Purification and Serology of Citrus Tristeza Virus

D. Gonsalves, D. E. Purcifull, and S. M. Garnsey

Agricultural Research and Education Center, University of Florida, Lake Alfred, FL 33850; Plant Virus Lab., University of Florida, Gainesville, FL 32611; and U. S. Horticultural Research Laboratory, Orlando, FL 32803, respectively.

We wish to thank Rodney Whidden, Horace Lasater, and Eugene Crawford for technical assistance.

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U. S. Department of Agriculture, and does not imply approval of it to the exclusion of other products that also may be suitable.

Florida Agricultural Experiment Stations Journal Series No. 631.

Accepted for publication 6 September 1977.

ABSTRACT

GONSALVES, D., D. E. PURCIFULL, and S. M. GARNSEY. 1978. Purification and serology of citrus tristeza virus. Phytopathology 68: 553-559.

Citrus tristeza virus (CTV) was purified from bark and leaf tissue of several citrus species. Tissue frozen in dry ice was pulverized and extracted several times with 0.10 M tris-HCl, pH 7.6 for bark or pH 8.4 for leaves. After several polyethylene glycol precipitation steps, concentrated viral suspensions were further purified by centrifugation in CsCl or Cs_2SO_4 density gradients. Antisera to formaldehyde-treated CTV, or to untreated CTV preparations reacted with purified CTV in microprecipitin tests. However, antiserum produced to untreated CTV reacted with viral antigen degraded in sodium dodecyl sulfate (SDS) in immunodiffusion tests in agar gels containing SDS and sodium azide, but antiserum to formaldehyde-treated CTV did not. Polyacrylamide gel electrophoresis of SDS-degraded CTV showed one prominent protein component which had a molecular weight of about 25,000 daltons, and a faster moving, fainter-staining region which probably was a degradation product(s) of CTV protein. Material eluted from the major protein zone reacted strongly with CTV-antiserum in SDS-immunodiffusion tests, while that from the faster-moving region reacted weakly. Some preparations of antiserum reacted weakly with extracts from healthy plants. Antiserum absorbed with healthy citrus tissue preparations was specific for CTV antigen. Citrus tristeza virus antigen was easily detected in extracts from young citrus bark and leaf tissue in SDS-immunodiffusion tests, thus indicating the usefulness of this technique for rapid diagnosis of CTV.

Tristeza has long been a serious disease of citrus. The damage caused by citrus tristeza virus (CTV) to the South American citrus industry in the 1940's is well documented (7), and CTV is now causing damage in other citrus-growing areas such as Spain (14) and Florida (11). Furthermore, considerable efforts are being expended to control the spread of the disease in California (19) and Israel (5).

In 1964, Kitajima et al. (13) consistently observed flexuous, threadlike particles approximately 2,000 × 10 to 12 nm associated with tristeza infected tissue. Until a few years ago, however, little information was avilable on other intrinsic properties of the virus. Bar-Joseph et al. (3) recently isolated and partly characterized these long, flexuous rods. These results suggested the inclusion of CTV in the closterovirus group (4, 20). More recently, CTV was mechanically transmitted from citrus to citrus and the infectivity of the flexuous rods purified from CTV infected tissues was established, thus providing the first biological evidence that the flexuous rods are the infectious agent (10).

A major inconvenience in working with CTV is the lack of a quick, easy way to diagnose CTV infection, which is symptomless in many citrus hosts. In the prevalent biological test, Mexican lime [*Citrus aurantifolia* (Christm.) Swingle] seedlings are graft-inoculated and show characteristic symptoms in 3 to 18 wk (6). Electron microscopy is more rapid and has been used successfully, in conjunction with the Mexican lime test, to monitor the spread of CTV in Israel (5). However, electron microscopes are expensive and not always available for routine use. Potentially, serology could be an ideal, practical way for diagnosing CTV. Indeed, antisera to CTV have been produced by using partially purified preparations as inject antigens (8, 15, 16). However, these antisera generally reacted strongly to antigens from healthy citrus and, to our knowledge, have not received widespread use.

The objectives of our work were first, to develop a routine purification procedure that would enable us to produce realtively large amounts of purified virus for serology and further characterization of the CTV particles and second, to develop serological tests for detecting CTV directly from citrus. We have found that purified CTV can be obtained in milligram amounts, and that antiserum produced to CTV reacted specifically with CTV antigen from either purified preparations or from crude extracts of infected citrus tissue in sodium dodecyl sulfate (SDS)-immunodiffusion tests.

MATERIALS AND METHODS

Virus isolates and propagation hosts.—A Florida isolate of CTV, designated as T-4, was used for purification throughout this work. The T-4 isolate has

00032-949X/78/000 092$03.00/0

been described previously (10) and was free of other known citrus viruses. Eureka lemon [*C. limon* (L.) Burm. f.], Etrog citron (*C. medica* L.), sweet orange [*C. sinensis* (L.) Osbeck], and Palestine sweet lime (*C. limettioides* Tan.) were used to propagate the virus. Plants were kept in a partially shaded, air-cooled glasshouse with temperatures ranging from 21 to 30 C.

Virus purification.—Tristeza particles were partially purified by a method similar to that described by Bar-Joseph et al. (3). Bark or leaf tissues in 50- to 300-g lots of new growth (2 to 3 wk old) of systemically infected source plants were frozen with dry ice and pulverized in a large mortar. Buffer (0.10 M tris-HCl, pH 7.6 for bark or pH 8.4 for leaves) was added at 2 ml/g tissue and the extract ground with a pestle for several minutes and filtered through four layers of moistened cheesecloth. Buffer (2 ml/g tissue) was again added to the debris, the extract was ground, and again filtered through the same cheesecloth. The debris in the cheesecloth was finally washed with buffer (1 ml/g tissue) and filtered. The combined filtrate was centrifuged at 5,300 *g* (avg) for 10 min. Polyethylene glycol (PEG) 6,000 MW and NaCl were added to the supernatant to make final concentrations of 4 and 0.8% (w/v), respectively. After stirring for at least 1 hr at 4 C, the suspension was centrifuged at 11,000 *g* for 15 min. Then the pellet was resuspended in 0.04 M potassium phosphate, pH 8.0 (2.5 ml/g tissue) for at least 1 hr and then centrifuged at 5,300 *g* for 10 min. Polyethylene glycol and NaCl were added to the supernatant to final concentrations of 4 and 1% (w/v), respectively. After 1 hr, the mixture was centrifuged at 11,000 *g* for 15 min and the pellet was resuspended for 1 hr in 0.015 M potassium phosphate, pH 8.0 at 1 ml/5 g tissue. The suspension was clarified by centrifugation at 2,800 *g* for 10 min.

The preparations were further purified by centrifugation in CsCl (3) or Cs_2SO_4 (9) density gradients using a Beckman SW 27.1 rotor. Preparations for CsCl centrifugation were fixed in 1.8% formaldehyde prior to centrifugation. For CsCl, the gradient was prepared by first mixing 4 ml of 60% (wt/wt) CsCl dissolved in 0.015 M potassium phosphate, pH 8.0 with 9.4 ml of virus solution, and then layering the mixture on 3.6 ml of the same CsCl solution. For Cs_2SO_4, 4 ml of 53% (wt/wt) Cs_2SO_4 dissolved in 0.05 M tris-HCl, final pH 8.0 was mixed with 9.4 ml of the virus preparation and layered on 3.6 ml of the same Cs_2SO_4 solution. The tubes were centrifuged at 23,000 rpm for 17 hr at 6 C. The gradients were scanned for UV-4 absorbing zones and fractionated with an ISCO UA-4 monitor and 640 fractionator. Preparations containing the CTV particles were dialyzed against 0.015 M potassium phosphate, pH 8.0, or 0.05 M tris-HCl, pH 8.0 and then subjected to a second cycle of CsCl or Cs_2SO_4 density gradient centrifugation. Preparations not immediately subjected to another density gradient centrifugation, or not used after the second density gradient cycle were stored frozen in buffer containing 5% sucrose. An extinction coefficient $E^{0.1\%}_{260\ nm/cm}$ of 2 was assumed for purified CTV preparations, based on work with sugar beet yellows virus (2).

Polyacrylamide gel electrophoresis.—Electrophoresis of SDS-degraded CTV in polyacrylamide gels was done by the method of Weber and Osborn (22). Virus or protein preparations suspended in 0.01 M sodium phosphate, pH 7.0 buffer which contained 1% SDS and 0.1% 2-mercaptoethanol were heated at 100 C for 1 min. Electrophoresis was carried out at 21 C for 3 to 4 hr at 8mA per gel. Protein molecular weight standards were bovine serum albumin (68,000 daltons), pepsin (35,000 daltons), trypsin (23,300 daltons), and tobacco mosaic virus protein (17,500 daltons). Healthy citrus antigens were prepared by the method of Purcifull et al. (18), or by processing citrus leaves according to the CTV purification schedule up to the density gradient step.

Serology.—Formaldehyde-treated virus (about 3 to 4 mg) purified by two cycles of CsCl or Cs_2SO_4 gradient centrifugation and suspended in 0.05 M tris-HCl, pH 8.0 was precipitated by adding formaldehyde to a concentration of 1.8% which brought the pH to approximately 4. The preparation was dialyzed against 0.015 M potassium phosphate, pH 8.0, centrifuged for 45 min at 36,000 rpm in a Beckman #40 rotor, and the pellet was resuspended in 1 ml of 0.05 M tris-HCl, pH 8.0. The preparation, which contained highly aggregated virus, was emulsified 1:1 with Freund's complete adjuvant and injected into thigh muscles on each hind leg of a rabbit. Antiserum was also prepared against untreated virus purified by two cycles of Cs_2SO_4 density gradient centrifugation. Three ml of virus (0.65 mg/ml) were emulsified 1:1 with Freund's complete adjuvant and injected intramuscularly into a second rabbit. Four wk later, 2 ml of virus (about 0.3 mg/ml) was mixed 1:1 with Freund's incomplete adjuvant and injected as before. Serum was collected from both rabbits periodically over a number of months and stored frozen or lyophilized.

Healthy citrus antigens for absorption of antiserum were prepared as described for gel electrophoresis except that preparations were not heated or treated with SDS. Citrus antigens were mixed with antiserum (1:4, v/v), incubated at 4 C overnight, and then centrifuged at 30,000 rpm for 1 hr in a Beckman 75 Ti rotor at 6 C. The supernatant was lyophilized and reconstituted to the original antiserum volume prior to use.

Immunodiffusion tests were usually done in agar plates containing SDS and sodium azide (12, 17). Plates were prepared by pouring 10 to 12 ml of Noble agar (0.8 g/100 ml) containing SDS (0.5 g/100 ml) and sodium azide (1.0 g/100 ml) into plastic petri plates. Wells were 7 mm in diameter and the outer wells were spaced 5 mm from the center well. Antiserum was used undiluted and tissues were ground in either water or in 1% SDS (17). Plates were read within 24 to 48 hr.

To assay polyacrylamide gels serologically following electrophoresis, the gels were measured, and one gel stained in Coomassie blue (22) to detect protein bands while a sister gel was sliced into disks 2 mm thick with a Bio-Rad gel slicer. Each gel disk was incubated in 0.1 ml of 0.1% SDS for 2 hr on a shaker at 24 C. The liquid was then withdrawn and tested serologically as detailed above. Then 0.2 ml of 0.1% SDS was added to the gel disks, shaken overnight, and the liquid again tested serologically. The stained gels were used to correlate serological reactions with positions of the protein zones.

Antisera were titered in microprecipitin (1) and SDS-immunodiffusion tests (17). Antisera were diluted with 0.85% NaCl dissolved in 0.05 M tris-HCl, pH 7.0 for

microprecipitin tests, and with normal serum for SDS-immunodiffusion tests. Purified virus ($A_{260} = 0.1$/ml) was used as the antigen.

RESULTS

Purification.—Using the described purification method, we could routinely process 300-g batches of leaf or bark tissue. It was necessary to process large amounts of tissue because CTV yields were quite low and variable. Yields ranged from 100 μg to 750 μg of virus per 100 g tissue with bark consistently yielding more virus than leaves. Yields of CTV were highest from tissue of shoots with fully expanded but not yet mature leaves. Electron microscopy indicated that tissues from these shoots contained many virus particles (Garnsey and Gonsalves, *unpublished*).

Partially purified virus preparations were often dark green prior to CsCl or Cs_2SO_4 centrifugation. However, this did not interfere with the scanning of gradients because during centrifugation, in Cs_2SO_4 gradients especially, the green material floated to the top of the gradient and formed a firm disk which was removed with a cotton swab prior to fractionation. Tristeza particles were stable in Cs_2SO_4 but unstable in CsCl unless fixed with formaldehyde, as previously reported (3, 9). Preparations of CTV formed a discrete UV-absorbing band, which contained numerous flexuous rods (Fig. 1), in both types of gradients (Fig. 2). In addition, small amounts of a second, more-dense component often was observed (Fig. 2). The dense component would

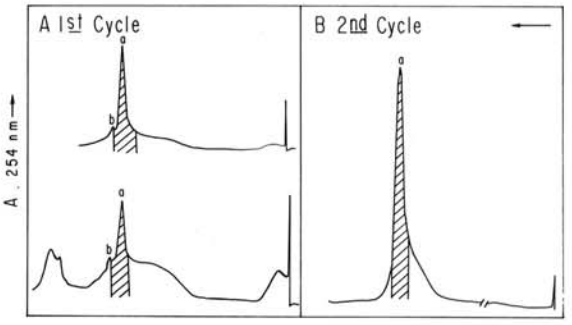

Fig. 2-(A, B). Ultraviolet absorbance profiles of citrus tristeza virus (CTV) centrifuged in Cs_2SO_4 gradients. **A)** Example of UV profiles of the first Cs_2SO_4 gradient centrifugation indicating variation in the quantity of host impurities. Top gradient contains less impurities than the bottom gradient. A denser CTV band (b) is almost always present along with the main CTV band (a). **B)** Ultraviolet profile of material collected from beneath band (a) in the first Cs_2SO_4 cycle (A) and centrifuged in a second Cs_2SO_4 gradient. The shaded areas indicate portions of the gradients from which material was collected. Arrow denotes direction of sedimentation.

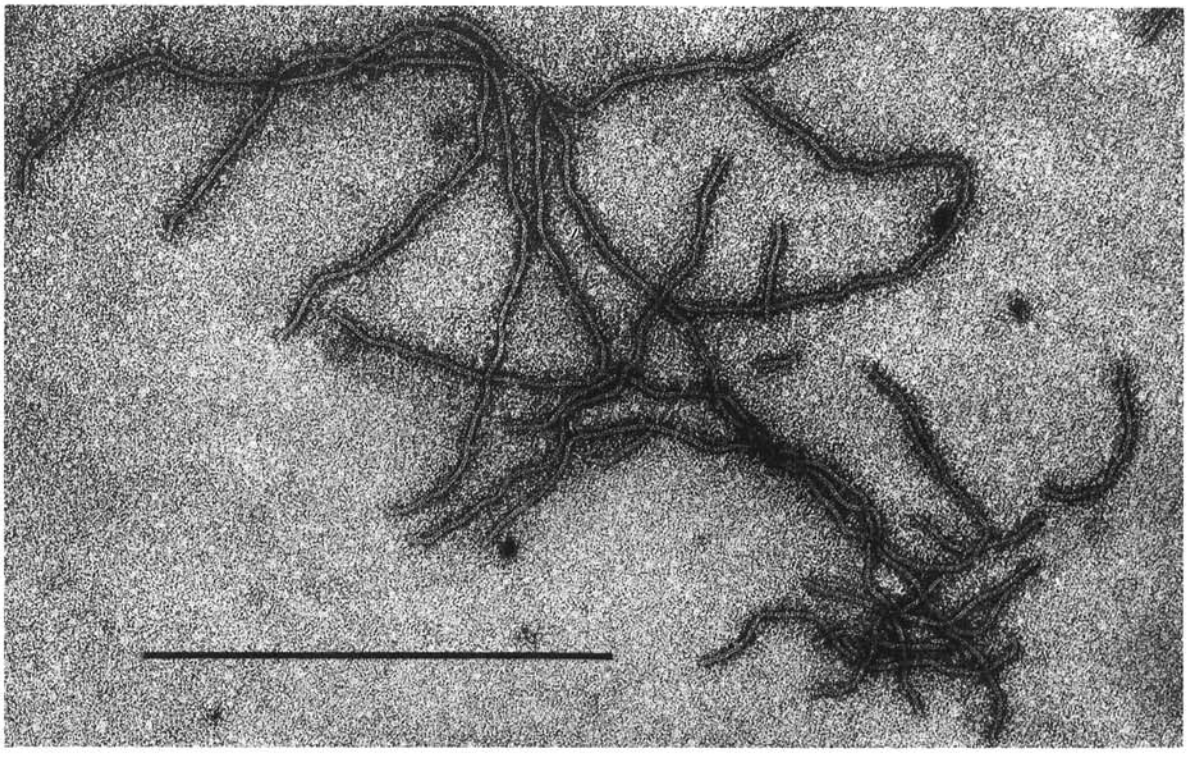

Fig. 1 Electron micrograph of citrus tristeza virus particles purified by Cs_2SO_4 density gradient centrifugation. Particles were negatively stained with phosphotungstic acid. Bar = 1 μm.

sometimes precipitate, but upon dialysis in either tris or phosphate buffer, it readily resuspended. This denser component contained threadlike particles that resembled CTV although preliminary electron microscopy observations showed that the diameter of the particles sometimes was irregular. After the first density gradient centrifugation, a broad opalescent region commonly was observed just above or more often merging into the virus band (Fig. 2). Similarly treated healthy preparations also showed this zone. A second cycle of gradient centrifugation largely eliminated the opalescent band (Fig. 2). Purified virus had a typical UV absorption spectrum with a 260/280 ratio of 1.31 uncorrected for light scattering.

Serology.—Antisera produced to both formaldehyde-treated, and to untreated virus preparations purified by two cycles of density gradient centrifugation reached titers of 1/256 in microprecipitin tests with whole, purified CTV. However, the two types of antisera behaved differently in SDS-immunodiffusion tests. Antisera prepared against formaldehyde-treated virus did not react, except very weakly in occasional tests, against purified virus which was degraded with SDS either before or after adding it to the reactant wells (Fig. 3). On the other hand, antisera prepared to untreated virus gave good serological reactions under similar conditions. Strong serological reactions also were observed when young infected bark or leaf tissue from greenhouse- or from field-grown plants was used as antigen sources. Furthermore, the observed reactions were identical to that of purified virus (Fig. 4). Antisera to CTV also reacted strongly to the T-3 and T-26 isolates of CTV which differ in the severity of their reaction on sweet orange on sour orange (*C. aurantium* L.) rootstock (10). Serological reactions were also obtained with a number of CTV field isolates. The titer of CTV antisera ranged from 1/4 to 1/8 in SDS-immunodiffusion tests.

Antisera from some bleedings of the rabbit which had been injected with untreated CTV reacted with antigens from healthy citrus, cucumber, and pumpkin plants in SDS-immunodiffusion tests. This reaction was eliminated, however, by absorption with proteins prepared from healthy citrus leaves (Fig. 4).

A nonspecific precipitin line also was observed occasionally with antisera collected from some bleedings

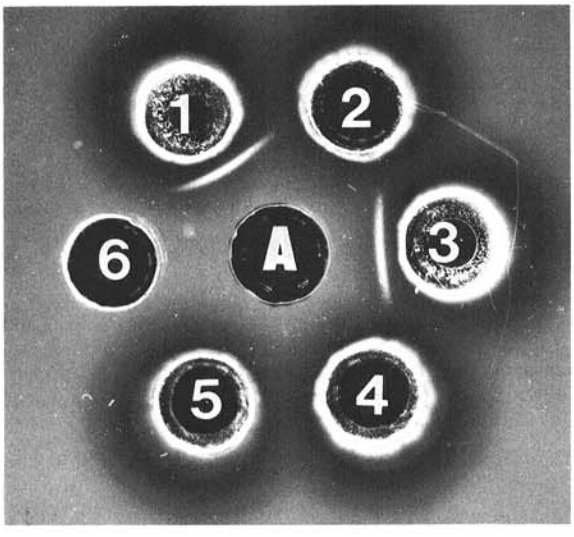

Fig. 3. Sodium dodecyl sulfate-immunodiffusion test of citrus tristeza virus (CTV) antisera with purified CTV (A_{260} = 0.1/ml). Well A = CTV; wells 1,3 = antiserum to untreated CTV; wells 2,4 = antiserum to formaldehyde-treated CTV; 5 = normal serum; and 6 = 0.05 M tris-HCl, pH 8.0.

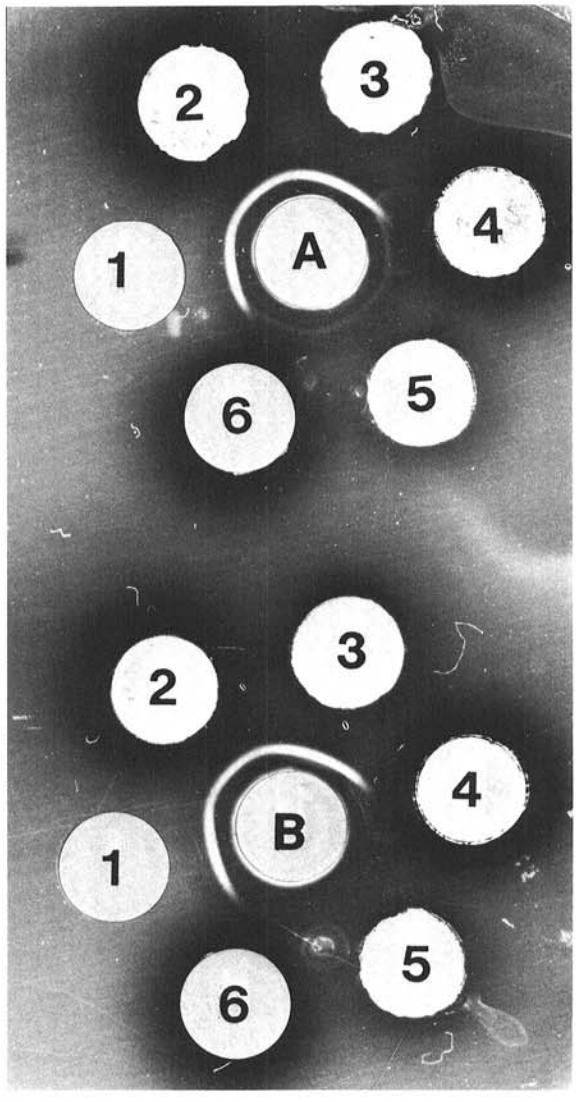

Fig. 4. Removal of reaction to healthy plant antigens in sodium dodecyl sulfate-immunodiffusion tests by absorption of antisera to untreated virus tristeza virus (CTV) with healthy citrus antigens. Well A = unabsorbed antiserum, well B = absorbed antiserum, wells 1 = purified CTV, 2 = CTV-infected bark extract, 3 = CTV infected leaf extract, 4 = healthy citrus bark extract, 5 = healthy citrus leaf extract, and 6 = healthy cucumber cotyledon extract. Note the absence of a reaction with healthy extracts when absorbed antiserum is used.

of the rabbit that had been injected with untreated CTV. This could be confused with a virus reaction if the antiserum was placed in the inner well. However, the nonspecific reaction could be distinguished from the virus reaction when antiserum was placed in the outer well (Fig. 5). A precipitin line appeared in the outer perimeter of the outside wells but not between the center and peripheral wells.

Since antisera prepared to only untreated CTV reacted in SDS-immunodiffusion tests, it seemed probable that the antiserum was reacting with the coat protein subunits of CTV. To test this supposition, purified CTV was degraded with SDS and electrophoresed in polyacrylamide gels. The gels then were sliced and the material eluted from each slice was tested serologically with

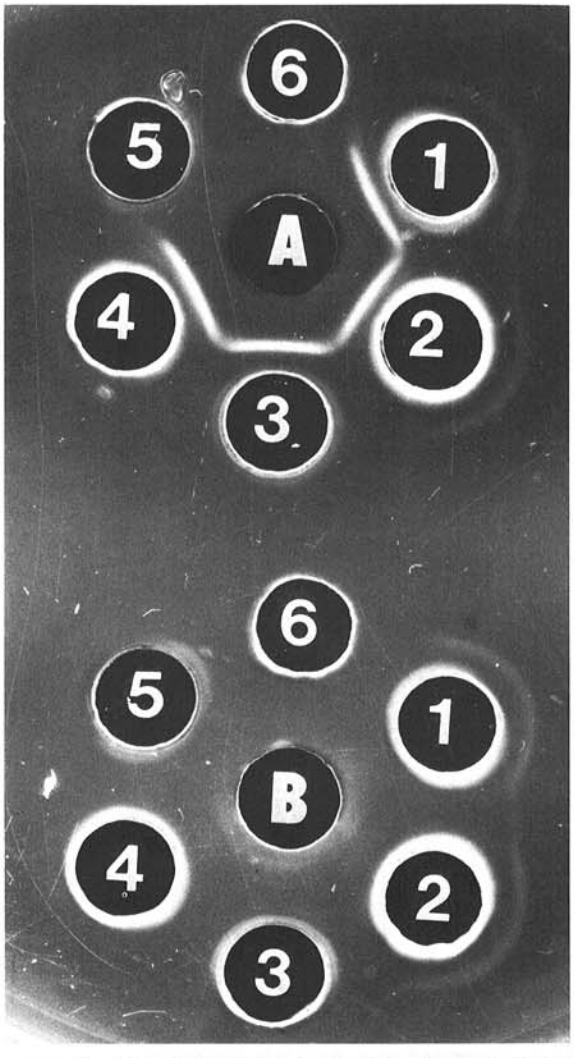

Fig. 5. Nonspecific reaction of antiserum produced to untreated citrus tristeza virus (CTV). Well A = purified CTV; B = healthy citrus leaf extract; 1,2 = antiserum batch showing nonspecific reaction; 3,4 = antiserum batch without nonspecific reaction; and 5,6 = absorbed and nonabsorbed normal serum, respectively.

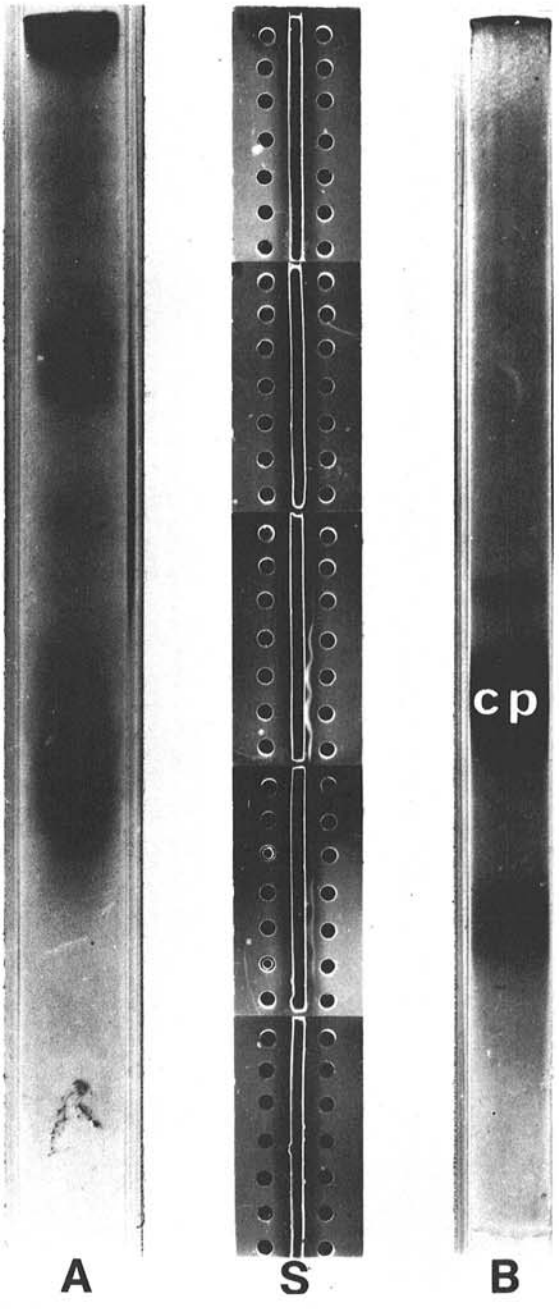

Fig. 6. Serological reaction of material eluated from 7.5% polyacrylamide gels. A and B are stained gels in which sodium dodecyl sulfate-degraded preparations of healthy citrus protein and citrus tristeza virus (CTV), respectively, were electrophoresed. The letter S denotes serological reactions of CTV-antiserum (center troughs) against eluates (wells adjacent to troughs) from the respective positions of the nonstained sister gels. Note the strong serological reaction with CTV coat protein (cp), and a weak reaction to the faster migrating protein band of gel B. Electrophoresis is from top to bottom. The slowest-migrating protein band of gel B is probably host protein since other preparations of CTV which did not contain this band showed similar serological reactions. The CTV preparation electrophoresed in gels was obtained from a single Cs_2SO_4 density gradient cycle.

antisera which reacted to healthy plant antigens, and also with antisera (absorbed and unabsorbed) which did not react to healthy plant antigens. Each of the antisera tested gave a strong serological reaction with a protein species having a molecular weight of about 25,000 daltons which corresponded to the CTV coat protein (3) (Fig. 6). In addition, a weaker serological reaction was observed with a faster-migrating, protein zone which may have been a degradation product(s) of the coat protein. Furthermore, these antisera did not react in similar tests with proteins prepared from healthy citrus leaves even though the stained gels showed numerous protein bands (Fig. 6). However, a very weak reaction against eluted material which migrated much slower than the CTV coat protein was observed when CTV antiserum which reacted with plant antigens was used (reaction not shown). Similar tests with antiserum prepared to formaldehyde-treated virus did not react. Thus, the evidence indicated that the serological reaction in SDS-immunodiffusion tests was with the CTV protein subunits.

Antiserum to CTV did not react with tobacco etch or potato virus X, in reciprocal SDS-immunodiffusion tests with crude extracts from infected plants. Also, antisera to these latter viruses did not react with purified preparations of CTV.

Monitoring the efficiency of purification steps by serology.—After the preparation of CTV antisera, SDS-immunodiffusion tests were used to determine loss of virus in the different purification steps. Apparently, much virus was being lost in the low-speed clarification steps and the PEG precipitation steps were very efficient in removing virus from the supernatant. Thus, a test was done to compare the amount of virus which could be recovered by Cs_2SO_4 centrifugation of the final 2,800 *g* supernatant (normal procedure) and of the resulting 2,800 *g* pellet which was normally discarded. Figure 7 clearly shows that relatively large amounts of CTV could be recovered from the resuspended pellet as compared to that recovered from the supernatant. This was especially true when bark was processed. Since CTV yields were low, the purification schedule was modified to increase virus yields. By omitting the final low-speed (2,800 *g*) clarification step, CTV yields were increased considerably. Preparations processed in this manner had more precipitated material dispersed in the gradient, but this was mainly below the viral zones and did not seriously interfere with fractionation of the gradients (Fig. 7).

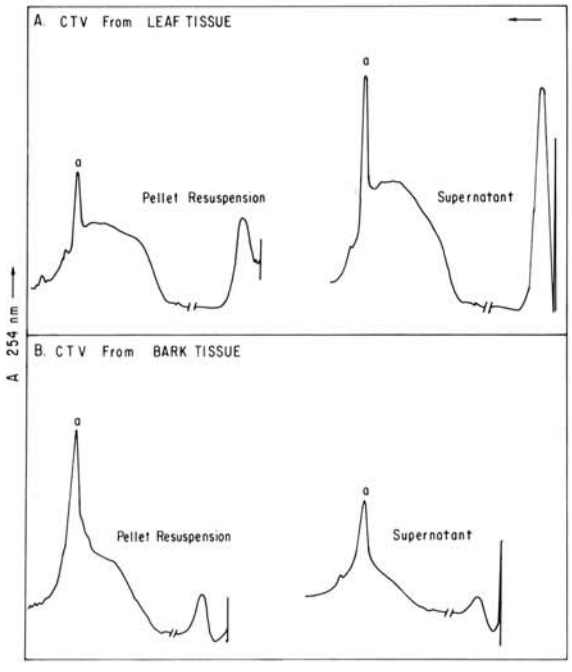

Fig. 7. Comparative citrus tristeza virus (CTV) yields from the final low-speed (2,800 *g*) supernatant and from the resulting 2,800-*g* pellet as monitored by UV absorbance of Cs_2SO_4 density gradients. The supernatant liquid was used in normal purification procedure and the pellet usually was discarded. Note the significant amount of CTV (a) recovered by resuspension of the pellet, especially from bark tissue. Arrow denotes direction of sedimentation.

DISCUSSION

Our purification schedule is similar to those previously reported for CTV (3, 9), but allows consistent recovery of virus from large quantities of citrus bark and leaf tissue. This, coupled with the use of virus source tissue that contained appreciable concentrations of CTV, enabled us to obtain milligram amounts of purified CTV.

Density gradient centrifugation in Cs_2SO_4 proved more desirable than centrifugation in CsCl because the virus preparations did not have to be fixed by formaldehyde. It was shown recently that CTV remains infectious even after prolonged exposure to Cs_2SO_4 (10). The modification of our purification scheme, which involves the elimination of the last clarification step prior to density gradient centrifugation, enables us to obtain even higher yields of CTV.

Apparently, more antigenic sites were exposed for antibody production in untreated CTV than in formaldehyde-treated CTV. The untreated virus probably was degraded to protein subunits in the rabbit, thus exposing additional antigenic sites which were different from those of the formaldehyde-treated virus. This concept is supported by the observation that antisera to both formaldehyde-treated and untreated virus reacted against intact virus particles, but only antiserum against untreated virus reacted with SDS-degraded protein subunits.

It appears that even two cycles of Cs_2SO_4 gradient centrifugation do not completely separate CTV particles from host proteins since some batches of antisera reacted with healthy material. It has been reported (21) that very small amounts of host material in virus preparations may elicit the production of significant amounts of antibodies to healthy antigens. Fortunately, CTV antisera absorbed with protein from healthy citrus leaves was specific for CTV and the titer was not appreciably reduced in SDS-immunodiffusion tests.

The fast-moving electrophoretic protein band observed in gels probably consists of degradation product(s) of CTV coat protein. This supposition is supported by the observation that healthy citrus antigens with similar

electrophoretic mobility to the fast-moving protein zone of CTV preparations did not react to unabsorbed CTV antiserum which showed a reaction to healthy plant preparations. Instead, a weak healthy reaction was obtained from a zone which moved much slower than the CTV protein band. Furthermore, CTV antisera (absorbed and unabsorbed), which did not react with healthy citrus antigens, reacted with material eluted from both the CTV coat protein, and the faster moving protein zones.

Primo et al. (16) produced an antiserum to partially purified CTV preparations obtained from field-collected citrus tissues with undefined virus contents. Therefore, one cannot be sure whether the antiserum was specific to CTV. Furthermore, their antisera reacted strongly to healthy material. The specificity of the antiserum obtained by Oliviera (15) is difficult to evaluate because no details are given regarding reactivity of the antiserum to healthy material.

The practical utility of the antiserum for detecting CTV in citrus has not been fully determined, but preliminary trials with bark tissue harvested from spring flush of field-grown citrus trees have given strong positive results using the SDS-immunodiffusion methods. Furthermore, our serological results have correlated well with the standard grafting test using Mexican lime. Work is now in progress to assess the usefulness of the SDS-immunodiffusion method for diagnosing CTV under field conditions.

LITERATURE CITED

1. BALL, E. M. 1974. Pages 5-7 *in* Serological tests for the identification of plant viruses. Plant Virology Committee, Am. Phytopathol. Soc., St. Paul, Minnesota. p.
2. BAR-JOSEPH, M., and R. HULL. 1974. Purification and partial characterization of sugar beet yellows virus. Virology 62:552-562.
3. BAR-JOSEPH, M., G. LOEBENSTEIN, and J. COHEN. 1972. Further purification and characterization of threadlike particles associated with the citrus tristeza disease. Virology 50:821-828.
4. BAR-JOSEPH, M., G. LOEBENSTEIN, and J. COHEN. 1976. Comparison of particle characteristics and cytopathology of citrus tristeza virus with other morphologically similar viruses. Pages 39-45. *in* E. C. Calavan, ed. Proc. 7th Conf. Int. Organ. Citrus Virol. Univ. Calif., Riverside. 227 p.
5. BAR-JOSEPH, M., G. LOEBENSTEIN, and Y. OREN. 1974. Use of electron microscopy in eradication of tristeza sources recently found in Israel. Pages 83-85 *in* L. G. Weathers, and M. Cohen, eds. Proc. 6th Conf. Int. Organ. Citrus Virol. Div. Agric Sci., Univ. Calif., Riverside. 232 p.
6. CHILDS, J. F. L., ed. 1968. Indexing procedures for 15 virus diseases of citrus trees. U. S. Dep. Agric., Agric. Handb. No. 333. 96 p.
7. COSTA, A. S. 1956. Present status of the tristeza disease of citrus in South America. FAO Plant Prot. Bull., Vol. IV, No. 7. p.
8. CUÑAT, P., E. HERNÁNDEZ, E. PRIMO, and R. VILA. 1973. Virosis de los cítricos. III. Técnicas serológicas para el diagnóstico de la tristeza de los cítricos. Rev. Agroquím. Technol. Aliment. 13:274-278.
9. FLORES, R., R. GARRO, V. CONEJERO, and P. CUÑAT. 1975. Purificacion en gradiente de densidad de sulfato de cesio de las particulas nucleoproteicas asociadas a la tristeza de los cítricos. Rev. Agroquím. Technol. Aliment. 15:93-97.
10. GARNSEY, S. M., D. GONSALVES, and D. E. PURCIFULL. 1977. Mechanical transmission of citrus tristeza virus. Phytopathology 67:965-968.
11. GARNSEY, S. M., and J. L. JACKSON, JR. 1975. A destructive outbreak of tristeza in central Florida. Proc. Fla. State Hortic. Soc. 88:65-69.
12. GOODING, G. V., JR., and W. W. BING. 1970. Serological identification of potato virus Y and tobacco etch virus using immunodiffusion plates containing sodium dodecyl sulfate. Phytopathology 60:1293.
13. KITAJIMA, E. W., D. M. SILVA, A. R. OLIVIERA, G. W. MÜLLER, and A. S. COSTA. 1964. Thread-like particles associated with tristeza disease of citrus. Nature 201:1011-1012.
14. MARTI, F. 1977. La enfermedad de la tristeza de los agrios en España. Pages 531-536 *in* I Congreso Mundial de Citricultura, 1973, Vol. II. Int. Soc. Citriculture, Murcia, Spain. 723 p.
15. OLIVIERA, A. R. 1975. Considracoes sobre antisoros obtidos pela technica de injecao de antigeno no linfonodulo. Summa Phytopathologica 1:61-64.
16. PRIMO, E., E. HERNÁNDEZ, J. HERNÁNDEZ, M. MARTINEZ, P. CUÑAT, and R. VILA. 1971. Diagnóstico precoz de la tristeza del naranjo. I. Separación y reacciones serológicas de partículas nucleoproteicas. Rev. Agroquím. Technol. Aliment. 11:229-235.
17. PURCIFULL, D. E., and D. L. BATCHELOR. 1977. Immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated plant viruses and plant viral inclusions. Fla. Agric. Exp. Stn. Tech. Bull. No. 788. 39 p.
18. PURCIFULL, D. E., E. HIEBERT, and J. G. MC DONALD. 1973. Immunochemical specificity of cytoplasmic inclusions induced by viruses in the potato Y group. Virology 55:275-279.
19. ROISTACHER, C. N. 1976. Tristeza in the central valley: a warning. Calif. Citrogr. 62:15-23.
20. SHEPHERD, R. J., R. I. B. FRANCKI, L. HIRTH, M. HOLLINGS, T. INOUYE, R. MACLEOD, D. E. PURCIFULL, R. C. SINHA, J. H. TREMAINE, V. VALENTA, and C. WETTER. 1976. New groups of plant viruses approved by the international committee on taxonomy of viruses, September 1975. Intervirology 6:181-184.
21. VAN REGENMORTEL, M. H. V. 1963. Serologically related plant contaminants in preparations of partially purified plant viruses. Virology 21:657-658.
22. WEBER, K., and M. OSBORN. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.