CONTINUOUS FILTER PAPER ELECTROPHORESIS OF TOBACCO MOSAIC VIRUS

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I. INTRODUCTION

Continuous electrophoresis on filter paper can be a useful technique in the preparation and purification of proteins. It differs significantly from either boundary electrophoresis or zone electrophoresis on filter paper in that it is principally a preparative rather than an analytical tool. In an earlier short communication[1] the reisolation of the individual strains from a synthetic mixture of two strains of purified tobacco mosaic virus (TMV) was reported. The separation of the two strains was a complete one as the biological integrity of each strain was regained after continuous electrophoresis. This paper extends the description of the electrophoresis technique and its effect on some biological and physical properties of the virus. In addition, the feasibility of purifying TMV without resorting to either chemical purification or high speed centrifugation has been investigated, and the purification of a low molecular weight protein associated with TMV infection is reported.

KARLER[2], in a brief abstract, has reported the isolation of poliomyelitis virus directly from its culture medium by continuous electrophoresis. He has also examined TMV, but no details are given.

II. MATERIALS AND METHODS

Virus preparations

Inoculum of Strains U1 (common) and U2 (mild mosaic) of TMV was obtained from Dr. S. G. WILDMAN. The origins and symptoms produced on tobacco plants are reported in detail by SIEGEL AND WILDMAN[3]. The virus was purified from infected *Nicotiana tabacum* var. Turkish Samsun plants in a manner analogous to that described by SIEGEL AND WILDMAN[3], but using water or dilute phosphate buffer rather than cacodylic acid buffer as the grinding medium. Generally, two or sometimes three cycles of high and low speed centrifugation were adequate to give a preparation

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suitable for continuous electrophoresis. The virus preparations were dialyzed at 2° C for at least 24 hours in a buffer similar to the buffer subsequently used when the virus was subjected to continuous electrophoresis.

Virus nitrogen was estimated by digestion and direct Nesslerization. The protein concentrations were calculated from the nitrogen determination, assuming 16% nitrogen for the proteins.

Virus bioassay

Except where indicated in the text, the half-leaf assay method using *Nicotiana glutinosa* plants was used to estimate relative virus activity. A randomized block design of 13 six-leaved plants, with each treatment replicated 24 times was employed to minimize the variation between plants, and between leaf levels on individual plants. The plants were kept in the dark for 18 hours before inoculation to increase their sensitivity to the virus. 50 mg of Celite was added to each ml of virus inoculum as an abrasive.

Continuous electrophoresis

The electrophoresis cabinet was purchased from the Shandon Scientific Company, London. Two additions to the basic apparatus enabled operation without attention for periods of up to 24 hours; *viz.*, a motor-driven syringe to apply the protein solutions to the filter paper, and a pumping system to recirculate the buffer through the dialysis tubing enclosing the platinum side electrodes.

The motor-driven syringe device (after HOLDSWORTH[4]) was bolted to the front panel of the cabinet. The protein solution was applied to the paper from an all-glass 5 ml hypodermic syringe through polythene tubing, at the rate of approximately 0.2 ml per hour. In an experiment where the rate of application of the protein to the paper was increased to 0.4 ml/h, a poor separation resulted as the applied protein solution moved faster than the buffer curtain. The syringe mounting was designed so that the position of the application of the protein to the paper could be varied Generally, with electrodes 40 cm apart, the protein was applied 13.5 cm from the cathode and about 5 cm above the top of the electrode wires which were clamped to the vertical edges of the paper. The effective length of the paper over which the protein migrated was approximately 50 cm.

The pumping system assured circulation of ample quantities of buffer through the dialysis tubing which encased the electrodes. About one liter per hour per electrode was required to flush properly the products of electrolysis (in this case hydrogen and oxygen). With rates much lower than this, pH changes at the electrodes caused a disturbance of the migration of the proteins and anomalies resulted. After flushing the electrodes, the buffer from both electrodes drained into a common vessel; it was then pumped into two large beakers above the apparatus which contained the buffer supplies for the individual electrodes. The two beakers were interconnected with a ¼" I.D. rubber tube to keep their levels equal. This interconnection represented a shunt to the high voltage system; approximately 30% of the total current was carried

here. A diaphragm-operated microswitch activated the pump according to the water pressure in the lower vessel.

In any experiment, the flushing buffer was always taken from the same source as the buffer which was allowed to flow down the filter paper (the buffer curtain).

The $M/90$, pH 7 phosphate buffer contained 0.0044 moles of KH_2PO_4 and 0.0066 moles of Na_2HPO_4 per liter. The ionic strength was 0.024.

A DC power supply, with full-wave rectification, operated as a constant voltage device, gave a regulated output of ± 1%. A potential of 460 volts was applied across the electrodes. With electrodes 40 cm apart, at 2^0 C the current across the paper was about 3 milliamperes. Because of the small amount of power to be dissipated, there was no apparent heating at the surface of the filter paper.

HOLDSWORTH[4] has investigated several Whatman filter papers suitable for the separation of proteins by continuous electrophoresis. From these, Whatman No. 3MM and Whatman No. 54 were examined for their suitability in the separation of TMV strains. No. 3MM has a very low wet strength and its use was abandoned in favour of No. 54, which is a very tough paper and can be handled easily when wet without much danger of tearing. In most experiments the paper was washed with 1 N HCl for one hour, and then rinsed for 24 hours in several changes of distilled water.

The buffer curtain which travelled down the paper collected into a series of 30 tubes placed at drip points cut into the bottom edge of the paper. After each electrophoretic run was completed, the tubes into which the protein had migrated were identified either by a precipitin test with TMV antiserum, by absorption at 260 and 280 mμ in a Beckman Model DU spectrophotometer, or by the staining of the paper with bromophenol blue[5] after heating for 20 minutes at 110° C[6].

In early experiments, the apparatus was run at room temperatures (about 20–25° C). Contaminations by microorganisms in the circulating buffer proved troublesome, and as will be shown below, the Strain U2 virus, after electrophoresis, retained only about 10% of its original infectivity. All subsequent experiments have been performed with the apparatus in a room held at 2° C.

III. EXPERIMENTAL RESULTS

The effect of continuous electrophoresis on the biological activity of the virus

The infectivity per unit protein which was retained by centrifugally purified Strain U2 after it had been subjected to continuous electrophoresis was determined by bioassay on *N. glutinosa*. After electrophoresis, the liquid in those tubes which contained the virus was pooled and the solution was dialyzed for at least two days at 2° C in $M/90$ pH 7 phosphate buffer. Another sample of the same lot of Strain U2 which had not been subjected to continuous electrophoresis, but which had been stored at 2° C, was dialyzed in the same buffer. In experiments where the filter paper had not been acid washed, significant quantities of a dialyzable, nitrogenous substance came from the paper. The dialysis of the U2 virus after electrophoresis was considered to be complete when no nitrogen could be detected in a control dialysis sample

consisting of buffer which had also travelled down the paper, but which contained no virus.

After dialysis, the two virus samples were diluted in $M/90$ pH 7 phosphate buffer to the levels shown in Table I. In all experiments, the infectivities were com-

TABLE I

THE EFFECT OF CONTINUOUS ELECTROPHORESIS ON THE INFECTIVITY OF STRAIN U2

Values expressed as the ratio of the number of lesions produced on *N. glutinosa* in response to virus that was subjected to continuous electrophoresis, to the number of lesions produced with the same preparation of virus that had not undergone electrophoresis.

Virus concentration at which the comparison was made (mg/ml)	Experiment number				
	1A*	1B	2	3	4**
	Electrophoresis at room temperature (20–25° C)		Electrophoresis at 2° C		
$5 \cdot 10^{-3}$			0.6	0.9	1.4
$2.5 \cdot 10^{-3}$		0.08			
$1 \cdot 10^{-3}$			0.7	0.9	1.0
$5 \cdot 10^{-4}$	0.05	0.08			
$2 \cdot 10^{-4}$			0.9	1.2	1.2
$1 \cdot 10^{-4}$	0.08	0.1			

* Experiments 1A and 1B represent separate bioassays on virus taken from one electrophoretic separation.
** Electrophoresis on acid-washed paper.

pared at three virus concentrations. In Experiment 1A and 1B the electrophoresis was at room temperature; only about 10% of the original infectivity remained after electrophoresis. Experiments 2, 3, and 4—performed at 2° C—suggested that Strain U2 retained full infectivity after continuous electrophoresis.

The trends seen in Experiments 1, 2, and 3, where the ratio of infectivity before electrophoresis to infectivity after electrophoresis increased with increasing dilution of the virus suggested that a substance inhibitory to virus infectivity might be originating from the filter paper. However, it was not possible to demonstrate such a substance in buffer extracts of the filter paper.

The electrophoretic separation of two TMV strains after isolation from mixedly infected plants

In an earlier report[1] the reisolation of the individual strains of a synthetic mixture of purified Strains U1 and U2 was reported. Similar experiments have now been performed with the two strains as isolated simultaneously from mixedly infected plants.

The strain composition of virus isolated from mature, directly inoculated tobacco leaves has been shown to approximate closely the strain composition of the inoculum used to infect them[7]. In order to obtain a strain mixture composed of approximately equal amounts of each component, the protein concentrations of U1 and U2 were adjusted to give equal levels of infectivity (as determined by prior

bioassay on *N. glutinosa*) and rubbed onto mature *N. tabacum* leaves. The virus was subsequently isolated from these leaves, purified centrifugally, and subjected to continuous electrophoresis. As in the case of the synthetic mixture[1], the two strains could be separated by continuous electrophoresis so that the biological integrity of the individual strains (symptoms on *N. rustica*) could be regained.

SINGER *et al.*[8] have examined virus purified from plants dually infected with Strains U1 and U2 (then named Severe and Mild, respectively) in the moving boundary electrophoresis cell and have observed in addition to U1 and U2 a third component of intermediate mobility. This component (I) is thought to be a centrifuge artifact consisting of an aggregation product of Strains U1 and U2. With continuous electrophoresis no component comparable with Component I reached the bottom of the filter paper, although in some of the experiments at least one other component appeared to be present between U1 and U2 immediately below the point of application of the protein to the paper.
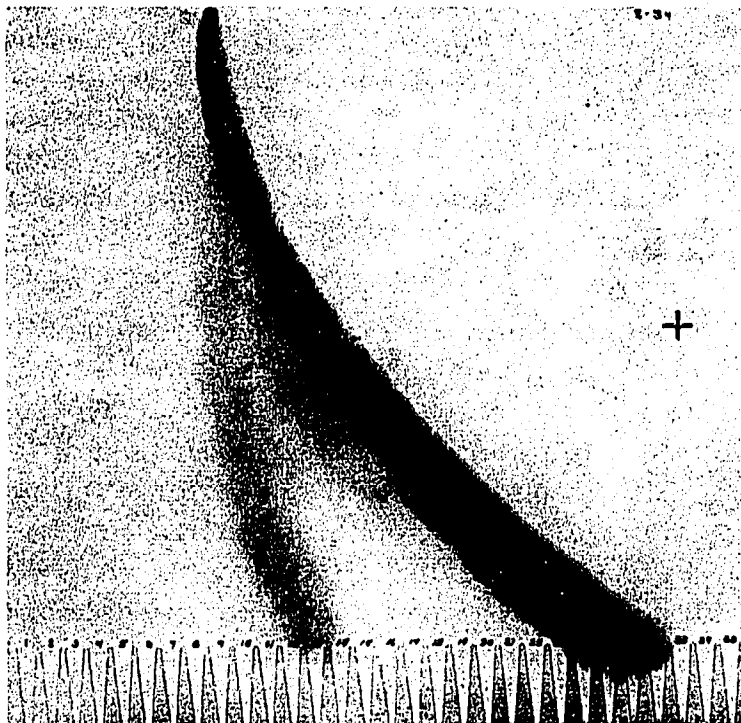

Fig. 1. Stained filter paper after continuous electrophoresis of a mixture of Strains U1 and U2 which were individually purified and then centrifuged together. The components from left to right are: Strain U2, Component I (see text), and Strain U1.

Strains U1 and U2 purified individually and then sedimented together in the high speed centrifuge also show the presence of Component I in the boundary electrophoresis diagram[8]. U1 and U2 mixtures prepared in this manner were also utilized in an attempt to isolate Component I by continuous electrophoresis—without success. Fig. 1 shows a stained filter paper from one of these experiments. This paper is somewhat atypical, in that the movement down the paper of the component assumed

to be I is the greatest ever observed, but it is depicted to illustrate a point in the discussion section of this paper.

Purification of virus from plant tissue homogenates

TMV is normally purified from leaf sap or leaf homogenates either by high speed centrifugation or salt precipitation. Continuous electrophoresis differs in principle from either of the above as a technique for the purification of proteins; hence, it was of interest to determine whether TMV could be isolated directly from leaf homogenates by continuous electrophoresis.

Virus-containing leaves of each strain (60 g) were ground in 150 ml of distilled water, the homogenates were filtered through glass wool and centrifuged for 30 minutes
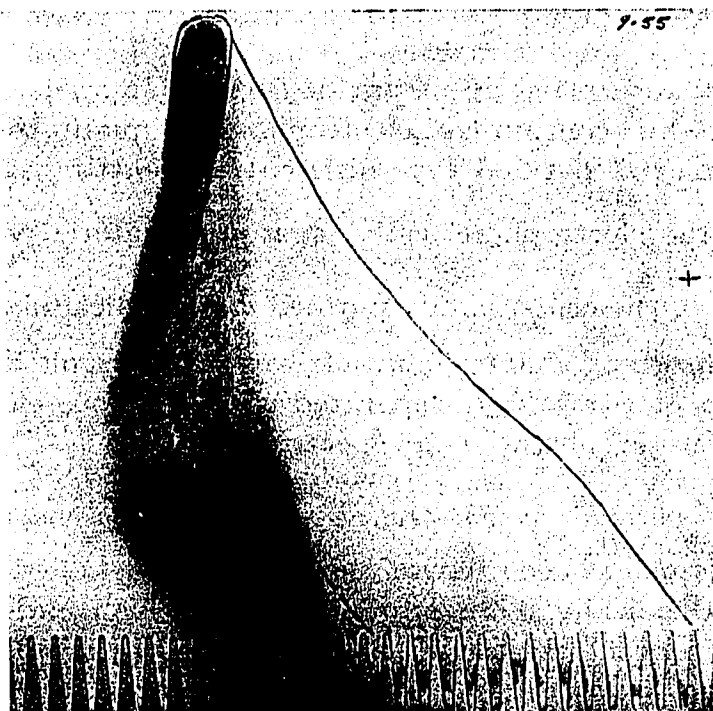

Fig. 2. Stained filter paper of Strain U2 purified directly from an homogenate of diseased leaves. The virus is represented by the streak on the left.

at 3500 g to remove cell debris. Before subjecting the homogenates to continuous electrophoresis it was necessary to reduce their volumes, as the maximum flow rate which could be handled on No. 54 paper was about 0.2 ml per hour. Concentration to about 20 ml was effected in a rotary evaporator operated so that the temperature of the water bath did not exceed 60° C*. The concentrated extract was centrifuged at 3500 g for 30 minutes, and the supernatant solution was dialyzed in the cold for 24

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* At the time of these experiments the deleterious effect of heating the virus, with the concomitant modification of its aggregation state and behaviour on the paper was not appreciated. However, virus did migrate on the filter paper, and heating probably did not affect the general conclusions from this type of experiment.

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hours in several changes of distilled water. Dialysis increased the volume of the concentrate, necessitating reconcentration to about 20 ml by rotary evaporation. A final dialysis in $M/90$ pH 7 phosphate buffer yielded a liquid, dark brown in colour, which was subsequently subjected to continuous electrophoresis.

A filter paper taken from a typical experiment with Strain U2 is shown in Fig. 2. The pencilled lines delineate the areas of the paper occupied by the brown material which is visible before staining with bromophenol blue. The trailing edge of the streak is dotted, as the edge of the boundary was not easily defined. Virus-containing material (determined by serological test) occurred in the tubes which collected liquid from beneath the streak on the left. In these experiments the virus exhibited much more spreading on the paper than experienced with centrifugally purified virus.

Strain U1 and the brown material migrated to a similar position on the paper. Electrophoresis at several pH's from 6 to 8 failed to separate the virus from the brown material.

The ultra-violet absorption spectrum of Strain U2 virus, isolated from plant tissue homogenates by continuous electrophoresis qualitatively resembled the spectrum of Strain U2 purified by differential centrifugation. However, the $E \frac{260 \text{m}\mu}{280 \text{m}\mu} = 1.11$ of the electrophoretically separated material when compared with the $E \frac{260 \text{m}\mu}{280 \text{m}\mu} = 1.21$ of the centrifugally purified virus indicated that the former contained ultra-violet absorbing contaminants. It was not possible to examine the UV absorption spectrum of Strain U1, separated by continuous electrophoresis, as the ubiquitous brown material obscured the spectrum.

Purification of a low molecular weight protein associated with virus infection

Virus-diseased leaf tissue has been shown to contain at least one low molecular weight protein which is antigenically related to TMV, and which under conditions of low pH and/or high salt concentration may be polymerized into rods[9] of the diameter of TMV rods, but of varying length[10]. The biological significance of this protein is not clear, but it has been shown not to be an artifact of preparation or a decomposition product of the virus[11]. Following the terminology of JEENER AND LEMOINE[12] the term "soluble antigen" (antigen) will be used to refer to this protein.

Soluble antigen-containing material was prepared by a method slightly modified from DELWICHE *et al.*[11]. Systemically infected leaves or leaves directly inoculated with Strain U1 some 17–52 days previously were employed. The leaves were ground in two times their weight of $M/90$ pH 7 phosphate buffer and the homogenates filtered through glass wool and subjected to low speed centrifugation (3500 g for 20 minutes). The resultant supernatant liquid was centrifuged at 100,000 g for 1 hour to remove the virus. In an attempt to minimize contamination by virus the supernatant fluid was carefully removed with a syringe, leaving about ½" of liquid over the pellet. Some of the preparations were frozen at this point without apparent effect on subsequent purification. The pH of the fluid (6.2–6.6) was then brought to 4.7 using

1 N hydrochloric acid. After standing for about 18 hours at 2° C, the flocculated material was removed at 3500 g (20 minutes) and was discarded. Centrifugation of the pH 4.7 supernatant at 100,000 g for one hour, followed by the rinsing of the pellet and tube with water, resuspension in $M/90$ pH 7 phosphate buffer, and dialysis yielded the soluble antigen-containing material which was subjected to continuous electrophoresis. Conditions of continuous electrophoresis (at 2° C) were identical with those used for the virus.

The antigen migrates to a position which would give an apparent mobility slightly less than that of U2 in the virus experiments (Fig. 1). Its behaviour on the paper, however, was quite different from that of the virus; the yields of antigen were much greater, indicating very little adsorption. An additional 12 hours electrophoresis after the last of the protein was applied to the paper was sufficient to elute it.

The tubes containing the soluble antigen (determined by spectrophotometric examination) were pooled, dialyzed for several hours in 0.1 M pH 4.7 acetate buffer, and then antigen was reconcentrated by centrifugation at 100,000 g for one hour.
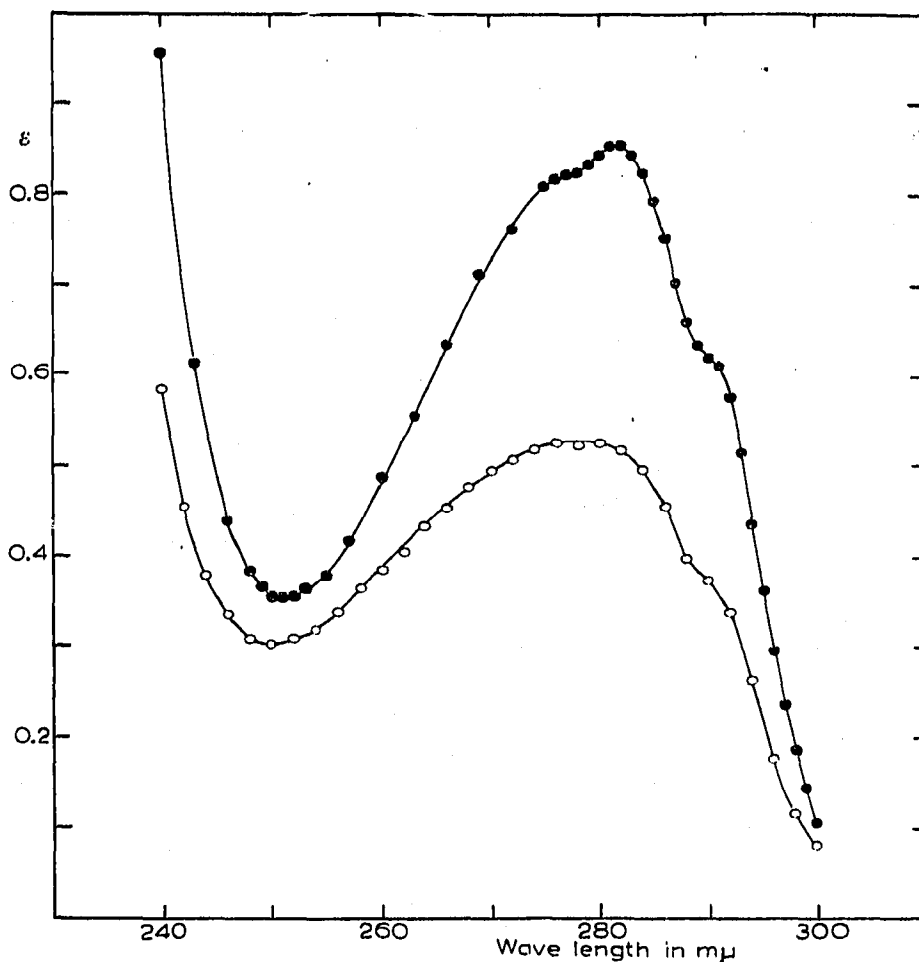

Fig. 3. Ultraviolet absorption spectra of purified soluble antigen of TMV — ●—●— and antigen-containing material as applied to the continuous electrophoresis filter paper --O--O--- in $M/90$ phosphate buffer, pH 7.

The subsequent pellets were usually clear and gelatinous and resuspended within a few hours in $M/90$ pH 7 phosphate buffer at 2° C. In some preparations, however, the pellet contained some opaque material which failed to resuspend. The solutions were clarified by centrifugation at 10,000 g for 30 minutes. Dialysis $vs.$ pH 4.7 buffer elicited the striking change in the light scattering properties of the antigen, originally observed by TAKAHASHI AND ISHII[9], and in all likelihood represented the aggregation of this protein into rod-shaped particles.

The UV absorption spectra of the purified soluble antigen and an example of the antigen-containing material applied to the electrophoresis filter paper are shown in Fig. 3. The $E\frac{1\%}{280m\mu}$ (1 cm light path) of the purified antigen, based on nitrogen determination, assuming 16% N, is 12.7–12.8 (three preparations). These values are not corrected for light scattering.

15 mg of soluble antigen was examined for nucleic acid content by hydrolysis and subsequent paper chromatographic isolation of bases and nucleotides; none were detected. Judging from TMV similarly examined as a control, the lower limit of detection establishes a maximum of 0.05% RNA in this preparation.

The soluble antigen was also examined for its infectivity by comparing the number of lesions produced on 24 half-leaves of $N.$ $glutinosa$ with lesions produced in response to TMV rubbed onto the opposite half-leaves. Comparing soluble antigen at 0.5 mg/ml with TMV (U1) at 10^{-3} mg/ml, the values obtained were 0 and 146 lesions per half-leaf respectively. In this laboratory, U1 may be detected with certainty on $N.$ $glutinosa$ at a minimum concentration of $5 \cdot 10^{-5}$ mg/ml. The upper limit of infectivity of the soluble antigen is, therefore, 0.01% of that of the virus.

In the Tiselius cell at pH 6.9 in 0.1 μ cacodylate–NaCl buffer[8] a 0.5% solution of the antigen migrated as a single component with no suggestion of heterogeneity.

Continuous electrophoresis of the soluble antigen-containing leaf extract prepared as above always yielded at least two components in addition to the soluble antigen. Small amounts of a fast-moving component were always found; this material did not stain when the paper was treated with acidic bromophenol blue, and from its absorption spectrum (E 260mμ/280mμ = 1.85–1.96) appeared to be rich in nucleic acid. Another component (or components) moving in front of the soluble antigen and spreading over a large number of tubes also appeared to be nucleoprotein in nature and probably corresponded to some host nucleoproteins. It was not determined whether these components were unique to infected leaves, but neither was infectious nor would react with U1 antiserum.

Soluble antigen is capable of aggregation—being sensitive to changes in ionic strength and pH[9]. In many of the experiments involving its purification by continuous electrophoresis, a portion of the antigen appeared to be in an aggregated form which migrated on the paper to a position slightly behind that normally experienced with U1 virus. The tendency to aggregate occurred principally when directly inoculated leaves were used as a source of antigen and was minimized with young, systemically

infected leaves*. Aggregation in effect resulted in a net loss of part of the soluble antigen during the process of purification, as the aggregated material was strongly adsorbed to the paper and little or none was eluted during the 1.5–3 days of the electrophoresis.

IV. DISCUSSION

Recently, virus which had migrated down the paper has been examined with the electron microscope. A marked heterogeneity of the virus contained in the several tubes into which each strain collects, and a significant qualitative difference in particle size of the virus after migration down the paper has prompted a further study of the fate of the virus on the paper. This work, in collaboration with Dr. J. N. PHILLIPS and Miss M. BRIGGS is in progress and will be published in detail later, but some of the conclusions drawn are important in discussing the data described above.

The purified Strain U1 virus material which is applied to the paper contains a proportion of rods which are much longer than 300mμ—the length of the TMV monomer. These long rods are conspicuously absent in the virus which is collected from the paper—no rods much longer than 300mμ have been observed. Long rods appear to be strongly adsorbed and treatments which aggregate the virus end to end would tend to restrict its movement on the paper. It has been observed that heat treating the TMV during purification, or allowing it to stand in phosphate buffer for a considerable time after purification reduces the rate of virus movement down the paper. In one striking case, a preparation of a U1/U2 mixture which migrated normally when subjected to electrophoresis shortly after centrifugal purification, failed to migrate from the point of application when examined after 9 months' storage at 2° C in pH 7 phosphate buffer.

The rate of migration down the paper of different preparations of freshy purified virus is in itself somewhat variable. It appears likely that some uncontrolled factor in the centrifugal purification of the virus, for example, the length of time the un purified virus remains in the presence of the leaf homogenate (suggested by PIRIE[13] may influence its state of aggregation, and, hence, its behaviour on the filter paper

TMV was examined by continuous electrophoresis principally in the vicinity o pH 7. Below pH 7 the protein becomes increasingly adsorbed to the paper, and a about pH 5 the virus does not migrate from the point of application to the paper The two strains studied differ widely in electrophoretic mobility between pH 5 and 8[14] and good separations with continuous electrophoresis are easily achieved at pH and above, where adsorption is minimized. In an earlier study of a naturally occurring mixture of viruses isolated by centrifugation from *Cattleya* orchid plants, moving boundary electrophoresis indicated a separation into two components at pH 5.8 o 5.1, whereas at pH 7 only one peak was in evidence[15]. Adsorption of the virus to th

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* This observation does not reveal whether aggregation exists within the host cell, or whethe it occurs after rupture of the cell, as a result of the conditions of pH or ionic strength resultin therefrom.

paper at low pH has precluded any separation of these two viruses by continuous electrophoresis.

Adsorption to the filter paper probably explains the failure to obtain Component I, the intermediate component between U_1 and U_2 originally observed in the Tiselius cell by SINGER et al.[8]. Component I is most easily interpreted to contain equal amounts of U_1 and U_2 protein. The fact that it is so strongly adsorbed to the filter paper (Fig. 1) suggests that it is aggregated to a dimer or higher.

The position to which a component will migrate on the filter paper appears to be influenced not only by its charge, but also by its relative adsorption to the paper. Where it has been possible to make comparative measurements the proteins investigated have a lower mobility on filter paper than would be expected from measurements of their mobilities in a Tiselius cell. For example, Strain U_2 and the soluble antigen exhibit almost a zero mobility (or on occasion a positive mobility) on the filter paper, whereas in boundary measurements their mobilities are $-4.9 \cdot 10^{-5}$ and $-3.6 \cdot 10^{-5}$ cm^{2}volt^{-1} sec^{-1}, respectively. Also, the position of migration of the components on the paper with respect to one another cannot be predicted from moving boundary measurements alone. The example of Strain U_2 and the soluble antigen may again be cited; a more direct comparison is provided in Fig. 1, where it may be seen that the mobility of the middle component assumed to be I is not midway between U_1 and U_2 as it is during electrophoresis in free solution[6,7], but is displaced towards the anode.

These facts suggest that the adsorption of the proteins to the filter paper influences the horizontal component of migration to a greater extent than the vertical component. This could arise from the interplay between the endosmotic and electrophoretic forces leading to an oriented adsorption of the virus particles on the paper*.

An apparently similar effect of adsorption may be seen in a diagram of a starch electrophoresis isolation of two strains of TMV and their intermediate component presented by PAIGEN[16].

The behaviour of TMV on filter paper has been investigated during chromatography[17,18,19] and zone electrophoresis[18,20]. With aqueous solvents the virus appears to show considerable adsorption to the paper. GRAY[18], using purified TMV and zone electrophoresis found that the virus remained at the origin at pH 7, although it migrated with considerable tailing at pH 8.6. The virus did migrate from the origin at pH 7 when extracts of diseased plants, rather than purified virus were examined. In this regard it is of interest to note that the virus has a low mobility on paper —appearing behind the bulk of the host protein[18,20] in contrast to its behaviour in free solution[21] where the virus has the greater mobility, demonstrating that the virus shows a greater adsorption than the host proteins. With paper chromatography, RAGETLI AND VAN DER WANT[19] found that TMV did not move from the origin in NaCl concentrations above 0.1 M, and in buffers of pH 3.6.

Electron microscopic examination of the U_1 virus collected from the filter

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* Endosmosis under the described conditions of continuous electrophoresis was confirmed by the use of dextran[5].

paper has revealed a higher proportion of small rod pieces than were present in the virus as applied to the paper. The distribution of rod lengths was non-random—a number of discrete rod lengths of less than 300mμ were observed in addition to the monomer. Several of the particle sizes observed were similar to those found by SCHRAMM, SCHUMACKER AND ZILLIG[22] in studies on the degradation of TMV at high pH. These findings will be reported in detail later.

Because of the low yield of U1 after continuous electrophoresis, resulting from a high percentage of the virus remaining on the paper, it is not possible to state unequivocally whether the short rods arose through breakage of larger rods, or were seen in greater amount because of their concentration due to favourable selection by passage down the filter paper. However, the short rod length found in greatest abundance in the material collected from the paper was too prevalent to be accounted for completely in the starting material, and it is not unreasonable to presume that there is considerable breakage of Strain U1 on the paper.

Strain U2 has been examined similarly in the electron microscope. Compared with U1, much fewer short rods have been found after continuous electrophoresis, suggesting less breakage on the paper. In this regard, it should be emphasized that the infectivity per unit protein of the virus recovered after continuous electrophoresis has been investigated with Strain U2 only. Considering the apparently greater breakage of Strain U1 on the filter paper, it may not be possible to extrapolate to Strain U1 the finding obtained with Strain U2, (Table I), that the virus infectivity per unit weight of protein is not diminished by passage down the paper during continuous electrophoresis.

The soluble antigen of TMV, as purified by continuous electrophoresis, may be a closer approach to a pure protein in a non-aggregated state than preparations of antigen hitherto obtained. Judging from phosphorus content[23], ultra-violet absorption spectra[24, 25], and ultra-violet absorption spectrum of material extracted from antigen with 10% perchloric acid[12], some of the antigen preparations reported may contain small amounts of nucleic acid. The ultra-violet absorption spectra presented by

TAKAHASHI AND ISHII[24] and COMMONER AND YAMADA[25] have much lower $E \frac{280 m\mu}{260 m\mu}$ and $E \frac{280 m\mu}{300 m\mu}$ values than those obtained with antigen purified by continuous elec-

trophoresis. The low $E \frac{280 m\mu}{300 m\mu}$ values suggest that the preparations contain material which is aggregated, and in fact, if corrections are made for light scattering with TAKAHASHI's data[24], the E (280mμ/260mμ) of his preparation approaches the ratio obtained with antigen purified as described in this paper.

Preparations of antigen which are apparently similar to antigen prepared in this laboratory have been obtained (after preliminary purification) by removal from a Tiselius cell[26], or by treatment at pH 10.7 and subsequent passage through columns of modified cellulose[27]. It appears that in order to obtain antigen with a high

$E \frac{280 m\mu}{260 m\mu}$ some purification technique in addition to acid or salt precipitation must be

employed to remove aggregated material, and possibly, nucleic acid-containing contaminants.

The ultra-violet absorption spectrum of the soluble antigen as purified by continuous electrophoresis (Fig. 3) is strikingly similar to that of the nucleic acid-free protein obtained from TMV[28]. This evidence gives strong support to the conclusion of SCHRAMM AND ZILLIG[10] that the antigen (X-protein) and the low molecular weight protein obtained upon degradation of TMV (A-protein) are identical.

Continuous electrophoresis as a direct preparative method for TMV yields a poor product when compared with TMV which is purified either chemically or centrifugally. Host proteins migrate over a wide area of the filter paper, which virtually precludes their separation from the virus. I am of the opinion that the unique contribution of continuous electrophoresis as a preparative technique for plant viruses and associated proteins lies in its use in conjunction with other physical or chemical methods, as demonstrated in the purification of the soluble antigen of TMV reported above. Frequently, existing methods such as differential centrifugation cannot in themselves enable a complete separation of virus from host constituents. This is particularly true of those plant viruses which closely approximate some host components in sedimentation behaviour. For example, with a stone-fruit tree virus isolated from cucumber, LINDNER et al.[29] and WILLISON et al.[30] found that high speed centrifugation of homogenates of tissue from infected plants resulted in a pellet which contained the virus along with some other host constituents. With continuous electrophoresis much of the contaminating substance which sediments along with the virus can be removed[31].

SUMMARY

1. The technique of continuous electrophoresis as applied to a study of tobacco mosaic virus (TMV) has been described.

2. Virus isolated by differential centrifugation from plants mixedly infected with two strains of TMV has been separated into two components with this technique. The components corresponded in biological activity to the strains used to infect the plants.

3. The biological activity per unit protein of one strain (U2) was unchanged by continuous electrophoresis. The other strain was not analysed in this way.

4. TMV purified directly from leaf homogenates by continuous electrophoresis was an inferior preparation when compared with TMV purified by differential centrifugation.

5. The low molecular weight protein associated with TMV infection (soluble antigen) has been purified and was found to be similar in ultra-violet absorption spectrum to the nucleic acid-free protein obtained by the degradation of TMV.

6. The usefulness of continuous electrophoresis as a technique for the purification of plan viruses and related proteins has been discussed.

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