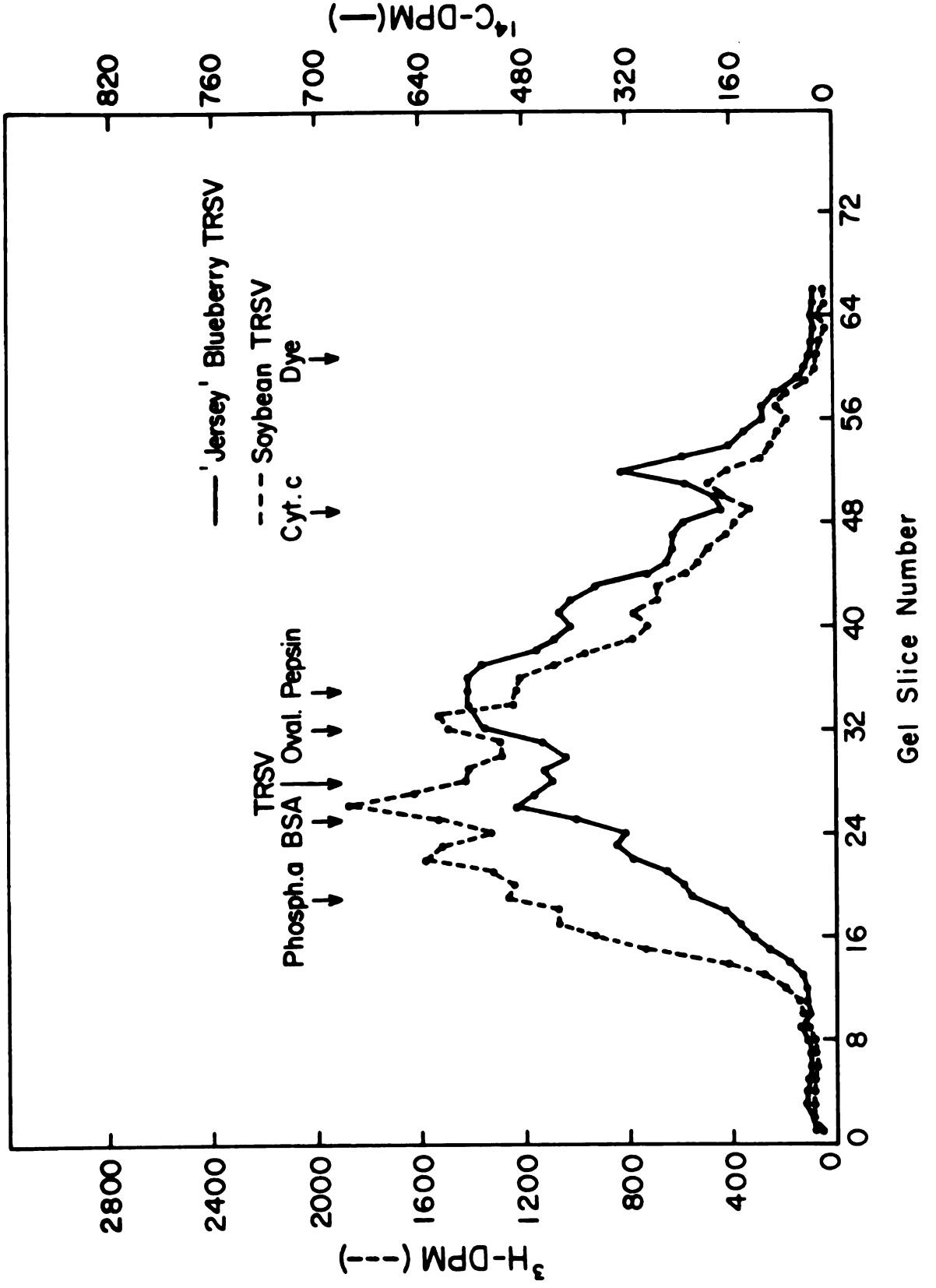

Tobacco TRSV
Soybean TRSV
Dye
Cyt.c
Oval. Pepsin
TRSV
BSA
Phosph. a
$^3$H-DPM (---)
$^{14}$C-DPM (—)
Gel Slice Number
0
400
800
1200
1600
2000
2400
2800
80
160
240
320
400
480
560
8
16
24
32
40
48
56
64
72

FIGURE 24.--SDS-polyacrylamide gel electrophoresis in *in vitro* translation products of TRSV-RNA from the 'Jersey' blueberry isolate (——) and the soybean isolate (---). *In vitro* protein products were made in the wheat embryo system using $^{3}H$-valine when translating the RNA from the soybean isolate, and $^{14}C$-valine when translating the RNA from the 'Jersey' blueberry isolate. The protein products from these two isolates were then mixed, denatured, and separated electrophoretically in the same gel for 60 to 90 minutes at 8 milliamps/gel. The migration position of the molecular weight markers is indicated by the arrows. Gels were sliced and counted simultaneously for $^{14}C$ and $^{3}H$ radioactivity. Migration is from left to right.


'Jersey' Blueberry TRSV
Soybean TRSV
Dye
Cyt.c
Oval. Pepsin
TRSV
BSA
Phosph.a
$^{14}$C-DPM(—)
$^{3}$H-DPM (---)
Gel Slice Number
0
160
320
480
640
700
760
820
400
800
1200
1600
2000
2400
2800
8
16
24
32
40
48
56
64
72

marker. The protein product of the tobacco isolate is slightly larger than the corresponding protein product of the 'Jersey' blueberry isolate. In the comparison between the 'Jersey' blueberry isolate and the soybean isolate, this protein product also differed in molecular weight, the protein product of the soybean isolate being slightly larger than that of the 'Jersey' blueberry isolate. Although the size of this protein product was smaller for the 'Jersey' blueberry isolate when compared with both the soybean and tobacco isolate product, there was no detectable difference in the molecular weight of this protein product when the soybean and tobacco isolates were compared (Figure 23).

The in vitro protein products of the different isolates of TRSV were sometimes strikingly different in the relative amounts of the proteins synthesized. For instance, a comparison of the profiles of the protein products from the 'Jersey' blueberry isolate and the soybean isolates of TRSV (Figure 24) shows that more of the smaller molecular weight products were synthesized when RNA from the 'Jersey' blueberry was used as messenger RNA in the wheat embryo cell-free synthetic system. The shifts in the protein product profiles to larger or smaller molecular weight proteins did not appear to be a function of the TRSV isolate used as the source of RNA.

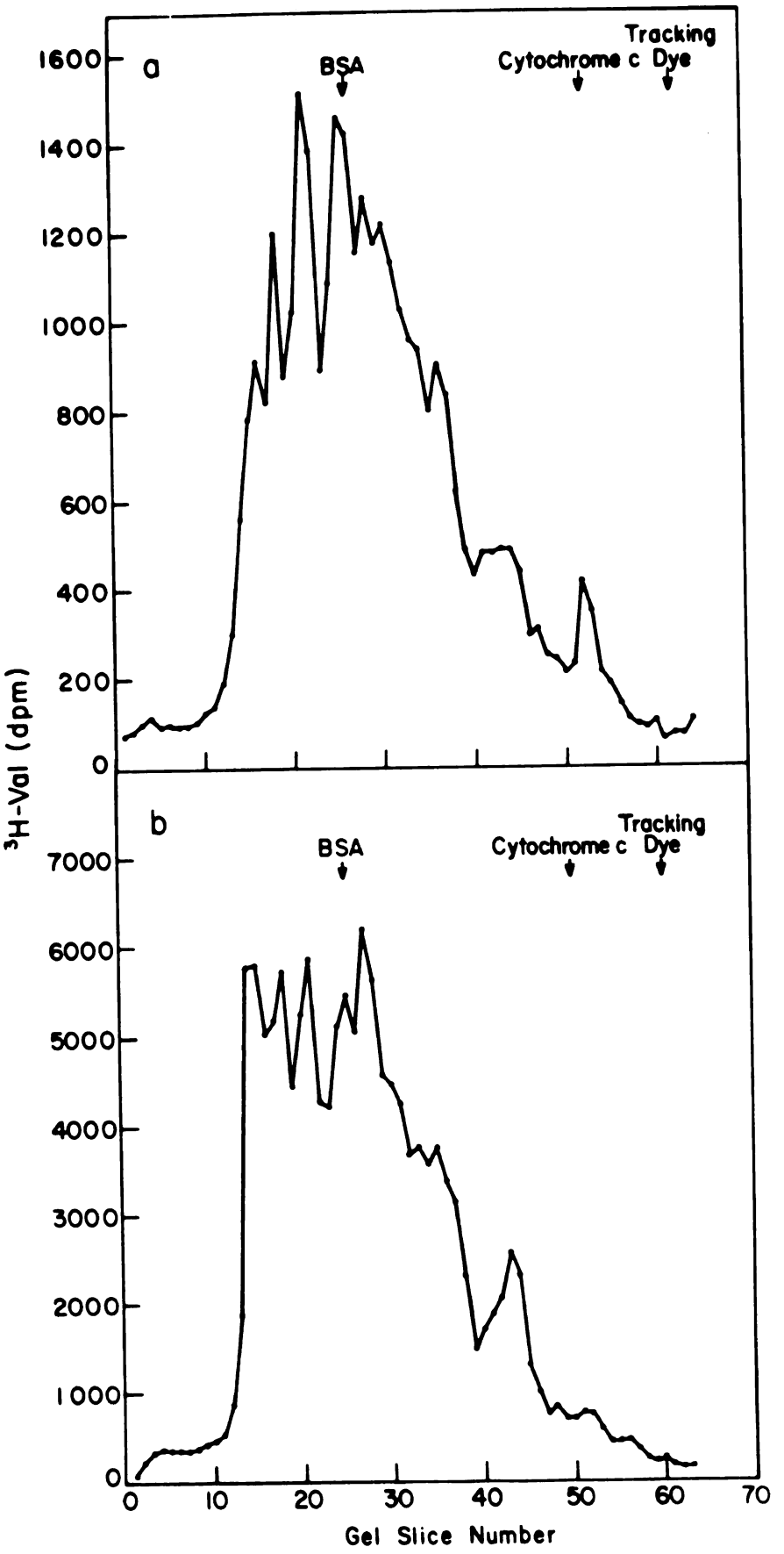
## Protein Products Made by RNA-2 of TRSV

When RNA-2 of TRSV was translated in the wheat embryo synthetic system, the translation efficiency of RNA-2 was about three times that for the unfractionated TRSV-RNA from which the

small RNA had been isolated, even though equal amounts of RNA were used. The SDS-polyacrylamide gel profiles of protein products made using unfractionated TRSV-RNA, and using RNA-2 are given in Figure 25. The unfractionated RNA used in this protein synthesis was from the same RNA preparation from which the RNA-2 was isolated.

One important feature of Figure 25 is that the largest translation product of RNA-2 has a molecular weight of about 130,000. A product of this size represents between 81 and 93% of the total coding capacity of RNA-2 of TRSV. Another important aspect of the profiles of protein products from unfractionated TRSV-RNA and the RNA-2 of TRSV is the absence, in the RNA-2 profile, of the protein product which migrates just ahead of the standard marker cytochrome-c. It appears that RNA-2 is capable of inducing the synthesis of the same polypeptides as unfractionated TRSV-RNA with the exception of one polypeptide of molecular weight about 10,500 daltons.

FIGURE 25.--SDS-polyacrylamide gel analysis of the products of translation of (a) unfractionated TRSV-RNA, and (b) RNA-2 of TRSV. Electrophoresis was carried out for 60 to 90 minutes at 8 milliamps/gel. The migration position of molecular weight markers is indicated by the arrows. Migration is from left to right.


a
BSA
Tracking
Cytochrome c Dye
1600
1400
1200
1000
800
600
400
200
0
b
BSA
Tracking
Cytochrome c Dye
7000
6000
5000
4000
3000
2000
1000
0
$^3$H-Val (dpm)
0
10
20
30
40
50
60
70
Gel Slice Number

# DISCUSSION

TRSV isolates can be clearly differentiated using several biological and physical-chemical criteria. This seems to be especially true for the isolates from 'Jersey' blueberry and from soybean which were found to be quite different from the other TRSV isolates in a number of properties studied.

The seven isolates of TRSV used in this study showed a range of virulence on the herbaceous indicator plants tested. The soybean isolate generally produced the mildest symptoms, and the 'Jersey' blueberry isolate produced the most severe symptoms. It is of interest to speculate on the natural selection pressures which might have caused the difference in virulence seen in these two isolates of TRSV. The occurrence of TRSV in commercial plantings of soybean is thought to be mostly due to the use of infected soybean seed (58). TRSV-infected soybean seeds germinate and grow into infected seedlings, which produce seed that may be TRSV-infected. The perpetuation of this disease cycle is dependent on the survival of the TRSV-infected seedling to a point where it produces viable seed. An extremely virulent isolate of TRSV would stunt and debilitate the soybean seedling to such an extent that production of infected seed would be unlikely. Relatively mild isolates of TRSV would be more likely to survive and reproduce in commercial soybean

production. On the other hand, transmission of TRSV in woody plants, such as blueberries, is thought to occur by nematode transmission, or through the use of infected propagating material; seed transmission is of little importance (64). The selection pressure on a TRSV isolate which is transmitted by nematodes from one plant to another, or by man through propagation, would be quite different from the selection pressure on a seed-borne TRSV isolate, and would not necessarily select for variants which produced a mild, non-lethal disease in the host plant.

Although there was only a small difference in the thermal inactivation temperatures of the seven isolates of TRSV (Table 2), it is interesting to note the correlation between the resistance to thermal inactivation and the virulence of the TRSV isolates on herbaceous hosts. The 'Jersey' blueberry, 'Riesling' grape, and watermelon isolates were the most resistant to inactivation by heating and were also the most virulent on most of the herbaceous plants tested. In contrast, the soybean isolate, which induced the mildest symptoms on the herbaceous hosts, was one of the isolates most sensitive to thermal inactivation.

The results of the spur formation tests (Table 3) indicate that the coat protein of the 'Jersey' blueberry isolate has at least one antigenic determinant which is not present on the other TRSV isolates. The watermelon isolate and the soybean isolate also appear to have unique antigenic sites in their coat proteins when compared to some of the other TRSV isolates. The lack of spur formation in the remaining isolates of TRSV indicates that these

isolates are not different in any surface antigenic determinants, at least that can be detected by spur formation tests.

The interpretation of the spur formation test results can best be explained by analyzing the comparison between the 'Jersey' blueberry isolate and the two isolates from soybean and cherry. Spurs were formed between the 'Jersey' blueberry antigen and the cherry antigen. Spurs were also formed between the 'Jersey' blueberry antigen and the soybean antigen when reacted with the 'Jersey' blueberry antiserum. This means that the 'Jersey' blueberry isolate has an antigenic site not shared with the cherry antigen, and that the 'Jersey' blueberry isolate has an antigenic site not shared by the soybean isolate. The possibility exists that the cherry isolate possesses an antigenic site not present on the 'Jersey' blueberry isolate. No spur was formed between the cherry antigen and the 'Jersey' blueberry antigen when reacted with the cherry antiserum. Therefore, all the antigenic sites found on the cherry isolate are also found on the 'Jersey' blueberry isolate.

The 'Jersey' blueberry antigen possesses an antigenic site not present on the soybean isolate. Again, the possibility exists that the soybean isolate possesses an antigenic determinant not present on the 'Jersey' antigen. A spur was formed between the soybean antigen and the 'Jersey' antigen when reacted with the soybean antiserum. Therefore the soybean isolate has an antigenic site which is not shared by the 'Jersey' blueberry isolate. This is in contrast to the cherry isolate which (as explained in the

preceding paragraph) possesses only antigenic sites which are also present on the 'Jersey' blueberry isolate.

From this example it is evident that an interpretation of spur formation tests to determine the serological relationship between isolates must include two tests for spur formation, i.e., both antisera to each of the isolates must be tested against the paired antigens. When dual comparisons such as this are made, it becomes evident that even though two isolates (such as the cherry and the soybean isolate) appear to be similar in that they both lack an antigenic determinant not present on a third isolate (such as the 'Jersey' blueberry isolate), they may also differ in that one of the two isolates may possess an antigenic determinant not present on the third isolate.

The results of the cross-absorption tests (Table 4) show that the blueberry isolate possesses at least one antigenic determinant which is not present on the soybean or tobacco isolates; this is in agreement with the results of the spur formation tests. The results of the cross-absorption tests also indicate that the tobacco isolate possesses at least one antigenic determinant not present on the soybean isolate. This conflicts with the results of the spur-formation tests which showed that the tobacco isolate did not possess any unique antigenic sites over those found on the soybean isolate. The reason for this discrepancy is not clear, but it may be due to the difference in the medium in which the antibody-antigen reaction takes place in these two tests. In the spur formation tests, the reaction takes place in an agar-gel

matrix, whereas in the cross-absorption tests the reaction takes place in an aqueous solution.

The 'Jersey' blueberry isolate and the watermelon isolate of TRSV both possess coat proteins which are serologically unique compared to the other TRSV isolates. These two isolates were also shown to be the most virulent on the different herbaceous hosts tested. It is not known whether the unique coat protein properties of these two isolates are responsible for their increased virulence in the herbaceous test plants.

Viruses which differ from one another in the composition of their coat proteins, as evident by serology, do not necessarily differ in the overall charge on their virus particles. When the electrophoretic mobility of the 'Jersey' blueberry isolate was compared to that of the soybean and tobacco isolates, it was evident that the virus particles of the 'Jersey' blueberry isolate were more negative in their overall charge than the virions of the soybean or tobacco isolates. Evidence from infection studies done with plant viruses in protoplasts indicates that the overall charge on the virus particle may be important in determining the virus-cell interaction during infection (25). The charge difference on the virions of the 'Jersey' blueberry isolate may be a contributing factor in the increased virulence of this isolate, especially if the charge of the virus particle is critical in determining cell-virion interactions in the plant. Although differences in electrophoretic mobiltiy have been used to characterize the strains of several viruses, such as BMV (28) and TMV (56), there is no

evidence to indicate that the overall charge of the virus particle influences its ability to cause disease in a host plant.

The RNA of TRSV is theoretically capable of coding for about seven times the amount of protein found in the coat protein of TRSV. For this reason, I synthesized the total protein products of TRSV-RNA in wheat embryo system, and then compared the *in vitro* protein products of three different isolates of TRSV. This allowed me to determine if there were protein differences, other than coat protein differences, which might be affecting biological properties of these TRSV isolates.

The results presented in this thesis for *in vitro* translation of TRSV-RNA in the wheat embryo system show that the RNA-2 of TRSV codes for a polypeptide of about 130,000 daltons. This accounts for roughtly 88% of the coding capacity of the RNA-2 of TRSV. There are as many as 9 other smaller polypeptides which are also produced when RNA-2 is translated in the wheat embryo system. Since the 130,000 dalton product accounts for about 88% of the coding capacity of RNA-2, the smaller polypeptides either represent proteolytic cleavage products of the 130,000 dalton product, or products from incomplete peptide synthesis. The addition of spermine and aurin tricarboxylic acid to the *in vitro* translation mix should have minimized the formation of incomplete synthesis of polypeptides.

An analysis of the protein products of tomato black ring virus (TBRV) (11), a nepovirus like TRSV, showed that the RNA-2 of TBRV was translated into a protein product of 160,000 daltons molecular weight. This represents an almost complete translation

product of the RNA-2 of this virus. As with other nepoviruses the RNA-2 is thought to code for coat protein (11,20), and if this is so, then the 130,000 dalton protein product of RNA-2 of TRSV must be processed by proteolytic cleavage to produce the 54,000 dalton coat protein.

Post-translational cleavage has been demonstrated in cowpea mosaic virus, a virus which resembles TRSV in many other respects (35). The existence of post-translational cleavage of the protein products of TRSV-RNA could be demonstrated by inhibiting protease activity through the addition of amino acid analogs or protease inhibitors to the wheat embryo translation system.

There does not seem to be a primary translation product of the RNA-1 ($2.2 \times 10^6$ daltons) of TRSV. Although it was not possible to separate RNA-1 from total TRSV-RNA, an examination of the protein products made with total TRSV-RNA shows a protein of maximum molecular weight of about 130,000 daltons, i.e., the primary translation product of RNA-2. When one compares the translation products made using RNA-2 (Figure 25), it is evident that the translation products of total TRSV-RNA contain only one additional protein product of about 10,500 daltons molecular weight. It appears that this 10,500 dalton product is coded by RNA-1 of TRSV when a mixture of the two RNAs from TRSV is translated in the wheat embryo system. This result must be interpreted with caution since RNA-1 has not yet been separated from total TRSV-RNA, and translated by itself in the wheat embryo system.

Schwinghamer and Symons (54) found that translation of unfractionated cucumber mosaic virus RNA gave essentially the same protein product profile as that found when RNA-4 (one of 4 RNA species of CMV) was translated alone. The translation products of the other 3 RNA species were produced only when these RNA species were translated separately in the wheat embryo system. By analogy, it may be that it is necessary to separate RNA-1 from total TRSV-RNA to produce the primary translation product of RNA-1 in the wheat embryo system.

In contrast to our results with TRSV, the translation of unfractionated TBRV-RNA (11) yielded a large protein of molecular weight about 200,000 daltons, much larger than any of the proteins produced when we translated unfractionated TRSV in the wheat embryo system. This 200,000 dalton protein product of TBRV may represent the primary translation product of the large RNA of TBRV. A corresponding protein representing the primary translation of the RNA-1 of TRSV was not detected when unfractionated TRSV-RNA was translated in the wheat embryo system. It appears that the 10,500 dalton protein product was the only protein product made by the RNA-1 of TRSV when RNA-1 and RNA-2 were translated together in the wheat embryo system.

Analysis of SDS-polyacrylamide gels separating proteins synthesized *in vitro* showed considerable variation in the relative amounts of the protein products when different RNA preparations were used as messenger RNA. There are several possible explanations which might account for shifts in the protein product profiles.

One possibility is that the RNA from some isolates of TRSV has properties which allow selective translation of certain proteins, resulting in a greater proportion of small or large molecular weight polypeptides. However, since two different RNA preparations from the same isolate of TRSV were capable of inducing the in vitro synthesis of polypeptides whose profiles were quite different (compare the soybean isolate product profiles in Figure 20 and Figure 23), this possibility seems unlikely.

A second possible explanation for the shifts in the relative amounts of the in vitro protein products is that the RNA is translated to produce a primary translation product which is subsequently cleaved to produce smaller molecular weight polypeptides. Variation in the amount of proteolytic cleavage of this primary translation product could account for changes in the relative amounts of the different protein products. Post-translational proteolytic cleavage has been demonstrated to be a function of proteases coded by the viral RNA, and also a function of endogenous proteases present in the in vitro protein synthetic system (43). Marcus, et al. (32) and Dr. James Asher (personal communication) both indicated that the amount of endogenous message in the wheat extract varies depending on the pH of the homogenate during the preparation of the wheat embryo extract. A higher pH resulted in release of the endogenous wheat embryo messenger RNA. If this endogenous messenger RNA is translated to produce an active protease, then varying amounts of endogenous messenger RNA could lead to varying amounts of proteolytic cleavage of other in vitro protein products.

A third possible explanation for the shifts in the in vitro protein product profiles involves the possibility that the RNA of TRSV has several initiation sites for protein synthesis. The availability of these sites for initiation may be dependent on secondary RNA structure, or on the presence of other factors which enhance or inhibit initiation at these sites (47). A subtle change in the secondary structure of the RNA, or in the concentration of these other factors which have been shown to affect initiation, could affect the availability of certain initiation sites for protein synthesis, and result in reduced or enhanced synthesis of a particular protein product. Selective degradation by ribonuclease could also lead to changes in the secondary structure of the RNA which might influence the availability of different initiation sites on the RNA.

A comparison of the molecular weights of the translation products from three isolates of TRSV showed that the molecular weight of the 10,500 dalton protein product was isolate-dependent. This protein product was detectably smaller when the protein products of the 'Jersey' blueberry isolate were compared to the protein products of either the tobacco or the soybean isolates (Figures 22-24). This isolate-dependent molecular weight difference in the 10,500 dalton protein product is a result of a difference in the RNA-1 of the TRSV isolates, since our evidence indicates that the 10,500 dalton product is coded by RNA-1. This is in contrast to the differences which are a function of coat protein differences and which are coded by RNA-2 of TRSV.

The 'Jersey' blueberry isolate of TRSV is more virulent than the soybean or tobacco isolates of TRSV. The 'Jersey' blueberry isolate also differs from the soybean and tobacco isolates of several properties which are a function of the coat protein of the virus. Since current evidence indicates that the genetic information for coat protein is found on RNA-2 of TRSV, these coat protein-dependent properties are probably a function of RNA-2 of the TRSV genome. In addition to the coat protein differences between these isolates, there is also an isolate dependent difference in the molecular weight of a gene product from RNA-1 of these isolates. It is not known which of the gene product differences, those from RNA-1 or those from RNA-2, is responsible for the increased virulence of the 'Jersey' blueberry isolate of TRSV.

Pseudorecombinant analysis has been used to determine which of the two RNAs of raspberry ringspot virus (RRSV) is responsible for various properties such as severity of systemic symptoms in various herbaceous hosts, serological specificity, and nematode transmissibility (20). In experiments with four naturally occurring strains of RRSV, virus hybrids were made by mixing RNA-1 and RNA-2 preparations from different strains. The genetic determinant for symptom severity in the herbaceous hosts was shown to be carried by RNA-1, the large RNA of RRSV, which does not carry the genetic information for coat protein. An analagous pseudorecombinant experiment with the isolates of TRSV in this study would reveal which of the 2 RNA species of the 'Jersey' blueberry isolate is responsible for the increased virulence of this isolate.

The manner in which the biological properties of viruses are determined by the virus genome is an important problem for plant virologists. To solve this problem it is necessary to know the number of proteins that a virus is capable of producing and also what the functions of these viral-coded proteins are. In this research I have identified some of the biological properties of several isolates of TRSV, and have also identified some of the unique biochemical properties of these TRSV isolates. It remains to be determined which of these unique biochemical properties that have been identified are responsible for the biological properties of these TRSV isolates.

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