THE ISOLATION OF DEFECTIVE TOBACCO MOSAIC VIRUS STRAINS*

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This paper describes a method for the isolation of strains of tobacco mosaic virus (TMV) which consist of infective particles that are labile under conditions where ordinary TMV is extremely stable. Two such strains have been isolated, PM1 and PM2, which are distinctly different in their properties. Evidence will be presented to demonstrate that tobacco plants infected with strain PM1 fail to synthesize viral protein and that plants infected with the PM2 strain synthesize a virus-like protein which fails to aggregate in vivo with the viral nucleic acid to form complete virus particles.

The possibility that defective strains of TMV could be recovered was suggested by the results of studies dealing with the induction of mutations with nitrous acid. It was noted that a class of survivors of nitrous acid treatment existed that would induce necrotic lesions on a local lesion host but from which infectivity could not be recovered. We postulated that perhaps some of these nontransferable lesions contained a labile infectious entity which was destroyed during transfer manipulation. Such defective strains have now been isolated by the direct inoculation of systemic-type host seedlings with nitrous acid-treated TMV preparations at limit dilution.

Isolation of the Defective Strains.—A preparation of the U1 strain of TMV was treated with nitrous acid according to the technique of Mundry and Gierer to three levels of survival: 4.2%, 0.28%, and 0.015%. The treated preparations were inoculated to tobacco (Nicotiana tabacum L. var. Samsun) seedlings at a concentration estimated to infect half or fewer of the seedlings with the expectation that many of the infections would result from a single infective particle. The infection present in those seedlings appearing diseased was tested for ease of transfer by macerating a small piece of leaf tissue and applying the macerate to a leaf of the local lesion host Xanthi (Nicotiana tabacum L. var. Xanthi-nc). Those seedlings yielding few or no lesions were set aside and allowed to grow for about a month in 6 in. pots before being retested. They were then tested for the presence of labile infectious material in the following manner: A mature leaf exhibiting symptoms symmetrically on each side of the midrib was divided along the midrib. One of the half-leaves was ground in a mortar with the addition of an approximately equal weight of M/15, pH 7, phosphate buffer, and the grindate was allowed to incubate for two hr at room temperature. The other half-leaf was extracted with phenol according to the method of Schlegel. The two leaf extracts were then applied to opposite halves of 6 Xanthi leaves. In a typical experiment, many (at least 40) lesions were induced on each of the Xanthi half-leaves rubbed with the phenol extract and an average of only 2.5 on the half-leaves rubbed with the buffer extract.

An attempt was made to demonstrate infectivity in the above lesions by cutting them out and macerating them between two ground glass spatulas with a drop of M/15, pH 7, phosphate buffer containing celite. The macerate was then rubbed
on a Xanthi half-leaf. Twenty-seven lesions induced by the phenol extract yielded no lesions on subsequent transfer. Of 14 lesions induced by the buffer extract, 13 yielded no lesions and one yielded a single lesion. Under the same circumstances, lesions induced by the parent U1 strain would yield 50–500 lesions on subsequent transfer to Xanthi half-leaves.

Two strains were isolated for further study from the experiment summarized in Table 1. Both of these strains, PM1 and PM2, have in common characteristics one would predict for TMV variants which consist of unprotected nucleic acid. In contrast to other strains of TMV, these are the first two which have been isolated that consist of a labile infective entity which is destroyed by the usual transfer techniques. We have only rarely been able to obtain infectivity from necrotic local lesions induced by these two strains. Transfer of infectivity from a systemic host is best accomplished by macerating infected tissue in the presence of ice-cold, M/15, pH 7, phosphate buffer and applying the macerate to leaves of another host, keeping the macerate cold.

**Symptomatology of strains PM1 and PM2:** Symptoms induced by strains PM1 and PM2 are similar and cannot be distinguished in the host plants we have examined. The symptoms of primary infection on mature tobacco leaves consist of yellow spots which appear about a week after inoculation. These yellow spots increase in diameter with time, and when a main vein is encountered, the yellowing develops preferentially along the sides of the vein in the direction of the midrib.

Mature tobacco leaves which become secondarily invaded first develop a light green to yellow band on either side of the midrib. This chlorosis spreads outward from the midrib more rapidly along the main side veins and less rapidly into the interveinal tissues so that finally the leaf has a yellow oak-leaf pattern bordered by green tissue.

The time between inoculation and the spread of the infection from the inoculated leaves to other tissues of the plant is slow and variable, and frequently the infection does not spread from the inoculated leaf. When secondary infection does occur, it is quite different from that of most TMV strains. The first leaf to become secondarily invaded by the defective strains is the leaf directly above the inoculated leaves, and the infection spreads slowly up the plant a leaf at a time. Young expanding leaves at the top of the plant at no time have been observed to have disease symptoms. In many systemically invaded plants, a yellowish streaking is seen along the main stem, suggesting the path of the infectious agent between leaves. A peculiar symptom sometimes seen is that of epinasty on the leaves above those inoculated.

When infected plants are topped, occasional shoots growing from the axils of infected leaves may exhibit symptoms. These symptoms are peculiar because they are seen only on the oldest and largest leaves of the shoot, the apical leaves appear-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Survival after HNO₃ treatment (%)</th>
<th>Virus conc. inoculated to seedlings (µg/ml)</th>
<th>No. of seedlings inoculated</th>
<th>No. showing disease symptoms</th>
<th>No. with defective transfer</th>
</tr>
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<tbody>
<tr>
<td>4.2</td>
<td>0.067</td>
<td>48</td>
<td>21</td>
<td>1 (PM1)</td>
</tr>
<tr>
<td>0.28</td>
<td>20</td>
<td>53</td>
<td>43</td>
<td>0</td>
</tr>
<tr>
<td>0.015</td>
<td>200</td>
<td>54</td>
<td>8</td>
<td>1 (PM2)</td>
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</table>
ing normal. These disease symptoms are similar to the light-green dark-green mosaic seen on plants infected with the more common strains of TMV, although tests have shown these leaves to be free of normal intact virus.

The unique symptom pattern of the defective strains in a systemic-type host suggests that the infective agent can only spread in the host from cell to cell and not through the conducting elements of the vascular system. It appears, however, that movement does take place in the vascular parenchyma. Neighboring cells are probably infected via the plasmodesmata, but the infective agent either fails to gain entrance to conducting elements or is inactivated in the sieve tubes where it may be exposed to degradative substances.

The observation that the infectious agent frequently fails to leave the inoculated leaf suggests that other defective strains may have been overlooked in the screening procedure used to isolate PM1 and PM2. It is quite possible that defective isolates which failed to leave the inoculated leaf would have been scored as not-infected in the experiment summarized in Table 1.

The local necrotic lesions induced by the two defective strains on local lesion hosts appear similar to U1 lesions. Although quantitative comparisons of lesion sizes induced by the two strains and the parent U1 strain have not yet been performed under carefully controlled conditions, visual observation has revealed no marked size difference. The lesions induced by the defective strains increase in size with time as do lesions induced by the parent U1 strain.

Properties of the Infectious Principle.—(a) Sensitivity to incubation and ribonuclease: When leaves showing symptoms are ground in the presence of buffer and the system is kept ice-cold, the grindate will induce lesions when rubbed on a local lesion host. If the grindate is allowed to incubate for an hour at 37°, however, the number of lesions induced is drastically reduced. Data demonstrating this point are shown in Table 2. In many experiments, pancreatic ribonuclease (Nutritional

<table>
<thead>
<tr>
<th>TABLE 2</th>
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<tbody>
<tr>
<td><strong>FRACTIONATION OF INFECTIVITY PRESENT IN HOMOGENATES OF PM2 INFECTED LEAVES</strong></td>
</tr>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Unfractionated homogenate</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>26,000 × g pellet (1 part pellet + 4 parts buffer)</td>
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<tr>
<td></td>
</tr>
<tr>
<td>26,000 × g supernatant</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>105,000 × g pellet (1 part pellet + ca. 20 parts buffer)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>105,000 × g supernatant</td>
</tr>
<tr>
<td>TMV, 0.1 μg/ml</td>
</tr>
</tbody>
</table>

* "Yes" indicates sample incubated for 1 hr at 37° with 0.1 μg/ml pancreatic ribonuclease added. Sample cooled in ice-bath prior to inoculation. "No" indicates sample kept ice-cold and inoculated immediately after preparation.

† This sample only was incubated without the addition of ribonuclease.

Biochemical Company, 5 × recrystallized) at a concentration of 0.1 μg/ml was added during the incubation with little additional effect. Probably, the infectious principle is attacked and destroyed by leaf ribonucleases when the infected tissue is
The fact that some infectivity survives incubation in the presence of ribonuclease is difficult to interpret. A similar phenomenon has been encountered in the study of free infectious nucleic acid present in early infections of the common strain of TMV and the winter or NM strain of tobacco rattle virus, which appears also to be a defective plant virus strain. Cadman has presented evidence that the infectious agent of the NM strain of tobacco rattle virus becomes bound to plant particulates where it is partially protected from the action of ribonuclease. Data obtained relative to the fractionation of the infectivity of the PM2 strain from a leaf homogenate indicate that the infectious material in this case is also associated with cellular particles.

Whatever the explanation for residual infectivity after incubation of the grindate, it is clear that lesions produced by the survivors of incubation and ribonuclease treatment are in no way different from lesions induced by susceptible particles. Repeated tests have revealed that little or no infectivity can be recovered from either type of lesion. Data bearing on this point are presented in Table 3.

(b) Sensitivity to heat: An aid to the production of purified preparations of TMV is a brief (10–20 min) heat treatment of infected leaf grindates at 55–60°. This treatment completely destroys the infectivity present in PM1 and PM2 leaf homogenates.

(c) Fractionation of leaf homogenates: It is a clear-cut observation that no virus-like pellet is obtained when attempts are made to purify the infectious principle present in PM1 and PM2 leaf homogenates by differential centrifugation.

In an effort to partially purify and concentrate the infective agent, homogenates of PM2-infected leaves were fractionated according to the scheme shown in Figure 1. At each stage in the fractionation, samples were taken for infectivity assay.

Data are shown in Table 2. Centrifugation of the homogenate at the relatively modest force of 26,000 × g for 15 min (#30 Spinco rotor, 15,000 rpm) sediments an appreciable portion of the infectivity although some is left in the supernatant fraction. The remainder of the infectivity is sedimented at 105,000 × g for one hr (#30 Spinco rotor, 30,000 rpm). Because much of the infectivity sediments at forces incapable of sedimenting complete virus particles, it is possible that a good deal
of the infective material is adsorbed onto or embedded in cellular particles or debris. Diener and Whitfeld et al. have observed that TMV infectious nucleic acid behaves in a similar manner when added to homogenates of uninfected leaves.

Infectivity contained in all of the fractions is sensitive to destruction by incubation at 37° in the presence of 0.1 μg/ml of pancreatic ribonuclease, although there is usually a low level of survival. As discussed previously, however, either no or only a very low level of infectivity can be recovered from lesions induced by the survivors. Data demonstrating this point are shown in Table 3.

Comparison of Strains PM1 and PM2.—Although both PM1 and PM2 induce similar symptoms on tobacco, a remarkable difference between PM1- and PM2-infected plants becomes obvious when clarified leaf homogenates are tested for the presence of X protein. In a plant infected with the common strain, this low-molecular-weight protein is present in addition to virus. It is indistinguishable from virus protein and has been demonstrated to be a precursor of virus protein. Upon testing, PM1-infected tissue proves to be devoid of X protein while PM2 tissue contains moderately large quantities.

The presence of X protein in PM2-infected tissue is demonstrated according to the method of Delwiche et al. as modified by Zaitlin. A filtered leaf homogenate, prepared by grinding infected leaves with 3 X their weight of M/15, pH7, phosphate buffer, is centrifuged at 105,000 X g for one hr. The supernatant solution is acidified to pH 4.7 and is kept overnight in order to aggregate X protein and to precipitate normal plant protein. Following slow-speed centrifugation to remove insoluble plant protein, the solution is centrifuged at 105,000 X g whereupon a glassy pellet is obtained. This pellet and the centrifuge tube are gently washed with water, and the pellet is resuspended in a small volume of water adjusted to pH 8 with 0.01 N NaOH. After clarification at 20,000 X g for 30 min and repetition of the procedure, the solution is crystal clear and behaves in a manner similar to a solution of TMV X protein. A Tyndall effect appears upon acidification of the solution to pH 5.5 which disappears upon adjustment of the pH back to pH 7. Upon reaction against TMV antiserum diluted 1/8, positive ring tests are obtained with solutions containing as little as 10 μg/ml of PM2 protein. The ultraviolet absorption spectrum of the PM2 protein is similar to the published absorption spectra for TMV protein and X protein and differs only slightly in detail near the absorption maximum.

The PM2 protein can be clearly distinguished from strain U1 protein on the basis of its serological specificity. Antiserum prepared to strain U1 will still react with U1 protein solutions after it has been absorbed with PM2 protein until it no longer reacts with PM2 protein solutions.
Although PM2 protein behaves in a manner similar to both X protein and TMV protein, no virus or virus-like material has been found in PM2-infected tissue. It is possible that the PM2 protein is defective in its ability to aggregate around TMV nucleic acid to form intact virus particles. This problem is currently being investigated by attempting to reconstitute strain U1 nucleic acid with PM2 protein. Preliminary attempts have been unsuccessful.

PM1-infected tissue yields no virus-like protein when the procedure for recovering PM2 protein is followed. Homogenates of PM1-infected tissue appear to be completely devoid of protein which reacts with TMV antiserum.

Discussion and Summary.—We have isolated two strains of TMV, PM1, and PM2, which are defective in their capacity to induce the formation of complete virus particles. The infective principle behaves in a manner similar to that of infectious nucleic acid isolated from ordinary strains of TMV both in its instability and in its manner of fractionation from leaf homogenates. The strain PM1 apparently has lost the capacity to induce the formation of a protein which would appear similar to virus protein by our methods of analysis. The PM2 strain does induce the formation of such a protein, but why complete virus is not formed in the host tissue remains a problem for further investigation.

The symptomatology of the two strains is compatible with the notion that "proteinless" viruses can spread in the host plant only from cell to cell, probably through the plasmodesmata, and either are incapable of entering or are rapidly inactivated in the conducting elements of the vascular system.

Several authors have speculated recently that certain types of tumors may have as etiologic agent incomplete viruses such as those described in this paper. These peculiar strains of TMV may, therefore, prove useful as a model system for study of infection induced by "proteinless" virus. A point to be noted is the lack of viral antigen associated with the infectious entity. The consequences of this feature for animal virus infection might be quite drastic because the infective agent would be immune to antibody neutralization. However, it may be difficult for such a labile agent to spread from cell to cell in tissue devoid of plasmodesmata-type structures.

The isolation of a strain which produces no viral protein and another which produces a defective viral protein is suggestive of certain Neurospora and Escherichia coli systems. For instance, some mutants at the tryptophane synthetase locus of these organisms produce an enzymatically inactive protein (CRM) which cross-reacts serologically with the enzyme protein, and other mutants are devoid of protein similar to the enzyme.

At least two other examples of infective agents which may lack the capacity to induce formation of complete virus particles have been described. These are the NM strain of tobacco rattle virus and the infective agent present in domestic rabbit papillomas.

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2 Siegel, A., Virology, 11, 156 (1960).
The CsCl density gradient centrifugation developed by Meselson, Stahl, and Vinograd\(^1\) can be used for estimating compositional heterogeneity as well as the average base composition of DNA.\(^2\)\(^,\)\(^3\)

During a survey of DNA samples from various organisms, it was found that the distribution of DNA in density gradient centrifugation is generally unimodal, but that there are a few exceptions.\(^4\)\(^-\)\(^6\) The most striking exception is the DNA isolated from marine crabs (\textit{Cancer borealis} and \textit{Cancer irroratus}), which has an extra ultraviolet absorbing band of lesser density distinct from its main DNA band.\(^4\)\(^,\)\(^7\) The relative amount of the "light DNA" is about 30 per cent in \textit{C. borealis} and about 10 per cent in \textit{C. irroratus}. The light DNA component was isolated and found to contain 97 mole per cent of deoxyadenylate and deoxythymidylate in predominantly alternating sequence.\(^8\)

\textit{Isolation of Total DNA.}—\textit{C. borealis} was obtained from Maine and Massachusetts. Most preparations of DNA were from testes and some were from \textit{vas deferens}. To isolate these tissues, the live crab was opened by cutting along the edge between the ventral and dorsal surfaces. Yellow digestive glands were first carefully removed and discarded. Testes were collected in cold versene-saline solution (0.1 \textit{M} ethylene diamine tetraacetate, 0.15 \textit{M} NaCl, pH 8.0), rinsed once, and then suspended in this solution (10 ml per crab). The tissue was gently macerated with a sintered glass tissue grinder and diluted with two volumes of versene-saline. Purification of DNA including ribonuclease treatment is essentially the same as that reported by Marmur for bacterial DNA.\(^9\)

The ultraviolet spectrum of the isolated DNA from \textit{C. borealis} is shown in Figure 1. Hyperchromic shift at 260 \textit{m}u of the total DNA as a function of elevated