THE PREPARATION AND USE OF TOBACCO MOSAIC VIRUS CONTAINING RADIOACTIVE PHOSPHORUS

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PLATES 6 AND 7

(Received for publication, April 8, 1942)

One of the major mysteries surrounding viruses is their manner of reproduction or growth. Despite the fact that the introduction of one unit of a virus within a living cell of a susceptible host is followed by the production of millions of virus units, practically nothing is known concerning the reproductive process. It is not known whether subsequent generations of virus are produced by some catalytic process in which none of the substance of the inoculum is incorporated into subsequently produced virus, or by a process similar to that involved in the growth of organisms in which a portion of the substance of the parent is transferred to the progeny, or by means of some new and unknown process. This problem is one of considerable importance, for it is related directly to the fundamental nature of viruses. It seemed possible that the preparation and isolation of tobacco mosaic virus containing radioactive phosphorus, the inoculation of plants with virus marked by radioactivity, and the determination of the distribution of the radioactivity in the diseased plants following extensive multiplication of virus should provide some measure of information concerning the process of virus reproduction. If, following the inoculation of the marked virus, radioactivity should be demonstrable in portions of the plant other than in the inoculated leaves, it might provide a basis for assuming that reproduction by fission had occurred. If, on the other hand, all of the radioactivity in the inoculum could be recovered from the inoculated leaves, the result might be interpreted to mean that reproduction had followed some catalytic process.

There is evidence that tobacco mosaic virus in mosaic-diseased plants acts as a foreign protein in that it does not appear to enter into catabolic processes and cannot be utilized in the synthesis of normal plant proteins (1, 2). It seemed possible, therefore, that theoretical objections to the method of approach outlined above, based on the known lability in vivo of components of proteins (3), might not necessarily prove valid in the present case. Although rapid movement of virus within plant stems has been noted (4), it is possible that a given unit of virus usually remains localized and does not move from place to place. Despite the number of theoretical and experimental difficulties, including those just mentioned and those discussed by Arnon, Stout, and...
Sipos (5), it appeared worthwhile to perform a few experiments in an attempt to determine the feasibility of this general method of approach to the question of the nature of virus reproduction.

In the present study, both normal and mosaic-diseased Turkish tobacco plants were fed a nutrient solution containing radioactive phosphorus and, after suitable periods of time, the distribution of radioactivity was determined in each set of plants. The tobacco mosaic virus which was present in the diseased plants was isolated and found to contain radioactive phosphorus. After purification, this virus was used as an inoculum for normal Turkish tobacco plants and the distribution of radioactivity and virus in inoculated and uninoculated portions of these plants was determined after 12 days. Although virus containing radioactive phosphorus was isolated from uninoculated portions of the plants, the results must be regarded as inconclusive because of reasons to be discussed later.

EXPERIMENTAL

The author is greatly indebted to Dr. John Lawrence and the Staff of the Radiation Laboratory of the University of California for the radioactive phosphorus that was used in the present experiments. The author also wishes to thank Dr. Robert Wilson and Mr. Ernest Frank of Princeton University for testing the samples obtained in the present work for radioactivity. These tests were made by placing each of the dried samples a given distance from a Lauritsen type electroscope or from the tube of a Geiger-Müller counter (5, 6).

A preliminary experiment was made in order to provide information regarding the nature and the rapidity of absorption of radioactive phosphorus by mosaic-diseased Turkish tobacco plants. A solution containing 51 mg. of disodium phosphate and 0.25 millicuries of radioactivity was added to 4 pots containing medium-sized mosaic-diseased Turkish tobacco plants which had been grown in sand and fed with a complete nutrient solution as described by Spencer (1). After 48 hours, the plants were removed, their roots well washed, and the entire plants frozen. The wash water from the roots and washings from the sand were combined and evaporated to dryness. The frozen plants were macerated and the virus was isolated by ultracentrifugation according to the method customarily employed in this laboratory (7). The various fractions into which the radioactivity might have been partitioned, namely, the washings from the sand and roots, the mash' or press cake, the supernatant liquid obtained on ultracentrifugation, and the isolated virus, were then incinerated and on being tested were found to contain radioactivity in the ratios of 60, 12.7, 26, and 1.3, respectively. Thus, the major portion of the radioactive phosphorus which had been made available to the mosaic-diseased plants had not been taken up by the plants during the 48 hour period, and of that absorbed only 3.2 per cent was incorporated in the virus protein. It
seemed likely, therefore, that, in order to secure virus possessing a reasonable amount of radioactivity, it would be necessary to feed radioactive phosphorus to mosaic-diseased plants over a considerable period of time.

In the next experiment, 12 seedling Turkish tobacco plants were started in sand and 12 days later 6 of the plants were inoculated with tobacco mosaic virus. Then each of 3 normal and 3 diseased plants from this group was fed 50 ml. of a nutrient solution prepared by adding 330 mg. of disodium phosphate containing 7 millicuries of radioactivity to 20 liters of the complete nutrient solution described by Spencer (1). The remaining diseased and normal plants were fed a nutrient solution prepared by adding 330 mg. of ordinary disodium phosphate to 20 liters of the complete nutrient solution. The nutrient solutions were added daily in amounts which were increased from 50 to 200 ml. per plant, so that during the 30 day feeding period approximately the same amount of radioactive phosphorus was added each day.

Following the feeding period, the 4 sets of plants were watered freely with tap water for 20 days. Some idea of the distribution of radioactivity after different periods of time may be gained from the contact “radiographs” shown in Figs. 1 and 2. These were prepared by the technique described by Arnon, Stout, and Sipos (5), except that the leaves were not detached from the plants and an exposure period of about 12 hours was used. It may be seen that there was considerable radioactivity dispersed throughout both normal and diseased leaves at the end of 1 week and that there does not appear to be much difference in appearance between the radiographs of the normal leaves and those of the diseased leaves. Most of the radioactivity appears to have been distributed in the major veins of the leaves. Although the light green areas of diseased leaves are known to contain much more virus than the dark green areas (8), there does not appear to have been a concentration of radioactivity in the light green areas. Because of the high concentration of virus nucleoprotein known to exist in diseased leaves (7), it might be expected that such leaves would contain more phosphorus than normal leaves. However, when leaves were removed from each of the 4 sets of plants described above and dried at 110°C, the dried leaves from normal plants fed radioactive and ordinary phosphorus were found to contain 0.94 and 0.78 per cent, respectively, of phosphorus, whereas the corresponding diseased leaves were found to contain 0.91 and 0.78 per cent, respectively. Comparable results were obtained with similar sets of plants as well as with plants grown in soil. It is obvious that there is no significant difference between the total phosphorus contents of normal and of mosaic-diseased Turkish tobacco leaves.

At the end of 50 days, the 4 sets of plants were cut, weighed, and placed in a room held at −12°C. The sets of normal plants fed ordinary and radioactive phosphorus weighed 590 gm. and 621 gm., respectively, whereas the
corresponding sets of diseased plants weighed 430 gm. and 385 gm., respectively. The roots, sand, and pots from the 2 sets of plants fed radioactive phosphorus were well washed and the wash waters concentrated and saved. The virus present in the diseased plants which had been fed with radioactive phosphorus was then isolated by means of differential centrifugation. The water-insoluble material which was obtained on ultracentrifugation was added to the mash or press cake. The supernatant liquids which were obtained from the first 3 ultracentrifugations were combined and divided into protein and protein-free fractions by means of precipitation of protein with hot 5 per cent trichloracetic acid. These latter fractions, together with the mash or press cake, the wash water from the sand and roots, and the supernatant liquid from the 4th ultracentrifugation, were each dried and incinerated. The 620 mg. of purified virus obtained from the plants fed radioactive phosphorus were prepared for analysis by simply freezing and drying. The various fractions were then examined for radioactivity by means of a Lauritsen type electroscope. The results which were obtained are summarized under Experiment 1 in Table I. It may be seen that 12.3 per cent of the total radioactivity which was recovered was associated with the virus. The fact that

### TABLE I

**Distribution of Radioactivity in Normal and Tobacco Mosaic-Diseased Plants***

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Virus</th>
<th>Supernatant liquids from first 3 ultracentrifugations</th>
<th>Supernatant liquid from 4th ultracentrifugation</th>
<th>Press cake plus insoluble material from ultracentrifugation</th>
<th>Wash of sand and roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Protein</td>
<td>Non-protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1‡ (diseased)</td>
<td>12.3</td>
<td>14.6</td>
<td>27.0</td>
<td>0</td>
<td>21.7</td>
</tr>
<tr>
<td>2‡ (diseased)</td>
<td>20.7</td>
<td>7.2</td>
<td>20.3</td>
<td>0</td>
<td>21.0</td>
</tr>
<tr>
<td>2§ (normal)</td>
<td>17.6</td>
<td>27.2</td>
<td></td>
<td>29.6</td>
<td>25.6</td>
</tr>
</tbody>
</table>

* The radioactivity was measured by means of a Lauritsen type electroscope. In some cases readings were also made by means of a Geiger-Müller counter. The numbers represent the per cent of radioactivity found in the various fractions obtained from a given set of plants.

‡ In this experiment the virus fraction was dried from the frozen state and tested. All other fractions were incinerated and tested.

§ In this experiment, preliminary tests were made on fractions prepared as in Experiment 1. However, the numbers given refer to tests made on the fractions following precipitation of phosphorus by ammonium molybdate. This was done to reduce self-absorption due to the great bulk of some of the samples. Nevertheless, for reasons unknown, the radioactivity estimated to have been recovered was only about one-fifth the amount that was estimated to have been present based on the known rate of disintegration of radioactive phosphorus. The virus isolated was estimated to contain about 3 microcuries of radioactivity.
the supernatant liquid from the 4th ultracentrifugation was found to be devoid of radioactivity, whereas radioactive phosphorus in the form of disodium phosphate may be separated readily from the virus, is good evidence that the radioactive phosphorus is integrally bound to the virus, presumably in the nucleic acid portion of the molecule.

A second experiment similar to the one just described was next carried out, except that a total of 450 mg. of disodium phosphate containing 11.4 millicuries of radioactivity was used and the feeding period was extended to 40 days, following which the plants were removed and frozen. The sets of normal plants fed ordinary and radioactive phosphorus weighed 403 gm. and 393 gm., respectively, whereas the corresponding sets of diseased plants weighed 222 gm. and 213 gm., respectively. The yield of purified virus from the 3 diseased plants fed radioactive phosphorus was 740 mg. and that from the plants fed ordinary phosphorus was 610 mg. In this experiment one-half of the purified virus containing radioactive phosphorus, together with the remaining fractions from both normal and diseased plants fed radioactive phosphorus, was incinerated. These preparations were then tested for radioactivity. In order to reduce inaccuracies in the tests due to self-absorption in the bulkier samples, all preparations were then taken into solution and the phosphorus was precipitated as phosphomolybdate. The phosphomolybdate precipitates were incinerated and tested for radioactivity. Following this treatment, the activities of the virus and of the two mash samples were approximately doubled, those of the protein components of the supernatant fluids were increased by about 20 per cent, and those of the remaining samples were essentially unchanged. The activities recorded in Table I for Experiment 2 are for the phosphomolybdate precipitates and probably provide a more accurate picture of the distribution of phosphorus than the values recorded for Experiment 1. It may be seen that 70 to 75 per cent of the radioactivity was found in the normal and diseased plants, and of that in the diseased plants about 30 per cent was in the virus. The supernatant fluid obtained on ultracentrifugation of the juice from normal plants contained more radioactivity than the corresponding fraction from the diseased plants. As in the previous experiment, the supernatant liquid from the 4th ultracentrifugation of the virus possessed no measurable amount of radioactivity. The experiments demonstrate that it is possible to secure purified tobacco mosaic virus containing considerable radioactivity.

In an effort to learn something of the distribution of radioactivity following inoculation of virus containing radioactive phosphorus, the lower leaves of 9 medium-sized normal Turkish tobacco plants were thoroughly rubbed by means of a bandage gauze pad with 15 ml. of a solution containing 58 mg. of the radioactive virus in 0.1 M phosphate buffer at pH 7. The plants were watered as usual and after 12 days the inoculated leaves were removed and
frozen. The upper uninoculated portions of the plants were also removed and frozen. The two portions were macerated and the virus was isolated from each by ultracentrifugation. The supernatant liquids obtained on ultracentrifugation as well as all insoluble material were added to the respective press cakes and these were incinerated, taken into solution, and the phosphorus precipitated as the phosphomolybdate. The 316 mg. of purified virus from the inoculated leaves, as well as the 204 mg. of virus from the uninoculated portions of the plants, were also incinerated and the phosphorus precipitated as the phosphomolybdate. Following ignition, the 4 samples were tested for radioactivity, and the results which were obtained are presented in Table II.

It may be seen that most of the radioactivity was found to be associated with non-virus components, of which about 40 per cent was in the inoculated and about 60 per cent in the uninoculated portions of the plants. A small but significant amount of radioactivity was found in virus isolated from the uninoculated portions of the plants. However, the fact that most of the radioactivity was found in the non-virus portion of the uninoculated parts of the plants and much radioactivity in the similar portions of the inoculated leaves proves that most of the radioactivity applied in the form of virus was converted into a non-virus form. Therefore, the isolation of radioactive virus from the uninoculated portions of the plants has no significance, for it could have been produced from radioactive phosphorus absorbed and transported in a non-virus form. Since it is likely that only a small portion of the virus in the inoculum was actually introduced within the cells as virus (7), it would follow that most of the radioactive phosphorus was actually absorbed later in the form of disintegration products of virus presumably formed on the surface of the leaves. It was determined that about 70 per cent of the radioactivity applied in the form of virus was actually isolated in the 4 fractions; hence, there was little loss due to washing of virus or of disintegration products from the leaves in the 12 day period during which the plants were watered.

### TABLE II

<table>
<thead>
<tr>
<th>Distribution of Radioactivity in Turkish Tobacco Plants 12 Days following Inoculation of Lower Leaves with 58 M.g. of Radioactive Tobacco Mosaic Virus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolated from inoculated leaves ................................</td>
</tr>
<tr>
<td>All material of inoculated leaves except virus .....................</td>
</tr>
<tr>
<td>Virus isolated from upper uninoculated leaves ......................</td>
</tr>
<tr>
<td>All material of uninoculated leaves except virus ..................</td>
</tr>
</tbody>
</table>

* The radioactivity was measured by means of a Geiger-Müller counter. The numbers represent the per cent of radioactivity found in the various fractions of the plants. The tests were made on the incinerated phosphomolybdate precipitates obtained from the various fractions. The radioactivity recovered represents 70 per cent of the 0.2 microcurie of radioactivity that was applied in the form of virus.
It appeared desirable to determine the extent to which virus can be removed from inoculated leaves by means of vigorous washing immediately following inoculation. Accordingly, all of the leaves of 2 sets of 7 plants each of normal medium-sized Turkish tobacco plants were inoculated with two 16 ml. portions of 0.1 M phosphate buffer at pH 7 containing 4 mg. of ordinary tobacco mosaic virus per ml. The leaves of one set of plants were then removed and each leaf was well washed in a running stream of tap water. The leaves of the other set of plants were also removed and both sets of leaves were placed in a room held at $-12^\circ$ within $\frac{1}{2}$ hour of the start of the experiment. After standing for 2 days at $-12^\circ$, the frozen leaves were macerated and the virus was isolated by differential centrifugation. The actual yield of virus from the unwashed leaves was 45 mg. or 70 per cent of the virus applied to the leaves. The actual yield of virus from the washed leaves was 27 mg. or 42 per cent of that applied to the leaves. These results indicate, in view of the losses known to occur during the purification of such small amounts of virus, that practically all of the virus applied was retained either on or within the unwashed leaves and that well over 50 per cent of the virus applied was retained in the case of the washed leaves. The fact that it is difficult to differentiate between radioactive phosphorus accepted in the form of virus and that absorbed in the form of virus disintegration products and the lack of information concerning the extent of the multiplication of virus immediately following inoculation make it exceedingly hazardous to attempt to determine the manner of virus reproduction by means of virus labeled with radioactive phosphorus.

DISCUSSION

When mosaic-diseased Turkish tobacco plants were fed a nutrient solution containing radioactive phosphorus in the form of disodium phosphate over a period of several weeks, about 30 per cent of the radioactive phosphorus taken up by the plants was isolated in the form of purified tobacco mosaic virus. The fact that the radioactive phosphorus was not separated from the virus on ultracentrifugation is an indication that it was integrally bound, presumably in the nucleic acid component of the virus. This result is in accord with that described in a preliminary paper by Born and coworkers (9), who noted that ordinary tobacco mosaic virus absorbed only an insignificant amount of radioactivity when allowed to stand for several days in a solution of sodium phosphate containing radioactive phosphorus. In the present work the proportion of radioactive phosphorus associated with the virus was considerably greater than that described by Born and coworkers, who found about 87 per cent of the radioactivity in the press cake and sediment of chloroplasts and only about 2.1 per cent with the purified virus.

The preparation and isolation of tobacco mosaic virus possessing high radioactivity made it possible to determine the distribution of radioactivity follow-
ing the inoculation of such virus to normal Turkish tobacco plants. Unfortunately, in this experiment most of the radioactivity was found to be associated with non-virus components in both inoculated and uninoculated portions of the plants; hence, it was impossible to be certain that the small amount of radioactive virus found in the uninoculated portions of the plants arose as a result of actual movement of inoculated virus. If, as has been generally assumed, only a very small part of virus applied to the surface of a leaf actually enters the cells of the leaf in the form of active virus, it would follow that most of the radioactive phosphorus taken up by the plants in this experiment was absorbed in a non-virus form. It seems likely that it will be exceedingly difficult to distinguish between radioactive phosphorus taken up by the plant in the form of virus and that taken up in the form of virus disintegration products. The present work indicates that there are major difficulties involved in the use of tobacco mosaic virus containing radioactive phosphorus as a means for determining the nature of virus reproduction.

The experiments described were conducted with the assistance of Mr. Marshall Barbour.

SUMMARY

Normal and tobacco mosaic-diseased Turkish tobacco plants were grown in sand for a period of several weeks, during which they were fed daily a complete nutrient solution to which had been added disodium phosphate containing radioactive phosphorus. Determinations were made of the distribution of radioactive phosphorus in different fractions such as the wash from the sand and roots, the press cake obtained on pressing the juice from the plants, the protein and protein-free portions of the supernatant liquids obtained on ultracentrifugation of the juices, and the purified tobacco mosaic virus isolated from the diseased plants. Chemical analyses as well as radiographs of the normal and diseased leaves indicated that they contained the same amount of phosphorus. Approximately 30 per cent of the radioactive phosphorus absorbed by the diseased plants was found to be combined with the purified tobacco mosaic virus that was isolated from these plants. Following the inoculation of purified tobacco mosaic virus possessing high radioactivity to normal Turkish tobacco plants, most of the radioactivity was found to be associated with non-virus components of which about 40 per cent was in the inoculated and 60 per cent in the uninoculated portions of the plants. Although a small amount of radioactive virus was isolated from the uninoculated portions of the plants, it was impossible, because of a number of complicating factors which have been discussed, to draw from the results any reliable conclusions regarding the mode of reproduction of tobacco mosaic virus.
BIBLIOGRAPHY

EXPLANATION OF PLATES
Photographed by J. A. Carlile

PLATE 6

Fig. 1. Contact radiographs of leaves of normal (a, c, e) and tobacco mosaic-diseased (b, d, f) Turkish tobacco plants 1 (a, b), 2 (c, d), and 3 (e, f) weeks, respectively, after the start of a daily application of a nutrient solution containing radioactive phosphorus. Although the radioactive phosphorus appears to be well dispersed throughout the leaves, the lightest areas, which are indicative of the greatest concentration of radioactivity, conform to the major veins of the leaves. The diseased leaves are from plants described in Experiment 1, Table I.
FIG. 1

(Stanley: Radioactive tobacco mosaic virus)
FIG. 2. Contact radiographs and photographs of leaves of normal (a, c) and tobacco mosaic-diseased (b, d) Turkish tobacco plant 7 weeks after the start of the experiment in which a nutrient solution containing radioactive phosphorus was fed for 30 days. The leaves are from the same plants used for Fig. 1.
Fig. 2

(Stanley: Radioactive tobacco mosaic virus)