Purification of the Tobacco Etch and Other Viruses of the Potato Y Group

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ABSTRACT

A procedure was developed for purification of the tobacco etch and other viruses of the potato Y group without marked aggregation as judged by behavior of the isolated product during sucrose density-gradient centrifugation. Using 0.5 M urea and 0.1% 2-mercaptoethanol in the resuspension buffer during purification was highly beneficial in preventing lateral aggregation. The results suggest that aggregation may be the consequence of hydrophobic interactions.

The purity of virus preparations was assessed by electron microscopy, sucrose density-gradient centrifugation, and polyacrylamide gel electrophoresis. With the latter technique, impure preparations yielded several migrating components in the running gel and heavy staining in the sample gel after electrophoresis. Protein from highly purified virus yielded two components in the running gel without detectable staining in the sample gel after electrophoresis, indicating dimerization due to formation of intermolecular disulfide bonds. Carboxymethylated viral protein migrated as a single component in polyacrylamide gel electrophoresis experiments with either cationic or anionic systems, demonstrating that only a single type of protein is present in the virus.

Also used as a preparative procedure was centrifugation to equilibrium in cesium chloride density gradients—to purify the viruses, to assess the purity of the products isolated, and to estimate buoyant densities. Estimation of buoyant densities by graphical methods gave values of 1.332 g/cc for the tobacco etch virus and 1.336 g/cc for turnip mosaic virus. Phytopathology 60:132-142.

The plant viruses of the potato virus Y (PVY) group have flexuous rod-shaped particles with a normal length ranging from 730 to 790 mμ (7), are transmitted by aphids in a stylet-borne manner, and induce diseases of the mosaic or mottle type in host plants. This group comprises the largest known category of plant viruses showing a common morphology, transmissibility, and serological relatedness.

Viruses of the potato Y group are characteristically difficult to manipulate in vitro because of their tendency to aggregate, both end-to-end and laterally (35). Aggregation, the most frequent and serious problem encountered during purification of these viruses, results in a physically heterogeneous population of virus particles, and sometimes proceeds to the point at which most or all of the virus loses solubility. Though several members of the group have been purified, none have been characterized to any extent as to degree of purity, since aggregation precludes such determination by conventional physical means. As a result, data are notably lacking on the chemical and physical properties of these viruses, and their tentative relatedness is based primarily on common morphological features and transmissibility and in some cases on serological relatedness (7).

Chemical and physical characterization of the potato Y group of viruses is desirable in order to confirm their postulated relatedness. Before meaningful data can be obtained on their chemical features, however, the viruses must be obtained in an essentially pure state and the degree of purity adequately assessed by reliable methods. To accomplish this objective, methods must be devised to prevent aggregation, so that purity can be determined by conventional physical methods, or, alternatively, methods must be developed for assessing the purity of physically heterogeneous preparations of these viruses. The methods described herein show promise of achieving both objectives.

Various methods and procedures were tested for purification of the tobacco etch or turnip mosaic viruses with the objective of obtaining pure virus in sufficient quantity for chemical characterization. The purified product, or that obtained at certain intermediate steps, was assessed for the amount of virus obtained, the degree of aggregation, and its purity—by sucrose density-gradient centrifugation (6) with whole virus, and by polyacrylamide gel electrophoresis experiments with protein from virus degraded in urea.

MATERIALS AND METHODS.—Most of the preliminary experiments carried out to evaluate the various preparative treatments were done with the tobacco etch virus. A stock culture of this virus was maintained in tobacco plants (*Nicotiana tabacum* L., 'Havana 425'), by mechanical transmission and occasional transmission by aphids. Virus for purification was propagated in tobacco grown in 5-inch plastic pots. Transplanted seedlings were inoculated mechanically in the 4- to 5-leaf stage, and the starting material was harvested 12-20 days after inoculation. Turnip mosaic virus was propagated in tendergreen mustard (*Brassica campestris* L.).

The infectivity of each of two or more inocula was compared by bioassay in 4-6 plants of *Chenopodium amaranticolor* Coste & Reyn., each with five well-developed leaves. Equal numbers of half-leaves were inoculated in a uniform manner after the leaves were dusted with corundum. Lesions were counted when well developed, 8-10 days later.

Preliminary fractionation.—Unless otherwise stated, infected tissue was homogenized in a Waring Blendor in 0.5 M phosphate buffer, ph 7.1, containing 1.0% 2-mercaptoethanol. Generally, 120 ml of the buffer were

used per 100 g tissue. The homogenized extract was strained through two layers of cheesecloth and clarified by adding *n*-butanol followed by slow-speed centrifugation (35). The *n*-butanol was washed thoroughly with bisulfite before use. The virus was precipitated from the clarified juice by dissolving 4.0 g of polyethylene glycol, mol wt 6,000-7,000 (PEG), per 100 ml of the extract (14), incubating the solution at 4°C for 60 to 90 min, and collecting the precipitated virus by low-speed centrifugation. The pellets were suspended in 0.025 M phosphate buffer, pH 7.4, and subjected to 2-3 cycles of differential centrifugation with suspension of the high-speed pellets in 0.025 M phosphate buffer. Occasionally the virus was precipitated by acidification to pH 4.8, followed by slow-speed centrifugation and resuspension of the precipitate in phosphate buffer. When the effects of various chemicals on the aggregation of TEV were tested, they were added to 0.025 M phosphate buffer, pH 7.4, which was used for suspending virus pellets, and were compared with the same solvent without additives. When it was desired to repeat precipitation of the virus by PEG from 0.025 M buffer, the solution was made 4% in NaCl.

High-speed centrifugations were made for 1.5 hr at 30,000 rpm in a Spinco Model L preparative ultracentrifuge in the No. 30 rotor. Low-speed centrifugations were made in a Sorvall refrigerated centrifuge—for 10-15 min at 9,000-10,000 rpm in the SS 34 rotor when volumes were small, and for 20-30 min at 7,500-8,500 rpm in the GSA rotor when volumes of solutions were large.

Rate zonal centrifugations were used to assess the degree of aggregation and purity of virus preparations (5, 6). These experiments were made in sucrose density gradients prepared by layering 4, 7, 7, and 7 ml of 0.025 M phosphate buffer, pH 7.4, containing 20, 30, 40, and 50 g sucrose, respectively, in 100 ml solution. The gradients were kept at 4°C for at least 16 hr before use. Centrifugations were made in the Spinco SW 25.1 rotor for 3 hr at 23,000 rpm. The results were then assessed with an ISCO density-gradient fractionator and ultraviolet scanner coupled to an external recorder.

Equilibrium density-gradient centrifugations were made in cesium chloride solutions with an initial density of 1.31 g/ml. These were prepared by mixing 1.8 ml of a saturated solution of cesium chloride (in 0.025 M phosphate buffer, pH 7.4, or in 0.01 M glycylglycine buffer, pH 7.8) with 3.2 ml of the virus solution in a 5-ml lusteroid tube. The virus reached its buoyant density zone in the gradient formed during 24 or 48 hr of centrifugation at 32,000 rpm in the SW 39 rotor. The gradient column was fractionated by puncturing the bottom of the tube and collecting 0.1-ml fractions. Even-numbered fractions, except near the virus zone, were used for estimating density with an Abbe refractometer (Basch & Lomb) and the formula of Ifft et al. (17). The odd-numbered fractions and all of those near major light-scattering bands were dialyzed individually against 0.02 M phosphate buffer, pH 7.4, or 0.005 M glycylglycine buffer, pH 7.8. The dialysis was performed in pieces of small glass tubing (one for each fraction) with one end sealed with a dialysis membrane. This end was dipped in the dialyzing medium. The tubes were supported in a perforated disc of Plexiglas rotated by an electrical motor in an Oxford multiple dialyzer. After dialysis, each fraction was made to 1.0 ml with distilled deionized water, and its optical density determined at 260 mμ in a Zeiss spectrophotometer. The infectivity of each fraction was assayed on *C. amaranticolor* and, in some cases, other plants.

Electron microscopy.—Virus preparations were examined in an RCA electron microscope, Model EMU-3. The virus was sprayed onto collodion-coated grids and shadowed with palladium. For negative staining with 2% potassium phosphotungstate, pH 7.0, the collodion membrane was coated with carbon.

Preparation of viral protein and nucleic acid.—To obtain TEV protein and nucleic acid in quantity, degradation was routinely carried out with guanidine hydrochloride in the presence of lithium chloride. The method is similar to that of Spitnik-Elson (40) for the preparation of bacterial ribosomal protein, except that urea is replaced by guanidine. The purified virus solution was mixed 1:1 with a solution containing 5.3 M guanidine hydrochloride, 3.3 M lithium chloride, 0.3 M borate (pH 8.5), and 0.1 M 2-mercaptoethanol, and the mixture was incubated at 4°C for 24 hr.

The viral RNA which precipitates under these conditions was recovered by low-speed centrifugation, suspended in 0.05 M barbital buffer, pH 7.0, and dialyzed against the same buffer at 0.01 M until dissolved. After estimation of the nucleic acid content by spectrophotometry, the RNA was precipitated by adding two volumes of cold ethanol and a few drops of 3.0 M acetate buffer, pH 4.5. The resulting precipitate was recovered by centrifugation for use in subsequent analyses reported elsewhere (12).

The protein was recovered from the supernatant after low-speed centrifugation by subjecting the solution to ultracentrifugation at 100,000 *g* for 90 min to remove undegraded virus, diluting the solution 1:1 with water, and adding an equal volume of saturated ammonium sulfate. After 1 hr at room temperature, the salted-out protein was collected by centrifugation, dissolved in 8.0 M urea, dialyzed exhaustively against water, and lyophilized.

Electrophoresis in polyacrylamide gel.—The purity of isolated virus and virus protein was tested by electrophoresis into 15% polyacrylamide gels. Both cationic and anionic systems were used, in which all the reagents were made up in 8 M urea. The cationic system used (15% running gel, pH 4.3) was described by Reisfeld et al. (33), and the anionic system was developed by G. E. Bruening and J. Dunning, Dept. of Biochemistry and Biophysics, Univ. of Calif., Davis. This system has a running pH of 10.5. It contains *n*-butylamine as the buffer, and employs chloride as the leading ion and β-alanine as the trailing ion.

The virus protein, 100-150 μg/tube, was dissolved in 8 M urea and applied in 0.15 ml of 2.5% polyacrylamide gel (sample gel) applied to the column and photopolymerized. Electrophoresis was carried out for 2-4 hr with a current of 5 ma/tube. The gels were removed, stained for 30-60 min with 0.05% aniline blue black in

7% acetic acid, and destained by repeated washing in 7% acetic acid.

The purity of virus was assessed also by direct electrophoresis in acrylamide gels. Volumes 100-200 μg virus were added to the sample gel before photopolymerization, or, alternatively, enough 8.0 M urea was added to the virus to make the solution at least 6 M in urea, followed by incubation for 15 min at room temperature to degrade the virus. Portions were then added to the sample gel. Electrophoresis, staining, and destaining were carried out as above.

Reduction and carboxymethylation of viral protein.—Reduction and carboxymethylation of viral protein was carried out by the procedure of Crestfield et al. (11). Five to 20 mg of lyophilized protein was dissolved in 5 ml of 8.0 M urea in a Warburg flask. To this was added 0.1 ml 2-mercaptoethanol, 0.1 ml of EDTA solution (50 mg of disodium EDTA/ml adjusted to pH 8.6), 2.0 ml of 0.1 M Tris HCl buffer (pH 8.6), 1.1 g deionized urea, and 1.0 ml of 16.0 mM dithiothreitol in 8.0 M urea (9). The atmosphere in the flask was then replaced with nitrogen, and the mixture was incubated in 37°C water bath for 10 to 16 hr for complete reduction. A solution of iodoacetate was prepared by dissolving 0.416 g of iodoacetic acid in 5.0 ml of 8.0 M urea and adjusting the pH to 8-9 with dilute sodium hydroxide. After reducing the protein, the iodoacetate solution was placed in the sidearm of the flask, the atmosphere in the flask was replaced again with nitrogen, and the flask was covered with aluminum foil to exclude light. The solutions were mixed by tilting the flask. The reaction was allowed to proceed for 30 min at room temperature. The reaction was stopped by adding 0.18 ml of 2-mercaptoethanol, and the mixture was dialyzed against several changes of cold water and then lyophilized.

RESULTS.—*Preliminary tests with the tobacco etch virus.*—In several initial experiments, the procedures used by others for purification of viruses of the potato Y group were tested for applicability to isolation of the tobacco etch virus. The results are summarized as follows:

Relatively impure preparations were obtained when material was homogenized in 0.1 M borate buffer, pH 8.5, containing 0.2% ascorbic acid, 0.2% thioglycol, and 0.3% sodium diethyldithiocarbamate (13), followed by cycles of differential centrifugation and suspension of the virus in 0.05 M borate buffer, pH 8.2. This procedure gave considerable amounts of material remaining at the meniscus or near the top of the sucrose density gradients after centrifugation. Precipitation of the virus at pH 4.8 in the presence of added salt, resuspension in borate, pH 8.2, followed by one or more differential cycles of centrifugation (30) gave much purer preparations, as judged by the amount of the material present near the meniscus of sucrose density-gradient tubes after centrifugation. During further fractionation, however, acid precipitation appeared to enhance aggregation of virus. Clarification with chloroform generally gave somewhat more infectious preparations of virus, but frequently did not give suitable clarification. Moreover, a high degree of impurity was indicated by the considerable amount of ultraviolet-absorbing material that appeared near the meniscus of sucrose density-gradient tubes after one or two cycles of differential centrifugation of chloroform-clarified samples.

Regardless of the medium used for clarification or as a suspension buffer after successive high-speed centrifugation cycles, all the preparations appeared to be badly aggregated as judged by the amount of light-scattering material present below the virus zone following sucrose density-gradient centrifugation. For this reason, alternative methods for initial fractionation of material were investigated. One of the most simple and successful methods tested was precipitation of virus with polyethylene glycol (PEG) as described by Gooding & Hebert (14). This was found to be a convenient procedure for the initial concentration of virus from clarified homogenates.

Frequently colorless and apparently unaggregated preparations of TEV were obtained by precipitation with PEG followed by two or three cycles of differential centrifugation, suspending the virus in 0.025 M phosphate buffer, pH 7.4. On other occasions, the virus aggregated so that no material corresponding to virus was obtained in the ultraviolet scanning patterns after density-gradient centrifugation in sucrose. Furthermore, aggregation of TEV was not prevented by the incorporation of EDTA in the buffers used for homogenization and suspension of virus following high-speed centrifugation.

Use of agents which potentially inhibit hydrophobic type interactions.—A number of detailed thermodynamic investigations on the polymerization of tobacco mosaic virus protein has revealed that specific aggregation of individual viral protein subunits to form helical rods is due primarily to the formation of new hydrophobic centers in the protein. These new hydrophobic regions presumably have a greater affinity for one another than for the aqueous medium, and thus interact in solution when conditions favor their formation (22, 39). In view of the effect of ionic strength and other factors on spontaneous aggregation of viruses of the potato Y group, it was postulated that both end-to-end and lateral aggregation may be largely due to a hydrophobic type of interaction. Therefore, various reagents which influence such interactions were tested for their effect on the aggregation of TEV.

The presence of ethanol at various concentrations during purification of TEV sometimes appeared to prevent or reverse aggregation, but its effect was not consistent. Aggregation of TEV, for example, appeared to be reversed when the virus was precipitated with 50-70% ethanol from a solution after one PEG precipitation and two cycles of differential centrifugation in 0.05 M or in 0.025 M phosphate buffer, pH 7.4, followed by resuspension in water. The virus lost infectivity and was degraded, however, when exposed to ethanol for more than a few minutes.

In other experiments, adding ethanol to a 10% concentration in the resuspension buffer prevented aggregation only slightly, and at 20% it caused some loss of virus as estimated by decreased absorption at 260 mμ.

Ethanol thus appeared to be of limited usefulness. Concentrations high enough to prevent or reverse aggregation appeared to denature or degrade TEV too rapidly for routine use.

The use of low concentrations of dioxane and formamide, which have been reported to cause depolymerization of TMV protein (39), were assessed for their effect on aggregation of TEV in a few experiments. Formamide at 10% concentration did not appreciably prevent or reverse aggregation of TEV in 0.025 M phosphate buffer, pH 7.4. Dioxane at 10% concentration in the phosphate buffer seemed more effective than formamide in reversing and preventing aggregation, but it was less effective than ethanol.

Urea at a concentration of 1.8 M will cause depolymerization and denaturation of TMV protein aggregated into rods, but at a concentration of 0.4 M it favors depolymerization without denaturation (39). These results suggested that urea at low concentrations might be useful in inhibiting TEV aggregation.

A preliminary experiment determined the concentration of urea which caused degradation of TEV. The loss of birefringence of partially purified virus with urea was observed in the manner described by Purcifull & Shepherd (31) in studies of the alkaline degradation of TEV and other rod-shaped viruses. In this experiment, concentrations of urea above 2 M caused rapid disappearance of flow birefringence.

In further tests, urea at lower concentrations was evaluated for its effect on aggregation of TEV during purification. In one experiment, infected material was homogenized in 0.5 M phosphate buffer, pH 7.1, containing 1.0% 2-mercaptoethanol and clarified with butanol in the usual manner. The homogenate was then divided into two equal portions, and the virus in both was precipitated with polyethylene glycol (PEG). The PEG precipitate in both lots was resuspended in 0.025 M phosphate buffer, pH 7.4, but the buffer of one lot contained 1.0 M urea and 0.1% 2-mercaptoethanol. Both lots of material were maintained in the same respective solutions throughout several cycles of differential centrifugation. The extent of aggregation at each step was assessed, as usual, by removing portions of resuspended material and subjecting it to sucrose density-gradient centrifugation followed by fractionation with the ISCO apparatus. As anticipated from previous trials, the virus in phosphate alone was aggregated after the first high-speed centrifugation, as judged by the spreading of ultraviolet-absorbing material through the lower portions of the density-gradient column following centrifugation. During two successive cycles of differential centrifugation, most of the virus in phosphate buffer alone was lost during low-speed centrifugation or sedimented to the bottom of the density-gradient tubes. Only very inconspicuous bands were apparent in the density-gradient tubes, and very little ultraviolet-absorbing material corresponding to virus was exhibited in the scanning patterns. In contrast, virus kept in the same buffer but containing 1.0 M urea and 0.1% 2-mercaptoethanol exhibited prominent virus bands and ultraviolet absorption corresponding to virus in the density-gradient tubes, and showed much less tendency to spread throughout the lower portion and to sediment to the bottom of the gradient columns during centrifugation.

Results were similar in repeated trials, and electron microscope examination of samples from the bands showed relatively unaggregated virus particles in the preparations purified in the presence of urea. Fig. 1 shows the results of one trial in which aggregation was monitored by sucrose density-gradient centrifugation after each cycle of differential centrifugation in the buffer, with or without 1.0 M urea and 0.1% 2-mercaptoethanol. In this particular experiment, the virus in the buffer alone was not highly aggregated, yet the spreading of the virus band(s) became more apparent with additional cycles of differential centrifugation (Fig. 1).

At this time, the question arose whether the urea induced a permanent change in some property of the virus that otherwise leads to aggregation. This was evaluated by preparation of relatively unaggregated TEV by fractionation in buffer containing urea as outlined above. This was then divided into two equal portions. One portion was dialyzed against several changes of water to remove the urea; the other lot was not dialyzed. Both lots were then subjected to one cycle of differential centrifugation, and the dialyzed portion was resuspended in 0.025 M phosphate buffer, whereas the pellets of the part that was not dialyzed were resuspended in 0.025 M phosphate buffer containing 1.0 M urea and 0.1% 2-mercaptoethanol. Comparison of the ultraviolet scanning patterns after sucrose density-gradient centrifugation indicated that virus aggregation occurred in the portion from which urea was removed.

In an experiment to evaluate the effect of mercaptoethanol alone on aggregation of TEV, suspending buffers were made to contain either 1.0 M urea or 0.1% 2-mercaptoethanol or both in 0.025 M phosphate buffer, pH 7.4. Virus pellets after PEG precipitation were suspended in either one of these buffer systems and maintained in it throughout purification. Results of sucrose density-gradient centrifugation after one PEG precipitation and two cycles of differential centrifugations suggested that 2-mercaptoethanol alone had little effect on aggregation. Urea was thus shown to be the main factor in preventing aggregation. Aggregation was least, however, when both urea and 2-mercaptoethanol were present in the buffer system (Fig. 2).

Ultraviolet scanning of the sucrose density-gradient columns with virus after centrifugation indicated ultraviolet-absorbing material near the meniscus (e.g., Fig. 1) when the solvents were solutions containing 1.0 M urea and 0.1% 2-mercaptoethanol. At least part of this absorption was due to 2-mercaptoethanol in the buffer, since floating samples of this buffer on sucrose gradient columns and scanning them in the ISCO apparatus gave similar, though less prominent, absorption near the meniscus. In some trials, variable amounts of absorption at the meniscus of centrifuged density-gradient columns suggested possible degradation of the virus by 1 M urea in the buffer. Therefore, the effect of lower concentrations of urea on aggregation was investigated. In three experiments, the virus was purified as usual

except for suspending it either in 0.025 M phosphate buffer or in buffer containing 0.5 M urea and 0.1% 2-mercaptoethanol. Aggregation was assessed by sucrose density-gradient centrifugation in the usual manner. Aggregation became apparent and increased after one PEG precipitation followed by 2 cycles of differential centrifugation in the buffer alone, whereas the virus was much less aggregated in the buffer containing 0.5 M urea and 0.1% 2-mercaptoethanol. After centrifugation, a small ultraviolet-absorbing peak was present near the top of sucrose density-gradient tubes used for virus suspended in the buffer containing 0.5 M urea and 0.1% 2-mercaptoethanol, but this was probably due to the 2-mercaptoethanol, not to degradation of TEV. In these experiments, the ultraviolet-absorption spectra after each step of purification were almost identical for the virus solution both in the buffer alone and in the buffer containing urea. Thus, with both treatments, estimated yield of virus per g tissue was the same after one PEG precipitation and three cycles of differential centrifugation.

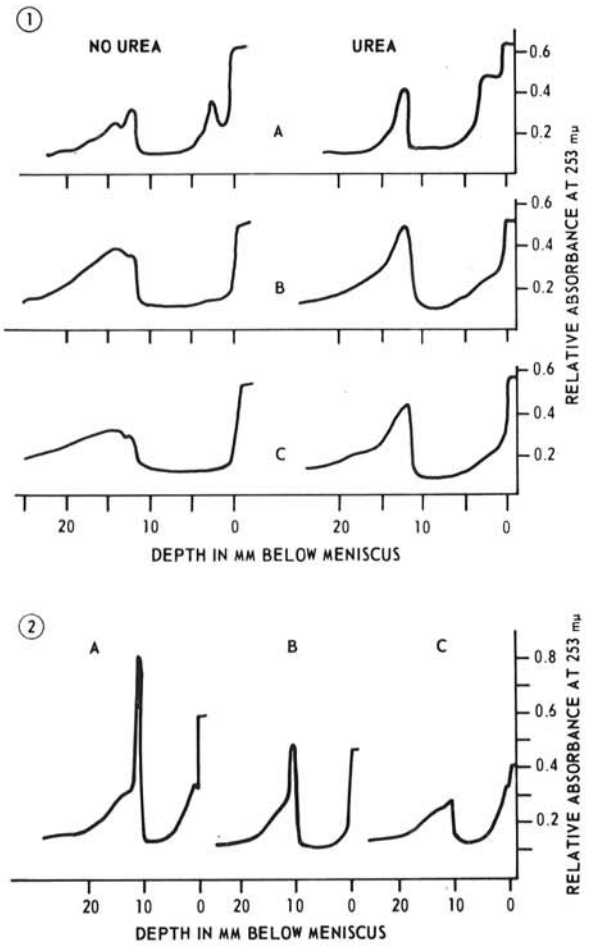

Fig. 1-2. 1) Effect of urea and mercaptoethanol on aggregation of the tobacco etch virus (TEV) during consecutive cycles of differential centrifugation. Ultraviolet scanning patterns after rate zonal centifugation of virus fractionated by one precipitation with polyethylene glycol followed by one (A), two (B), or three (C) cycles of differential centrifugation with resuspension in either 0.025 M phosphate, pH 7.4 (no urea), or in the same buffer containing 1.0 M urea and 0.1% 2-mercaptoethanol (urea). The sucrose density gradients were centrifuged for 3 hr at 23,000 rpm. 2) Effect of mercaptoethanol in a urea-containing solvent on the aggregation of TEV. Ultraviolet scanning patterns after rate zonal centrifugation of virus subjected to one precipitation with polyethylene glycol and two cycles of differential centrifugation. (A) Virus fractionated in 0.025 M phosphate, pH 7.4, containing 1.0 M urea and 0.1% 2-mercaptoethanol; (B) virus fractionated in the same buffer containing 1.0 M urea only; (C) virus processed in the same buffer without either urea or mercaptoethanol.

In various experiments, yields of virus ranged between 40 and 60 mg/kg fresh tissue, based on the extinction coefficient obtained by Purcifull (30); 2.4/cm for a 0.1% solution at 261 mμ.

Some degradation of virus resulted in attempts to purify TEV by homogenization of infected tissue in 0.2 M borate buffer followed by precipitation with PEG and resuspension in 0.02 M borate, pH 8.8, containing 1.0 M urea and 0.1% 2-mercaptoethanol. This was indicated by the large amount of ultraviolet-absorbing material at the meniscus and a small sedimenting component corresponding to virus after sucrose density-gradient centrifugation, and the yield as estimated by absorbance at 260 mμ was about one-half of that suspended in the buffer alone. This effect is similar to that observed by Bawden & Pirie (2) and Lauffer (21), which was that higher pH values enhance the action of urea in degrading tobacco mosaic virus.

Effect of urea on infectivity of TEV.—Although urea at concentrations of 0.5-1.0 M apparently prevented aggregation of TEV during purification, it was necessary to test its effect on the infectivity of the virus to *C. amaranticolor*. Results of several experients suggested that urea at a concentration of 0.5-1.0 M affects the susceptibility of the host without reducing the intrinsic infectivity of the virus. In one experiment, for example, in which TEV did not become markedly aggregated after purification by precipitation with PEG and three cycles of differential centrifugation in the absence of urea, the final product was assayed at the same concentration (based on absorbance at 260 mμ) with a portion of the same preparation fractionated in the presence of 1.0 M urea and 0.1% 2-mercaptoethanol. Both preparations at an $A_{260} = 0.56$ in 0.025 M phosphate gave 30 lesions/half leaf on *C. amaranticolor*. The concentration of urea in the treated portion during assay in this case was 0.1 M, which may have had no effect on the assay host, or limited aggregation may have led to partial loss of infectivity of the portion fractionated in the absence of urea. In another trial, virus purified by one precipitation with PEG and two cycles of differential centrifugation in the presence of 0.5 M urea and 0.1% 2-mercaptoethanol was dialyzed against three changes of 1-liter batches of dilute phosphate (0.02 M, pH 7.4) over a 36-hr period. One portion of this dialyzed preparation was then diluted one-fortieth in the same buffer solution, and another was similarly diluted in the same buffer containing 0.5 M urea and 0.1% mercaptoethanol. The portion without urea gave 70 lesions/half leaf, whereas the portion with urea produced only 23 lesions/half leaf.

Thus, 0.5-1.0 M urea may reduce host susceptibility, but probably does not markedly affect the infectivity of the virus.

Use of urea in purification of other viruses in PVY group.—The use of PEG precipitation and 1.0 M urea in 0.025 M phosphate buffer containing 0.1% 2-mercaptoethanol was also tested as a method for purification for several other viruses of the potato Y group. This was done in one or more trials with potato virus Y in Havana 425 tobacco; watermelon mosaic virus-2 in pumpkin (*Cucurbita pepo* L.) 'Small Sugar'; lettuce mosaic virus in lettuce (*Lactuca sativa* L.); the Johnson grass strain of sugar cane mosaic virus in naturally infected corn (*Zea mays* L.); and turnip mosaic virus in tender green mustard (*Brassica perviridis* Bailey). Results, as assessed by sucrose density-gradient centrifugation of virus preparations after one PEG precipitation and one or more cycles of differential centrifugation, indicated that the method was applicable with these viruses. A sharp, prominent virus band was obtained in the sucrose gradient after centrifugation when the virus was suspended in buffer containing 1.0 M urea and 0.1% 2-mercaptoethanol, but was less prominent or absent when the buffer did not include urea.

Identification of the sedimenting components in sucrose density-gradient centrifugation.—The identity of the sedimenting components obtained during density-gradient centrifugation was routinely confirmed by infectivity tests, and occasionally by serology and electron microscopy. As shown in the ultraviolet scanning patterns (Fig. 1, 2), bimodal curves were frequent, suggesting two components with similar rates of sedimentation. In some cases with prolonged centrifugation these were so separated that small portions of each could be removed with relatively little contamination with the other. This was done by puncture through the walls of the tubes with a hypodermic needle and removal of a few droplets of solution for infectivity and serological tests. Several of these bands were collected and pooled separately and concentrated by high-speed centrifugation and resuspension in 0.025 M phosphate buffer, pH 7.4, for serological testing. Both fractions reacted with TEV antiserum in microprecipitin tests. This indicated that the lower band was simply an aggregate of the virus. Both of these components were infective and produced similar symptoms in the plants tested. The marked downward skewing at the leading edge and the sharp trailing boundary which the virus zones showed in most of the scanning patterns are probably a reflection of the viscosity effect that long, flexible particles exhibit in solution.

Electron microscope examination provided further evidence that the lower band consisted of end-to-end aggregates of the virus particles (Fig. 4), for the slower-sedimenting band contained comparatively more dispersed particles when viewed in the electron microscope. End-to-end aggregation appeared early during purification (Fig. 3, 4) and presumably was not reversed by urea. From repeated examinations it appeared that the faster-sedimenting band contained fewer individual rods and many extremely long particles (Fig. 4). The presence of such particles in the faster sedimenting band is in agreement with the prediction that end-to-end aggregates sediment only slightly faster than unaggregated particles in sucrose density-gradient columns (26). Delgado-Sanchez & Grogan (13) encountered similar problems of obtaining two virus bands for potato virus Y in sucrose density-gradient centrifugation. They also found that the more rapidly sedimenting component was an aggregate of the virus.

Electron microscopy of purified virus (Fig. 3, 4) indicated that relatively little extraneous material was present in these preparations.

Evaluation of purity of the isolated virus by polyacrylamide gel electrophoresis.—Polyacrylamide gel electrophoresis was used in addition to sucrose density-gradient centrifugation and electron microscopy to assess the purity of isolated TEV.

Since polyacrylamide gel separation of proteins is done most commonly in the presence of 8 M urea as the solvent to abolish secondary and tertiary structure, and since TEV has been found to be readily degraded by exposure to urea in excess of 2 M, the method adopted for routine use was the procedure of Reisfeld et al. (33), with all the reagents prepared in freshly deionized 8 M urea. Similar procedures have been used by others in evaluating the homogeneity of virus coat proteins (18, 24, 27, 28, 36).

In most experiments with whole virus, small samples of the preparations at various steps during purification or after a particular treatment were made 6 to 8 M in deionized urea and held for 10-20 min at room temperature to allow complete degradation of the virus and solubilization of other proteins present. Portions were then added to a suitable volume of sample gel. A measured amount of this was pipetted onto the surface of running gel columns previously overlaid with polymerized spacer gel, and the sample gels were then photopolymerized. The columns were then filled with tray buffer containing 8 M urea to fill the top portions of the tubes placed in the apparatus for the electrophoretic separations. For routine use, the cationic system was used. Virus protein obtained by degradation of purified virus was dissolved in 8 M urea and was analyzed by the same procedure.

In preliminary experiments to evaluate the behavior of TEV proteins in the system, highly purified virus (after two precipitations with PEG and one cycle of differential centrifugation) taken from sucrose density-gradient bands and concentrated by high-speed centrifugation, was tested in 15% polyacrylamide gel system. This yielded two characteristic bands which migrated to distances of 8 mm and 13 mm after electrophoresis for 2 hr (Fig. 6-D). In all experiments with partially purified virus, the most rapidly migrating prominent component was present and varying amounts of the more slowly migrating material. As described later, additional experiments provided evidence that the material in the trailing band probably represented dimerization as a result of spontaneous oxidation of sulfhydryl groups in the viral protein, accompanied by the formation of intermolecular disulfide bonds, rather than other viral or host protein.

In several experiments with virus that was partially

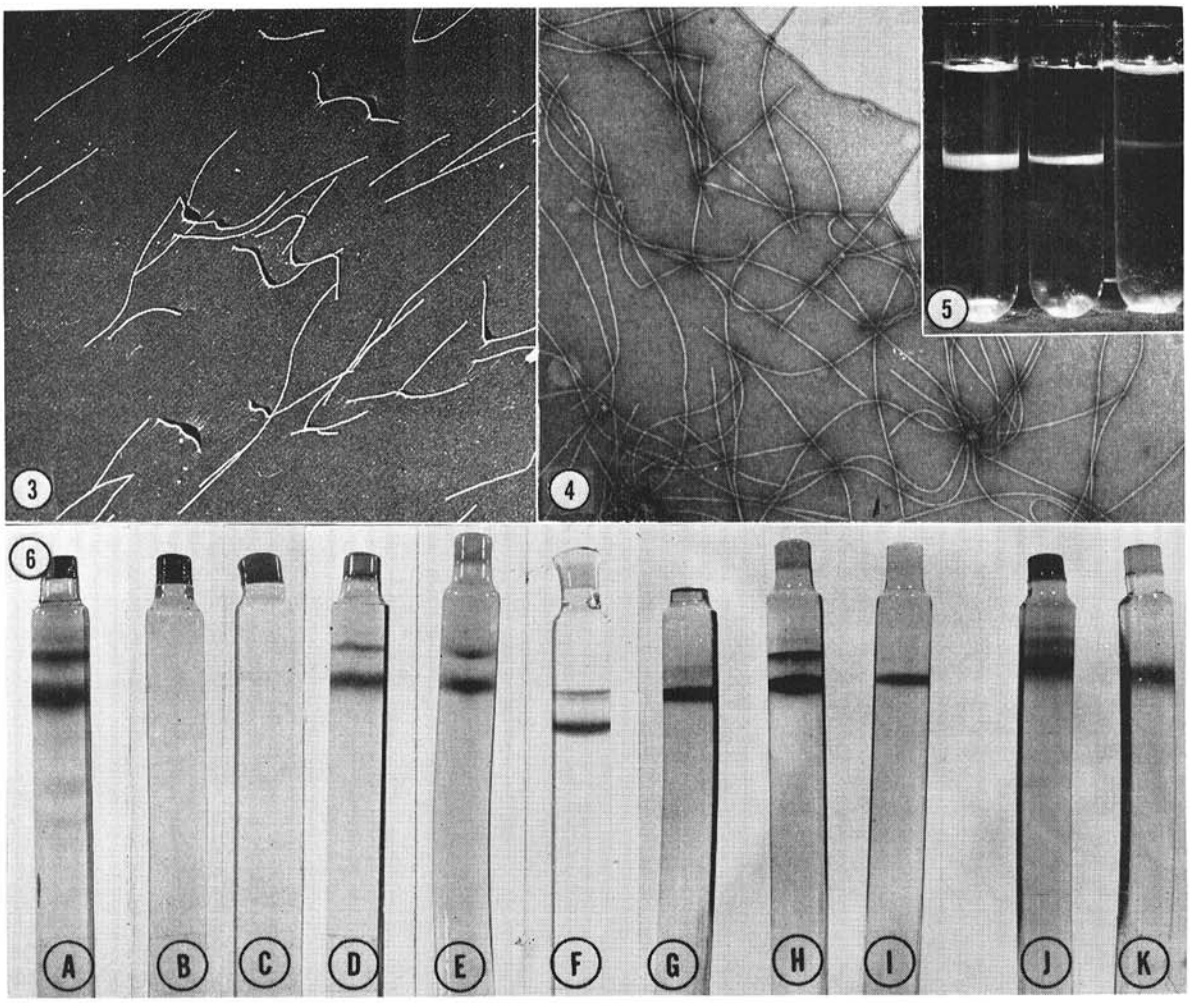

Fig. 3-6. **3)** An electron micrograph of a shadowed preparation of TEV (tobacco etch virus) (× 21,000) after one precipitation with polyethylene glycol and one cycle of differential centrifugation using 0.025 M phosphate, pH 7.4, containing 0.5 M urea and 0.1% 2-mercaptoethanol as solvent. **4)** An electron micrograph of a negatively stained preparation of TEV (× 24,000) showing marked end-to-end aggregation with material taken from the more rapidly sedimenting component during zonal centrifugation into sucrose density gradients. This preparation was subjected to one precipitation with polyethylene glycol and two cycles of differential centrifugation in 0.025 M phosphate containing 0.5 M urea and 0.1% 2-mercaptoethanol. **5)** Equilibrium density gradient separations of TEV (left), turnip mosaic virus (center), and beet yellows virus (right) in cesium chloride. Each virus was purified by one precipitation with polyethylene glycol and two cycles of differential centrifugation before mixing with cesium chloride (initial density = 1.31 g/cc) and centrifuging for 40 hr at 33,000 rpm in the Spinco SW39 rotor. **6)** Results of polyacrylamide gel electrophoresis with TEV protein with material taken at various stages of purification or with fraction I protein. Gels labeled A through I electrophoresed for 2.5-3.5 hr in the cationic system. (A) Impure virus from tissue homogenized in 0.1 M borate followed by chloroform clarification and three differential cycles of centrifugation; (B) Fraction I protein of tobacco taken from the meniscus of sucrose density-gradient columns after centrifugation with virus prepared as in A; (C) Fraction-I protein of tobacco prepared by homogenizing healthy tissue in 0.5 M phosphate followed by clarification with butanol, one precipitation with polyethylene glycol (PEG), and one cycle of differential centrifugation; (D) Virus from sucrose density-gradient columns after centrifugation with virus prepared by two precipitations with PEG and one cycle of differential centrifugation; (E) Virus purified by two precipitations with PEG and one differential cycle of centrifugation followed by sucrose density-gradient electrophoresis (24 hr) and one additional cycle of differential centrifugation; (F) Virus purified by one precipitation with PEG, three cycles of differential centrifugation, and isopycnic separation in cesium chloride density gradients; (G) Virus purified by three cycles of differential centrifugation after one precipitation with PEG, followed by isoelectric precipitation at pH 4.8 and degradation with urea immediately before electrophoresis; (H) Virus protein prepared with guanidine HCl from virus purified as in G; (I) Virus protein prepared as in H after carboxymethylation; (J) The same protein as in H electrophoresed for 3 hr in the anionic system; (K) The same protein as in H after carboxymethylation and electrophoresis for 3 hr in the anionic system. Unless otherwise stated, all experiments were done with virus degraded with 8 M urea.

purified in various ways, electrophoretic components were obtained in multiplicity, demonstrating the presence of host proteins in the virus preparations. For example, with material partially purified by homogenization of infected tissue in 0.1 M borate, pH 8.2, followed by clarification with chloroform and one differential cycle of centrifugation, at least six components were obtained besides the two virus-associated components (Fig. 6-A).

Impure preparations of TEV were found to be commonly associated with prominently staining sample gels in polyacrylamide electrophoresis experiments. This is clearly demonstrated in the gel columns in Fig. 6-A, B, and C, in which the intensely stained sample gel overlies a similar disc of clear spacer gel, which in turn overlies the running gel column. The results of several experiments indicated that host fraction I protein (38) was responsible for this. Material after several cycles of differential centrifugation alone, for example, and the material remaining at or near the top of the sucrose density-gradient columns after centrifugation, gave intensely staining sample gels after acrylamide electrophoresis (Fig. 6-A, B, C). Virus taken from sucrose density gradients after centrifugation of after zone electrophoresis, or from cesium chloride column after equilibrium density-gradient centrifugation, all yielded only the two staining components characteristic of virus in the running gel columns, and very faintly staining or clear sample gels (Fig 6-E, F) after electrophoresis. When tissue of healthy plants were homogenized in 0.5 M phosphate buffer, the juice clarified with 8% butanol, followed by precipitation with PEG and one high-speed centrifugation, yielded only small pellets. When these pellets were suspended in buffer with 8 M urea and analyzed electrophoretically in polyacrylamide gels, they gave intense staining only in the sample gel and no detectable components in the running gel (Fig. 6-C).

As mentioned previously, purified virus and its degradation products gave rise to two closely spaced bands after polyacrylamide gel electrophoresis. The slower-moving band was usually rather inconspicuous compared with the faster-moving band. It was noted that the slower-moving band was even less conspicuous when the virus was degraded just before electrophoresis (Fig. 6-G). On the contrary, when the protein was isolated from the virus by the guanidine hydrochloride method, this minor band was more prominent (Fig. 6-H). This suggested that the slower-moving band was perhaps a covalently bonded dimer, or alternatively, possessed a configurational difference with greater frictional coefficient accompanied by retardation because of sieving of the molecules in the gel. The possibility of disulfide bond formation was tested by treating the protein to cleave and block disulfide bonds. Reduction and carboxymethylation of the protein (11) obtained by the guanidine hydrochloride method from virus (after one PEG precipitation and three cycles of differential centrifugation and one acid precipitation at pH 4.8 in 0.025 M phosphate buffer containing 1 M urea and 0.1% 2-mercaptoethanol) in 8.0 M urea gave a single heavily stained band, both in cationic and in anionic systems, after electrophoresis (Fig. 6-I, K). This provided conclusive evidence of the homogeneity of the protein that showed two components in gels before reduction and carboxymethylation. Therefore, the characteristic presence of a prominent band along with a lighter-stained, slower-moving band was considered good evidence for the homogeneity of purified virus or its protein and for the presence of a single protein in the virus.

Further convincing evidence came from the fact that highly purified virus preparations almost always yielded these two bands. These preparations included virus from sucrose density gradients (after two precipitations with PEG and one cycle of centrifugation, and another cycle of differential centrifugation after removal of virus from the density-gradient columns); virus from sucrose density-gradient zonal electrophoresis (after two precipitations with PEG and two cycles of differential centrifugation in 0.025 M phosphate buffer, pH 7.4, containing 1.0 M urea and 0.1% 2-mercaptoethanol); and virus from cesium chloride density-gradient columns centrifuged to equilibrium (after one PEG precipitation and three cycles of differential centrifugation in 0.025 M phosphate buffer containing 0.5 M urea and 0.1% 2-mercaptoethanol) (Fig. 6-D, E, F).

Use of equilibrium density-gradient centrifugation for purification, evaluation of purity, and determination of buoyant density of TEV.—Equilibrium density-gradient centrifugation was tested as a method for further purification of TEV and as another independent means of characterizing preparations of virus for purity. Though exposure of rod-shaped viruses to strong salt solutions has been commonly assumed to be undesirable and to cause serious aggregation or precipitation, its usefulness has apparently not been tested with viruses of the potato Y group.

In several preliminary tests with cesium chloride as the gradient-producing salt and highly purified TEV, or turnip mosaic virus, a very prominent light-scattering band 2.6-2.8 cm beneath the meniscus (of a 4.2-cm high-density-gradient column) was present (Fig. 5) after 46 hr of centrifugation at 32,000 rpm. The material in this band was found to be associated with infectivity in tests on *C. amaranticolor,* tobacco, and Tabasco pepper (*Capsicum frutescens* L.). Results were similar with glycylglycine or phosphate as the buffer.

Tests of other materials as the gradient-producing salt included potassium tartarate, potassium bromide, and potassium citrate. Potassium tartarate in 0.01 M glycylglycine buffer, pH 7.8, caused immediate aggregation and precipitation of TEV (in 0.01 M glycylglycine buffer, pH 7.8, containing 0.5 M urea and 0.1% 2-mercaptoethanol) even at about one-fifth saturation. In solutions of potassium citrate at concentrations sufficient for isopycnic banding, TEV was degraded, and gave discernible bands only at the meniscus after centrifugation. After 38 hr of centrifugation at 32,000 rpm of TEV in solutions of potassium bromide, three light-scattering bands were present in the density-gradient column. The ultraviolet-absorption spectra of these components indicated that they were devoid of nucleic

acid, and no rods were found by electron microscope examination of samples from these bands.

In other experiments, equilibrium density-gradient centrifugation in cesium chloride was used to evaluate the purity of the isolated virus and to determine its buoyant density. The results of one of these experiments are shown in Fig. 7. Essentially no ultraviolet-absorbing material was present except in the virus band, indicating a high degree of buoyant homogeneity of the purified product. In addition, infectivity was associated only with the prominent ultraviolet-absorbing band, which contained rod-shaped particles typical of TEV.

The densities estimated for TEV by graphical methods in four experiments were: 1.342 (in cesium chloride dissolved in 0.05 M phosphate buffer, pH 7.4, containing 0.5 M urea), 1.330, 1.332, and 1.336 g/cc (Fig. 7). Ignoring the first value, which was probably spuriously high, an average value of 1.332 g/cc was obtained for TEV. However, a lower estimate of the density for the virus was obtained by using micule spheres (15) and Bio-Gel of known density. One of the micule spheres (purchased from Microspheres, Inc., Palo Alto, Calif.), with a density of 1.300 g/cc, was found within the virus band after centrifugation for 24 or 48 hr at 32,000 rpm in a cesium chloride density gradient. A similar sphere, with a density of 1.200 g/cc, was observed to be about 11 mm above the virus band; another with a density of 1.400 g/cc was found about 10 mm below the virus band. On the other hand, Bio-Gel beads with a density of 1.315 g/cc were positioned immediately below or in the lower boundary of the virus band, suggesting a density of about 1.314 g/cc for TEV.

The reliability of the refractive index and graphical method for estimating buoyant densities was evaluated by carrying out an identical experiment with purified U1 strain of tobacco mosaic virus (TMV). The results

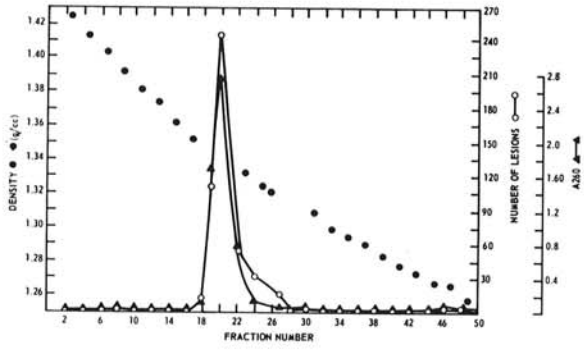

Fig. 7. Use of equilibrium density-gradient centrifugation to assess purity and buoyant density of the tobacco etch virus. Purified virus (after one precipitation with polyethylene glycol and three cycles of differential centrifugation) was mixed with saturated cesium chloride (1.8 ml) and centrifuged for 40 hr at 33,000 rpm in the Spinco SW39 rotor, followed by collection of 0.1 ml fractions from the bottom of the tube. Even-numbered fractions were dialyzed for 2 days against 0.02 M phosphate, pH 7.4, and made to a volume of 1.0 ml for the absorbance readings and infectivity assays. Odd-numbered fractions except in the region of the virus zone were used for refractometer readings to estimate density.

gave a buoyant density for TMV of 1.325 g/cc, identical to the value of Siegel & Hudson (37).

In similar experiments to test the usefulness of buoyant separations of other viruses of the potato Y group, the method was found to be successful with the turnip mosaic virus (Fig. 5). A value of 1.336 g/cc was obtained in a single experiment for the buoyant density of this virus using the refractive index-graphical method.

DISCUSSION.—In previous investigations of the purification of the filamentous plant viruses, a variety of procedures has been reported to be helpful in avoiding aggregation, but none has been universally applicable. Boedtker & Simmons (3), for example, described a method for the purification of TMV which prevented end-to-end aggregation of the final product. Similarly, Reichmann (32) showed that prolonged dialysis of potato virus X containing extracts against dilute citrate previous to fractionation prevented aggregation. Neither method, however, prevented aggregation of turnip mosaic virus (35), although citrate was found helpful in the isolation of the watermelon mosaic (41) and sugar cane mosaic (34) viruses. Aggregated virus X was solubilized by incubation with borate or trypsin (1), but electron micrographs of these soluble preparations showed highly aggregated structures (20) similar to those found with insoluble preparations. Brakke (4) found that several detergents, notably Igepon T-73, would disperse aggregated barley stripe mosaic virus, which could then be separated by density-gradient centrifugation. This result suggested that aggregation may result from hydrophobic-type forces. Corbett (10) and others have prepared unaggregated linear viruses by zonal density-gradient methods, but not in quantities sufficient for extensive characterization studies. Similarly, Shepherd & Pound (35) reported a method for the purification of turnip mosaic without significant losses through aggregation, and Delgado-Sanchez and Grogan (13) adapted a particular method for extraction and purification of potato virus Y with the same considerations in mind. Those and other investigations emphasized the detrimental effect of many commonly used procedures for purification when employed with the rod-shaped viruses, reflecting a loss of part or all of the virus by insolubility as the result of aggregation. Purcifull (30) obtained highly purified preparations of TEV which exhibited a single boundary in the analytical centrifuge and showed no antigenicity with antiserum to host-plant protein in precipitin and immunodiffusion tests. This procedure relies in part on isoelectric precipitation of virus for removal of contaminating host material. Although used with considerable success in earlier phases of this study, it occasionally resulted in considerable losses of virus because of aggregation unless the solvent used for resuspension contained urea. It should be emphasized that the use of urea and mercaptoethanol in fractionation does not prevent end-to-end aggregation, but is largely effective in preventing the lateral type of aggregation, with concomitant insolubility.

Little is known concerning the actual process that results in aggregation, though various suggestions have

been made on the basis of treatments which initiate the process (1). The possible involvement of divalent cations has been suggested by Reichmann (32) and Loring et al. (25), but some viruses are known to aggregate markedly when fractionated in citrate or EDTA (36), though these factors may be of significance in individual cases with certain viruses. It appears that interactions of a hydrophobic type may be responsible for the lateral type of polymerization of some filamentous plant viruses. If so, it seems likely that the role of urea is to weaken the affinity of nonpolar areas on the surface of the virus coat and thus to weaken hydrophobic-type interactions. This action of urea may be due to increased solubility of nonpolar groups on the protein rather than solely as a "hydrogen-bond-breaking" reagent, as was previously assumed. This point has been made in discussions of the effect of urea by Kauzmann (19), Bruning & Holtzer (8), and Smith & Lauffer (39).

The detrimental effect of urea on viruses has been known for some time (29). Lauffer & Stanley (23), for example, reported that urea at relatively high concentrations degraded tobacco mosaic virus, and many subsequent studies have dealt with its effect on this and other viruses. It should be pointed out that the effectiveness of urea and similar reagents in reducing the aggregation of different viruses will probably be highly variable, and that suitable concentrations will need to be determined experimentally for each individual virus before the reagent can be used in a preparative procedure as described herein. It should also be pointed out that urea is a potent denaturing agent, probably by virtue of the same processes by which it prevents aggregation. At concentrations in excess of 2.0 M, it may be detrimental through destruction of the secondary and tertiary structure of proteins. Thus, in some cases levels high enough to prevent aggregation may denature viral proteins or lead to degradation of intact viruses.

The tentative role of 2-mercaptoethanol in preventing aggregation is more difficult to assess, but may be related to its capacity to reduce disulfide bonds. Conceivably, the polymerization process leading to lateral aggregation could originate through the formation of intermolecular disulfide bonds by exchange or oxidation of closely adjacent groups on separate virus particles, or by participation of metallic ions to form such bridges between particles. A mechanism of this sort, however, seems less feasible unless the sulfhydryl groups were optimumly positioned on the surface. Dimerization of other proteins, however, to produce more rapidly sedimenting components, is known to be due to the formation of intermolecular disulfide bridges by oxidation of sulfhydryl groups. The common dimer encountered with bovine serum albumin, which contains a single sulfhydryl group, for example, is reversible by mercaptoethanol (16). Measurement and comparison of the sulfhydryl content of highly aggregated and unaggregated virus might provide interesting information on this point with the filamentous plant viruses. Such information as there is on the role of 2-mercaptoethanol indicates that it exerts a relatively limited influence on the aggregation process, for no prominent differences in degree of aggregation were observed with its use alone. It thus appears to serve primarily to reinforce the beneficial function of urea in solubilizing virus during fractionation.

The utility of polyacrylamide electrophoresis deserves mention as an accessory method for assessment of the purity of isolated virus. The method is convenient to use, particularly with viruses readily degraded by urea used in the electrophoresis system, and possesses a high degree of sensitivity. The method should be applicable to a wide variety of other filamentous viruses which aggregate to such an extent that purity cannot be evaluated by conventional means. The use of equilibrium density-gradient centrifugation in cesium chloride as used herein is of similar usefulness both as a means of purification and as a method for assessing purity.

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