# A Comparison of Two Strains of Apple Chlorotic Leaf Spot Virus

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ABSTRACT

On the basis of biological and serological properties, two apple chlorotic leaf spot virus (CLSV) isolates, CLSV-A (from apple), and CLSV-P (from peach), were differentiated as strains. They differed in symptoms on woody indicator hosts and in the severity of symptoms caused on *Chenopodium quinoa*. In serological ring precipitin tests, CLSV-A antiserum had a homologous titer of 1/128 to 1/256 and a heterologous titer of 1/32. CLSV-P antiserum had homologous and heterologous titers of 1/128 and 1/64, respectively. In serological gel diffusion tests, partial identity of the two strains was indicated by spur formation when the two strains were reacted with either antiserum. Differences in physical properties were minor. The yield of CLSV-P was increased by including 1% polyethylene glycol (MW=6,000) in the extraction buffer.

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*Additional key words:* serology, virus purification, virus properties.

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Apple chlorotic leaf spot virus (CLSV) (8) appears to be worldwide in occurrence in deciduous tree fruits and ornamentals. Thus, in many locations viruses identified as CLSV are commonly isolable from commercial rosaceous crops, including apple, pear, peach, cherry, apricot, and also from ornamental *Pyrus, Prunus*, and *Malus* species. In apple, these viruses are frequently symptomless, but they are associated with a range of disease syndromes in other woody hosts (6, 11).

Due to difficulties in virus purification, and hence serological testing, many reported isolations are based solely on symptomatology in grafted woody indicators or in mechanically infected *Chenopodium* species (notably *C. quinoa* Willd.). Therefore, some isolates classified as CLSV may be incorrectly identified. Adding to the difficulty and importance of accurate diagnosis, the literature also suggests that important biological differences exist among isolates of CLSV, including variations in symptoms and in the range of woody hosts in which pathogenesis occurs (1, 5).

Currently available methods for purifying CLSV, and thus preparing highly specific antisera (7, 10), now provide means for serological classification and critical investigation of the relationships of various isolates. The work reported here was done to determine whether biological diversity in selected isolates was related to differences in their general in vitro properties, and particularly to differences in their serological properties.

MATERIALS AND METHODS.—*Viruses used.*—The two distinctive strains of CLSV used for most work were selected as representative of field isolations from apple, peach, and cherry from several sources (Table 1). For convenience, the strains are designated as CLSV-A (=apple strain "B-38E") and CLSV-P (=peach strain "Anzac 1").

TABLE 1. Classification of some apple chlorotic leaf spot virus (CLSV) isolates

| Woody plant source | Isolate Designation | Origin | Symptomatology on *Chenopodium quinoa* | Reference |
|---|---|---|---|---|
| Apple | B-38E[a] = "CLSV-A" | Apple; Dr. R. M. Gilmer Geneva, New York | A type[b] | Lister & Hadidi (10) |
| | C-8 | Apple; Virginia Crab; Purdue clonal selection | A type | Lister et al, (9) |
| | 710-EL2 | Apple; Dr. Kegler Aschersleben, E. Germany (DDR) | A type | Personal communication |
| | 15-17-45 | Cherry; Dr. R. M. Gilmer Geneva, New York | A type | Personal communication |
| Peach | Anzac 1[a] = "CLSV-P" | Peach; Dr. P. R. Smith Victoria, Australia | P type[c] | Personal communication |
| | Anzac 2 | Peach; Dr. P. R. Smith Victoria, Australia | P type | Personal communication |
| | "Smith's seedling peach" | Peach; Dr. P. R. Smith Victoria, Australia | Mild P type | Personal communication |

[a] Isolates selected for detailed study in present work.
[b] A-type symptoms were large (2-mm diam) chlorotic primary lesions 3-4 days after inoculation which then became necrotic; after 5-7 days a severe systemic chlorotic mottle developed.
[c] P-type symptoms were fewer primary lesions developing 3 days later than the A-type lesions, and followed by a much milder chlorotic mottle.

Techniques for handling viruses were essentially those of Lister & Hadidi (10). Virus strains were maintained, cultured, and their infectivity assayed, in *C. quinoa*. Extraction of virus from infected leaves and mechanical inoculation of *C. quinoa* plants was done with buffers and inocula at 4 C.

*Virus purification.*—Highly purified virus preparations of the two CLSV strains were made by a modification of the bentonite/polyethylene glycol method described by Lister & Hadidi (10). All steps were carried out at 4 C. Leaf extracts, made by homogenizing chilled tissue (1:2, w/v) in cold 0.01 M Tris-HCl + 0.01 M $MgSO_4$, pH 8.2, were clarified by cautious addition of bentonite suspension. The virus was further clarified and concentrated by precipitation with 8% (w/v) polyethylene glycol (M W 6,000 = PEG) and resuspension in buffer without $MgSO_4$. Subsequent purification was by one cycle of differential ultracentrifugation, again resuspending in buffer, followed by rate-zonal sucrose density-gradient centrifugation.

*Density-gradient centrifugation.*—Rate-zonal sucrose density-gradient ultracentrifugation (3) was used in purification, and in analyzing virus preparations during purification or after dialysis under various conditions (see results). Density gradients were made in Spinco SW 27 tubes by layering 9 ml, respectively, of 100, 200, 300, and 400 mg of sucrose per ml of 0.01 M Tris-HCl, pH 8.2, and storing at 4 C for 20 hours. Gradients were loaded with 1-2 ml lots of the virus preparations to be purified or analyzed, and centrifuged for 2.5 hours at 25,000 rpm.

Ultraviolet absorbance profiles of the gradients were obtained at 254 nm with an ISCO density-gradient fractionator and ultraviolet analyzer (4), equipped with an ISCO Model 170 Servographic recorder. The amounts of absorbing material were estimated by summation of the areas under the absorbance curves.

*Serology.*—Antisera to CLSV-A and CLSV-P were prepared by injecting each into a rabbit. The highly purified virus preparations were free of all serologically detectable normal host proteins. Purified virus obtained directly from density gradients was injected both intravenously, and also intramuscularly after emulsification with Freund's incomplete adjuvant. Sucrose was not removed. Standard procedures were used for ring precipitin and agar gel double-diffusion tests. For the latter, gel diffusion plates were made with 0.6% Ionagar No. 2 in 0.85% NaCl made in 0.01 M phosphate buffer, pH 7.0 and containing 0.22% sodium azide. Most gel diffusion plates were stained to improve resolution for photography. Plates were first washed several times with 1% saline during 2 days, then stained 10-15 min in amido black (9 g in 1.5 liters methanol: acetic acid: water, 45:10:45, v/v) and destained in the solvent.

*Ultraviolet absorbance spectra.*—Ultraviolet

absorbance spectra were obtained with a Perkin Elmer-Hitachi continuous scanning spectrophotometer, and individual absorbance readings were made with a Beckman DU spectrophotometer.

RESULTS.–*Symptomatology*.–Unlike CLSV-A, CLSV-P did not induce the typical chlorotic leaf spot symptoms on the Russian apple indicator R-12740-7A, but it did produce a dark green sunken mottle symptom in peach (Dr. P. R. Smith, *personal communication*). We do not know if CLSV-A will cause dark-green, sunken mottle symptoms in peach.

The two strains also differed in the symptoms produced on *Chenopodium* spp., those of CLSV-A being more severe. CLSV-A induced abundant, large (1- to 2-mm diam), necrotic lesions on the inoculated

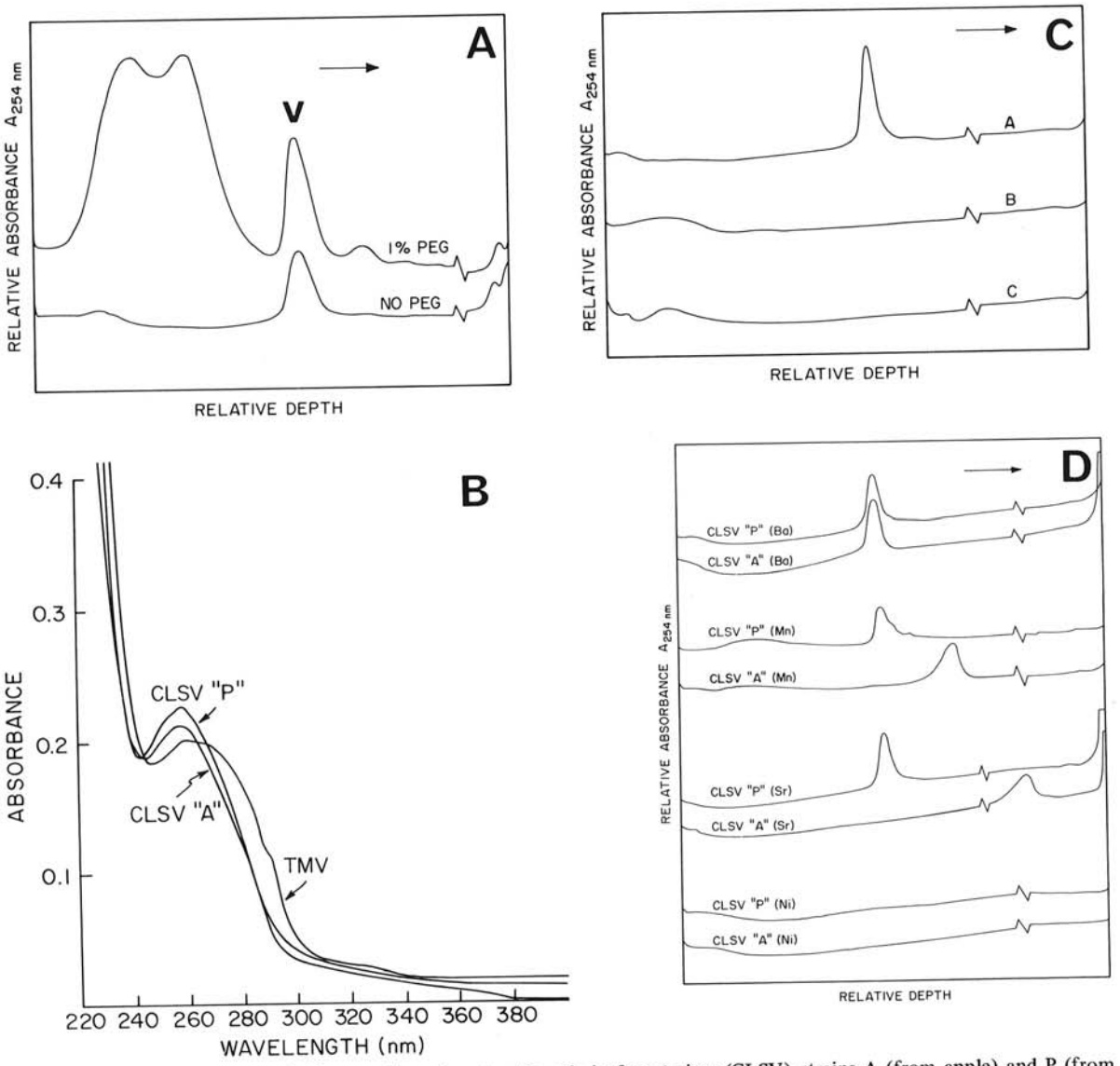


**Fig. 1.** Comparison of physical properties of apple chlorotic leaf spot virus (CLSV) strains A (from apple) and P (from peach). **A)** UV absorbance profiles of rate zonal density gradient centrifugations of products from purifications of extracts from CLSV-P infected *Chenopodium quinoa* leaves. Extractions were in 0.01 M Tris-HCl, 0.01 M $MgSO_4$, pH 8.2 (lower), and the same buffer containing 1% polyethylene glycol MW 6000 (upper). The peaks represent yields from comparable wieghts of leaves. Sedimentation direction is indicated by arrow. Virus peak is indicated by a V. **B)** Comparative UV absorbance curves for CLSV-A, CLSV-P and tobacco mosaic virus (TMV). In each case, the viruses were in 0.01 M Tris-HCl, 20% sucrose, pH 8.2. **C)** UV absorbance profiles of density-gradient centrifugations of equal aliquots of a preparation of CLSV-P dialyzed at pH 7.8 for 12 hr against A, 0.05 M Tris-HCl containing 0.005 M $MgCl_2$; B, 0.05 M Tris-HCl; C, 0.05 M Tris-HCl containing 0.05 M NaCl. Arrow indicates direction of sedimentation. **D)** UV absorbance profiles of density-gradient centrifugations of the same preparations of CLSV-A or CLSV-P dialyzed for 18 hr at pH 7.6 against 0.05 M Tris-HCl containing $BaCl_2$ ("Ba"); $MnCl_2$ ("Mn"); $SrCl_2$ ("Sr"); or $NiCl_2$ ("Ni"), all at 0.005 M. Arrow indicates direction of sedimentation.

leaves of *C. hybridum*, while CLSV-P did not cause readily identifiable lesions. Both strains induced a systemic chlorotic mottle. On *C. quinoa*, 3-4 days after inoculation, CLSV-A caused large (2-mm diam), chlorotic, primary lesions, which later became necrotic. A systemic chlorotic mottle developed 5-7 days after inoculation. CLSV-P caused relatively fewer, though similar, lesions 3 days later than did CLSV-A, and a milder systemic chlorotic mottle developed. Used at comparable concentrations, purified preparations of CLSV-P caused about one-tenth the number of lesions caused by CLSV-A. Indeed, in transfers using relatively low virus concentrations, for example crude sap inocula, CLSV-P frequently induced no discernible lesions, and infection was evident only when systemic symptoms developed. Interestingly, repeated subculturing of CLSV-P in *C. quinoa* resulted in some increase in symptom severity.

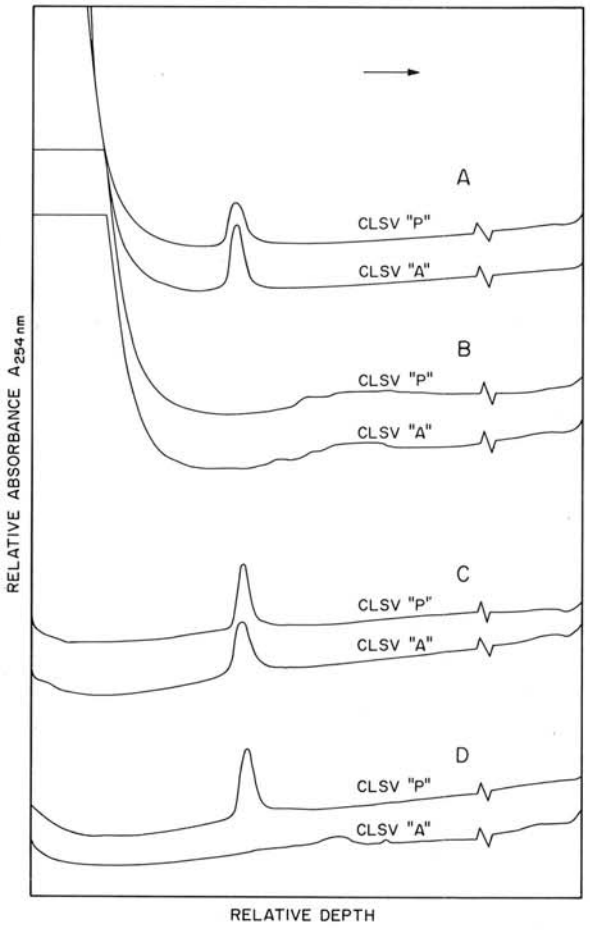


**Fig. 2.** UV absorbance profiles of density-gradient centrifugations of aliquots of the same preparations of apple chlorotic leaf spot virus from apple (CLSV-A) and apple chlorotic leaf spot virus from peach (CLSV-P) dialyzed for 18 hr against **A)** normal rabbit serum at 4 C; **B)** normal rabbit serum at 20 C; **C)** 0.05 M Tris-HCl containing 0.005 M $MgCl_2$, pH 7.8 at 4 C; **D)** 0.05 M Tris-HCl, 0.005 M $MgCl_2$, pH 7.8 at 20 C. Arrow indicates direction of sedimentation.

The other isolates tested could also be categorized as Type "A" or Type "P" on the basis of symptomatology in *C. quinoa* (Table 1).

*Virus yields.*—Summation of the areas under absorbance profiles of density-gradient centrifugations showed that equal quantities of tissue yielded only one-fifth as much CLSV-P as CLSV-A when the standard purification procedure was used. However, the yield of CLSV-P but not CLSV-A, could be increased by about 75% by the addition of 1% PEG to the extraction buffer (a procedure suggested to us by Dr. J. M. Thresh, East Malling Research Station, England). Although the use of PEG introduced more low molecular weight impurities into the virus preparation, these were separable by density-gradient centrifugation (Fig. 1A). The results of an experiment comparing yields of CLSV-P obtained by adding PEG at 0%, 0.5%, 1.0%, 1.5%, 2.0%, and 2.5% indicated that addition of more or less than 1% PEG resulted in a decrease in virus yield.

In contrast to the situation with CLSV-A, yields of which may be increased fivefold including 0.01 M $MgSO_4$ in the extraction buffer (10), extraction of CLSV-P in the presence of 0.01 M $MgSO_4$ did not appear to enhance the virus yield.

*Physical properties.—Particle characteristics.*—When centrifuged in sister rate-zonal density gradient tubes, preparations of CLSV-A and CLSV-P sedimented to exactly the same level. Preliminary comparative particle measurements of preparations of the two strains stained in uranyl acetate, indicated that on the basis of particle length and width the two strains were indistinguishable; they both showed numerous long flexuous rods with cross-banding, which suggested helical construction (9). We conclude that particles of each strain have the same size and general characteristics.

*Physical properties.—Ultraviolet light absorption.*—The unusual $A_{260/280}$ ratio of 1.85 previously reported for isolates of CLSV from apple (10) was also obtained for several of the purified preparations of CLSV-P used in this study. A comparison of the absorption spectrum for a preparation of tobacco mosaic virus with those for CLSV-A and CLSV-P showed that a tryptophan shoulder was lacking for the CLSV strains (Fig. 2B). Also the absorption maximum and minimum of both CLSV strains was shifted to the left of the TMV absorption profile. Possibly the absence of tryptophan may contribute to the unusually high $A_{260/280}$ ratio.

*Physical properties.—Thermal inactivation tests.*—Lister et al. (9) reported an infectivity half-life at 45 C of 12 ± 1 min for bentonite-clarified sap from *C. quinoa* infected with strain C-8, an apple isolate of CLSV, and the infectivity of the samples used reached zero within 80 min. In a similar study of both CLSV-A and CLSV-P, we found that purified preparations were still quite infectious after 160 min at 45 C. Restricted applicability of local lesion assay prevented a half-life determination for CLSV-P, but for purified preparations of CLSV-A, the half-life determined graphically from plots of log lesions against time at

45 C was approximately 42 min.

This value remained substantially unchanged with the addition of equal volumes of either partially or very thoroughly bentonite-clarified extracts from healthy *C. quinoa* plants; and similarly, these treatments did not noticeably affect the infectivity of CLSV-P, as judged by systemic infections. Apparently, the bentonite treatment readily removed unreacted or free inhibitors present in the sap (12).

In contrast, when crude leaf extracts (with or without 0.005 M $MgCl_2$) were added to purified virus, the infectivity of both strains was virtually eliminated within 10 min at 45 C or at 4 C, a result consistent with the known inhibitory effect of *C. quinoa* sap on CLSV.

The relatively low half-life reported by Lister et al. (9) may have resulted from the presence of inhibitors already attached to the virus during extraction. Inhibitors occurring in *Chenopodium* species can be removed by density-gradient centrifugation of viruses (2). Our results did not indicate any marked difference in thermal sensitivity between CLSV-A and CLSV-P.

*Physical properties.—Relative stability during dialysis.*—Lister & Hadidi (10) found that there is a structural requirement for divalent cations in two CLSV strains of Type A. Unless $Mg^{2+}$ or $Ca^{2+}$ was supplied during dialysis against buffers, these viruses disassembled.

In our studies, CLSV-P was also degraded when dialyzed overnight at 4 C against 0.05 M Tris-HCl at pH 7.8 in the absence of divalent cations or in the presence of $Na^{+}$ as 0.05 M NaCl, and was no longer detectable as a peak in ultraviolet absorbance profiles or density-gradients (Fig. 1C). Indeed, the results of a series of such experiments which compared the products after dialysis in the presence or absence of various selected cations, indicated that both strains showed a similar structural requirement for divalent cations. All the cations were supplied as 0.005 M chlorides except for $CdSO_4$, and dissolved in 0.05 M Tris-HCl at pH 7.8. Magnesium ion ($Mg^{2+}$), $Ca^{2+}$, or $Ba^{2+}$ stabilized both strains, whereas $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Fe^{2+}$, $Hg^{2+}$, and $Cd^{2+}$ caused loss, either through aggregation or degradation (Fig. 1D). Both strains were preserved during dialysis in the presence of $Mn^{2+}$ or $Sr^{2+}$, although after these treatments, CLSV-A sedimented more rapidly (Fig. 1D). A further difference between the two strains was revealed by dialysis against buffer containing 0.005 M $MgCl_2$ at 20 C, after which CLSV-P sedimented normally but CLSV-A was lost, probably through aggregation (Fig. 2). No infectivity tests were made in these experiments.

Because of indications of differential stability revealed in serological testing, the effects of dialyzing the two strains against normal rabbit serum were investigated. Both strains remained intact upon overnight dialysis against normal serum at 4 C, but each was lost, probably by aggregation, by similar dialysis at 20 C (Fig. 2).

*Serological relationships.*—To check purity, purified virus preparations as used for antiserum production were tested against antiserum prepared against normal proteins of cucumber, primarily Fraction 1 protein (13), which was kindly supplied by Dr. R. H. Converse, Oregon State University. No reactions occurred between the virus preparations and the normal host protein antiserum. Also, antisera to both strains of CLSV did not react with proteins extracted from healthy *C. quinoa*, obtained as components sedimenting above the position expected for virus in rate-zonal density-gradient centrifugations, or with fractions sampled at the position expected for virus. These results show that the virus preparations used were essentially free from contaminating host proteins.

In ring interface precipitin tests, the antiserum to CLSV-A had a homologous titer of 1/128-256, and a heterologous titer to CLSV-P of 1/32. CLSV-P antiserum had a homologous titer of 1/128 and a heterologous titer of 1/64.

Gel double-diffusion tests between CLSV-A and

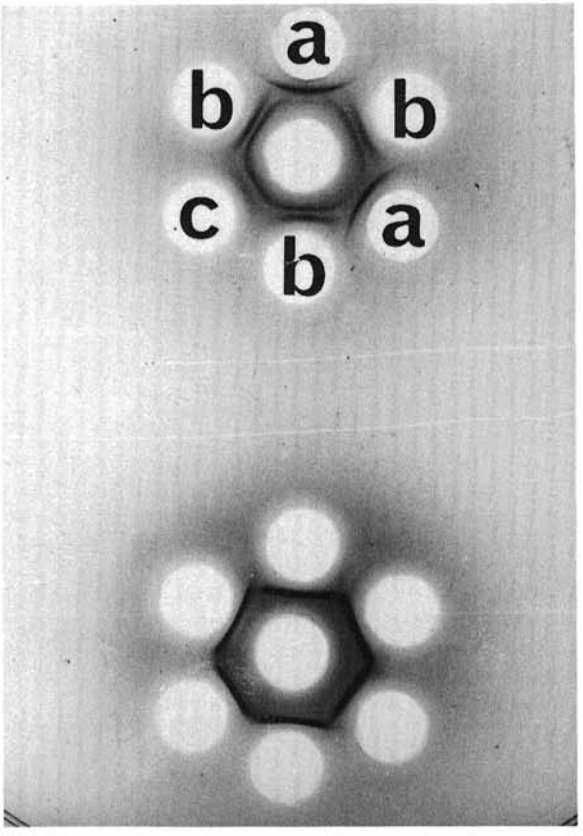


**Fig. 3.** Gel diffusion tests showing reactions of apple chlorotic leaf spot virus from apple (CLSV-A) and apple chlorotic leaf spot virus from peach (CLSV-P) antisera against various antigens. Central wells contain undiluted antisera to CLSV-A (top) and CLSV-P (bottom). Other wells contain: **a)** purified CLSV-A; **b)** purified CLSV-P; **c)** purified CLSV (Anzac 2 isolate). Note formation of spurs indicating partial identity. Concentrations of each antigen was approximately $A_{260\ nm} = 0.03$.

TABLE 2. Homologous and heterologous titers of antisera to two apple chlorotic leaf spot virus (CLSV) strains in ring interface tests with various antigens

| Antigen | Antisera[a] | |
|---|---|---|
| "A-type" CLSV isolates | CLSV-A[b] | CLSV-P[c] |
| B38-E ("CLSV-A") | 1/128-256 | 1/64 |
| C-8 | 1/128-256 | 1/64 |
| 710-EL2 | 1/128-256 | 1/64 |
| Cherry 15-17-45 | 1/128 | 1/64 |
| "P-type" CLSV isolates | | |
| Anzac 1 ("CLSV-P") | 1/32 | 1/128 |
| Anzac 2 | 1/8 | 1/128 |
| "Smith's seedling peach" | 1/16 | 1/32 |

[a] Reciprocal titers.
[b] CLSV-A = antiserum against B38-E.
[c] CLSV-P = antiserum against Anzac 1.

CLSV-P and antiserum to CLSV-A resulted in the formation of two obvious precipitation lines representing relatively slow-diffusing and fast-diffusing antigens (Fig. 3). With antiserum to CLSV-P, a faint precipitation line to fast-diffusing antigen was seen only when concentrated antigen samples were used. The significance of the two precipitation lines as indicating the presence of "soluble antigen" will be discussed elsewhere (Chairez & Lister, *in press*). Here, it is of interest that the presence in antiserum to CLSV-A of relatively more antibody to the fast-diffusing antigen suggested a differential stability of CLSV-A and CLSV-P, at least in the particular rabbits used to prepare antisera.

In appropriate arrangements comparing the reaction of the two strains against one antiserum (Fig. 3) the more slowly diffusing antigens formed a spur, indicating partial identity. This spur formation and the difference in ring interface titers were the serological bases for strain differentiation. Cross-absorption tests were not attempted because of limitations in virus yields.

Antisera to both CLSV-A and CLSV-P were tested against all isolates in Table 1 in both ring precipitin and gel diffusion tests (Table 2). In gel diffusion tests CLSV-A, and 710-EL2 showed no spur formation when reacted against CLSV-A antiserum. Cherry 15-17-45 isolate did not appear to show spur formation against CLSV-A antiserum, although the precipitin line was faint due to limited virus yields with this isolate. Isolates Anzac 2 and "Smith's seedling peach" from peach reacted like CLSV-P in respect to spur formation, but the "Smith's seedling peach" isolate showed a low ring test titer (1/16-32) to both antisera.

DISCUSSION.—Biologically distinctive isolates of CLSV appear to be common, but the evidence presented here establishes unequivocally for the first time the existence of serologically distinguishable strains. Interestingly, though the two strains primarily worked with differ so much in symptoms as to make diagnosis of both as CLSV difficult with the test plants used, their physical properties are sufficiently similar and distinctive to confirm relationship.

The physical differences noted, including the differential effects of PEG and $Mg^{2+}$ in virus extraction, and the slight difference in stability when dialyzed at 20 C against 0.005 M $MgCl_2$, could well be related to minor factors, such as electrophoretic charge differences, which commonly occur between virus strains. However, more substantial differences may occur between other CLSV strains, and serological testing clearly takes precedence as the most convenient and reliable procedure for identifying CLSV. It is thus important to assess the degree to which CLSV isolates fit into the CLSV-A and CLSV-P serotype groupings, and whether widely differing serotypes are common. Such information would be highly relevant to the choice of antisera used in diagnosis. In this regard, although all the isolates from peach examined here resembled CLSV-P in origin and symptoms, one of them differed serologically.

CLSV-A and CLSV-P differed in woody host origin, symptomatology, and virulence. Infected woody hosts may typically harbor mixtures of strains of CLSV, and local lesion isolates from the same source can differ in virulence (9). Indeed, it has been suggested that culturing CLSV in specific woody hosts can result in biological modification, presumably by strain selection (1). However, in herbaceous test plants, the isolates studied so far seem to retain their characteristics, and although CLSV-P tended to become somewhat more virulent after much subculturing, its serological characteristics were unchanged.

No natural vector of CLSV is known, and epidemiologically and from the standpoint of control, it would be interesting to know if serological differentiation reflects such characteristics as natural host origin, geographical occurrence, symptomatology, and virulence.

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