THE NON-VIRUS PROTEINS ASSOCIATED WITH TOBACCO MOSAIC VIRUS*

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INTRODUCTION

That tobacco leaf infected with tobacco mosaic virus (TMV) produces a multiplicity of proteins was first indicated by the work of Bawden and Pirie (1) who found non-infectious components serologically related to TMV in extracts of such tissue. Later, Commoner et al. (2) and Takahashi and Ishii (3) identified in extracts of infected leaf a non-normal low molecular weight protein characterized by a relatively low electrophoretic mobility \( (ca. -3.4 \times 10^{-8} \text{ cm}^2/\text{v.}/\text{sec.}) \) at pH 7.0. Jeener and Lemoine (4) reported that infected leaf yields a non-virus nucleic acid-free protein not present in normal leaf which cross-reacts with anti-TMV serum and which can be crystallized as microneedles resembling those of TMV. Further investigations by Commoner et al. (5) showed that at least three low molecular weight proteins capable of cross-reacting with anti-TMV serum could be found in infected leaf though absent from healthy tissue.

These observations indicated that in infected plants TMV may be accompanied by several related proteins and suggested that investigation of these non-virus proteins might provide new insight into the biochemistry of TMV reduplication.

As a step toward accomplishing this aim, the experiments described in the present paper were designed to establish the identities and origins of the non-virus proteins, and to determine whether they are, equally with TMV, consistent products of infected leaf. The general approach was to compare electrophoretically the total protein complements of systemically infected leaf and otherwise identical uninfected leaf. This approach required that (a) non-virus proteins occurring consistently in extracts be detected and characterized; and (b) it be determined which of these non-virus proteins represent components actually present in the living infected cell.

The second of these requirements bears some examination at this point. It can be safely assumed that TMV nucleoprotein found in a leaf extract is pro-

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duced in the living leaf, rather than in the extract. This conclusion is justified by ample evidence that the virus nucleoprotein isolated by lengthy purification procedures is present in the initial extracts as soon as they are prepared, and that different isolation methods yield virus of constant composition and properties. Unfortunately, among proteins extractable from tissues, TMV is somewhat unusual in this respect. Normal tissue proteins, once extracted from the cell and brought into solution, may form dissociation systems of varying degrees of complexity, which respond to different extract conditions by yielding different products (6). This being the case it cannot be assumed that any non-virus protein detected in extracts of infected leaf represents a primary component actually present as such in the leaf cells. It becomes necessary to determine whether such a protein is secondarily derived from an original component by processes induced in vitro during the course of the frequently lengthy manipulations employed in such work. As shown below, this problem enters significantly into experiments with the non-virus proteins related to TMV.

**Methods**

1. **Plants.**—The plants used were an inbred strain of *Nicotiana tabacum* (variety White Burley). These were grown in the greenhouse under conditions of maximum nitrogen supply according to a fixed culture schedule. When healthy and infected leaves were to be compared, these were obtained from plants of the same culture generation grown under identical conditions. Plants were infected with an electrophoretically homogeneous strain of common TMV by rubbing all fully enlarged leaves. These plants were maintained in the greenhouse for a 3 week period and then harvested. For maximum yield of TMV, and to insure the presence of non-virus proteins in readily detectable amounts, it is essential that infected plants be given a maximal supply of supplementary nitrogen during their entire period of growth and during the time between inoculation and harvesting. During the winter months the low level of natural illumination is inadequate for full plant growth; under these conditions production of TMV and the non-virus proteins is limited. Consequently, plants grown at this time (both healthy and infected) were given approximately 1000 foot-candles of illumination from combined natural and fluorescent light for a 12 hour period daily.

2. **Fractionation and Analytical Procedures.**—Protein preparations started with leaf blade tissue from 15 to 25 plants, weighing 500 to 1500 gm. wet. The tissue was washed, homogenized in the cold in a Waring blender, and thereafter treated as described below. Low speed centrifugation was at 4,000 to 5,000 r.p.m. at 4°C. Ultracentrifugation was carried out in a Spinco ultracentrifuge at 40,000 r.p.m. (104,500 X g). All protein preparations and electrophoresis runs were made in pH 7.0, 0.05 M phosphate buffer (ionic strength, 0.13) except when a range of pH values was desired for mobility curves. Electrophoretic analyses were carried out in a Perkin-Elmer model 38 Tiselius apparatus in a 2.4 ml. cell. Analytical ultracentrifuge data were obtained with a Spinco analytical centrifuge. The immunochemical methods used to determine qualitatively whether non-virus proteins related to TMV were present in various extracts are described in a following paper (7).
EXPERIMENTAL

1. The Non-Virus Proteins Unique to Infected Leaf

(a) Exhaustive Fractionation of Soluble Leaf Proteins.—In order to determine the number of detectable non-normal proteins occurring in TMV-infected leaf, fractional analyses of the entire soluble protein complement of comparable healthy and infected tobacco leaf were undertaken. Preliminary experiments suggested that all components unique to infected leaf were precipitable by 0.4 saturated ammonium sulfate. To confirm this observation, the residual protein (i.e. soluble in 0.4 saturated ammonium sulfate) was concentrated by salting out with 0.7 saturated ammonium sulfate, redissolved by dialysis against pH 7.0 phosphate buffer, and examined electrophoretically. Typical results are shown in Fig. 1 (I). These indicate that in both infected and uninfected tissue this fraction contains three detectable components, and that no differences associated with infection can be observed. This was further confirmed by the finding that no protein capable of immunochemical cross-reaction with anti-TMV serum occurred in this fraction (8). It was concluded that the sought for non-virus proteins were restricted to that fraction of the total extractable protein capable of being salted out with 0.4 saturated ammonium sulfate.

About 30 to 50 per cent of the soluble protein of both normal and infected leaf consists of a component containing a variable amount of pentose nucleic acid which exhibits a sedimentation constant of 19–20 (2, 9). This component is precipitated by 0.4 saturated ammonium sulfate. When such a precipitate is redissolved, the normal nucleoprotein can be removed by prolonged ultracentrifugation. Fig. 1 (II) shows electrophoresis patterns of the material isolated by ultracentrifugation of the redissolved 0.4 saturated ammonium sulfate precipitate obtained from infected and uninfected leaf. Uninfected tissue yields the normal high molecular weight nucleoprotein (NP); infected tissue yields this component and a fast moving protein which can readily be identified as TMV. This finding shows that TMV is the only high molecular weight component which occurs uniquely in these extracts of infected tissue.

When the low molecular weight proteins remaining in the ultracentrifuge supernatant are brought to pH 3.4, a sharp difference between the infected and uninfected preparations is observed. Infected extracts yield a considerable protein precipitate which readily dissolves in pH 7.0 buffer, while uninfected extracts do not yield such protein (Fig. 1, IV). The protein precipitated from extracts of infected tissue at pH 3.4 contains two distinguishable components. One of these, with an electrophoretic mobility of about −3.4 (at pH 7.0) has been designated component B3 (5). It is identical with component “B” previously reported by us (2) and with component “X” of Takahashi and Ishii (3). Also present is a faster moving component (μ = −6.7 at pH 7.0) which has been given the designation B6.

The low molecular weight components soluble at pH 3.4 (Fig. 1, III) from
FIG. 1. Fractionation of soluble proteins from leaves of tobacco plants systemically infected with TMV, and from leaves of uninfected plants grown under identical culture conditions. Diagrams are descending electrophoresis patterns; migration is to the left from the indicated starting boundaries. Electrophoresis in pH 7.0 phosphate buffer, for 90 minutes at 8 ma.
infected and uninfected tissue yield apparently identical electrophoresis patterns. However, when these fractions are tested with anti-TMV serum, it is found that cross-reacting material is present in the infected extracts and absent from uninfected extracts. In order to characterize the components responsible for the antigenic activity of this fraction, the crude pH 3.4 soluble material from infected tissue was fractionated by zone electrophoresis at pH 7 on a starch column (10). The column was cut into 1 cm. strips, each of which was extracted and washed with pH 7.0 phosphate buffer. The protein content of each of the fractions was estimated from the optical density at 280 m\(\mu\). Aliquots of the fractions were then precipitated with 0.01 ml. of anti-TMV rabbit serum, according to a procedure described elsewhere (7). The relative antigen content of each tube was then determined from the quotient, precipitin value/protein concentration of tube. The results, which are plotted in Fig. 2, show that two components of approximate mobilities -4 and -8 are responsible for the antigen activity of this material.

This result agrees with the previous report (7) of the isolation of a pH 3.4 soluble component of mobility -4.7 from the low molecular weight fraction.

![Fractionation of pH 3.4 soluble non-virus protein from infected leaf by electrophoresis on a starch column. The points represent the relative amounts of precipitate given by the protein contained in successive 1 cm. sections of the column when reacted with anti-TMV rabbit serum. Migration time, 25 hours; current, 55 ma.](image)

**Fig. 2.** Fractionation of pH 3.4 soluble non-virus protein from infected leaf by electrophoresis on a starch column. The points represent the relative amounts of precipitate given by the protein contained in successive 1 cm. sections of the column when reacted with anti-TMV rabbit serum. Migration time, 25 hours; current, 55 ma.
of infected tissue extracts. This component, which has been given the designation A4, has not yet been isolated from the crude pH 3.4 soluble material in sufficient amounts to permit extensive characterization. For this reason, its regular occurrence in infected material is not well established. As suggested by further evidence cited below, the second component present in Fig. 2, which we designate A8, is probably a secondary polymer derived from A4.

These results show that: (a) the non-virus proteins present in the original extracts of infected leaf are all of relatively small size; no protein of the size of TMV occurs, (b) the non-virus material unique to infected leaf is comprised of two major components, B3 and B6, (c) a third low molecular weight component, A4, can be detected in the material soluble at pH 3.4.

(b) Evidence That B3 and B6 Are Consistently Produced by Infected Tissue.—The results which follow, show that B3 and B6 satisfy criteria which establish them as primary protein components of infected cells. Because of the difficulties involved in the isolation of A4, it has not been possible to make a comparable study of this component.

(1) Components B3 and B6 May Be Isolated from the Leaf by More Than One Method.—A second isolation method was developed from the observation of Takahashi and Ishii (11) that "component X" tends to polymerize at low pH's into a high molecular weight protein readily sedimented in the ultracentrifuge. The scheme employed is shown in Fig. 3. Comparison of these results with those shown in Fig. 1 reveals that both procedures yield B3 and B6. Since the isolation of these proteins does not appear to be a function of the method used, it may be concluded that they represent actual tissue components rather than secondary products. Additional support for this conclusion, at least with regard to B3, is the fact that this protein has been detected by electrophoretic analysis of the total soluble protein of the leaf after concentration by salting out (2). Fig. 3 shows that the new procedure also yields in addition to B3 and B6, another component (B8) not found in uninfected tissue. The significance of this observation is discussed below.

(2) B3 and B6 Are Consistently Obtained from Different Crops of Infected Plants.—Fractional analyses of ten approximately kilogram batches of infected leaves obtained from plants grown under standardized nutritional conditions were carried out at intervals over an 18 month period. Both the fractionation procedures described above were used. The results of these analyses, with respect to the occurrence of components B3 and B6, are summarized in Fig. 4. All ten batches of infected plants grown in all seasons of the year, yielded two components with electrophoretic mobilities characteristic of B3 and B6 (−3.3 to −3.5 and −5.9 to −6.9 respectively). The observed range of variation in mobilities is expected from the fact that no attempt was made to control the protein concentration in these electrophoresis runs, and that this factor has a secondary effect on the characteristic mobility of a protein.
Fractionation of low molecular weight non-virus proteins from infected and otherwise identical uninfected tobacco leaf. Electrophoresis details as for Fig. 1.

**Components Found**

<table>
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<tr>
<td></td>
<td>b</td>
<td>May 54</td>
</tr>
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</table>

**Mobility**

Fig. 4. The descending electrophoretic mobilities of non-virus proteins found in systemically infected tobacco plants grown at intervals between October, 1952, and May, 1954. Method a indicates that fractionation was according to the scheme illustrated in Fig. 1. Method b indicates that the procedure outlined in Fig. 3 was used.
It will be noted that the electrophoretic properties of the products are independent of the isolation method employed. It can be concluded that components B3 and B6 are consistently produced by leaf systemically infected with TMV.

(3) B3 and B6 Are Consistently Absent from Uninfected Leaf.—About half the experiments summarized in Fig. 4 were accompanied by parallel analyses of comparable uninfected leaf obtained from the same plant culture generation. In no case were components B3, B6, or A4 detected in the uninfected preparations. Indeed, these components have never been found in the course of a large number of electrophoretic analyses of normal tobacco leaf proteins undertaken for various purposes over a 3 year period. B3 and B6 are therefore consistently present in infected leaf and consistently absent from normal tobacco leaf.

From the foregoing results, we conclude that B3 and B6, are along with TMV itself, characteristic protein products of the infected tobacco leaf.

(c) The Properties of Components B3 and B6.—Since repeated precipitation of B6 at its isoelectric point causes irreversible denaturation of this protein (see below), mixtures of B3 and B6 can be freed of the latter component in this manner. Several electrophoretically homogeneous preparations of B3 have been made in this way and partially characterized.

Such preparations of B3 are colorless solutions, and are relatively stable when stored in the cold. The preparations give characteristic protein tests and the Kjeldahl nitrogen can be recovered quantitatively from the hydrolysate as amino acid nitrogen. The absorption spectrum of B3 (see Fig. 5) shows a maximum at 280 mμ and a minimum at 253 mμ. Extraction with hot 0.5 m
perchloric acid, and determination of the optical density of the extract at 260
mg show that the protein does not contain nucleic acid. From determinations
of its sedimentation velocity in pH 7.0 buffer, the sedimentation constant of
B3 appears to be 2.9. When an effort was made to determine the electrophoretic
mobility of B3 at various pH's below 7.0, a striking anomaly was observed.
Although the mobility of this protein at pH 7.0 is about −3.4, at pH 5.85 the
mobility is about −7.8. This reversal in the expected effect of pH on mobility
indicates a profound change in the protein's ionic configuration. The effect
appears to be associated with polymerization (see below).

Component B6 can be isolated from mixtures of B3 and B6 by rapidly con-
verting the former to its high molecular weight polymer (see below) and re-

![Graph showing electrophoretic mobilities of TMV and proteins B6 and B8 at various pH's.](image)

**Fig. 6.** Descending electrophoretic mobilities of TMV and proteins B6 and B8 at various pH's in a series of common ion buffers (12).

moving it by ultracentrifugation. Several electrophoretically homogeneous
preparations of B6 have been prepared in this way. Such preparations are
relatively unstable, tending to form insoluble precipitates when stored in the
cold. B6 is a protein, yielding amino acids quantitatively on hydrolysis. Its
absorption spectrum (see Fig. 5) shows a maximum at 275 mμ and a minimum
at 250 mμ. Extraction with hot perchloric acid shows that no nucleic acid is
present. The sedimentation constant of this protein at pH 7.0 is 3, as deter-
mined from its sedimentation velocity. The electrophoretic mobility of puri-
fied B6 has been determined at various pH's using a common ion series of
buffers (12). The results, which are shown in Fig. 6, reveal no anomalies in the
behavior of B6. It has a mobility of about zero at pH 4.7.

Purified preparations of B3 and B6 have been repeatedly tested for infec-
tivity on leaves of *N. glutinosa*. No infectivity was detected, aside from an occasional lesion attributable to trace contamination with TMV.

2. Secondary Components

Fig. 1 shows that the non-virus components, B3, B6, and A4, occur as low molecular weight components, and that direct ultracentrifugation of leaf extracts fails to reveal the presence of any abnormal high molecular weight components other than TMV itself. However, Takahashi and Ishii (11) reported the occurrence of a high molecular weight component ("X") in extracts of infected tissue.

The origin of such high molecular weight material is described in Fig. 7. The starting material is a pH 7.0 phosphate buffer extract of infected leaf tissue. This is concentrated and freed of extraneous normal protein by precipitation with 0.4 saturated ammonium sulfate. After dialysis against pH 7.0 phosphate buffer, the redissolved protein is freed of TMV by ultracentrifugation and brought to pH 5.0. A heavy precipitate of normal nucleoprotein forms and is readily removed by low speed centrifugation. The supernate is then ultracentrifuged for one-half hour at 40,000 R.P.M. A glassy pellet appears in the centrifuge tubes. An electrophoretic analysis typical of this pellet (redissolved in pH 7.0 phosphate buffer) is shown in Fig. 7 (I).

The pellet contains components B3, B6, and two new components which have been designated B7 and B8. Considerable amounts of B8 occur in such preparations, while the concentration of B7 is usually relatively low.

If the pellet is redissolved in pH 7.0 buffer and again ultracentrifuged, it can be shown that components B7 and B8 are sedimentable (Fig. 7, II) and therefore of high molecular weight. The low molecular weight supernate contains B3 and B6 (Fig. 7, III). However, when the solution of remaining B3 and B6 is permitted to stand for several days in the cold, more high molecular weight material is formed, for as shown by pattern IV of Fig. 7, ultracentrifugation again removes B7 and B8.

These effects show that B3 and B6 polymerize to form high molecular weight proteins at pH 5.0. If the material has been freshly isolated, the polymerization is partially reversed when the proteins are returned to pH 7.0, so that the latter solution shows both low and high molecular weight components. After this process has been repeated several times, the ability to depolymerize is lost and the proteins remain in the aggregated state. This condition is more quickly attained by B3 than by B6, and the latter can frequently be isolated from mixtures by rapid conversion of B3 to its polymer. This behavior is reminiscent of the properties exhibited by the muscle protein, actin (13). Another parallel between the non-virus and muscle proteins is the fact that in both cases, the polymerized proteins differ markedly from the starting material in electrophoretic behavior. This phenomenon means that the aggregation process involves considerable changes in the ionic configuration of the protein.
That component B8 is a polymer of B3 is shown by the following observations. Preparations of purified B3, on standing, or after being brought to a low pH invariably show the new component (mobility = -8.0 at pH 7.0). Since B8 sediments in the ultracentrifuge at pH 7.0 (one-half hour at 40,000 R.P.M.), purified preparations can be readily made. Such preparations are stable, retaining a fixed sedimentation constant (ca. 200), and electrophoretic mobility on storage in the cold. Preparations of B8 electrophoretically homogeneous at
pH 7.0 retain their homogeneity when examined electrophoretically at various pH's down to 3.0. The results of such analyses, plotted in Fig. 6, show that B8 exhibits a typical variation of mobility with pH, being isoelectric at about pH 3.2. As noted above, when a purified preparation of B3 is brought to pH 5.85, its mobility is -7.8. This value is identical with the mobility of purified B8 at this pH. Furthermore, after dialysis at pH 5.85, the mobility of B3 in pH 7.0 buffer shifts from its original value (-3.4) to the mobility characteristic of B8 (-8.0). It is evident, therefore, that B8 is a polymer of B3.

As shown previously (5, 11), B8 is a rod which resembles TMV in size and shape. Like the starting protein (B3), it lacks nucleic acid, and is not infectious. B8 cross-reacts with serum prepared against TMV, and TMV cross-reacts with serum prepared against B8 (5). B8 can be precipitated as microneedles resembling those of TMV at its isoelectric point.

The polymerization properties of B6 are more complex than those of B3. The analytical procedure described above shows that this component, although of low molecular weight at pH 7.0, polymerizes to a unit (B7) sufficiently large to be sedimented in one-half hour at 40,000 r.p.m. at pH 5.0. This effect is complicated by the fact that with time B6 tends to become irreversibly denatured at pH 5.0. As a result it has not yet been possible to produce B7 by treatment of purified B6 at pH 5.0. However, such treatment of initial preparations of B3 and B6 invariably yields B7 as well as B8. Furthermore, the amount of B7 obtained is generally proportional to the amount of B6 in the starting material. This suggests that B7 is a polymer of B6. However, in the absence of more rigorous data it is also possible that B7 represents a copolymer of B3 and B6.

A trace of a third secondary component can be detected in the mixture which comprises the original pH 5.0 ultracentrifuge pellet. If the redissolved pellet is brought to pH 3.4 and the resulting precipitate and supernate examined electrophoretically, components B3, B6, B7, and B8 are found in the precipitate, while the supernate contains a very small amount of component with a mobility of about -8.2 at pH 7.0. This component (A8) remains soluble when again brought to pH 3.4, and is thereby distinguishable from component B8. Component A8 cross-reacts with sera prepared against TMV and B8. It has never been found in extracts of uninfected leaf. We infer from these results, and from the data described in Fig. 2 that A8 is a secondary component, which is probably derived from component A4 by polymerization. The amount of A8 yielded by extracts of infected leaf is so slight that no further information on its origin or properties is available at present.

3. The Amounts of the Non-Virus Proteins Produced in Systemically Infected Leaf

From the areas of the various peaks in the electrophoresis patterns yielded by the fractionation experiments described earlier it is possible to estimate
the amounts of the non-virus proteins found in infected leaf. Typical results are presented in Table I. The amounts of B6 found in systemically infected leaf range from 20 to 40 micrograms per gm. of tissue, or about 2 per cent of the value for TMV content. The corresponding values for B3 are 70 to 140 micrograms per gm. or about 7 per cent of the TMV value. Because of the considerable losses involved in isolating A4, corresponding determinations of the amounts of this non-virus protein which occur in infected leaf are not accurate. The A4 content is certainly the lowest of the three non-virus proteins and is roughly 10 to 20 micrograms per gm.

TABLE I

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<th>Preparation</th>
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<tr>
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<tr>
<td>1</td>
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<tr>
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<td>69</td>
</tr>
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<td>3</td>
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* Calculated from areas of electrophoretic peaks. Relation between peak area and protein concentration is based on empirical determinations with known amounts of TMV.

DISCUSSION

The chief conclusion which can be derived from our results is that leaf systemically infected with TMV yields a multiplicity of interrelated protein products. A sharp distinction must be made between those non-virus proteins (primary) which are demonstrably produced as such in the infected leaf, and the secondary proteins which result from polymerization processes induced by the procedures used in handling the extracts. In the data presented above, we find evidence which establishes proteins B3 and B6 and probably A4, as primary non-virus products of the infected leaf. These proteins have in common their relatively small size (sedimentation constants ca. 3) and lack of nucleic acid. None is infectious. Their close relation to TMV is demonstrated by immunological cross-reactions (7) and by their unique occurrence in TMV-infected plants. These proteins are as characteristically associated with the infection process as TMV itself. It follows that any proposed explanation of the biochemistry of TMV synthesis must also account for the occurrence of the non-virus proteins in infected leaf.

The secondary proteins, A8, B7, and B8, do not appear to bear so closely upon the problem of TMV biosynthesis. Since their formation is induced in leaf extracts, there is no evidence that these high molecular weight proteins occur as such in the infected leaf. In a sense these proteins are artefacts. It may be argued that since B8 is a macromolecular rod quite similar to TMV in appearance, the conversion of B3 to B8 represents a true model of the path of
TMV formation in the infected cell. However, the polymerization of an isodiametric protein into a rod is by no means unique to this system. The transformation of muscle proteins, for example, is quite similar, and like B8, the fibrous form of actin has an electrophoretic mobility markedly different from that of the low molecular weight starting material (13).

The consistent occurrence of at least three distinct non-virus proteins in infected tobacco leaf demands a certain degree of additional rigor of the proposals which can be advanced to describe the mode of reduplication of TMV. The bearing of the above observations on such proposals is discussed in the paper which follows.

SUMMARY

1. Exhaustive fractionation of leaves from tobacco plants systemically infected with TMV has led to the isolation of two non-virus proteins, B3 and B6, and the detection of a third, A4, which do not occur in comparable uninfected plants.

2. Components B3 and B6 have been found consistently in a series of ten extracts from plants grown over an 18 month period in all seasons of the year. It is concluded that these components are as characteristic of the infected plant as TMV itself.

3. As they occur in the initial extracts, the non-virus proteins are of low molecular weight ($S_{20}$ ca. 3). On treatment, each component tends to form a high molecular weight polymer with an electrophoretic mobility considerably greater than that of the starting material. The high molecular weight derivatives of A4, B3, and B6 have been designated A8, B8, and B7 respectively. There is no evidence that these high molecular weight components occur as such in the infected leaf.

4. The non-virus proteins are free of nucleic acid and are not infectious. They cross-react immunochemically with TMV.

5. Compared with TMV content, the amounts of the non-virus proteins found in infected leaf are relatively small, falling in the range of 10 to 150 micrograms per gm. of tissue.

REFERENCES