RELATIONSHIPS BETWEEN TOBACCO MOSAIC VIRUS 
BIOSYNTHESIS AND THE NITROGEN METABOLISM 
OF THE HOST*

BY BARRY COMMONER, DOROTHY L. SCHIEBER, AND PHYLLIS M. DIETZ
(From the Henry Shaw School of Botany, Washington University, 
St. Louis)

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INTRODUCTION

The processes which transform source materials into tobacco mosaic virus
(TMV) may be described by relating the amount of TMV formed in infected
tissue to the concurrent changes in host metabolism. In previous investigations
it was shown that TMV synthesis withdraws nitrogen from the host’s pool of
non-protein nitrogen components (1, 2), significantly alters host protein metab-
olism, and results in the appearance of at least one other abnormal protein in
addition to TMV itself (3). The experiments reported in the present paper were
designed to specify the non-protein source of TMV nitrogen and to elucidate
the relationships between TMV biosynthesis and the protein metabolism of
the host.

In these investigations, the chief experimental approach was to determine
which of the many processes encompassed by the leaf’s nitrogen metabolism
are specifically associated with TMV synthesis. Since this aim required quan-
titative comparison of the changes with time in the composition of otherwise
identical infected and uninfected tissue, experiments were based on uninfected
and infected tissue derived from opposite halves of the same tobacco leaf.

Methods

1. Culture Procedure.—Leaves were obtained from an inbred strain of Nicotiana

tabacum (var. White Burley) grown in the greenhouse with maximum nitrogen supply.
To attain optimal nutritional conditions single plants were grown in large flats con-
taining heavily manured soil to which weekly supplements of organic nitrogen were
supplied. Plants attained the size of field-grown plants; the leaves used in investiga-
tions were nearly mature and about 24 inches long.

Leaves were removed from uninfected plants, washed with distilled water, and
split along the midrib. One leaf-half was inoculated by rubbing with a gauze pad

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soaked in a purified TMV preparation containing 200 μg. of virus per milliliter of pH 7.0 phosphate buffer. The opposite leaf-half was rubbed with a pad containing pure buffer and supplied the control material. The leaf-halves were kept overnight in separate glass-topped moist boxes at 23°C. and 100 foot-candles of constant illumination from daylight fluorescent lamps.

Discs 12 mm. in diameter were then punched from each leaf-half and washed in sterile distilled water. Sets of about 15 discs totalling about 350 mg. taken from all positions of the leaf-half, were made up and floated on nutrient medium in sterile Petri dishes. Discs were prevented from sinking by coarse pyrex cloth which covered the bottom of the dish. Discs were maintained at 23°C. and 100 foot-candles of constant illumination until removed for analysis.

Two types of nutrient were used.

(a) N nutrient. This was half-strength Vickery's solution (4) which contained: 0.071 gm. KH₂PO₄, 0.116 gm. CaCl₂, 0.437 gm. MgSO₄·7 H₂O, and 0.278 gm. (NH₄)₂ SO₄ per liter. The solution was adjusted to pH 6.4 before use.

(b) N-free nutrient. This was identical with the above except that the ammonium sulfate was replaced by an equivalent amount of potassium chloride.

2. Fractionation Procedures.—Samples of discs were removed from the dishes, washed, weighed, and homogenized in a glass homogenizer with cold pH 7.0 phosphate or phthalate buffer. The homogenate was made up to 5 ml. in a volumetric flask and aliquots removed for analyses.

Two 0.5 ml. aliquots were made up to 70 per cent ethanol, centrifuged, and the precipitate washed. Kjeldahl analyses of these precipitates yielded values for total protein nitrogen. Two 1.0 ml. aliquots were centrifuged for 1 hour at 2500 r.c.f., supernatants removed, and the precipitates washed with pH 7.0 buffer and discarded. The supernatants plus washes were made up to 70 per cent ethanol and centrifuged after 24 hours in the cold. Kjeldahl analyses of the alcohol precipitates yielded values for soluble protein nitrogen (SPN); the supernatants yielded non-protein nitrogen (NPN). Two 0.5 ml. aliquots were analyzed for TMV. Values for insoluble protein nitrogen (IPN) were obtained from the difference between total protein and soluble protein. Total nitrogen was obtained from the sum of total protein and non-protein nitrogen. In N¹⁴ experiments IPN was isolated directly.

3. Analytical Methods.—(a) Kjeldahl nitrogen: Nitrogen values for NPN, SPN, IPN, and in some cases for TMV were obtained by a micro Kjeldahl procedure involving 18 hours of digestion and the use of H₂O₂ and mercury catalyst. Ammonia was distilled into borate-indicator mixture (methyl red and methylene blue) and titrated with 0.01 N HCl. Analyses were made in duplicate. For the 246 analyses reported below mean difference between duplicate samples was 6.0 per cent.

(b) TMV analysis: The method of Commoner et al. (5) was used. Where N¹⁴ determinations were desired, the TMV was subjected to Kjeldahl digestion and the distilled ammonia analyzed for N¹⁴ as described below.

(c) Ammonia, amide, and amino nitrogen: Ammonia was isolated from the NPN fraction by vacuum distillation (6). Amide nitrogen was obtained from NPN by hydrolysis in 2 N HCl for 2 hours at 100° followed by ammonia distillation. Amino nitrogen was released as ammonia by the ninhydrin method of MacFadyen (7).
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(d) N¹⁴ analyses: After titration, Kjeldahl samples were boiled down to about 2 ml., placed in a vacuum tube similar to that described by Sprinson and Rittenberg (8), evacuated, and the ammonia nitrogen released as N₂ gas into a mass spectrometer by reaction with hypobromite.

EXPERIMENTAL

1. Changes in Protein and Non-Protein Components

(A) Discs Cultured in N Nutrient.—Infected and uninfected discs cut from opposite halves of the same leaf were cultured in N nutrient and analyzed for TMV, IPN, SPN, and NPN at approximately daily intervals for a 400 hour period after the time of inoculation. The results are described in Figs. 1 and 2. Fig. 1 shows that the infected discs formed virus in a typical manner, the final virus content reaching about 0.3 mg. of TMV nitrogen per gram of leaf. The bulk of the virus was formed during the period 180 to 300 hours after inoculation. The final virus concentration was maintained without change between 300 and 400 hours after inoculation. Fig. 2 shows that both infected and uninfected discs absorbed nitrogen from the nutrient; the total nitrogen content of the tissues increased by about 50 per cent over the 400 hour period.

Infection with TMV induced significant changes in the sizes of the several fractions. Although non-protein nitrogen rose in both infected and uninfected discs, a significant deficiency in non-protein nitrogen developed in the infected discs (as compared to control discs) during the time when virus was being synthesized.

The insoluble protein content of uninfected discs fell somewhat until 180 hours after inoculation. This period was followed by a sharp rise which more than doubled the amount of insoluble protein nitrogen. The comparable curve for the infected tissue was generally similar to the control except for an excess of insoluble protein during the 100 hour period preceding the time of maximum virus synthesis (i.e. 100 to 200 hours after inoculation).

The soluble protein nitrogen content of uninfected discs showed a decline to 300 hours after inoculation, followed by a rise which almost regained the initial value. The corresponding curve for infected discs was similar but exceeded the control during the period in which virus was being synthesized.

The changes in total protein nitrogen (upper curve of Fig. 1) show that previous to and during the time of virus formation, the total protein content of infected discs exceeded that of uninfected discs by an amount which was greater than the virus present in the former. Thus, for a time, infected discs contain excess non-virus protein. At all times TMV represents an excess over the protein content of the controls.

(B) Discs Cultured in N-Free Nutrient.—In an experiment otherwise identical with (A) above, the ammonium sulfate of the nutrient was replaced by an equivalent amount of potassium chloride. Consequently, virus formation was
Fig. 1. Changes in amounts of non-protein nitrogen, TMV, protein insoluble in pH 7.0 buffer, protein soluble in pH 7.0 buffer, and total protein of uninfected tobacco leaf discs (open circles, broken lines) and TMV-infected discs (closed circles, solid lines) during culture in N nutrient. Soluble protein nitrogen includes virus. Necessarily at the expense of intrinsic host nitrogen. The results are shown in Figs. 3 and 4.
Under these circumstances TMV was formed slowly; the final virus content, at 380 hours after inoculation, was about half of that formed in N nutrient. Virus formation began at about 160 hours after inoculation and continued at a more or less constant rate throughout the remainder of the experiment.

Both infected and uninfected discs lost nitrogen during the first 70 hours after inoculation. Nitrogen excreted in that time was reabsorbed in the next 70 hours and at 140 hours after inoculation both infected and control discs had regained their initial nitrogen content. Subsequently, the uninfected discs again lost nitrogen until an apparent equilibrium level, about 20 per cent lower than the initial values, was attained in the period 240 to 380 hours after inoculation. In the infected discs this final fall in total nitrogen was smaller and did not occur until 300 hours after inoculation.

These fluctuations in total nitrogen were accompanied by similar changes in wet weight of the discs. Thus, as shown in Fig. 4, the wet weights of infected and uninfected discs agreed over the period in which nitrogen contents were identical (i.e. the first 200 hours after inoculation). Thereafter the infected discs showed a concurrent excess in both wet weight and in total nitrogen.

Fig. 3 shows that the effects of TMV infection on nitrogen distribution found in the previous experiment also occurred in the absence of outside nitrogen. The deficiency in non-protein nitrogen occurring in the infected discs was relatively small and short lived (280 to 360 hours after inoculation). We have previously observed that discs cultured in N-free nutrient, which form virus more slowly than discs supplied with external nitrogen, develop a correspondingly
smaller non-protein nitrogen deficiency during the time when virus is synthesized (1).

An excess in insoluble protein nitrogen occurred in the infected discs soon after inoculation. This resulted from the failure of the insoluble protein content of infected discs to decline in the face of a rapid fall in the control series. Soluble protein nitrogen content fell steadily in the control discs. The infected discs showed a parallel decline which halted temporarily at the time when virus formation began. This resulted in a considerable excess in soluble protein nitrogen in the infected series during the latter half of the culture period.
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The total protein content of infected and uninfected discs was practically identical up to the time when virus formation began. Subsequently, the protein content of infected discs exceeded that of the control; at its maximum this excess was 1.2 mg. of nitrogen per gram or about six times the amount of virus formed. The excess disappeared gradually until in the last 60 hours of the experiment it was reduced nearly to zero.

These results show that the effects of TMV infection are superimposed on the intrinsic metabolic patterns of the uninfected discs. The effects are largely limited to the time during which TMV synthesis occurs and to a period just preceding it.

![Graph](image)

**Fig. 4.** Changes in total nitrogen content and wet weight of discs described in Fig. 3. The lowermost curve represents TMV nitrogen in infected discs.

Figs. 5 and 6 show that despite the large differences between the nitrogen levels attained by discs cultured in N-free nutrient, and those cultured in N nutrient, the effects of infection are similar. In both experiments, the major differences between uninfected and infected discs are of the same sign and occur at similar times with respect to the TMV synthesis curve.

In both culture conditions, infected discs as compared with controls show: (1) A non-protein nitrogen deficiency which ends with cessation of TMV synthesis. (2) An excess in insoluble protein which ends at least 80 to 100 hours before cessation of TMV synthesis. (3) An excess in soluble protein which appears a little later than the insoluble protein excess and which ends at about the time when TMV formation ceases.

The presence of ammonia in the nutrient results in a relatively early cessation of TMV synthesis (at 300 hours); in N-free nutrient, discs continue to synthesize virus (although at a lower rate) until at least 385 hours after inoculation.
lation. There is a corresponding shift in the times at which the above differences between infected and uninfected discs appear. It can be concluded, therefore, that the observed differences in nitrogen content between infected and control discs are due to processes specifically associated with TMV synthesis.

Fig. 5. Differences between uninfected and infected discs cultured in N nutrient with respect to amounts of non-protein nitrogen (NPN), insoluble protein (IPN), soluble protein (SPN), and TMV. Data from Fig. 1.

2. The Distribution of $N^{15}$ in Various Protein Fractions

Several experiments were carried out in which discs obtained from infected and uninfected halves of the same leaf were cultured in N medium containing 32 atom per cent $N^{15}$ until a significant amount of TMV had been synthesized in the infected half. Both sets of tissue were then homogenized, fractionated, as described, and nitrogen and $N^{15}$ analyses made.

(A) Insoluble Protein.—Protein associated with particulate matter insoluble in pH 7.0 phosphate buffer (i.e. precipitated by centrifugation of the homogenate for 1 hour at 2500 r.c.f.) was fractionated crudely by a specific gravity method. Solutions of glycerol and ethanol producing a graded series of specific gravities were prepared. Aliquots of the insoluble material were suspended in
TABLE I

Fractionation of Insoluble Protein

<table>
<thead>
<tr>
<th>Specific gravity interval</th>
<th>Protein nitrogen precipitated (μg/100 mg)</th>
<th>Atom per cent N₁⁴ in precipitate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninfected</td>
<td>Infected</td>
</tr>
<tr>
<td>1.32–1.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.21–1.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.17–1.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.13–1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein not precipitated at specific gravity = 1.32</td>
<td>136</td>
<td>70</td>
</tr>
<tr>
<td>Free ammonia nitrogen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMV</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean error in these values is ±1 atom per cent N₁⁴.

these solutions and centrifuged for 1 hour at 2500 r.c.f. The supernatants were then poured off and each precipitate analyzed for total Kjeldahl nitrogen and for N₁⁴.

Table I shows the results of this fractionation. The N₁⁴ enrichment of the
total IPN from infected discs is only slightly greater than that from uninfected tissue. However, the relatively small fractions of infected IPN which precipitate at specific gravities between 1.00 and 1.17 contain a considerably higher proportion of isotope than the comparable fractions from uninfected material. In fact the atom per cent \( \text{N}^{15} \) in these highly labelled fractions is about equal to that found in TMV and in free ammonia. Thus, the effects of infection on IPN appear to be associated with a specific cell particulate. Since the isotope labelling of this component is equivalent to that of ammonia, it must be either of extraordinary metabolic activity, or newly synthesized in infected tissue. As will be shown below, it is likely that this insoluble component is associated with an early stage in TMV synthesis.

**TABLE II**

<table>
<thead>
<tr>
<th>pH interval</th>
<th>Protein nitrogen precipitated</th>
<th>Atom per cent ( \text{N}^{15} ) in precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu g/100 \text{ mg.} )</td>
<td>( \mu g/100 \text{ mg.} )</td>
</tr>
<tr>
<td>Uninfected</td>
<td>Infected</td>
<td>Uninfected</td>
</tr>
<tr>
<td>7.0-5.2</td>
<td>72</td>
<td>81</td>
</tr>
<tr>
<td>5.2-5.0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>5.0-4.6</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>4.6-4.4</td>
<td>2.3</td>
<td>2.4</td>
</tr>
<tr>
<td>4.4-4.2</td>
<td>2.0</td>
<td>2.6</td>
</tr>
<tr>
<td>4.2-3.4</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>7.0-3.4</td>
<td>108</td>
<td>124</td>
</tr>
</tbody>
</table>

* Difference between infected and uninfected values is significant.

**(B) Soluble Protein.—**Fractionation of protein soluble in pH 7.0 buffer was accomplished by successively lowering the pH of the solution and collecting the protein fractions which precipitated at each stage. TMV was separated from the precipitates by extraction with pH 7.0 buffer and a series of isoelectric precipitations of the virus. The results (Table II) indicate that the \( \text{N}^{15} \) content of certain fractions isolated in this was significantly affected by infection. The protein fractions precipitating between pH 4.4 and 3.4 attain a significantly higher isotope level in infected tissue than they do in uninfected tissue. Therefore, these fractions include material which is either newly synthesized from NPN or which turns over more rapidly in the infected tissue than in the control. Since the sizes of these fractions are larger in the infected tissue than in the controls, it is likely that in infected tissue they contain protein newly synthesized from NPN.
Table II also shows that a highly labelled protein fraction (pH 5.2-5.0 precipitate) occurs in uninfected tissue but is absent in infected discs. Further evidence concerning this protein is presented in section 4 below.

3. Isotope Relations between NPN Fractions and TMV

Experiments were carried out in which it was possible to describe the changes in \(^{15}N\) content of various NPN components and to compare them with the \(^{15}N\) incorporated into the TMV synthesized concurrently. This was done by inoculating two tobacco leaves with TMV, dividing each into longitudinal halves, and culturing discs obtained from both leaf-halves in unlabelled N nutrient. After some days the tissue from one leaf-half was homogenized, fractionated, and the size of each fraction determined. At the same time the tissue from the opposite leaf-half was transferred to N nutrient in which the ammonium N contained 60 atom per cent \(^{15}N\). On the following day the tissue was removed, washed, homogenized, and analyzed for free ammonia, amide, and amino nitrogen. The isotope content of each fraction was determined in the usual way. The atom per cent \(^{15}N\) of the TMV newly synthesized while the half-leaves were in labelled nutrient was calculated from the quotient: micrograms of \(^{15}N\) in final TMV sample/difference in TMV contents of initial and final half-leaves.

The results obtained from two leaves treated in this way are presented in Table III. In both cases the isotope enrichment of the newly synthesized TMV is considerably higher than that of the free amino acids or amides. It is not possible therefore that the nitrogen of the TMV amino acid residues can be derived from the corresponding free amino acids unless it can be shown that these components comprise a small, very highly labelled fraction of the entire free amino acid pool. However, the latter alternative is ruled out by the fact that the amino acids represented in TMV protein comprise at least 70 per cent of the free amino acid pool in cultured tobacco leaf discs (2).

On the other hand, the atom per cent \(^{15}N\) of the newly synthesized TMV falls within the isotope level attained by the ammonia. Thus, ammonia is the only source of nitrogen sufficiently rich in \(^{15}N\) to account for the isotope content of the virus. It can be concluded, therefore, that ammonia serves as the primary source of the bulk of TMV nitrogen, and that most of the amino acid residues of TMV are not derived from the corresponding free amino acids.

4. Time-Course with \(^{15}N\)-Labelled Nutrient

The results just described suggest that specific fractions of the insoluble and soluble protein components of the tobacco leaf are involved in TMV synthesis. In order to investigate these relationships in more detail, a time course similar to that described in section 1A above was carried out using \(^{15}N\)-labelled nutrient.

Infected and uninfected discs were prepared from opposite halves of a
single leaf in the usual manner and cultured in N nutrient containing 32 atom per cent N\textsuperscript{15} in ammonium ion. Uninfected samples were fractionated as in the previous experiments, but fractionation of infected samples was modified to separate TMV from the buffer-soluble proteins. This was accomplished by repeated precipitation of the virus at its isoelectric point (pH 3.4); hence the soluble protein fraction differs from the comparable fraction in previous experiments in that it does not include TMV. Each fraction was subjected to Kjeldahl digestion, titrated, and analyzed for N\textsuperscript{14}. Determination of isolated TMV was carried out by the Folin method (5), followed by Kjeldahl digestion, and N\textsuperscript{15} determination. From these analyses the changes with time in the nitrogen content and atom per cent N\textsuperscript{15} in each fraction from both infected and uninfected halves of the leaf, were obtained. These results are plotted in Figs. 7 and 8.

The effects of infection on nitrogen fractions were similar to those reported above, with the exception that the excess in non-virus soluble protein previously noted was not observed. This is probably associated with the fact that in both infected and uninfected discs from this leaf, the soluble protein content de-

### TABLE III

<table>
<thead>
<tr>
<th>Component</th>
<th>Initial leaf-half</th>
<th>Final leaf-half</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Atom per cent N\textsuperscript{15}</td>
</tr>
<tr>
<td>Ammonia</td>
<td>21.97</td>
<td>0.384</td>
</tr>
<tr>
<td>Amino</td>
<td>25.01</td>
<td>0.384</td>
</tr>
<tr>
<td>Amide</td>
<td>20.08</td>
<td>0.384</td>
</tr>
<tr>
<td>TMV</td>
<td>1.03</td>
<td>0.384</td>
</tr>
</tbody>
</table>

TMV newly formed (final - initial) \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 14.1 \pm 2.0

Nitrogen values refer to 100 mg. wet weight of original leaf tissue.

Leaf I, initial leaf-half analyzed at 96 hours after inoculation; final leaf-half analyzed at 116 hours after inoculation.

Leaf II, initial leaf-half analyzed at 185 hours after inoculation; final leaf-half analyzed at 214 hours after inoculation.

* Carrier nitrogen added for N\textsuperscript{14} analysis.
clined at an unusually rapid rate. This behavior appears to be characteristic of discs obtained from plants grown in the winter months, even when flowering is suppressed by extra illumination.

![Image](image_url)

**Fig. 7.** Changes in amounts of TMV, non-protein nitrogen (NPN), insoluble protein (IPN), and soluble protein (SPN) of uninfected tobacco leaf discs (open circles, broken lines) and TMV-infected discs (closed circles, solid lines) during culture in N nutrient containing 30 atom per cent N\(^{15}\). SPN does not include virus; dotted line in SPN curve represents sum of SPN and TMV.

Significant differences between the N\(^{14}\)-labelling of infected and uninfected discs occurred. Previous to the period of virus formation the infected discs attained a greater enrichment in NPN N\(^{15}\) than the uninfected discs; this excess disappeared during the period of virus formation so that at the end of that time the N\(^{14}\) enrichment of the NPN fractions in both sets of discs became about equal. The IPN of infected and control discs showed almost identical changes.
in N\textsuperscript{16} level until 140 hours after inoculation. After 140 hours, the atom per cent N\textsuperscript{16} in IPN of infected tissue was consistently greater than the comparable value for the control. The N\textsuperscript{15} level of the SPN fraction was also unaffected by

infection until the time when virus synthesis began. Then an excess N\textsuperscript{16} enrichment occurred in the infected tissue. This gradually disappeared until the values became equal at 300 hours.

Analysis of these data lead to the following conclusions concerning the metabolic path of TMV synthesis.

(A) An NPN Component (Ammonia) Is the Initial Source of TMV Nitrogen
TMV sufficient for isotope analysis (using a 1:10 dilution with carrier nitrogen) was obtained beginning at 160 hours after inoculation. Fig. 8 shows that the N\(^{15}\) enrichment of the virus was relatively constant (12 to 13 atom per cent) in the period 160 to 285 hours. During this time the isotope content of IPN rose from 5 to 8 atom per cent N\(^{15}\); that of SPN rose from 6 to 7 atom per cent N\(^{15}\), while the N\(^{15}\) of NPN fell from 12 to 11 atom per cent. The isotope level found in TMV is consistent with the previous observation that the bulk of TMV nitrogen is derived from NPN ammonia. Since N\(^{15}\) enters the tissue as ammonia, this component tends to exceed other NPN components in isotope content. Thus, TMV synthesized from ammonia nitrogen necessarily attains an atom per cent N\(^{15}\) somewhat higher than that of the whole NPN pool, and considerably in excess of the isotope level of the bulk SPN and IPN.

Additional evidence that TMV nitrogen is obtained from ammonia N is the fact that the atom per cent N\(^{15}\) in NPN declines as TMV is formed and that the actual amount of N\(^{15}\) present in NPN (see Fig. 9) of infected discs drops significantly below the corresponding value for uninfected discs during the period of TMV synthesis.

(B) TMV Is Synthesized from an Insoluble Protein Precursor.—The previous isotope experiments implicated an insoluble protein component in TMV synthesis. Analysis of the changes in isotope content of IPN during TMV synthesis describes the role of this component in virus synthesis.

Fig. 9 shows that at about 200 hours the N\(^{15}\) found in IPN of infected discs rises sharply over the isotope content of uninfected IPN, reaching a maximum excess of 5 \(\mu\)g. of N\(^{15}\) at 234 hours, and declining rapidly thereafter. These changes in the steady state isotope content would be expected if the IPN fraction accumulated a limited amount of a TMV precursor, which was finally depleted by conversion to virus protein.

To test this proposal it is necessary to account for the observed steady state changes in IPN N\(^{15}\) in terms of the amounts of N\(^{15}\) added to and removed from this fraction. This can be done by calculating the amounts of IPN synthesized and removed from equations based on the treatment of Sprinson and Rittenberg (9).

It is assumed that IPN is synthesized from NPN and concurrently converted to unspecified components. It can then be shown that for a given period of time, the amounts of protein synthesized and removed are related to the observed data by the following equations:

\[
B = \frac{dN^{15} - dN \times a}{a - b}, \quad \text{and}
\]

\[
S = dN + B,
\]

in which for the given time period, \(B\) is the amount of protein nitrogen removed, \(S\) is the amount of protein nitrogen synthesized from NPN, \(dN\) is the change in
the net amount of protein nitrogen present, $dN^{15}$ is the change in the amount of protein $N^{15}$ present, $a$ is the average atom per cent $N^{16}$ in NPN, and $b$ is the average atom per cent $N^{16}$ in the protein fraction in question. It is also apparent that the $N^{15}$ entering IPN = $S \times a$, and the $N^{15}$ leaving IPN = $B \times b$. 

Fig. 9. $N^{15}$ content of various fractions; data derived from values given in Figs. 7 and 8. Open circles, broken lines, uninfected discs. Closed circles, solid lines, infected discs.
The calculated values for the amounts of N\(^{15}\) entering and leaving IPN are shown, as integrals, in Fig. 10.

At 115 hours, when TMV synthesis begins, the amount of N\(^{15}\) which has passed into IPN is identical in infected and uninfected discs. Thereafter, the rate of N\(^{15}\) entry into IPN of infected discs rises sharply over the rate for uninfected discs. By 211 hours, the infected material attains an excess of 5 \(\mu\)g. of IPN N\(^{15}\) which is more or less maintained thereafter.

Fig. 9 shows that a total of 5 \(\mu\)g. of N\(^{16}\) appears in TMV, chiefly in the period 211 to 285 hours. Thus, the amount of N\(^{16}\) which is finally found in TMV can be accounted for by the extra isotope incorporated into IPN of infected discs. Fig. 10 shows that TMV begins to appear at about the time at which the rate of N\(^{15}\) incorporation into infected IPN begins to exceed that of the control. However, in this period the rate of TMV appearance is lower than the rate of accumulation of excess IPN N\(^{15}\). Thus, infected discs synthesize an excess of labelled insoluble protein which coincides in amount of N\(^{15}\) with the TMV finally produced. Synthesis of this new IPN protein precedes in time the formation of
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These observations coincide with the behavior expected of an insoluble protein precursor of TMV.

This conclusion requires that the amount of excess N\textsuperscript{15} leaving the IPN fraction be sufficient to account for the isotope found in TMV. Fig. 10 shows that this is the case. It will be noted that the extra IPN loss in infected discs is greater than the 5 \textmu g. of N\textsuperscript{15} incorporated in TMV. Hence, transformation of the IPN precursor of TMV into virus is accompanied by degradation of some insoluble protein, presumably into NPN.

An alternative explanation of the above data must be considered; i.e., the excess IPN accumulated before TMV appears is actually virus which has been incompletely extracted in the fractionation process. We have shown previously (5) that the extraction of TMV from homogenates of the dilution employed is nearly complete. Furthermore, if extraction were incomplete, the unextracted TMV retained in the IPN fraction should be a constant fraction of the total TMV. This is not the case; the ratio of N\textsuperscript{15} present in the excess IPN to N\textsuperscript{15} in extracted TMV becomes increasingly smaller and approaches zero as the TMV content increases. It is very unlikely, therefore, that the observed excess in IPN represents unextracted TMV.

(C) Soon after Inoculation, Infected Tissue Synthesizes an Insoluble Protein Component, Which Temporarily Resists Metabolic Degradation.—The experiments described in section 1 show that within the 100 or so hours which intervene between inoculation and the appearance of TMV, infected discs accumulate an excess of insoluble protein which is lost when virus begins to appear. Fig. 10 shows that during the accumulation of this early excess in IPN, the rates at which nitrogen enters IPN of infected and uninfected discs are about the same. However, in this period the rate of nitrogen removal from IPN is considerably lower in infected discs than in uninfected discs. When the excess IPN disappears, removal of nitrogen from IPN of infected discs shows a corresponding increase in rate.

Thus the excess IPN which appears in infected discs soon after inoculation is due to the synthesis of insoluble protein which is temporarily more stable than the IPN synthesized in uninfected discs at this time. It will be noted from Fig. 11 that this process is accompanied by a temporary reduction in the amount of soluble protein synthesized by infected discs. As will be shown below, there is reason to believe that at least part of the soluble protein is derived from nitrogen removed from IPN, and that infection-induced suppression of IPN degradation reduces the synthesis of SPN.

(D) Synthesis of the Insoluble Protein Precursor of TMV Prevents the Synthesis of a Comparable Amount of Normal Soluble Protein.—The differences in the isotope content of SPN from infected and uninfected discs indicate that infection induces metabolic changes in this protein fraction. These effects can be analyzed by the methods used for IPN; i.e., by calculating the rates of entry and departure of nitrogen and N\textsuperscript{15}. These results are presented in Fig. 11.
These data show that between 191 and 211 hours after inoculation, the amount of N\textsuperscript{15} incorporated into SPN in infected tissue falls about 3 \mu g. below the comparable value for uninfected tissue; at the same time a 40 \mu g. deficiency in total SPN nitrogen develops. This effect occurs precisely when comparable quantities of N\textsuperscript{16} and total nitrogen are removed from NPN and converted to the insoluble protein precursor of TMV. It is reasonable to conclude, therefore, that synthesis of the TMV precursor prevents the synthesis of a like amount of normal soluble protein.

It is possible that this effect is a result of the general reduction in available NPN resulting from the drain imposed by withdrawal of nitrogen for synthesis of TMV precursor. Alternatively, the synthesis of the insoluble protein precursor of TMV may suppress formation of a normal protein with which it competes in a more specific manner, for example by involving the same site of synthesis. Evidence favoring the second of these alternatives is the fact that

![Graph showing amounts of N\textsuperscript{15} incorporated into and withdrawn from SPN fraction. Values calculated from data of Figs. 7 and 8 according to equations given in text. Open circles, broken lines, uninfected discs. Closed circles, solid lines, infected discs.](image-url)
infected leaf fails to synthesize a highly labelled soluble protein which appears in uninfected leaf (see Table II). The fraction which contains this protein component (precipitate between pH 5.2 and 5.0) is the only one of the number of SPN fractions obtained which contained a higher isotope level in uninfected than in infected discs. Furthermore, the N\textsuperscript{14} contained in this fraction (in uninfected tissue) is about 10 per cent of the total N\textsuperscript{14} of SPN; this agrees with the observation (see Fig. 11) that the deficiency in SPN N\textsuperscript{14} resulting from the suppressed SPN synthesis due to TMV formation is also about 10 per cent of the concurrent N\textsuperscript{14} content of the total SPN.

It is likely, therefore, that the protein which infected tissue fails to synthesize when the TMV precursor is formed is a specific component of the normal complement of soluble protein, which is characterized by an unusually high isotope level. The specificity of this effect suggests a close relation between the mechanism involved in synthesis of this normal protein and that engaged in synthesis of the insoluble protein precursor of TMV. This competition may serve as a useful point of attack on the synthesis of the TMV precursor. It may also help to explain some of the metabolic effects of infection on the host.

**DISCUSSION**

The results permit description of certain phases of the sequence of events which is precipitated by the entry of TMV into the leaf and which culminates in the appearance of newly synthesized virus. Since virus nucleic acid represents but 5 per cent of the total nitrogen, the present experiments are incapable of detecting effects bearing on the origin of TMV nucleic acid nitrogen. The conclusions deal only with the protein moiety of TMV.

In agreement with previous investigations (1, 2, 10) the results reported above show that the initial source of the bulk of TMV nitrogen is the non-protein nitrogen pool of the leaf. It has been suggested that TMV is synthesized from a preexisting normal protein which comprises 30 to 50 per cent of the normal soluble protein of the leaf (11). This proposal is ruled out by (a) direct evidence relating the isotope contents of TMV and NPN, (b) the observation that the non-virus soluble protein content of infected discs exceeds the SPN of uninfected discs, and (c) isotope data which show that TMV synthesis is not associated with an increase in the rate of nitrogen removal from SPN. Nitrogen originally contained in normal proteins may serve as a source of TMV nitrogen, but only by first being degraded and joining the NPN pool. A normal protein which so serves as a “source” of TMV nitrogen does not have a specific role in TMV synthesis.

The isotope experiments show that ammonia is the major and perhaps the only component of the pool of free NPN which participates in *de novo* synthesis of TMV or its precursor. The evidence of Table III rules out the possibility...
that the bulk of the free amino acid pool can serve as the major source of TMV protein nitrogen. This means that synthesis of TMV (or a precursor protein) cannot be based on withdrawal of the requisite amino acids from the NPN pool. Additional evidence which supports this conclusion has been reported previously (2).

It is possible, but not likely, that a small fraction of the free amino acid pool, perhaps one or two amino acids, attains an N\textsuperscript{16} enrichment sufficiently above the total amino acid average to permit it to serve as a source of the heavily labelled TMV nitrogen. If this were the case such an amino acid(s) would serve as a \textit{non-specific} source of all the peptide nitrogen atoms present in TMV; this possibility again excludes TMV synthesis by condensation of free amino acids in the order and frequency required by the composition of the virus protein.

It should be emphasized that these observations do not exclude the possibility that TMV or a precursor protein is indeed synthesized by the long postulated peptide condensation of appropriate amino acids. The data require only that amino acids involved in such a process not be in equilibrium with the free amino acid pool. Thus, TMV synthesis might involve formation from free ammonia of amino acids at a site (e.g. a specific protein or particulate surface) to which they remain bound until incorporated into TMV protein by peptide condensation. In any event it must be concluded that, regardless of details, the initial incorporation of ammonia nitrogen into the carbon residues required for the amino acid constituents of the virus protein yields products which are sequestered from the pool of free amino acids and amides. In this sense, the specificity of the biochemical mechanism involved in TMV synthesis must begin at the point at which ammonia enters into it. It will be of considerable interest to describe in detail these initial events; efforts in this direction are in progress.

The early incorporation of N\textsuperscript{16} into a cell particulate, and its subsequent removal as soluble TMV appears, suggest that this particulate may be the site of the primary step in TMV biosynthesis (i.e. ammonia withdrawal). Since this process is necessarily induced by the inoculum, it is likely that this cell particulate is also the primary point of attack of the entering virus. Electron micrographs published by Black et al. (12) suggest association of TMV with chloroplasts, and Woods and duBuy (13) have, at least on theoretical grounds, implicated the mitochondria in this process.

On the basis of the evidence shown in Table I, it does not seem likely that the component with which the insoluble protein precursor of TMV is associated is the chloroplast. The excess N\textsuperscript{16} found in infected IPN is associated with a component which represents no more than 12 per cent of the total IPN; chloroplasts comprise most of the IPN.

The fact that infected tissue fails to synthesize a normal rapidly metabolized
soluble protein component provides another clue to the mechanism of TMV biosynthesis. This observation suggests that the sequestration of ammonia for TMV synthesis deprives the host of material required for synthesis of normal protein. The results imply that the cell particulate involved in the initial stages of TMV synthesis is also concerned with the synthesis of the normal protein which fails to appear in infected tissue, and that the two processes are competitive. It may be anticipated, therefore, that this normal protein is closely related to the primary insoluble protein precursor of TMV.

The isotope data reported in Table II show that certain small fractions of the complement of soluble leaf proteins contain significantly more isotope in infected leaf than they do in uninfected leaf. We have found (14) that the pH range in which this protein precipitates (pH 4.4-3.4) also causes isoelectric precipitation of a new non-virus protein previously detected in electrophoretic analyses of proteins from infected leaf (and absent from uninfected leaf). The present isotope data, in agreement with the earlier electrophoretic observations indicate that this non-virus protein (component “B” in reference 3) is, in addition to TMV itself, synthesized as a specific consequence of the infection process. Recent electrophoretic analyses of highly concentrated preparations of soluble proteins from infected tissue (14) have led to the detection of at least two other low molecular weight proteins, which like component B are not found in uninfected plants. A protein probably identical with component B has recently been reported by Takahashi (15, 16).

These observations suggest the possibility that the new low molecular weight proteins may represent intermediates between the primary insoluble TMV precursor and the virus proper. Alternatively, the accessory non-virus proteins and TMV may be parallel rather than sequential products of the insoluble precursor, or the two products may have independent origins.

Finally, it is apparent that the processes directly associated with TMV synthesis, i.e. incorporation of NPN ammonia into an insoluble protein precursor and the subsequent appearance of TMV and the accessory non-virus proteins, are preceded by metabolic changes in some IPN component. The significance of the unusual IPN stability detected soon after inoculation is not evident from the present data. However, since the transitory accumulation of IPN which results from this effect has been consistently observed in infected leaf, there is reason to believe that the phenomenon is a fundamental part of the infection process.

It is evident from the multiplicity of processes and products involved in the synthesis of TMV, that reduplication of this virus is not accomplished by simple replication of the inoculum. It is true that the processes which have been described begin with inoculation of TMV and culminate in the appearance of new protein identical with the inoculum. However, between these limits lies a complex series of steps which effectively dissociates the inoculum from direct par-
participation in the final event. The intervening processes appear to resemble those responsible for replication of normal host protein, and the unique biology of the virus seems to arise from its ability to divert these processes from their normal course. The fundamental question to be answered is how the inoculum can impress on the protein-synthesizing apparatus of the host a specificity which weaves from the simplest of raw materials the complex structure of the virus protein.

SUMMARY

1. Comparisons of the nitrogen content of TMV-infected and uninfected tobacco leaf discs at various times after inoculation show that virus synthesis is associated with a net increase in protein content. This excess protein is due to: (a) TMV, (b) an excess in insoluble protein which develops soon after inoculation and ends about 100 hours before cessation of TMV synthesis, and (c) an excess in soluble non-virus protein, which is variable in size and which only occurs during the time of virus synthesis. A deficiency in non-protein nitrogen occurs during the time when virus appears.

2. Isotope experiments with N\textsuperscript{15}-labelled nutrient show that: (a) The bulk of TMV nitrogen is derived from the free ammonia of the host tissue. (b) Amino acid residues of TMV protein are not derived from the corresponding free amino acids in the host. (c) The appearance of TMV is preceded by the synthesis of an insoluble precursor of the virus which is then converted into TMV or some soluble intermediate protein. This effect is associated with a cell particulate which represents a small fraction of the total insoluble protein. (d) Infected tissue synthesizes \textit{de novo} small amounts of soluble non-virus protein, which may represent intermediates in TMV synthesis. (e) Infected tissue fails to synthesize a rapidly turned-over soluble protein which is synthesized in comparable uninfected tissue. (f) TMV synthesis is preceded by a temporary enhancement of the metabolic stability of an insoluble protein component.

3. The results lead to the conclusion that TMV formation is due to diversion of some part of the host’s protein-synthesizing apparatus from its normal course.

BIBLIOGRAPHY