Alteration of Tobacco Streak Virus Component Ratios as Influenced by Host and Extraction Procedure

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ABSTRACT

For two strains of tobacco streak virus, the proportions of the nucleoprotein components and the total yield of nucleoprotein varied with host and the precise extraction procedures. For the bean red node strain, *Chenopodium quinoa* tissue extracted directly at pH 5 yielded more total nucleoprotein and a greater proportion of a slowly-sedimenting "top component" than did tissue extracted at pH 7 and subsequently adjusted to pH 5 for clarification. With *Phaseolus vulgaris* as the source, precisely the reverse situation applied. The ratio of nucleoprotein components could be varied by adding healthy leaves to infected leaves of the heterologous host before extraction, but not by mixing virus with various leaf-extracts or by blending purified virus with leaves. The virus was electrophoretically homogeneous. Nucleoprotein component proportions were unaffected by the use of detergent, prolonged extraction, or re-extracting fiber. Oxidative reactions and possible direct effects of pH on virus during extraction did not appear to be a factor. All the evidence suggests that unexplained pH-mediated and host-regulated effects governed extraction per se. Phytopathology 60:689-694.

In the course of investigations on the spherical multicomponent tobacco streak virus (TSV) (7) and the symptomatologically-distinguishable bean red node strain (TSV-RN) (12), we have noted that the nucleoprotein component ratios of purified preparations may vary in uncommon ways. Fulton (7), using a purification schedule involving clarification by hydrated calcium phosphate and antiserum to host protein, found that TSV component ratios were strain-specific, but were not influenced by the host (tobacco or cucumber), the period of infection, or the age of the purified preparation. With TSV-RN obtained from unbuffered bean sap after charcoal treatment, Mink et al. (12) reported variation in nucleoprotein content and possibly ratio after different infection times. Our results with these viruses suggest a potentially more complex situation regarding the yields of various components than was previously realized. Using a simple purification schedule capable of producing high yields of TSV from various hosts, we have found that, at least with TSV-RN, the proportions of various virus-specific nucleoproteins can be varied not only by the choice of host but also by the exact protocol used for extraction and clarification with a particular host. We cannot explain the effect, but describe it in view of the special interest of TSV, in that its components exhibit a host-dependent infectivity-enhancement (7). Some of our results have been summarized in an earlier abstract (10).

MATERIALS AND METHODS.—TSV and TSV-RN were supplied by R. W. Fulton and G. I. Mink, respectively. TSV and TSV-RN affected some hosts differently under our conditions. Both TSV and TSV-RN infected inoculated leaves of tobacco and *C. quinoa,* but TSV more readily infected tobacco systemically and TSV-RN more readily infected *C. quinoa* systemically. Both strains infected bean, but TSV gave local lesions only, while TSV-RN caused the typical severe systemic "red node" disease (17). TSV was usually grown in bean (*Phaseolus vulgaris* L. 'Bountiful') and tobacco (*Nicotiana tabacum* L. 'Burley' and 'Xanthi'), and assayed on bean. TSV-RN was usually grown in bean and *Chenopodium quinoa* Willd., and assayed on *C. quinoa. Chenopodium quinoa* was not entirely satisfactory, as lesions spread readily and became uncountable, but bean (12) did not produce lesions consistently under our conditions.

Purification, detailed under RESULTS, involved extraction in buffers (0.1 M phosphate or 0.2 M acetate) at various pH values, followed by clarification by adjustment to pH 5 and cycles of differential ultracentrifugation of the clarified extracts. Enough buffer (generally 1:2.5 w/v) was used to maintain pH within 0.2 pH units of the nominal pH value. The extraction medium always contained 0.01 M diethyldithiocarbamate and 0.02 M thioglycollate or mercaptoethanol. The best yields of specific nucleoprotein obtained (see RESULTS) were about 10-15 mg/100 g of leaf, assuming $A^{0.1\%}_{260\ m\mu} = 5$. The proportions of the different nucleoprotein components were determined by Schlieren observation in a Spinco Model E analytical centrifuge. Preparations were also examined, and fractions collected when required, by using an ISCO analyzer to scan rate-zonal density-gradient centrifugations of samples on 10-40% sucrose gradients in Spinco SW 25 tubes (3).

Free boundary electrophoresis was done in a Perkin-Elmer Model 38-A electrophoresis apparatus using the 2-ml cell. Other procedures used are noted by the appropriate experiment for clarity.

RESULTS.—*General characteristics of purified preparations.*—Preparations of either virus were resolvable as three overlapping but distinct nucleoprotein components sedimenting at 90, 98 and 113 *S* (top, middle and bottom, Fig. 1). Electron-microscopic examination showed that each contained spherical particles, but absorbancy measurements for a typical preparation (Table 1), showing slight light scattering at A_{320}, showed $A_{260/280}$ ratios of 1.56, 1.60, and 1.65 for top, middle, and bottom components, respectively. Despite the limitations imposed by poor separation of

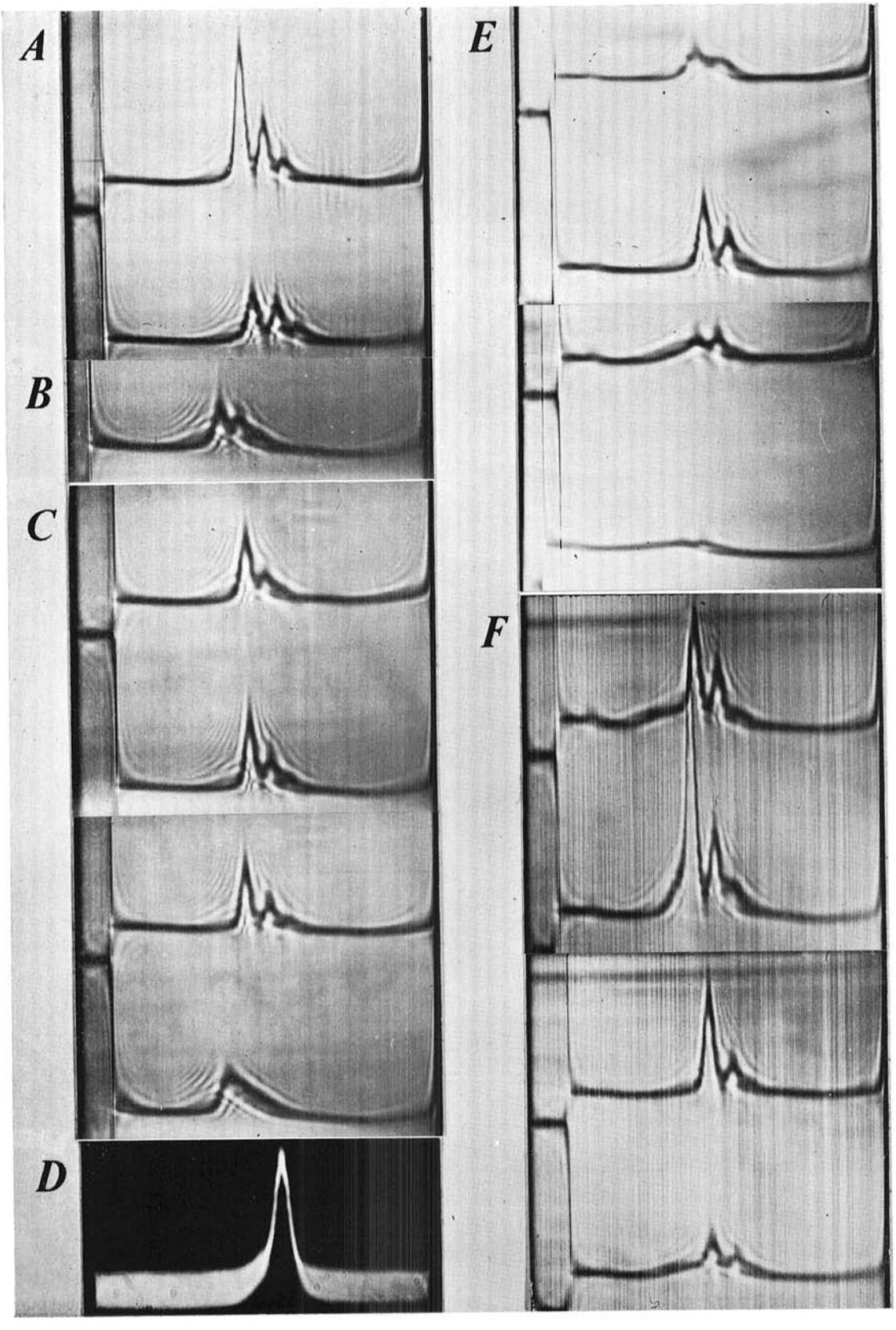
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Fig. 1. Schlieren patterns of analytical ultracentrifugations of purified preparations of tobacco streak virus—bean red node strain, showing effects of **A)** extraction at pH 7 (upper) or pH 5 (lower); **B)** dialyzing product from pH 5 extraction to pH 7; **C)** dialyzing product from pH 7 extraction to (top to bottom) pH 8.5, pH 7.5, pH 4, or pH 3; **E)** extraction at (top to bottom) pH 8, pH 7, pH 5, or pH 4; **F)** extraction from *Chenopodium quinoa* (upper patterns) at pH 7.0 (top) or pH 5.0 (bottom), or bean (lower patterns) at pH 7.0 (top) or pH 5.0 (bottom). All samples were centrifuged in 0.1 M acetate or 0.05 M phosphate as appropriate. Photographs were taken between 9 and 11 min after reaching a speed of 35,600 rpm. Bar angle used was 50 degrees. Comparisons of yields are valid within each sample set. **D)** The electrophoretic pattern (descending limb) shown by TSV-RN, prepared using pH 7 extraction, after 4-hr electrophoresis at 5.96 v/cm in 0.1 μ pH 5 acetate buffer.

TABLE 1. Infectivities of various tobacco streak virus components used alone and mixed

Inoculum[a]	Lesions[b]/sample tested		
	Exp. 1	Exp. 2	Exp. 3
Top + buffer	0	0	0
Middle + buffer	10	0	28
Bottom + buffer	3	2	0
Top + Middle	92	40	103
Top + Bottom	35	9	1
Middle + Bottom	57	60	47
Unfractionated + buffer		6	10

[a] Inocula were whole peak-area samples used diluted to 0.05 A_{260}. (The final concentration of each component in the mixed samples was thus $A_{260} = 0.025$.) $A_{260/280}$ ratios for top, middle, and bottom components were 1.56, 1.60, and 1.65, respectively, and the unfractionated preparations contained about twice as much top component as middle or bottom component.

[b] Lesion numbers are totals for eight half-leaves of *Phaseolus vulgaris* 'Bountiful'.

components, such values suggested a reduced RNA content in the top and middle components as compared to bottom component.

The relationship between our three components and the two showing infectivity enhancement described by Fulton (7) is not clear, but with TSV we noted infectivity enhancement when samples of noninfective top component were mixed with samples of either middle or bottom components, and also when the latter were mixed together (Table 1). However, here again, since the components sediment so closely together, critical separation was probably not achieved.

The effects of pH on the quantity and quality of TSV-RN isolated from bean.—The initial observation that TSV-RN might respond in a unique fashion to the extraction regime was made on impure extracts from infected bean (Table 2, exp. 1). The infectivities of extracts that had been made initially at pH 5.0 rather than at 7.0 were lower than those made at 7.0, even if the latter were subsequently adjusted to pH 5.0. This observation suggested that the actual release of virus from tissue, rather than its stability or infectivity at pH 5.0, was involved.

This idea was checked by blending two split samples of infected bean leaves for 45 sec each in a 1:2.5(w/v) ratio of tissue to either 0.1 M pH 7.0 phosphate buffer or 0.2 M pH 5.0 acetate buffer. The pH of the material extracted at pH 7.0 was lowered to pH 5.0 with 10% acetic acid after 1 hr, and both preparations were left overnight at 4 C before two cycles of differential ultracentrifugation, the pellets being suspended in 0.1 M pH 5.0 acetate buffer at each step. The results in this typical experiment showed that extraction at pH 7.0 yielded 10-15 mg of virus nucleoprotein/100 g of leaf, about twice that from extraction at pH 5.0; but more striking was that the component ratios, as observed in the analytical centrifuge, were greatly different (Fig. 1-A). Nucleoprotein from the extraction at pH 7.0 contained about four times the amount of 90 *S* top component than found using extraction at pH 5.0, as well as more of the 98 *S* and 113 *S* components. Infectivity assays showed that the pH 5.0-extracted product was not infective at the same dilutions at which the pH 7.0-extracted product was (Table 2, exp. 2), although both purified preparations were stored at pH 5.

Component ratios in the purified preparations were not changed simply by changing the pH. Virus extracted at pH 5.0 before purification was dialyzed to

TABLE 2. Influence of pH of extraction on infectivity of the bean red node strain of tobacco streak virus from bean plants[a]

Exp.	pH of		Whether dialyzed before inoculation	Diluent used before inoculation	Lesion no.[b]
	Extraction	Purification			
1.	5	[c]	No	Water	16
	7		No	Water	70
	5		pH 7	Water	4
	7		pH 5	Water	45
2.	5	5	No	pH 5	0
	7	5	No	pH 5	47
	5	5	No	pH 7	0
	7	5	No	pH 7	174
3.	7	5	pH 3	pH 7	0
	7	5	pH 4	pH 7	114
	7	5	pH 7.5	pH 7	123
	7	5	pH 8.5	pH 7	32
4.	4	5	No	pH 7	7
	5	5	No	pH 7	6
	7	5	No	pH 7	207
	8	5	No	pH 7	40

[a] TSV-RN was extracted from infected bean leaves using 0.1 M phosphate or 0.2 M acetate buffer, as appropriate, at the pH values indicated. For purification procedure, see text. Subsequent adjustment, dialysis, or dilution before inoculation was in 0.05 M phosphate or 0.1 M acetate.

[b] Lesion numbers are totals for 8-12 leaves of *Chenopodium quinoa,* inoculated either with the various preparations similarly diluted (Exp. 1-3), or a standard concentration as indicated by A_{260} (Exp. 4).

[c] Samples used in Exp. 1 were not purified.

pH 7.0 and re-examined in the analytical centrifuge (Fig. 1-B). The component ratio remained unchanged, signifying that one sedimenting form could not be so converted to another in vitro, and that the difference in ratios between the two initial preparations resulted from differences in extractibility. Further tests with the purified virus extracted at pH 7 showed that all components were stable and the virus remained equally infective between at least pH 4 and 7.5, though degradation was noticeable at pH 8.5 and obvious at pH 3.0 (Fig. 1-C; Table 2, exp. 3).

Attempts were also made to determine if other component ratios could be obtained if pH levels other than 5 and 7 were used in extraction. Tissue was blended at pH 4, 5, 7, and 8 (using acetate or phosphate, as appropriate) and left 1 hr or more at these pH levels before adjustment to 5.0, overnight storage, and final purification and concentration by the usual method. The results showed the usual ratios after pH 7 and 5 extraction, that almost no virus was obtained at pH 4 (although the pure virus does not precipitate and is stable at this pH), and that virus at pH 8.0 showed signs of degradation (Fig. 1-E). In this experiment, specific infectivity assays of products also showed highest infectivity for the virus obtained using the pH 7 to 5 regime (Table 2, exp. 4).

This relatively greater infectivity could be a consequence of the greater amount of top component in the pH 7.0 extracts, because of its effect in augmenting infectivity. Although virus infectivity was unaffected over the pH range 4-7.5 (Table 2, exp. 3), so that the differences cannot have been due to a direct effect of pH, it remains possible that pH-mediated extraction effects can impair the infectivity of products made at the lower pH values in other ways.

The question as to why more top component, particularly, would be preferentially extracted at pH 7.0, could conceivably be answered if the virus system were electrophoretically heterogeneous. Thus, virus extracted at pH 7 and exhibiting the usual heterogeneous pattern in the analytical centrifuge was examined electrophoretically after 48-hr dialysis against 0.1 μ pH 5 acetate electrophoresis buffer (11). A single component (Fig. 1-D) migrating at 2.7×10^{-5} cm^2/volt per sec was observed, indicating homogeneity and the unlikelihood of explaining the extraction results on a basis of charge difference.

The efficiency of different extraction procedures was investigated in a number of ways. The length of the blending period at pH 7.0 was increased from the usual 45 sec to 2 min, 45 sec, with no change in yield. The time samples were kept at pH 7.0 before adjustment to pH 5.0 did not affect yield, 15 min resulting in the same yields and component ratios as 8 hr at pH 7.0. Addition of Triton X-100 to 0.5% during extraction did not affect yields. Re-extractions at pH 7.0 of the green fibrous residue from a pH 5.0 extraction of bean leaf yielded no further virus nucleoprotein. Further, "reciprocal" readjustments of the pH of extracts made at pH 7.0 (subsequently adjusted to 5.0, then to 7.0, then to 5.0) or pH 5.0 (subsequently adjusted to 7.0, then to 5.0), before final clarification and purification, had no effect on yield or component ratio.

Experiments in which buffered bean-leaf extracts were added to purified virus showed no change in component ratios. For example, high speed supernatants of sap from infected leaves initially prepared at pH 7.0 or 5.0 had no effect on the component ratio of purified virus, nor did freshly made crude sap from healthy leaves buffered at these levels. Thus, it again appeared that as previously implied, the problem centered on extraction, in the strict sense, of virus from tissue.

The type of tissue used for extraction did not matter in terms of component ratio, the same ratios being found from both bean leaves and mixtures of stems and petioles. Yields, however, did differ, the product from leaves being about twice that from the stem and petiole mixtures, and not pigmented, contrary to Mink et al. (12). Nor did the age of infection affect component ratio, preparations from beans infected for 1 and 4 weeks being the same in this regard, again contrary to previous observations made using a different purification procedure (12).

The influence of host on the products of purification of TSV-RN.—The results of many experiments showed that the extraction effect varied strikingly according to the host used. Bean and *C. quinoa* were grown together, inoculated and harvested at the same time, and virus was purified from them by the usual pH 7.0 and 5.0 protocols. The results of a typical experiment (Fig. 1-F) show that the greatest total nucleoprotein yield and amount of top component was found with pH 7 extraction from bean and pH 5 extraction from *C. quinoa*. The same result was also obtained with a second isolate of the bean red node strain (7, supplied by R. Fulton). Surprisingly, no virus nucleoprotein was detectable when 50-100 g lots of leaves of Xanthi tobacco infected with TSV-RN were processed following either the pH 5 or the pH 7 protocols, and the products concentrated into 2-ml volumes for analysis on density gradients.

Since TSV-RN-infected bean and *C. quinoa* responded differently to extraction, the effect of added tissues from the 2 species in virus preparation was investigated by (a) blending pure virus nucleoprotein (made from bean leaves extracted at pH 7.0) with healthy bean or *C. quinoa* leaf at pH 7.0 or 5; (b) separately adding equal wt of healthy bean or *C. quinoa* to the same wt of infected bean leaf prior to extraction at pH 5 or 7; and (c) separately adding equal wt of healthy bean or *C. quinoa* to the same wt of infected *C. quinoa* leaf prior to extraction at pH 5 or 7. In (a), only about one-fifth of the added virus was recovered when pure virus was blended with bean or *C. quinoa* leaves at pH 7.0 or 5.0 and then "repurified" by adjustment to pH 5 and ultracentrifugation, but the component ratios were unchanged. In (b), the yield of nucleoprotein from infected bean and healthy bean leaf extracted at pH 7 was about twice that from pH 5 extraction. Addition of *C. quinoa* leaf instead of bean leaf altered yields somewhat and reduced the yield differential by about one-fourth (Table 3). Differences

TABLE 3. The effect of mixing bean and *Chenopodium quinoa* on the yield of tobacco streak virus, bean red node strain

Added leaf tissue	pH of extraction	Relative yields in A_{260} units[a]	
		Infected bean	Infected *C. quinoa*
Bean	7	1.435	.536
C. quinoa	7	1.290	.172
Bean	5	0.728	.576
C. quinoa	5	0.840	.411

[a] In each case, 25 g of infected and 25 g of healthy leaf were blended together and virus purified by clarification at pH 5 and concentration by 2 cycles of differential ultracentrifugation. Final pellets were each suspended in 1.5 ml of 0.1 M pH 5 acetate buffer and A_{260} readings were made on 10-fold dilutions of the products.

in nucleoprotein component content were obvious, if not marked, and the ratios found in the preparations also accorded with expectation. That is, the presence of healthy *C. quinoa* leaf when extracting infected bean leaf at pH 7 reduced the proportion of top component as compared with extraction with added healthy bean leaf (Fig. 2). For the (c) series, the results were still more striking (Table 3, Fig. 2). Not only did the added bean leaf improve, as expected, the yield of nucleoprotein from infected *C. quinoa* at pH 7 (by about three times), but it actually reversed the relative proportions of the top and middle components.

The effect of pH and host on the quality and quantity of TSV.—It was not known if TSV would show extraction characteristics similar to those found with TSV-RN. The strains are very closely related serologically. Several antisera made to TSV-RN showed the same titer in ring-interface precipitin tests to TSV as were found in the homologous reaction. In gel-diffusion tests between TSV-RN antiserum and the two strains, the precipitation lines fused completely with no spur-formation. Extraction experiments with TSV were carried out with inoculated leaves of bean and with mixtures of inoculated and systemically infected leaves of tobacco, and also of *C. quinoa*.

Application of the pH 7 and 5 procedures with TSV in bean showed that extraction at neutrality before adjustment to pH 5 yielded more than fifty times (i.e., about 10 mg/100 g of leaf) the yield found when extraction was at pH 5. Unlike the results found with TSV-RN extracted from bean, the component ratios remained about the same regardless of the extraction protocol. Extraction of tobacco (both varieties) at pH 7 gave substantial yields of virus (about one-half those from bean extracted at pH 7), but yields from extracts made at pH 5 were undetectable. The component ratios from tobacco were markedly different from those obtained from bean under the same conditions (Fig. 3), and those of TSV from bean were different from those of TSV-RN from the same host (see Fig. 2, 3). Yields of TSV from *C. quinoa* were low, reflecting poor invasion of this host as judged from symptoms. As with TSV-RN, extraction at pH 5 gave 2 to 3 times the nucleoprotein yield of extraction at pH 7, but with

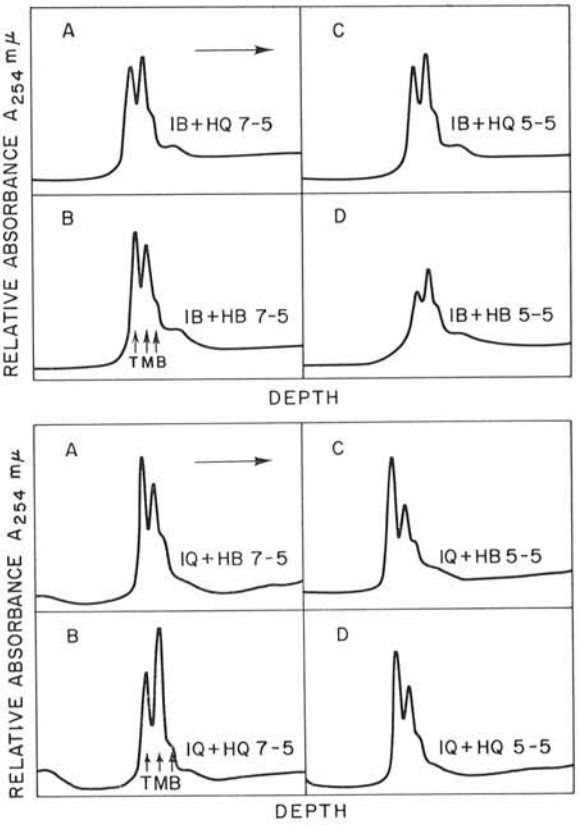

Fig. 2. Ultraviolet absorbancy profiles of rate-density gradient ultracentrifugations (3.5 hr at 23,000 rpm on 10-40% sucrose gradients in Spinco SW 25-L tubes) of tobacco streak virus—bean node strain, made by extracting infected bean leaves (IB series—upper 4 curves) or infected *Chenopodium quinoa* leaves (IQ series—lower 4 curves) mixed with healthy bean (HB) or healthy *C. quinoa* (HQ) leaves. In each case, extraction was done at pH 5 or 7, as indicated, and clarification and subsequent concentration was at pH 5. "T", "M", and "B" indicate positions of top, middle, and bottom components, respectively. Loadings of total nucleoprotein for each set of tubes were adjusted to be approximately equivalent at A_{260}, so only the component ratios are comparable. Arrow indicates direction of sedimentation.

TSV, top component did not predominate in either product. Ultraviolet absorbancy profiles for rate density-gradient centrifugations of both products were in fact closely similar to those for the virus purified from bean (Fig. 3).

DISCUSSION.—The variations in viral component ratios related to host and extraction procedure described here seem unusual. With various multicomponent plant virus systems, enrichment of purified preparations for specific components has been described, but these are based, for example, on differential adsorption to bentonite, varying with pH and Mg^{2+} concentration (5), and differential solubilities in the presence of Mg^{2+} (8), in polyethylene glycol (4), or in sucrose solutions (2). Differential effects can also occur during clarification procedures, as with the use of either organic solvents or polyethylene glycol to

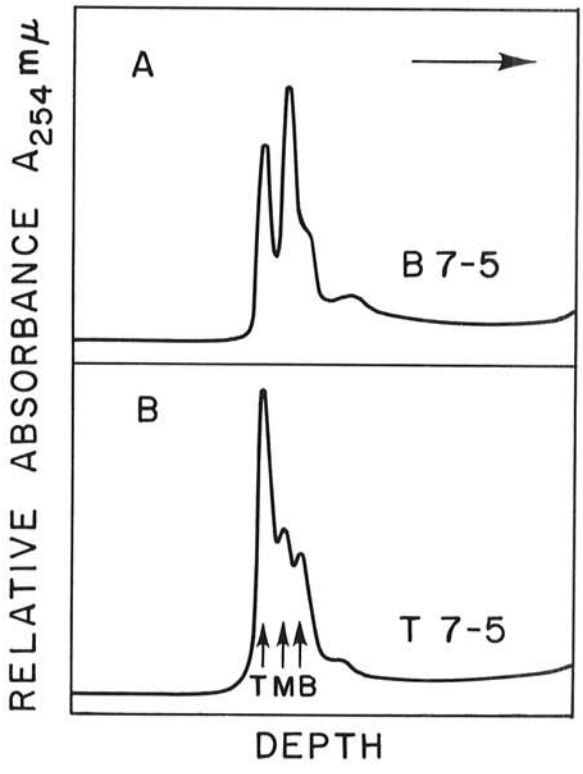

Fig. 3. Ultraviolet absorbancy profiles of rate-density gradient ultracentrifugations (3.5 hr at 23,000 rpm on 10-40% sucrose gradients in Spinco SW 25-L tubes) of tobacco streak virus, made by extracting infected bean (B) or tobacco (T) leaves at pH 7, before clarifying at pH 5.

purify cowpea mosaic virus (18) and alkaline-chloroform emulsions to purify bean pod mottle virus (15). Differential component ratios apparently related to strains, host, or culture time have also been observed (9, 13, 14, 16, 18).

The effect we have described, however, is certainly distinct from all these, since it specifically relates to host-mediated, indirect effects of pH during extraction, under conditions in which the purified virus is unaffected. The existence of differences in the component ratios obtained for different strains is less unexpected than the differences in the component ratios that can be obtained for one strain simply by varying extraction procedure or host. With TSV-RN, transformation of bottom or middle component to top component particles by nucleases during extraction, as invoked to explain the origin of a 70 *S* component in preparations of ϕX 174 (1), appears to be excluded by the results of our experiments with prepared virus and leaf extracts. Also, though TSV-RN was extensively degraded at either pH 5 or pH 7 by added pancreatic ribonuclease at 40 μg/ml, there was no indication of preferential effects on any particular component.

The central unanswered question raised thus relates to the nature of the compounds in various host species that behave in such distinct fashions according to pH that they modify the viral nucleoprotein products of extraction from these hosts. TSV is readily inactivated by oxidized phenolic compounds in tissue extracts (6, 7). Our results may simply indicate differential sensitivity of different components to this process, though this seems unlikely in view of the use of efficient antioxidants during extraction.

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