DISC ELECTROPHORESIS STUDIES OF HEALTHY AND TOBACCO MOSAIC
VIRUS INFECTED NICOTIANA TABACUM L. PLANTS

by

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The techniques of disc electrophoresis and of radioactive labeling were used to study protein changes in a young leaf of a small tobacco plant following infection by TMV. Proteins were separated into bands which were detected by staining with a general protein stain, aniline blue black; radioactivity of the bands was detected by autoradiography of the stained electropherogram. Approximately twenty protein bands were seen in stained electropherograms of ultracentrifuged extracts from young, normal tissue. The specific radioactivities of most bands were nearly the same but two and sometimes three bands had high specific radioactivities.

Following infection by TMV, the only protein change which was found was the appearance of a labeled area with \( R_f \) value of 0.33 in autoradiographs of electropherograms of extracts from plants infected for four days. A band with the same \( R_f \) value was not detected by staining until eight days after infection, when the protein had accumulated. This band was tentatively identified as X or subunit protein on the basis of an identical \( R_f \) value. The high specific activity of this band indicated that subunit protein was being rapidly synthesized.

Triton X dissolved chloroplasts of both healthy and TMV infected leaves showed six bands on stained electropherograms. The same bands could be seen in autoradiographs.
Some bands in electropherograms of extracts from mature leaves were clearer and appeared to have more protein associated with them than corresponding bands in extracts from young leaves. Such differences indicated that changes in proteins accompanying maturation as well as changes following infection could be detected by this technique.
INTRODUCTION

Neither the process of tobacco mosaic virus (TMV) biosynthesis nor the mechanism by which it initiates a disease condition in the host plant is fully understood. The aim of this study is to gain insight into these processes by determining whether any changes occur in the proteins of the plant following infection.

Changes in the protein composition might be expected because enzymes are intimately involved in virus formation. New proteins which are not found in uninfected tissue might appear following virus infection. The appearance of at least one new protein, viral coat protein, would be expected; other new proteins coded for either by the viral RNA or host DNA might also appear. A viral RNA dependent synthetase enzyme is an example of a new protein which could be induced by virus. Such an enzyme is likely to be necessary for viral reproduction because normal plants appear to lack an enzyme requiring an RNA template. An RNA synthetase has been found in E. coli which has been infected by an RNA virus (Nathans et al., 1966, Ochoa et al., 1964). However, no convincing reports which characterize such an enzyme for TMV-RNA have appeared.

A recently developed technique, disc electrophoresis on poly-acrylamide gels was used to separate the proteins found in tissue extracts. With this technique, a large number of protein components in a complex mixture can be separated. These can be detected by staining with dye, by the presence of enzymatic activity, or by the presence
of radioactive label. In the present study, $^{14}C$ labeled proteins were detected by autoradiography of the polyacrylamide gel column and the protein pattern was detected by staining. New virus specified proteins would be expected to have high specific radioactivity because there would be no endogenous protein. However, the normal proteins of the cell, except those which have a high turnover rate, will have relatively low specific activity.

The type of leaf chosen for this study was the top leaf of a tobacco plant eight weeks old and less than four inches tall. It had been shown that when the lower leaves of a very small plant were inoculated with TMV, the virus could be detected in the upper leaf within 18 hours and that at this time, the virus was distributed throughout the whole leaf (Wildman, unpublished). This top leaf was chosen with the expectation that the massive systemic virus invasion might produce a corresponding and detectable change in the proteins of the infected cells. It was found that a very rapidly labeled protein which probably is viral coat protein could be detected four days after infection. In addition, it was found that uninfected leaves contain two or three proteins which have a high turnover rate compared with the rest of the proteins in the cell.
LITERATURE REVIEW

Previous electrophoretic studies of the proteins of tobacco plants following infection by TMV showed that TMV itself was the principal new protein which appeared after infection (Frampton and Takahashi 1944, Wildman, Cheo and Bonner 1949). Moving boundary electrophoresis in a Tiselius apparatus was utilized in those early studies of the proteins of TMV infected plants. Four components were distinguished in extracts containing the soluble cytoplasmic proteins of healthy leaves. One of these components, called fraction I (Wildman, et al. 1949), made up approximately 75% of the total cytoplasmic proteins. In addition to the components found in healthy plants a new component, V, appeared in extracts from plants infected for 15 days. The narrowness and height of the area indicated that V had a small diffusion constant, characteristic of TMV itself (Frampton and Takahashi 1944). Wildman, et al. (1944) gave evidence that virus protein was synthesized at the direct expense of the normal cytoplasmic protein, fraction I. In contrast, another study (Bawden and Kleczkowski 1957) indicated that there was no consistent correlation between the concentration of normal proteins and the amount of virus produced or the severity of the symptoms shown by the leaves. The number and concentration of electrophoretically distinguishable components of the leaf were shown to vary with the position on the stem, the age of the plants, their nutrition and conditions of growth.
In addition to virus itself, other new proteins have been found in infected plants. These proteins were noninfective but immunologically related to TMV and aggregated into RNA free virus-like particles in vitro. The presence of these proteins, termed X or subunit protein, was reported by several groups who sought to elucidate their role in virus synthesis (Takahashi and Ishii 1953, Commoner, et al. 1952, 1953, 1955, Jenner, Lemoine, and Lavand'homme 1954). Takahashi and Ishii (1953) reported that X protein could be detected in three days following inoculation and that it increased rapidly up to about the seventh day, after which it remained constant. However, Commoner, et al. (1952) did not detect this protein until seven days after inoculation. Early workers had suggested that the protein could be a degradation product of the virus, a rejected imperfect virus protein, or a precursor to virus. However, the results of van Rysselberge and Jenner (1955, 1957) who used C\(^{14}\) labeling supported the hypothesis that the antigen immunologically related to TMV (X protein) was a precursor to virus protein. Later, Takahashi (1959) found that the X protein was identical with TMV coat protein; it could be reconstituted with TMV RNA to produce infectious virus.

More recent comparisons of the proteins from healthy and TMV infected plants have been done using agar gel electrophoresis (Townsley 1959). Only four components could be distinguished in extracts from healthy leaves. Electrophoresis on agar gel was an effective tool for gentle fractionation of crude sap from infected leaves, prior to electron
microscopic examination. A "fast" band serologically related to TMV and a band of nonaggregated TMV were separated and were characterized in electron micrographs as being composed of particles about 1000 Å and 3000 Å, respectively. Concomitant labeling studies indicated that the "fast" band represented an intermediate in virus synthesis.

A significant improvement in the separation of proteins was presented by electrophoresis on polyacrylamide gel. With this technique proteins can be separated by their size as well as by their charge because of the sieving effect of the "pores" in the polymerized acrylamide.

Polyacrylamide gels are nonionic, thermostable, transparent, strong, relatively inert chemically and can be prepared in a large range of pore sizes. They are easier to prepare than starch gels and easier to handle than agar gels. The superiority of gel electrophoresis over previous techniques was demonstrated by Ornstein and Davis (1962). They introduced a sharp discontinuity in voltage gradient, pH, and average pore size to achieve sharper boundaries and increase resolution and sensitivity. The polyacrylamide gel column was composed of two layers, a large pore, spacer gel and a small pore, running gel. The proteins were concentrated into thin starting zones in the large pore, spacer gel, then they separated according to their size and charge in the small pore, running gel. The proteins appeared to be stacked one on top of the other; hence the name "disc" electrophoresis.

By the use of this technique, qualitative changes in plant host proteins as a result of infection by a disease organism have been demonstrated. Proteins of beans, infected by a fungus, a bacteria, and a
virus have been studied. In all cases, when gels were stained with a general protein stain, no visible difference between the protein profiles of healthy and infected plants was noted. On the other hand, enzyme specific stains revealed differences in the isozymic patterns produced by extracts from healthy and Uromyces phaseoli infected tissue (Staples and Stahmann 1964, Williams and Staples 1964). Isozyme patterns for malate dehydrogenase, soluble acid phosphatase, aldolase, and succinic dehydrogenase from healthy leaves, rusted tissue, and uredospores were each different. When bands of the same mobility appeared in extracts of both infected plants and uredospores, the presence of the isozyme in rusted tissue was attributed to the presence of fungus.

Rudolph and Stahmann (1964, 1966) extended these studies to bean plants infected with the halo blight disease, Pseudomonas phaseolicola. They found several isozyme changes following infection. Evidence for the bacterial origin of these changes was the high concentration of activity in the "greasy infection centers", low activity in the surrounding yellow halos, no activity in the green tissue, and presence in bacterial sonic extracts.

Changes in isozyme patterns of bean leaf peroxidases were associated with infection by southern bean mosaic virus. Peroxidases from healthy plant tissues were resolved into two bands, whereas four were found in virus infected lesion bearing leaves. One of the new bands was detected as soon as lesions became detectable, while the other appeared later. Aged but healthy plants also showed the latter
peroxidase; thus its presence in SBMV infected plants was attributed to accelerated aging (Farkas and Stahmann 1966).

The properties of X or subunit protein have been studied by moving boundary and agar gel electrophoresis and the results of such investigations have been presented in the foregoing literature review. The use of disc electrophoresis in demonstrating qualitative changes in host proteins following infection by a disease organism have also been reviewed. Since the technique of disc electrophoresis has not been utilized previously for the detection of new proteins in TMV infected tobacco plants, the present work was undertaken.
MATERIALS AND METHODS

I. Preparation of Infected Plants

Greenhouse grown, potted *Nicotiana tabacum* L. var. Turkish Samsun plants that were eight weeks old, about three inches tall, and had at least four leaves were used. The upper surface of the second, third, and fourth leaves were inoculated with a solution containing $10^{-1}$ mg/ml TMV strain U₁ in M/15 phosphate buffer, pH 7.0 or buffer alone, in the case of the control. Both solutions contained 50 mg/ml Celite. The plants were maintained under artificial lights which consisted of fluorescent tubes and 60-watt incandescent bulbs giving an intensity of 400 foot candles 20 inches above the bench. The average temperature was 30° F. In plants inoculated with virus, the first or top leaf, which was approximately 4 cm in length, became systemically invaded by virus.

II. Preparation of C¹⁴ Labeled Proteins

From each of three infected and three control plants, the top leaf was removed and weighed at intervals of one day after inoculation. Leaf proteins were labeled with C¹⁴ as follows: 0.03 ml of a solution containing 3 μc of C¹⁴ labeled amino acids (New England Corp.) was diluted with 0.6 ml deionized water; 0.1 ml of the diluted amino acid solution (0.5 microcurie) and a single leaf were placed into each of six small test tubes. The leaves were allowed to take up the label through the petiole at room temperature with a fluorescent light one
foot above them for about an hour. Water was added if needed to prevent wilting.

III. Extraction and Fractionation of Proteins

Extraction of proteins was accomplished by grinding leaves in cold mortars with four times their weight of sucrose-Tris buffer pH 8.2 (0.5 M sucrose, 0.01 M MgCl₂·6H₂O, 0.05 M Tris 1L, 0.025 M KCl, and 0.005 M 2-mercaptoethanol, adjusted to pH 8.2 with HCl). (Marcus and Feeley 1964). The grindate was filtered through Miracloth (Chicopee Manufacturing Co., Miltown, N.J.) and the resulting green solution was fractionated by differential centrifugation. The first centrifugation was performed at 3000 rpm (1000 x g), for 5 minutes in a Sorvall SS-34 refrigerated centrifuge. The supernatants were centrifuged at 40,000 rpm (105,000 x g) for one hour in a Spinco Model L analytical ultracentrifuge using a #40 rotor. The supernatant of this centrifugation will be referred to as the cytoplasmic fraction. The 1000 x g pellets were resuspended in 2 ml sucrose-Tris buffer containing 3.5% Triton X-100 (Rohm and Haas) which solubilized the chloroplasts, and centrifuged again at 3000 rpm for 5 minutes. The supernatant of this centrifugation will be called the chloroplast fraction. The supernatants from these two centrifugations were used for the electrophoretic studies. In some cases further fractionations were performed.

One of these steps consisted of a 13,000 rpm (10,000 x g) centrifugation for 30 min. of the 3000 rpm supernatant prior to that at 40,000 rpm; the resulting pellet was considered to be the mitochondrial fraction. A nuclear fraction was obtained from the Triton insoluble
residue in the 1000 x g pellet. These pellets were resuspended in sucrose-Tris buffer. The fractions were assayed for protein and radioactivity.

Total proteins were estimated by the method of Lowry, et al. (1951). Bovine serum albumin was used as a standard and the absorbance was measured at 660 μm. For radioactivity assay, 0.1 ml of each sample was plated on a disposable planchet, allowed to dry and counted in a thin window gas flow counter (Nuclear Measurements of Chicago, Model 186).

The infectivity of the 105,000 x g (40,000 rpm) pellet was determined to correlate the presence of virus with the possible appearance of a new protein band. The pellet was resuspended in M/15 phosphate buffer pH 7.0 containing 50 mg/ml Celite and rubbed onto half leaves of N. tabacum var. Xanthi. Local lesions were counted three days later.

IV. Procedure for Disc Electrophoresis

Polyacrylamide gel electrophoresis was carried out following the procedures described by Ornstein and Davis (1962) with some modifications. A 5.6% small pore running gel and 7 mm I.D. x 120 mm columns were used instead of a 7.5% small pore running gel and 4 mm x 63 mm columns. The polyacrylamide gel column was composed of two layers: 1) a large pore, spacer gel in which electrophoretic concentration of the sample proteins was accomplished and 2) a 5.6% small pore running gel in which electrophoretic separation took place. The large pore sample gel used by Ornstein and Davis as an anti-convection medium could
not be utilized because photopolymerization was inhibited by the plant extract. In place of a sample gel, a 44% acrylamide solution was mixed with the sample to make a dense solution.

Stock solutions were prepared as follows:

a. Small pore gel buffer (pH 8.8 - 9.0)

1N HCl - 48 ml
TRIS (hydroxy methyl aminomethane) - 36.3 gm
TEMED (N,N,N',N'-tetramethylethylenediamine) - 0.23 ml
H₂O to 200 ml

b. Small pore monomer solution

Acrylamide monomer - 40.0 gm
N,N'-Methylenebisacrylamide - 1.6 gm
H₂O to 200 ml

c. Ammonium persulfate solution

Ammonium persulfate, reagent grade - 0.1400 gm
H₂O to 100 ml

d. Large pore monomer solution (pH 6.7)

1M H₃PO₄ - 25.6 ml
TRIS - 5.7 gm
Acrylamide monomer - 20.0 gm
N,N'-Methylenebisacrylamide - 5.0 gm
Riboflavin - 4.0 mgm
H₂O to 400 ml

e. 40% sucrose solution

Sucrose - 40 gm
H₂O to 100 ml

f. 44% acrylamide solution

Acrylamide monomer - 44 gm
N,N' - Methylenebisacrylamide - 0.29 gm
H₂O to 100 ml

g. TRIS - Glycine buffer (pH 8.2)

TRIS - 6.0 gm
Glycine - 23.8 gm
H₂O to 1 liter
All the solutions were stored in the refrigerator ($4^\circ$ C) except solution G which was prepared just prior to use.

The gels were made up as follows:

a. 5.6% small pore gel

Stock small pore buffer (pH 8.8-9.0) - 1 part
Small pore monomer solution - 1.5 parts
Ammonium persulfate - 1.65 parts

b. Large pore gel

Large pore monomer solution - 1 part
40% sucrose solution - 1 part

The small pore gel solution was mixed, degassed by suction and put into the stoppered glass columns to within 2 cm of the top, layered with about 2 mm deionized water, and allowed to polymerize at room temperature. After polymerization, the water was shaken off and the top of the gel was rinsed with large pore gel solution. Large pore gel was stacked to a height of about 1.5 cm and layered with water as before. After photopolymerization of the large pore gel with a fluorescent light, the columns were inserted into the Canalco Model 6 electrophoresis apparatus which was in a 5$^\circ$ cold room. Tris-glycine buffer (solution g) was used without dilution, in the upper electrode vessel with a few drops of bromophenol blue as tracking dye and in the lower electrode vessel without dye. The gel columns were suspended between the two electrode vessels which completed the circuit. The plant protein solutions, the 3000 rpm or 40,000 rpm supernatants, or resuspended nuclear or mitochondrial pellets, were mixed in a ratio of 3 to 1 with 44% acrylamide gel solution. Approximately 0.4 ml of the resulting dense solution containing approximately 1200 cpm in 0.5 mg protein, was
layered under the buffer, on top of the columns. A Spinco Duostat regulated D.C. power supply provided the 3 - 4 ma per column which accomplished electrophoretic separation in two to four hours. The direction of migration was downwards toward the anode. Upon completion of electrophoresis, the gels were removed from the glass tubes using a blunt needle and were fixed and stained in 0.55% Aniline Blue Black (Matheson, Coleman and Bell) in 7.5% acetic acid. They were destained in larger diameter (8 mm I.D.) tubes with small ends. Destaining was complete in about 3 hours using 7.5% acetic acid in a Model 6 apparatus at 50V per column.

V. Procedure for Autoradiography

Directions by Fairbanks, Levinthal and Reeder (1965) were followed for longitudinal slicing and drying of the gels and development of the pattern of radioactivity present. Gels were placed in the cavity of a plexiglass holder and sliced with a device similar to a cheese cutter, which consisted of three 5 mil stainless steel wires stretched taut in an aluminum jig. When the wires were drawn through a gel column while it was firmly supported by the holder, four longitudinal slices were produced. One of the outer slices was used to obtain the densitometric trace of the electropherogram. The two inner slices, 1/16 inch thick with two flat faces, were then dried onto filter paper supported by the bottom plate of a sterilizing filter (Van Waters and Rogers). The gel slices were covered by Saran Wrap which made a vacuum seal at the periphery when the vacuum was applied from below. The uniform pressure on the slices caused them to adhere to the filter paper and
prevented them from shrinking in length or width during drying. Heating with an infrared lamp from a distance of about one foot hastened the drying. The pattern of radioactivity in the gels was developed by autoradiography of the dried slices using Kodak No-Screen X-ray film. A heavy lead block was placed upon the x-ray exposure holders to assure adequate contact of film and slices. Film exposed for a month was processed using Kodak x-ray developer and fixer as directed by the manufacturer.

Densitometric tracings of the autoradiographs and the gel columns were made using a Jarrell-Ash recording microphotometer, Model 24-300.
RESULTS

I. Electropherograms of Young Uninfected Leaf Fractions

A. Cytoplasmic fraction

1. Cytoplasmic proteins detected by staining with aniline blue black.

The 105,000 x g supernatant fraction of young uninfected plants was examined by the disc electrophoresis technique to serve as the standard control. This fraction contains most of the soluble proteins of the plant. An electropherogram prepared as outlined in the materials and methods section is presented in Figure 1. Figure 1a is a photograph of a polyacrylamide column in which the proteins have been separated and stained with aniline blue black. Figure 1b is the author’s idealized diagram of the photograph; Figure 1c is a microdensitometric trace of the stained gel. This procedure for presenting the results will be followed because many of the bands are too faint to be clearly distinguished in the photograph. Due to the high background of residual stain, the position of bands whose intensity is only slightly greater than background is difficult to ascertain in the microdensitometer trace. Usually, twelve major bands can be easily seen by cursory observation (Fig. 1a). Careful scrutiny will reveal other faint bands so that about twenty bands are seen altogether. Different faint bands are seen in different electropherograms. The author’s representation shown in Figure 2 shows a composite which includes the maximum number of
Figure 1: Electropherogram and autoradiograph of extract from young uninfected tobacco leaf cytoplasm.

a: photograph of electropherogram
b: idealized diagram
c: microdensitometric trace of electropherogram
d: autoradiograph
e: microdensitometric trace of autoradiograph (dotted line) and its corresponding electropherogram (solid line)
Figure 2: Idealized composite diagram of electropherogram of extract from young uninfected leaf cytoplasm. Bands are numbered in the order of their relative mobilities and are presented with average $R_f$ values and standard errors.
bands found in the extract of proteins from young uninfected leaves. The bands are numbered in the order of increasing mobility. The slight variations in the number or intensity of bands may be caused by the following: inherent slight differences in the proteins present, as well as inconsistencies in gel polymerization, slight variations in gel or protein concentration, differences in length of time for electrophoresis or heating as a result of the voltage used.

The separation of proteins obtained by the disc electrophoresis technique is clearly superior to that obtained either by the Tiselius moving boundary or agar gel electrophoresis, each of which differentiates approximately four components. In this procedure, approximately twenty protein bands were detected and other proteins can be detected by adjustment of the procedure. The samples used in the study were differentially centrifuged so that only a portion of the total protein composition was used. In addition, there are other reasons why more bands were not visible. Proteins which were not soluble in the buffer used for extraction would not appear. Other proteins which were too large to enter the small pores made by the acrylamide and its accompanying crosslinking agent, methylenebisacrylamide would have remained in the large pore spacer gel or in the Tris glycine buffer above. It is also possible that some of the bands represent more than one species of protein which have not been resolved. Proteins that are present in the leaf extract in low concentration would not be stained sufficiently to be seen. In general, the type of pattern presented in Figure 2 was obtained in several independent experiments, although the distance
between the start of the running gel and the protein traveling with the marker band fluctuated from experiment to experiment in an uncontrolled fashion. Also, the presence or absence of a band preceding the "front" could not be controlled. However, the relative distance traveled by the different proteins when calculated as \( R_f \) value was fairly consistent. The \( R_f \) values obtained by averaging values for ten different electropherograms are presented in Figure 2 together with their standard errors.

2. Cytoplasmic proteins detected by autoradiography.

In order to determine which of the proteins in the control uninfected young leaves were rapidly labeled, the \(^{14}C\) labeled extract was subjected to disc electrophoresis and autoradiography. Figure 1d showing the result of such an experiment is an autoradiograph that has been in contact with the gel for four weeks. The size and shape of the gel slice shows little distortion upon drying. It can be seen that most of the stained bands have radioactivity. In Figure 1e a microdensitometric trace of the autoradiograph and its corresponding electrophrogram are superimposed, so that the degree of labeling can be compared with the degree of staining.

One band with an approximate \( R_f \) value of 0.7 was not detectable at all by staining with aniline blue black, but was often found by its labeling; this band was designated 22r (Fig. 1d, Fig. 2). The proteins in bands 6, 7 and 12 were found to have high specific radioactivities (Fig. 1e). The proteins with high specific radioactivity are probably turning over rapidly, or being rapidly synthesized in the young
expanding leaves. Although band 3 is very heavily labeled in the autoradiograph, in contrast to bands 6, 7, and 12, there is a corresponding heavily stained protein; thus, the protein in band 3 does not have high specific activity.

Bands 6 and 7 sometimes are found to have low specific activity; that is, the band appearing on the autoradiograph is not proportionately darker than the stained band. That bands 6 and 7 are not always heavily labeled in the autoradiographs is due to many factors such as uncontrolled growing conditions, different sizes of amino acid pools within the cells, variable time of C\(^{14}\) uptake and different amounts of protein put on the column. Attempts were made to minimize the effects of these factors by using comparable conditions for control and infected leaves when experiments were performed.

B. Chloroplast fraction

1. Chloroplast proteins detected by staining with aniline blue black.

The chloroplast fraction of young leaves was examined by disc electrophoresis followed by staining with aniline blue black. The 1000 x g pellet resuspended in buffer containing 3.5% Triton-X-100, which solubilizes the chloroplasts, was considered the chloroplast fraction. Six distinct bands whose R\(_f\) values are 0, 0.05, 0.09, 0.46, 0.52, and 1.0, respectively were often found (Figure 3a). Four bands including the one traveling with the front appear consistently, but the bands with R\(_f\) 0.46 and 0.52 were sometimes very faint. These two bands compare favorably in R\(_f\) value with bands 16 and 18 of the cytoplasmic fraction. It is possible that they may be contaminants from
Figure 3: Electropherogram and autoradiograph of the chloroplast fraction from young uninfected tobacco leaf.

a: photograph of electropherogram
b: idealized diagram
c: microdensitometric trace of electropherogram
d: autoradiograph
e: microdensitometric trace of autoradiograph (dotted line) and its corresponding electropherogram (solid line)
that fraction. Triton-X treatment may release these proteins if they were bound to the membranes. It is also possible that these six bands are truly chloroplast proteins or that they represent the only proteins which are stable in the detergent.

2. Chloroplast proteins detected by autoradiography.

Autoradiographs of the electropherograms of the chloroplast fraction of uninfected young leaves were very similar in their appearance with the stained electropherograms themselves. This can be seen in Figure 3e in which the microdensitometric traces of the electropherogram and the autoradiograph are superimposed. Usually only the three slowest bands could be distinguished clearly. Although there is enough protein at the solvent front to be stained well, there is rarely a corresponding labeled band. This band is a good example of differential labeling and staining which indicates that some proteins are not turning over. The two bands with \( R_f \) 0.46 and 0.52 (Figure 3a) are often not seen in autoradiographs (Figure 3d).

C. Nuclear and mitochondrial fraction - proteins detected by staining with aniline blue black.

The 1000 x g residue which was not solubilized by Triton X-100 was regarded as the nuclear fraction. When it was resuspended in sucrose-Tris buffer and subjected to disc electrophoresis and staining, no bands could be found. The same was true for the electropherograms of the mitochondrial fraction, which was the 13,000 rpm (10,000 x g) pellet. It is possible that the organelles were too large to penetrate
into the gel or that they were already completely disrupted and had no proteins left in them to release. After these preliminary experiments, no further investigation into the electrophoretic characteristics of the proteins in these fractions was made.

II. Electropherograms of Young Infected Leaf Fractions

A. Cytoplasmic fraction

1. Cytoplasmic proteins detected by staining with aniline blue black.

The 105,000 x g supernatant fraction of young *N. tabacum* leaves systemically infected by tobacco mosaic virus was examined by disc electrophoresis in order to see whether protein changes occurred as a result of invasion by the virus. These changes could reflect enzymes induced by the virus to reproduce itself, or could reflect products made by the host as the result of altered metabolism due to the presence of virus. In these studies, the lower leaves of plants were infected and homogenates of upper leaves were obtained at various periods after infection. No differences in the proteins were detected until the fourth day after infection. On the fourth day, changes in autoradiographic patterns were obtained, but no new aniline blue black stained protein was detected (Fig. 4a). By comparing Figure 4a with Figure 1a, one can see that they are nearly identical. However, when the systemically infected leaves of plants infected for eight days were used, a new band with $R_f$ 0.33 was found (Fig. 5a). Since the supernatant from
Figure 4: Electropherogram and autoradiograph of the extract from young tobacco leaf cytoplasm, infected with TMV for 4 days.

a: photograph of electropherogram
b: idealized diagram
c: microdensitometric trace of electropherogram
d: autoradiograph
e: microdensitometric trace of autoradiograph (dotted line) and its corresponding electropherogram (solid line)
Figure 5: Electropherogram and autoradiograph of the extract from young tobacco leaf cytoplasm, infected with TMV for 8 days.

a: photograph of electropherogram
b: idealized diagram
c: microdensitometric trace of electropherogram
d: autoradiograph
e: microdensitometric trace of autoradiograph (dotted line) and its corresponding electropherogram (solid line)
the 40,000 rpm centrifugation was used, intact TMV could not have been
the source of the new protein.

2. Cytoplasmic proteins detected by autoradiography.

An autoradiograph of the 105,000 x g supernatant fraction of
systemically infected leaves taken from plants four days after inocula-
tion is presented in Figure 4d. A heavily labeled band with Rf 0.33 is
particularly noticeable in the cytoplasm of plants infected four days
and thereafter. This band corresponds to the band detectable by stain-
ing only after eight days and is absent from extracts of uninfected plants
(Fig. 1d). Simultaneous with the appearance of this rapidly labeled
protein was a rather high concentration of virus as determined by local
lesion assay. The stained band corresponding in position to the heavily
exposed bands in the autoradiograph was not noticeably darker than the
other bands in the extract from plants infected four days. Comparison
of the microdensitometric traces of the electropherogram and autoradio-
graph (Fig. 4e) indicates that the band has high specific activity. By
eight days after inoculation, enough of this protein had accumulated so
that the band could be detected by staining (Fig. 5a). Even so, the
band detected by labeling was heavier (Fig. 5d), indicating that the
protein was being rapidly synthesized.

3. Identification of the new band appearing upon infection.

Experiments were performed to determine whether the new band
could be TMV subunit protein. Electrophoresis of purified TMV subunit
protein (Fraenkel-Conrat 1957) revealed that its mobility in the gel
was similar to that of the new band. Also, an attempt was made to determine whether the new band was subunit protein by using the immunological procedure of Poulik (1964). The gel column of separated proteins from the infected plant was embedded in 1% agar in barbital buffer and a trough cut about 2 mm from the gel. The trough was filled with antiserum to subunit protein. On one occasion, a faint precipitin line was discerned after some 48 hours but the results were not conclusive.

An experiment was performed in which unlabeled uninfected plant extract was mixed with purified labeled subunit protein and subsequently subjected to disc electrophoresis. The labeled purified subunit protein was found to have the same \( R_f \) value as the newly formed protein found in tissue infected for eight days. Also, a combination of labeled infected plant extract and unlabeled subunit protein resulted in a heavily exposed band at a position equivalent to the heavily stained one. Thus it was concluded that the rapidly synthesized protein in recently infected very small leaves was probably subunit or \( X \) protein.

B. Chloroplast fraction

Both the stained electropherogram and the autoradiograph of the chloroplast fraction from infected leaves resembled closely those obtained from uninfected leaves (Fig. 3).
III. Electropherograms of the Cytoplasmic Fraction From Mature Leaves.

Tissue from mature leaves (Fig. 6) gave electropherograms more well-defined than those obtained from young tissue. The bands were more distinct and those with $R_f$ value greater than 0.47 were more clearly stained. These bands appeared to have more protein associated with them than corresponding bands in electropherograms of young leaves (compare Fig. 6a and 1a). The quality of the electropherogram was noticeably affected by the buffer in which the leaves were ground. A buffer with sucrose which osmotically stabilized the plant organelles and with -SH which prevented oxidation products from forming gave results superior to those obtained by grinding in a simple Tris-phosphate pH 8.6 or phosphate M/15 buffer alone.
Figure 6: Electropherogram of the extract from mature uninfected tobacco leaf cytoplasm.

a: photograph of electropherogram
b: idealized diagram
c: microdensitometric trace
DISCUSSION

The purpose of this study was to determine changes which might occur in the soluble protein components of leaves infected with a plant virus. The techniques chosen to characterize the protein composition in this study were disc electrophoresis in combination with autoradiography. By these techniques twenty components were separated and defined in normal tobacco tissue extracts. Undoubtedly, additional protein species are present but these could not be detected by the methods employed. Comparisons were made between electropherograms of young uninfected tissue and tissues infected systemically for various time periods. Electropherograms of young infected tissue were remarkably similar to those obtained from non-infected tissue, except for the appearance of a new stainable band with an $R_f$ value of 0.33 which appeared eight days after inoculation. This band has been tentatively identified as X protein, identical with subunits of TMV coat protein. This tentative identification was made on the basis of an identical $R_f$ value exhibited by subunits of TMV protein.

The above comparison of healthy and infected leaf proteins was made on the basis of their staining capacity with a general protein stain, aniline blue black. In order to extend the efficiency of the technique the electropherograms were subjected to autoradiography. Three bands in extracts from uninfected tissue had relatively higher specific activity than all other bands. Autoradiographs of infected
plant electropherograms were similar in all cases to those obtained with uninfected controls, except for a heavily labeled band with $R_f$ value of 0.33 which appeared four days after inoculation and thereafter. A band with this $R_f$ value had been tentatively identified as subunit protein, and because of its high specific activity it was thought to be rapidly synthesized.

A similar conclusion was reached by Engler and Schramm (1959) who concluded that the rate of TMV protein synthesis was much greater than that of its nucleic acid on the basis that 2000 peptide units were required to envelope one "free" TMV-RNA molecule. The supply of protein exceeded that of RNA and the excess accumulated as X or subunit protein. Other workers (Takahashi and Ishii 1952, Jeener, et al. 1954) have reported that subunit protein is associated with TMV infection.

It is possible that improvements in technique would permit detection of other virus specific proteins in infected plants. Several such improvements are suggested by the recent publication of Nathans, et al. (1966) who studied the appearance of new proteins in bacteria infected with an RNA containing virus. They found four phage-specific proteins in lysates of actinomycin D treated E. coli. They incorporated small amounts of urea and detergent in the gel in order to prevent protein interaction and to facilitate destaining. They eliminated host protein synthesis by treatment with actinomycin D, so that the bulk of protein made after infection would be coded by virus. Such treatment may not be as effective in a plant system because there is evidence that the mRNA in plants is relatively stable. Another useful technique
was to use as the source of label an amino acid not present in the virus coat, such as methionine or histidine in the case of TMV, so that non-coat proteins can be selectively labeled post infection. Gels of different pH's, monomer concentrations, and lengths were used for profitable comparison of patterns and for determination of the optimum conditions for distinct separations and minimal background of staining and radioactivity. Another worthwhile procedure, although not utilized by Nathans, et al. (1966), would be to employ enzyme specific stains to detect changes in the enzyme pattern of the host as a consequence of infection. Another point of improvement can be to increase the amount of label fed to the leaves under study in order to increase the specific activity of the proteins and thus shorten the time necessary for development of the autoradiograph.

It is interesting to note that the number of protein bands found in extracts from tobacco plants in the present study is similar to the number of soluble plant proteins found using this technique in extracts from other plants. Galvanico (1964) using 5% gel found twenty bands in bean extract. Neurospora crassa extract was fractionated on 7½% gel into some twenty bands (Steward and Barber 1964). Only about thirteen bands were found in wheat extract using 8½% gel (Stroebel and Sharp 1965).

One further point of interest is the relationship of the bands with previously identified plant proteins. Galvanico (1964), using extracts from bean leaves, designated the faster moving bands as fraction II (Wildman, et al. 1949) and the slower ones beginning with the darkly stained one as fraction I on the basis of their centrifugal
separations. The \( R_f \) value of fraction I in 5% gel is 0.38 (Racunisen and Foote 1965) but in the 5.6% gel used in this study, it is about 0.04.

It has been mentioned that the faster moving bands of fraction II (bands with \( R_f \) value greater than 0.4), in electropherograms of the extracts of mature leaves appeared to have more protein associated with them than corresponding bands in electropherograms of young leaves. It remains to be shown whether the difference is due to the actual increase in fraction II as leaves mature and expand or whether it is an artifact of solubility or extraction. A band (22r) which can be seen in young leaf extract only in autoradiographs is seen in the extract of mature leaves by staining, indicating perhaps that the protein was being rapidly synthesized and had accumulated to a relatively high concentration in mature leaves.

A suggested technical improvement for future experiments would be the use of the recently marketed preparative polyacrylamide gel electrophoresis apparatus or the polyacrylamide gel slabs. In comparison with the separate columns used for disc electrophoresis in which the distance travelled by the protein is sometimes different for each column, electropherograms on slabs might be more uniform and larger quantities of material could be fractionated.
SUMMARY AND CONCLUSIONS

The proteins of young TMV infected and uninfected plants have been studied using the techniques of disc electrophoresis on polyacrylamide gel in combination with radioactive labeling. In ultracentrifuged extracts from young, uninfected leaves, approximately twenty protein bands could be distinguished by staining electropherograms with a general protein stain. Three of these bands were heavily labeled in autoradiographs when compared with the stained bands; the other bands were comparably labeled and stained. The three bands with high specific activity represented proteins which were rapidly turning over or were being synthesized rapidly in the young expanding leaves.

The only change in the soluble protein components which could be detected in stained electropherograms following infection was the appearance in extracts of plants which had been infected for eight days of a band with $R_f$ value of 0.33. This band was tentatively identified as X or subunit protein on the basis of an identical $R_f$ value exhibited under the same conditions. In extracts of plants which had been infected for four days, a band with an $R_f$ value of 0.33 was detected as a heavily labeled band in autoradiographs; a corresponding band was not found in stained electropherograms. Simultaneous with the appearance of this heavily labeled band was a rather high level of infectivity as determined by local lesion assay. It was concluded from the high
specific activity of this band that the subunit protein it represented was being rapidly synthesized or was rapidly turning over.

Only six protein bands could be detected in electropherograms of the Triton-X dissolved chloroplasts. In autoradiographs, the lack of labeling of one band in this fraction showed that the protein in that band was turning over slowly or not at all.

Some bands present in extracts from mature leaves appeared to have proportionately more protein associated with them than corresponding bands in extracts from young leaves. Such observations indicated that changes in protein accompanying maturation can be detected by this technique.

This system offers much opportunity for further investigation of protein metabolism following infection by TMV. Perhaps some of the suggested improvements will be useful for such studies.
LIST OF REFERENCES


