Influence of Host and Seasonal Variation on the
Components of Tobacco Ringspot Virus

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ABSTRACT

When purified preparations of tobacco ringspot virus were centrifuged for 2.5 hr on buffered sucrose density gradients, each separated into four, three, or two components depending on the host in which the virus was increased and the season of the year when the host plant was infected. These components were designated 1, 2, 3, and 4 from top to bottom. Within a single season of the year, the type and the relative amounts of the components were determined by the five hosts used. The bulk of the infectivity was associated with component 3, and component 1 was not infectious. Unfractionated virus preparations were more infectious than component 3 when they were both diluted to the same A_{260} reading.

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Some spherical plant viruses consist of more than one component. Markham & Smith (9) first reported this when they found that purified preparations of turnip yellow mosaic virus (TYMV) produced two peaks when examined in the analytical ultracentrifuge. Since then the heterogeneity of purified preparations of other spherical plant viruses has been noted (1, 8, 14, 16). Tobacco ringspot virus (TRSV) belongs in the group of such multicomponent plant viruses. Steere (16) obtained two centrifugally separable components designated a major and infectious component and a minor but noninfectious component. In 1965, Stace-Smith et al. (15) obtained three components when he centrifuged purified preparations of TRSV on sucrose density gradients. Only the bottom component was infectious. They pointed out that the two top components originated from the degradation of the bottom component. Diener & Schneider (4) and Schneider & Diener (11, 12) increased TRSV in *Phaseolus vulgaris* 'Black Valentine' and purified the virus essentially by Steere's method (16). They obtained three components on sucrose density gradients. The relative amounts of the three were influenced by the age of infection. Only the bottom component was infectious. In contrast to the notion of Stace-Smith et al. (15) on the origin of the middle component, they concluded that the components were formed concurrently. Gilmer et al. (6) obtained a TRSV isolate from grapevine. They increased the virus in cucumber, and obtained only two components from purified preparations by sucrose density gradients.

Unquestionably, the number and the relative amounts of the components of TRSV or any of the other multicomponent spherical plant viruses are strain specific (3, 5). However, the variation of these properties within a strain as influenced by the host species and the season of the year the virus is increased has not been investigated for TRSV. This study was conducted, therefore, to determine what influence the host species and season of the year have on the number and relative amounts of the components in the purified preparations of TRSV isolated from gladiolus (*Gladiolus* sp.).

MATERIALS AND METHODS.—A strain of tobacco ringspot virus serologically related but not identical to the TRSV type strain isolated from a naturally infected gladiolus plant and designated TRSV(g) was used. The virus was maintained in Xanthi tobacco, which served as the source of inoculum throughout the investigation.

Host species and culture.—Representatives of three plant families were selected as host species for increasing the virus. These were tobacco (*Nicotiana tabacum* L. 'T. I. 787' and *Xanthi-nc*), Solanaceae; cucumber (*Cucumis sativus* L. 'Chicago Pickling') and Caserta squash (*Cucurbita pepo* L.), Cucurbitaceae; and Bountiful bean (*Phaseolus vulgaris* L.), Leguminosae. The composition and treatment of the soil used in this study were standardized (7). The two tobacco cultivars were grown in pots, and the other species were grown in flats.

Inoculation of plants.—Plants were grown so that the five plant species were ready for inoculation at the same time. In order to avoid variability in inocula, we inoculated representatives of all five plant species each time the inoculum was prepared. The inoculated leaves were harvested 5 days later. In certain cases, half an inoculated leaf was harvested on various days after inoculation, depending on the host species. The harvested leaves were frozen and the virus was purified.

Virus purification.—Steere's method (16, 17) of purification was adopted, but modified slightly in order to obtain a more stable and higher yield of virus. Frozen infected leaves were ground in a meat grinder and squeezed through two layers of cheesecloth. The residue was homogenized in a Waring Blendor in 0.02 M phosphate buffer, pH 7.2 to which 0.02 M sodium diethyldithiocarbamate (DIECA) and 0.02 M 2-Mercaptoethanol (2-MerEt) were added as stabilizers. The volume of buffer used was equivalent to half the fresh weight of the plant

tissue in milliliters. The two extracts were immediately combined and, from this stage on, Steere's method was followed.

Infectivity of sap from inoculated leaves of various host plants.—Sap infectivity was assayed on lima bean (*Phaseolus lunatus* L. 'Early Thorogreen'). Bountiful bean was used as the source of standard inoculum. Thus, several bean plants were inoculated (one plant each day) over a period of 9 consecutive days so that at the time of assay one bean plant inoculated 5 days previous to the assay was available to provide standard inoculum. One plant each in the case of Xanthi, T. I. 787, and bean, and two plants each in the case of squash and cucumber were later inoculated. Three days after inoculation, the infectivity assay was begun. One disc from one inoculated leaf of each of the plant species and one from the source of standard inoculum were cut with a No. 7 (1-cm-diam) cork-borer. Between samples, the cork-borer was sterilized by flaming. Each disc was ground in 1 ml 0.03 M phosphate buffer, pH 8.0. One half-leaf of lima bean was inoculated with the standard inoculum; the other half, with test inoculum. A total of four half-leaves were inoculated per test. Lesions were counted 5 days after inoculation, and infectivity was determined relative to the number of lesions produced by the standard.

Density-gradient centrifugation.—Linear gradients of 7-30% sucrose solutions in 0.02 M phosphate buffer, pH 7.2, were prepared by a modification of Stace-Smith's method (13). One ml of purified virus ($A_{260} = 10$) was layered on top of each of the gradients. The preparations were centrifuged for 2.5 hr at 25,000 rpm in a Spinco Model L2-65 ultracentrifuge at 18 C, using an SW 25.1 rotor. The gradients were then fractionated, and the fractions collected by means of an ISCO density gradient fractionator (2). Each fraction was given a high speed centrifugation and the pellets were resuspended in 0.02 M phosphate buffer, pH 7.2. The infectivity of each fraction was tested on lima bean without further density gradient centrifugation. Estimates of the relative amounts of the components in the fractions were made by measuring the height of the peaks from a common base line.

RESULTS.—Except when otherwise stated, these results are those of virus preparations from leaves of different species that had been inoculated the same day and harvested the same day. This was done in order to avoid variability that might be due to differences in age of infection.

When purified preparations of TRSV(g) from inoculated leaves were centrifuged for 2.5 hr on buffered sucrose density gradients, each separated into four, three, or two components, depending on the host used and season of the year. These components are designated 1, 2, 3, and 4 from top to bottom (Fig. 1). Components 1, 2, and 3 had approximately the same sedimentation coefficients as those reported by Stace-Smith (14) and Schneider & Diener (11, 12) for the top, middle, and bottom components, respectively, of TRSV. The ultraviolet light source in the analyzer used in all fractionation
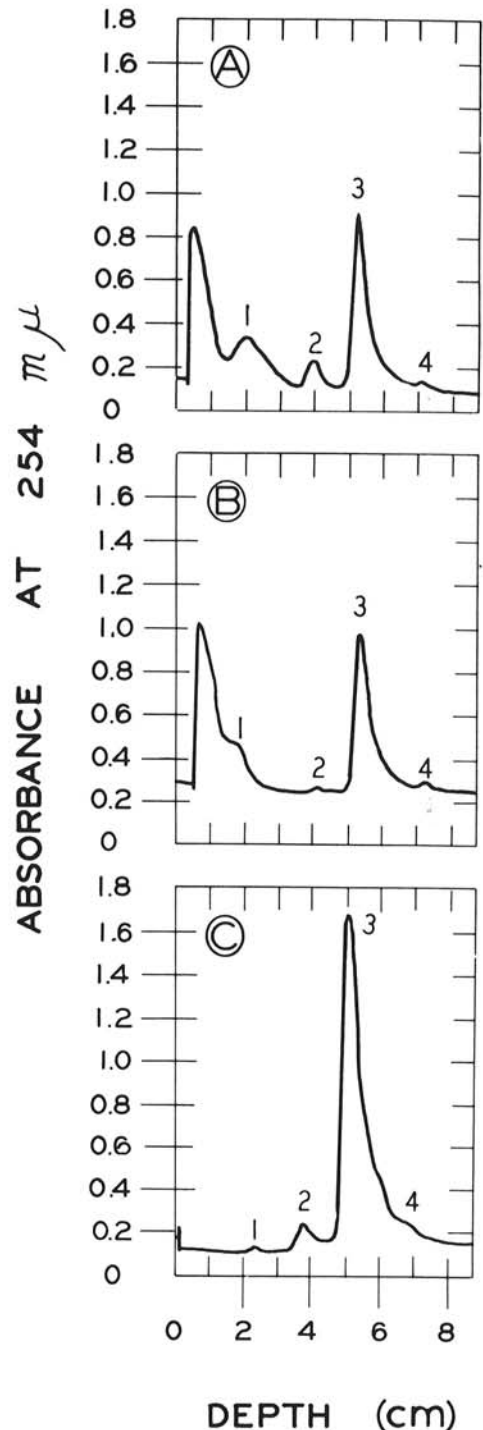

Fig. 1. Profiles of the four components in purified preparations of tobacco ringspot virus [TRSV(g)] from **A)** T.I. 787 tobacco; **B)** Xanthi tobacco; and **C)** Bountiful Bean in early spring.

experiments operated at 254 nm. At this wave length, the effect of light scattering on quantitation was determined, following the method of Treiber &
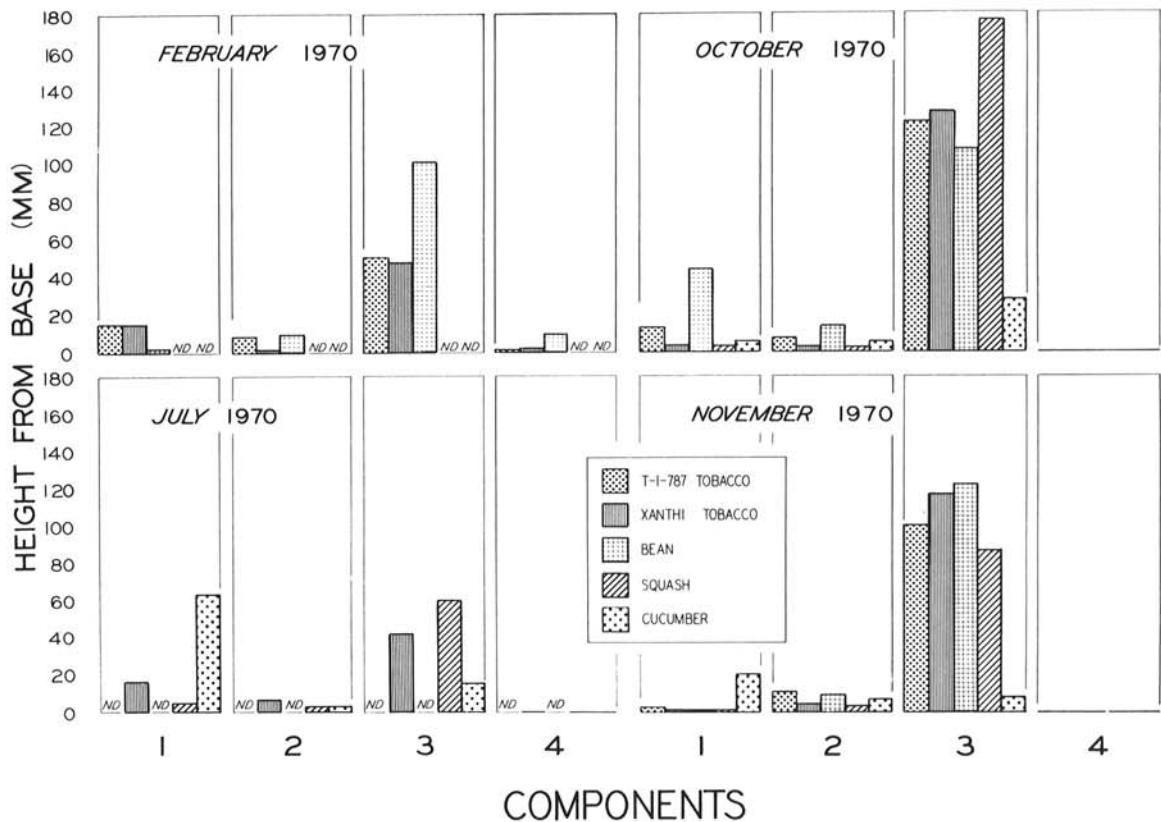
Fig. 2. Relative amounts of components in purified preparations of tobacco ringspot virus [TRSV(g)] from five host species during four periods of the year. ND = data not included because of the unsatisfactory virus recovery from the infected plants.

Schauenstein (18), and found to be negligible at the virus concentrations used (10). The results reported here are based on three to six experiments in each of the four seasons. Representative data were selected for the illustrations.

Virus.—The $A_{260/280}$ ratios of the virus preparations from the different hosts were between 1.60 and 1.85. The ratios always increased from component 1 to 4, with component 4 always having the highest ratio.

The ratio of component 1 $A_{260/280}$ was often higher than 1.3. It was assumed that this was due only to RNA contamination, because the sedimentation coefficient and lack of infectivity indicated similarity with the top component of TRSV reported by others (11, 12, 14, 15).

Comparison of components among hosts.—At any one period of the year, the amount of each component varied from host to host. This variation was more striking between virus preparations from squash and cucumber, and less striking between preparations from the two cultivars of tobacco (T.I. 787 and Xanthi). At any one period, component 3 accounted for the highest peak in preparations from all of the host species except cucumber. Figures 1 and 2 show representative results obtained for virus preparations from different host species at different periods. Data for preparations from certain hosts

were not included because the virus concentration in each of them was less than the standard absorbancy of 10 at 260 nm that was used throughout the experiment.

Comparison of components within host and among periods of the year.—In any one host and at one season of the year, the amount of individual components varied from preparation to preparation, but in most cases, the further apart the preparations, the bigger the variations. This variation was more striking in preparations from certain hosts (T.I. 787 and Xanthi tobacco) than from other hosts, in that components 1 and 3 showed greater variability among seasons than did component 2. Figure 2 shows representative data for four periods. Data for preparations in certain periods of the year were not included because of unsatisfactory virus recovery from infected plants. Within any one host, the type of components present and their amounts were determined by the period of the year when the particular host was inoculated. Figure 3 shows representative data for preparations from T. I. 787 tobacco.

Infectivity of various components.—When the components were diluted to the same virus concentration of $A_{260} = 0.005$, it was found that while other components were infectious, component 1 was not infectious even at absorbancy readings
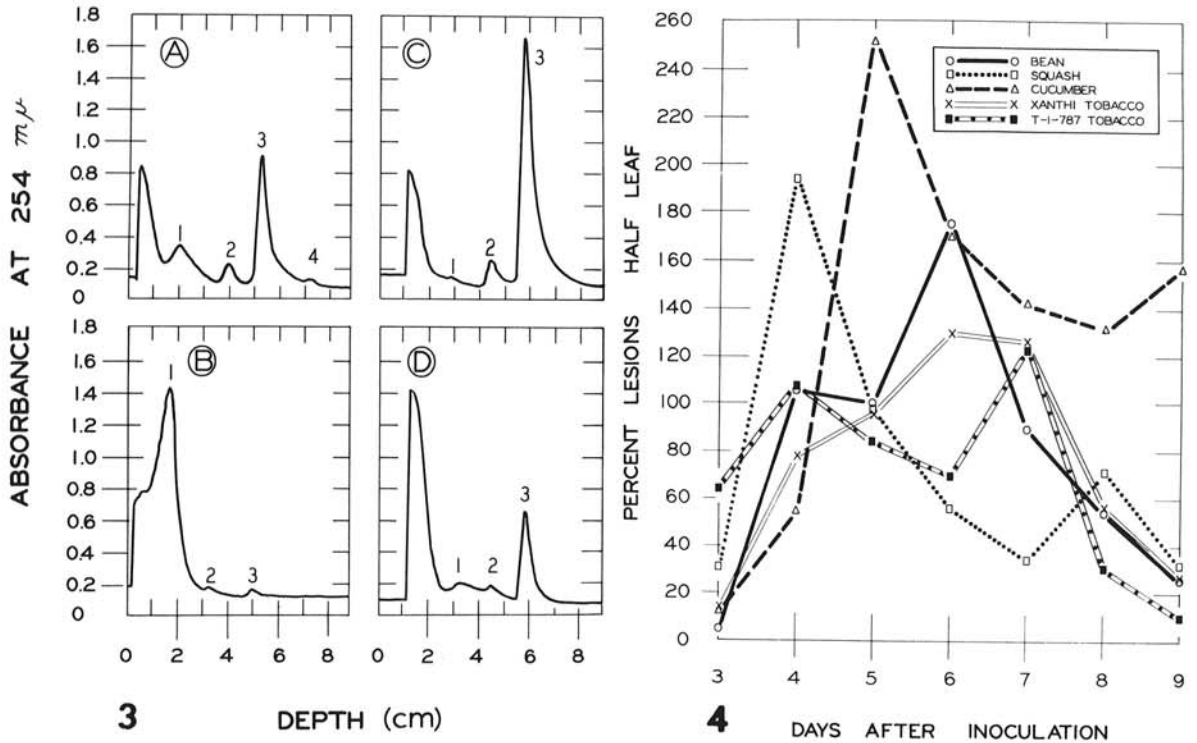
Fig. 3-4. 3) Profiles of the components in purified preparations of tobacco ringspot virus [TRSV(g)] from T.I. 787 tobacco in **A)** February 1970; **B)** August 1970; **C)** November 1970; **D)** January 1971. **4)** Trends in infectivity of sap from leaves of various host plants inoculated with TRSV(g) observed during a 9-day period. These results are averages of two trials.

higher than 0.005. The bulk of the infectivity was associated with component 3 whereas components 2 and 4 were equally infectious. In all of these infectivity tests, A_{260} = 0.005, of corresponding unfractionated virus preparations were used as standards. In all the five hosts except cucumber, the unfractionated preparation was more infectious than any of the components as shown in Table 1. In cucumber, however, it was found that component 3 was always more infectious than the unfractionated virus preparation (Table 1).

Infectivity of sap from inoculated leaves of various host plants.—In summer, two problems were encountered: (i) the failure to obtain sufficient virus in many of the purifications; and (ii) that the $A_{260/280}$ ratios of many of the preparations were consistently 2.0 or more at these times. In the latter case, when these preparations ($A_{260/280}$ = 2.0) were centrifuged on density gradients, the amounts of components 2 and 3 were greatly reduced, whereas the bulk of the material layered on the gradients remained at the meniscus or separated as component 1 as shown in Fig. 3-B. Subsequently, attempts were made to find out to what degree infectious virus was produced in inoculated leaves of different hosts in the summer months. Bountiful bean was used as the standard in this investigation because it was found earlier that purified preparation of TRSV(g) from this host subjected to density-gradient centrifugation separated into four components, with infectious component 3 being very large, with a lack of

appreciable absorption at the meniscus. Titers of infectious virus in different hosts were high in all inoculated leaves while the peaks of infectivity fell on different days after inoculation (Fig. 4).

Comparison of components in preparations from leaves harvested on different days.—In view of the above, an attempt was made to compare 2 harvesting days within a host with regard to virus yield. As indicated previously, half of each inoculated leaf was harvested 5 days after inoculation, and the other half on days corresponding to the day of highest infectivity (Fig. 4) of the sap from such a host. Table 2 shows a representative result. In the case of cucumber, however, the day of highest sap infectivity happened to be 5 days after inoculation, and therefore the same data for preparations from that host were presented in Table 2. Components 2 and 3 in tobacco (T.I. 787 and Xanthi) and bean were larger in amount when purified preparations were from inoculated leaves harvested on days of highest sap infectivity than when such preparations were from leaves harvested 5 days after inoculation. The same trend was found for component 1 in beans, whereas the reverse was the case with T.I. 787 tobacco. Since the bulk of infectivity is associated with component 3, the large amount of this component in preparations from leaves harvested on days of highest sap infectivity may account for the high sap infectivity recorded for such days.

DISCUSSION.—It is common to find communications which differ in the number of

TABLE 1. Representative data on infectivities of various components of tobacco ringspot virus from gladiolus

Virus components	Average lesions/half leaf. Inoculum A_{260} = .005				
	T.I. 787 tobacco	Xanthi tobacco	Bean	Squash	Cucumber
1	0	0	0	0	0
2	10	10	3	1	0
3	34	43	29	24	16
4	9	16	3		
Unfractionated virus	116	171	105	47	6

TABLE 2. Heights (mm) of component peaks in density gradient scans of tobacco ringspot virus preparations from leaves of four hosts harvested on different days

Host species	Components			
	1	2	3	4
Data for leaves harvested 5 days after inoculation				
T. I. 787 tobacco	11	5	19	
Xanthi tobacco		3	99	
Bountiful bean	2	3	79	
Cucumber	60	7	17	
Data for leaves harvested on days of highest sap infectivity				
T. I. 787 tobacco	7	6	37	
Xanthi tobacco		5	104	
Bountiful bean	15	9	93	
Cucumber[a]	60	7	17	

[a] Data presented twice because the day of highest sap infectivity was 5 days after inoculation.

components reported for purified preparations of a particular multicomponent plant virus. Granting that the number and in fact the relative amounts per unit concentration of purified preparations of such a virus may be strain-specific, it is often overlooked that, working with the same strain of a multicomponent plant virus, host species in which the virus is increased and the period of the year when such hosts are inoculated may determine which components are present and in what amounts. This study shows that with the same strain of TRSV, (i) the type of components that are present and their amounts, in particular periods of the year, are determined by the hosts that are used to increase the virus; and (ii) when using the same host to increase the virus, the type of components that are present and their amounts are determined by the period of the year when the particular host is inoculated.

The fact that preparations from squash and cucumber in early spring had less virus than the standard (A_{260} = 10) made the inclusion of data from them unrealistic. As a result of this, no categorical statement can be made on the presence or absence of any of the four components in these two hosts, at least for that period of the year. However, the absence of component 1 in preparations from

Xanthi tobacco and its presence in preparations from other hosts (Table 2) indicate that within a particular period of the year, the type of components present is determined by the host in which the virus is increased. The presence of four components in preparations from tobacco (T.I. 787 and Xanthi) and bean in one period of the year and the absence of the fourth component in the preparations from these same hosts during other periods of the year are regarded as sufficient evidence in support of the reverse statement that, in a particular host species, the type of component that is present is determined by the period of the year when the host species is inoculated.

Although the selection of the host species used for this study was based broadly on earlier results of the host range studies (7), the final selection was made so that it would be possible to study the variation in component production between members of a plant species, members of a plant family, and members of different families. If the influence of host species on relative amounts of various components of TRSV(g) is real, results obtained for preparations from the two tobacco varieties should show close similarities, whereas fewer similarities would be expected for preparations from squash and cucumber. If the results

in early spring are considered, it will be seen that about equal amounts of components 1, 3, and 4 were recorded for the two tobacco varieties, whereas figures for corresponding components in bean were either lower or higher. In other seasons where data for squash and cucumber are included, it will be seen that less similarities existed between those results. In fact, results for preparations from cucumber were in most cases peculiar, and were at variance with the results for preparations from squash. The existence of these similarities between cultivars, and the lack of similarity between representatives of two different genera are taken as evidence that the influence of the host on the relative amounts of components of TRSV(g) is real, using our purification methods.

The peculiarity of the results for preparations from cucumber deserves some consideration. In all seasons of the year, preparations from cucumber had $A_{260/280}$ ratios of 2.0, a figure considered to be too high for TRSV (expected $A_{260/280} = 1.55$-1.88). Also, when such preparations from cucumber were centrifuged on sucrose gradients, the bulk contributed to absorption at the meniscus and of component 1, while very low amounts of components 2 and 3 were present in the preparations. As a, consequence of this, the infectivity of component 3 from cucumber was higher than the infectivity of the unfractionated preparation diluted to the same A_{260} reading ($A260 = 0.005$). This was in contrast to results from other hosts, where the unfractionated virus preparation was more infectious than the component 3 fraction diluted to the same A_{260} reading. This suggests that the bulk of the virus particles produced in cucumber were the noninfectious component-1 particles. It could, therefore, be expected that sap from inoculated cucumber leaves would show a lower infectivity. However, results of studies of sap infectivity (Fig. 4) show that sap from cucumber was the most infectious, as compared with similarly extracted juice from other hosts. This indicates that large numbers of infectious virus particles were produced in the inoculated leaves of cucumber that were not extracted quantitatively during the purification procedure. However, it fails to explain why the high $A_{260/280}$ ratios occur. Possibly a large amount of host ribonucleic acid (RNA) was extracted along with the virus particles during the purification process. This host RNA diluted the virus greatly, and thus resulted in false absorption readings which were taken as a measure of virus concentration. If this is correct, it then explains the high $A_{260/280}$ ratios and the high absorption in the meniscus. The question as to why the method used in the purification of this strain of TRSV should preferentially extract large amounts of RNA from cucumber and not from other hosts remains unanswered. A different method of virus purification may be required for TRSV(g) increased in cucumber.

The low amounts of virus extracted in summer may indicate a failure of each of the hosts to produce sufficient virus particles during that period. Infectivity studies do not support this view. However,

the virus particles may not have been extracted quantitatively during the normal purification process. This indicates the need for a different method of purification for TRSV(g) during the summer months. Where the amounts of components 2 and 3 are reduced in summer, a large amount of host RNA seems to be extracted together with virus particles during those months of the year.

Since there was no further purification of each of the components on sucrose gradients, we could not be sure that any one of them was free of contamination from any of the others. In view of this, we are not able to say which of the components was actually infectious, but the bulk of the infectivity was associated with component 3. The unfractionated preparations were more infectious than the most infectious component 3 except in the case of cucumber. This indicates that enhancement of infectivity occurred in mixed component preparations, but in which component combinations was not studied.

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