Antiserum Preparation and Partial Purification of Potato Virus A

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ABSTRACT

Potato virus A (PVA) was partially purified employing three different buffer systems (borate, citrate, and phosphate). During initial extraction, 0.01 M 2-mercaptoethanol plus 0.01 M ascorbic acid in 0.05 M phosphate buffer pH 7 followed by dialysis against the same buffer stabilized the virus. Aluminum oxide added before the grinding of the tissues also gave a high degree of stabilization. These results show that an oxidative degradation of PVA may be at work during the first steps in purification. Carbon tetrachloride was used for clarification in a 1:1 sap solvent ratio. Infectivity of the preparations was best maintained when borate buffer was used for resuspension. Density-gradient centrifugation in a borate buffer system did not produce infectious fractions in the sucrose gradients, but gave infectious pellets of PVA at the bottom of the gradient tubes. Electron microscopy showed that degradation and loss of protein from the virus particles may account for the loss of PVA infectivity. An antiserum against PVA was produced with a homologous titer of 1/512 and a heterologous titer against a PVY isolate of 1/64. Phytopathology 60:1415-1421.

Purification of labile viruses presents a special problem, since initial clarification and concn usually requires several hr, during which time much of the infectivity is destroyed.

Many labile viruses have been stabilized by using reducing agents like sodium sulfite (3), cysteine alone (17) or in combination with sodium cyanide (12), 2-mercaptoethanol (13, 19), ascorbic acid (3), dithiothreitol (19), thioglycollic acid (27), and others.

Polyphenoloxidase inhibitors like DIECA (13, 14, 15, 16, 20), sodium cyanide (14), and phenylthiourea (14) are known to stabilize some viruses. Substances that combine with tannins, like caffeine (10) and alumina (8), have also been employed in stabilization of viruses.

Potato virus A is a labile virus that belongs in the potato virus Y group according to Brandes' (6) system of classification. There is no report of its purification, although the size of its particle has been measured by Brandes & Paul (7), and antiserum has been prepared by Roland (22) and Bartels (1).

Most of the procedures used for the purification of viruses in this group involve differential centrifugation and differ mainly in the process of clarification of the extracts. Wetter (29) used ether and carbon tetrachloride to purify potato virus Y, bean common mosaic, and bean yellow mosaic. Tomlinson (27), Shepherd & Pound (24), and Purcifull (21) purified lettuce mosaic, turnip mosaic, and tobacco etch, respectively, by using *n*-butanol. Shepherd (23) and Delgado-Sanchez & Grogan (9) used chloroform alone to purify sugar cane mosaic and potato virus Y, respectively.

The present study reports the stabilization of potato virus A in sap and its partial purification by differential centrifugation, using carbon tetrachloride to clarify the extracts.

MATERIALS AND METHODS.—*Source of virus.*—The isolate of potato virus A (PVA) used in this study was obtained from a single local lesion produced on the test plant *Solanum demissum* × *Solanum tuberosum* "A6" (18) by inoculation with the original isolate supplied by Robert Akeley, USDA, Beltsville, via H. Darling. The host range and physical properties of this isolate were similar to those reported in the literature (4, 25) for PVA.

Greenhouse handling of the plants used in the study.—The local lesion host "A6" was used for assaying the virus, and PVA was purified from *Nicotiana debneyi*. Both were grown in a greenhouse at 20-24 C.

"A6" plants were propagated using stolons grown in 6-inch pots filled with a muck soil. They were fertilized every 2-3 weeks with 1.4 g of a 10:10:10 NPK fertilizer. To prevent early flowering, the seasonal light regime was augmented with 100 w of artificial light to make a day length of 18 hr. Only one plant was allowed to grow in each pot, and after most of the leaves had been used, the stem was cut at its base and a new stolen allowed to grow.

N. debneyi was transplanted to 4-inch pots containing a mixture of 2:1:1; soil:peat moss:sand. After they were established and after inoculation they were fertilized with approximately 0.7 g of the same fertilizer used for the "A6" plants. Three weeks after transplanting, the apex was cut and only the three middle leaves allowed to grow until they were between 18-22 cm long; they were then dusted with Carborundum and inoculated with potato virus A. The inoculum was prepared by grinding infected *N. debneyi* leaves in 0.03 M phosphate buffer, pH 8. New inoculum was used after 6 to 8 plants had been inoculated.

Assay.—For assaying, the leaves of "A6" were detached (28) and placed in petri dishes containing

damp filter paper. Leaves were dusted with Carborundum and inoculated using a glass spatula. The plates were then put in polyethylene bags and placed under artificial light (1,000 lux) at a temp of 24 C.

To minimize variability, only the fully expanded leaves were selected for experimentation. Unless otherwise stated, they were distributed in such a way that all the treatments had four leaves and the same number of leaves of the same age. Lesion counts are expressed as number of lesions per unit area, a parameter which is found by the formula:

$$\frac{\text{Total no. of lesions per leaf (2).}}{\text{length} \times \text{width in cm}}$$

Centrifugation.—High-speed centrifugation was executed in a Spinco Model L ultracentrifuge using No. 30 (90 min at 30,000 rpm) and No. 40 (45 min at 40,000 rpm) rotors. Low-speed centrifugation for clarification of the homogenates or resuspended pellets was done in the GSA rotor of the Servall RC-2 refrigerated centrifuge (20 min at 5,000 rpm) or in the No. 30 or 40 rotor (20 min at 8,000 rpm), respectively.

Density-gradient centrifugation was used to study the partially purified preparation. Linear gradients consisting of 7 to 30% buffered sucrose were prepared using a modification of the method described by Stace-Smith (26). One ml of the virus preparation with an OD of 10-30 units (at a wavelength of 260 mμ) was floated on top of the gradients and subsequently centrifuged in the SW 25.1 rotor at 25,000 rpm for 2 hr. Fractions were collected by means of an ISCO density-gradient fractionator (5).

Infectivity assays were carried out after each step in the purification.

Sedimentation coefficients were determined from partially purified PVA preparations using a Spinco Model E analytical centrifuge.

For electron microscopy, partially purified preparations or the collected fractions from the density gradient were used. A small drop was placed on grids, allowed to dry, and a mixture of carbon and platinum was evaporated under acute angles over the specimen. The specimens were subsequently examined in the JEM7 electron microscope.

Preparation of antiserum to Nicotiana debneyi protein.—An antiserum to *N. debneyi* protein was prepared following a modification of the method described by Purcifull (21). Tissue was homogenized in 0.1 M Tris[tris(hydroxymethyl) aminomethane]-HCl pH 7.2 and 0.1 M $MgSO_4$ in proportion of 2:1, w/v. Two-hundredth M 2-mercaptoethanol and 0.03 M ascorbic acid were added immediately before processing the tissue. The homogenate was filtered through cheesecloth and centrifuged at 3,000 rpm for 20 min. The supernatant was centrifuged at 30,000 rpm for 3 hr, and the resulting pellets were resuspended in distilled water. The high-speed supernatant was made up to 60% saturation with ammonium sulfate. The precipitate formed was pelleted by centrifugation, resuspended in distilled water, and dialyzed against distilled water for 48 hr. After dialysis and low-speed centrifugation, the supernatant was reprecipitated with ammonium sulfate and resuspended with distilled water ⅓ of the preceding volume. The suspension was dialyzed again to free the preparation of the salt and then mixed with the resuspended pellets. The pH of the final preparation was raised to 9 with 0.5 M NaOH to solubilize all the protein, which was dispensed in small test tubes and stored frozen.

Rabbits were injected intramuscularly every 3 days with 1 ml of the protein preparation (E_{280} ca. 36) emulsified in Freund's incomplete adjuvant. After 12 injections, antiserum diluted 1:256 precipitated appropriately diluted protein. The gamma globulin was precipitated from the antiserum with 40% sat. ammonium sulfate. The precipitate was resuspended and centrifuged twice in fresh ammonium sulfate, dissolved in saline equal to one-fourth of the original volume, and stored frozen in 2-ml lots.

Serology.—Antisera prepared during the course of this study were tested for their homologous and heterologous titers in microprecipitin tests under mineral oil.

RESULTS.—*Rate of virus increase.*—To determine the conditions under which directly inoculated leaves produce the highest concn of virus, two plants of *N. debneyi* were trimmed to three leaves each and inoculated heavily with PVA using a glass spatula. The leaves, then, were divided into 4 equal areas, and one disc was removed from each using a cork-borer. A composite sample totalling 0.7 g was ground in 2 ml of 0.03 M phosphate buffer, pH 8.2, and inoculated to 4 leaves of "A6" every 2 days starting from the 2nd day after inoculation of *N. debneyi* plants.

The $\log_{10}$ of the number of local lesions per unit area obtained on "A6" leaves was plotted as a function of time after inoculation (Fig. 1). The infectivity showed a logarithmic increase until the 10th day, and decreased following the same pattern. On the basis of these results, inoculated leaves were harvested for virus purification 8-12 days after inoculation.

Stability of PVA in extracts.—Since the infectivity of PVA is lost in undiluted plant extracts in a few hr, a number of chemicals were tested for their ability to stabilize this virus.

Extracts were prepared by grinding 2 g of infected tissue in 10 ml of 0.05 M potassium phosphate buffer, pH 7. To get a uniform inoculum, the infected leaves were cut in small squares and these were randomized before weighing. Chemicals were dissolved or suspended in the buffer immediately before the tissue was ground. Inoculations were made to four leaves of "A6" immediately after the tissue was ground, and again 4 hr later. Leaves were not rinsed after inoculation.

Infectivity was best stabilized by 2-mercaptoethanol plus ascorbic acid followed by dialysis of the extract for 1 hr in the same buffer (Table 1), although complete stabilization was not possible. Use of aluminum oxide was preferred since it did not involve the time-consuming process of dialysis. Although at the end of 4 hr there is still a fairly high percentage of infectivity in most of the treatments, it should be mentioned that this is not the case in undiluted plant extracts.

Clarification of extracts.—Infected tissue was ground

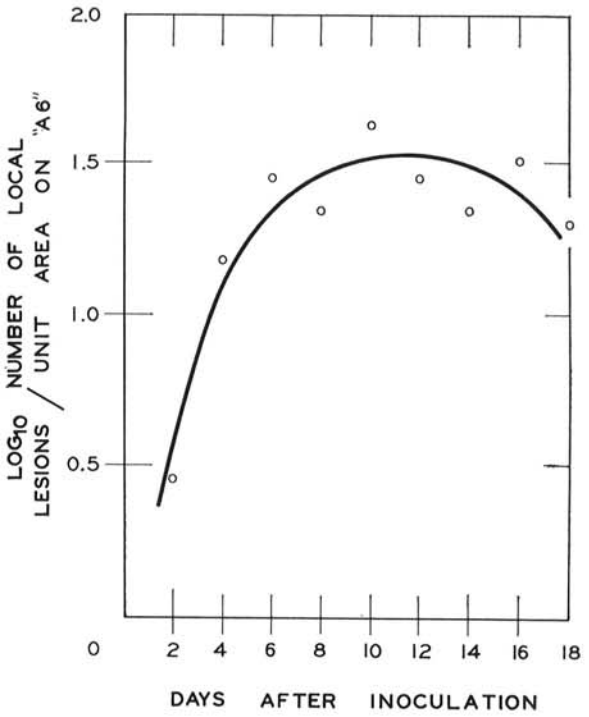

Fig. 1. Rate of PVA increase in directly inoculated leaves of *Nicotiana debneyi* at 20-24 C.

TABLE 1. Effect of various chemicals on the infectivity of PVA in crude extracts on *Nicotiana debneyi*[a]

Chemical and concn	Lesions/unit area 0 hr	Lesions/unit area 4 hr	Avg of 2 trials: % Infectivity retained for 4 hr
Distilled water	49	20	41
0.05 M K phosphate buffer, pH 7	47	22	45
Na sulfite 0.01 M	46	26	56
2-mercaptoethanol 0.01 M	42	24	58
Cysteine HCl 0.01 M	55	22	41
Ascorbic acid 0.01 M	44	30	70
2-mercaptoethanol 0.01 M + ascorbic acid 0.01 M	23	11	48
2-mercaptoethanol 0.01 M +ascorbic acid 0.01 M followed by dialysis of extract in same buffer	28	23	81
Caffeine 0.01 M	27	15	54
Sodium diethyldithiocarbamate 0.01 M	28	19	68
Polyvinyl pyrrolidone[b]	23	13	57
Aluminum oxide[b]	31	24	78

[a] Comparisons between treatments cannot be made because of difference in age of "A6" leaves. All the chemicals were in 0.05 M phosphate buffer, pH 7, except where noted otherwise.

[b] Insoluble materials tested at the proportion of 10% of wt of tissue.

with 0.05 M potassium phosphate buffer pH 7 in the proportion of 100 g of tissue to 100 ml of buffer. Before grinding, aluminum oxide was added at the rate of 10 g Al_2O_3/100 g of tissue. The ground pulp was squeezed through cheesecloth and divided into four parts. Two solvents (chloroform and carbon tetrachloride) in two concentrations were compared for their ability to clarify the plant extracts containing PVA. Each aliquot was shaken in a separatory funnel with the appropriate solvent in a 1:1 or 1:5 solvent sap ratio. The mixtures were then centrifuged at 5,000 rpm for 20 min. The supernatants were left at room temp for 4 hr and inoculated to four leaves of "A6".

Infectivity was best maintained by using chloroform in a 1:5 solvent:sap ratio (Table 2) or by carbon tetrachloride in either proportion, but carbon tetrachloride was preferred since relatively more normal host material was removed without appreciable loss of infectivity using this solvent in a 1:1 solvent:sap ratio.

Resuspending media.—PVA-containing sap, extracted from infected tissues using phosphate buffer, aluminum oxide, and carbon tetrachloride clarification was centrifuged. The resulting pellets were resuspended in 0.02 M, pH 7 borate, citrate, and phosphate buffers, respectively. The resuspension was faster in the citrate buffer than in the phosphate or borate buffers, but the infectivity after 1 week of storage at 2 C expressed in number of local lesions per unit area was 29 for borate, 13 for citrate, and 12 for phosphate. The infectivity of clarified extracts diluted 1/25 was 3 (lesions per unit area in "A6"), while the infectivity after the second high-speed centrifugation and resuspension in borate buffer of a similarly diluted sample was 9. This indicated that a concn of the infective agent was obtained. On the basis of these results, the use of borate was preferred.

Analysis of the tubes subjected to density-gradient centrifugation in the ISCO fractionator showed strong absorption only in the meniscus in the case of pellets resuspended in citrate buffer. Absorption (254 mμ) peaks were present below the meniscus in the cases where borate and phosphate were used.

Resuspended pellets obtained after the first high-speed centrifugation using borate or phosphate buffers were put through a second cycle of high- and low-speed centrifugation using the same buffers as resuspension media. These suspensions were analyzed as before on sucrose-density gradients. In all cases, a small pellet

TABLE 2. Infectivity of PVA after clarification with two different solvents

Solvent	No. lesions/unit area, avg of two trials
Chloroform 1 Vol/sap 1 vol	19
Chloroform 1 Vol/sap 5 vol	30
Carbon tetrachloride 1 Vol/sap 1 vol	29
Carbon tetrachloride 1 Vol/sap 5 vol	25

TABLE 3. Scheme for the purification of potato virus A

—Tissue ground in 0.05 M phosphate buffer pH 7 in proportion of 1:1, w/v, + 10% w/w aluminum oxide
—homogenization for 15 sec
—filtration through cheesecloth

Filtrate
—+ CCl_4 1:1
—shaking in sep. funnel for 2 min
—centrifugation at 5,000 rpm for 20 min

Supernatant
—centrifugation at 30,000 rpm for 90 min

Pellet
—resuspension in 1 cc of 0.02 M borate buffer pH 7
—centrifugation for 15 min at 8,000 rpm

Supernatant
—centrifugation at 40,000 rpm for 45 min

Pellet
—resuspension in 1 cc of 0.02 M borate buffer, pH 7
—centrifugation for 15 min at 8,000 rpm

partially purified virus

was formed at the bottom of the density-gradient tubes after centrifugation, indicating probable aggregation of the virus. Two peaks were present where phosphate was used, and only one in the case of borate.

High absorption in the meniscus and the absence of a peak in the citrate system and the presence of two peaks, both containing rodlike particles of small sizes, in the case where phosphate was used, indicated breakdown of the virus.

On the basis of the initial infectivity studies and the appearance of a single absorption peak when borate was used, it was decided to analyze this system further. Using the Spinco analytical centrifuge Model E with ultraviolet optics, we found that the sedimentation coefficient of the band in the borate system was 83 S. Potato virus Y (PVY) has been reported (25) to have a sedimentation coefficient of 200 S, and since PVA and PVY are similar in shape and size, a similar sedimentation coefficient was expected for PVA. Concentrated collected peaks gave a typical nucleoprotein absorption spectrum with an $E_{260}:E_{280}$ of 1.46, but as already expected from the analytical centrifuge data, no infectivity was present in these preparations. Observations in the electron microscope showed the presence of broken and degraded rods with exposed RNA cores (Fig. 2-B, D, E).

Resuspensions of the pellets found in the density-gradient tubes, when the borate system was employed, resulted in preparations with $E_{260}:E_{280}$ ratios of 1.2. When the optical densities of these preparations were adjusted to that of the material originally layered on the sucrose gradients and subsequently tested for infectivity, it was found that no increase of infectivity over the original material was obtained.

For maintenance of max infectivity it was, therefore, decided not to use the density-gradient centrifugation but to follow the procedure of purification shown in Table 3.

Serology.—For preparation of antiserum, the citrate buffer was used since the virus pellets resuspended readily in this buffer and produced suspensions with a high absorption at 260 mμ (± 70 OD units/ml) after the second cycle of differential centrifugation if compared with similar pellets resuspended in the other buffers. Infectivity of the preparations was not increased during repeated cycles of centrifugation as in the case of borate.

Removal of host protein from the viral preparations was achieved by absorbing the resuspended second high-speed pellets with our antibody preparation against *N. debneyi* protein. Initial addition of antiserum resulted in clouding of the preparations. After

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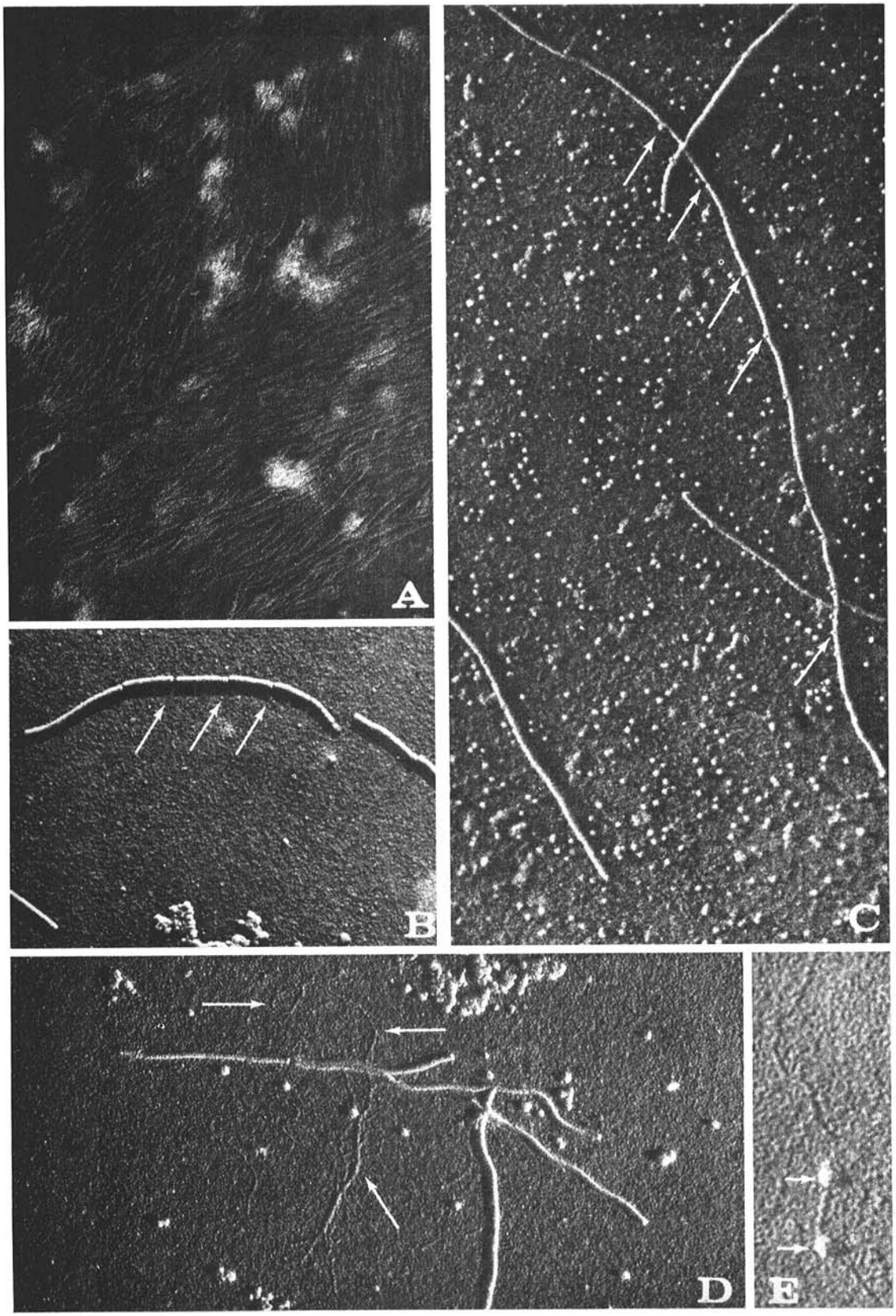
Fig. 2. A) An aged virus suspension prepared from a first high-speed centrifugation showed virus aggregates (×14,200). **B)** Virus collected from bands in density-gradient tubes. The borate system was employed. Note protein loss and breaks in particles as observed in the shadows (arrows) (×56,000). **C)** Virus particles in association with viral protein in a solution used for antiserum preparation. Unaggregated viral protein is seen in the background. Note the protein loss of the virus particles (arrows) (×56,000). **D)** PVA prepared using the borate system after density-gradient centrifugation and collection of the virus fraction. Note the strands of viral RNA (arrows) (×56,000). **E)** A close-up of an RNA strand from a similar preparation as in Fig. 2-D. Note the protein still attached to the RNA (arrows) (×120,000).


A
B
C
D
E

30-60 min of incubation, the preparations were centrifuged for 15 min at 8,000 rpm. A pellet was formed and the supernatant was placed in another tube, and more of the antibody preparation was added. The process was repeated until no pellet was formed. Electron microscopy of these preparations showed the presence of extraneous materials (Fig. 2-C) which were believed to be of viral nature since they were not removed by the antiserum treatment. Besides particles of normal length as reported by Brandes (6), particle fragments could be observed as well as end to end aggregated particles and particles in the process of breakdown (loss of protein). After removal of precipitated host protein, the preparations were injected into the rabbit without separating virus from extraneous rabbit gamma globulin. One ml of a virus preparation with an E_{260} value of 10 OD units/ml was emulsified with 1 ml of Freund's incomplete adjuvant and injected intramuscularly. After the first injection, 1 ml of a preparation with the same OD or higher, without adjuvant, was injected intravenously every 3 days. After 10 injections, the rabbit was bled. The titer of the antiserum was $\frac{1}{512}$ using as antigen partially purified PVA ($E_{260} = 75$ OD units/ml) in a microprecipitin test. In a similar test, there was no reaction of the antiserum against normal host protein ($E_{280} = 36$ OD units/ml). When the PVA antiserum was tested against purified PVY, the titer was $\frac{1}{64}$; however, there was no reaction when antiserum prepared to this PVY isolate with a homologous titer of $\frac{1}{125}$ was tested against partially purified PVA.

Discussion.—One of the main problems in the purification of PVA is its low concn in systemically infected plants. It has been shown, however, that the concn of PVY (9) and a number of other viruses is higher in directly inoculated leaves than in those systemically invaded after inoculation. It was, therefore, decided that only directly inoculated leaves would be used for purification purposes. The large increase of PVA in a 10-day period in these directly inoculated leaves and the high OD values of the final virus preparations indicate that a sizable virus concn was reached during this time.

The first of a number of processes involved in the loss of infectivity and the breakdown of PVA in virus preparations can be recognized and inhibited during the initial virus extraction from infected tissues. The high degree of stabilization of PVA during virus extraction using antioxidants followed immediately by dialysis of the crude sap suggests that virus inactivation in crude sap is, at least in part, due to oxidation of polyphenols, as has been shown for cucumber mosaic virus (CMV) by Harrison & Pierpoint (16), who found evidence that when polyphenol oxidase is inhibited in plant sap by DIECA and the extract is subsequently dialyzed to remove polyphenol oxidase substrates, a high degree of virus stabilization is achieved. The importance of the removal of polyphenol oxidase substrates in the initial step of PVA purification is confirmed by the fact that aluminum oxide, which is known to bind polyphenols and tannins, also stabilizes this virus.

Irreversible aggregation of PVA in suspension (Fig. 2-A) of the virus pellets can be considered the second process contributing to the loss of infectivity during purification.

When infectious virus in borate was subjected to density-gradient centrifugation, no infectivity was observed in the collected peaks. Electron microscopy from these preparations gave evidence of the third process that contributes to PVA inactivation. From Fig. 2-B, D, and E, it is seen that the loss of protein from intact particles exposes the RNA core of the particles which results in particle breakage. Sometimes RNA strands can be visualized (Fig. 2-D, E, arrows). Protein loss from flexuous rods as a mechanism of inactivation has been reported earlier for maize dwarf mosaic virus (11). The high $E_{260}:E_{280}$ ratios of our preparations indicated that an excess of RNA was present in the virus suspensions.

The possible explanation for the partial loss of infectivity in a density-gradient system containing borate may be found in the fact that dislodged viral protein is separated from the particles. When the protein in the fully assembled particle is in equilibrium with the dislodged viral protein in solution, the removal of this protein from the solution during density-gradient centrifugation tends to dislodge more protein from the particles resulting in increased breakdown of PVA. Therefore, density-gradient centrifugation may be self-defeating where PVA is concerned.

Pellets always appearing in density-gradient centrifugation would tend to show that aggregation of PVA has taken place. The infectivity per OD unit of the pellets formed in the sucrose gradients compared with the infectivity per OD unit of the material originally layered on these gradients did not increase. Infectivity, therefore, must have been lost during the density-gradient centrifugation.

Electron microscopy showed that the citrate buffer removes protein from the PVA particle (Fig. 2-C), seemingly in discrete aggregates that do not reaggregate. Since citrate has a chelating effect, it seems appropriate to assume that a metal plays a role in stabilizing the PVA particle. This matter needs more attention before conclusions can be drawn.

The relationship between PVA and PVY described earlier by Bartels (1) was confirmed. Our PVA antiserum reacted with a PVY isolate from a Peruvian potato variety.

It has been shown that PVA can be purified, and that antisera can be produced from these partially purified preparations. The breakdown of PVA constitutes an interesting phenomenon in plant virus inactivation. This type of viral breakdown, resulting in the loss of infectivity, may constitute a general pattern of inactivation of the labile viruses in the group of the flexuous rods.

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